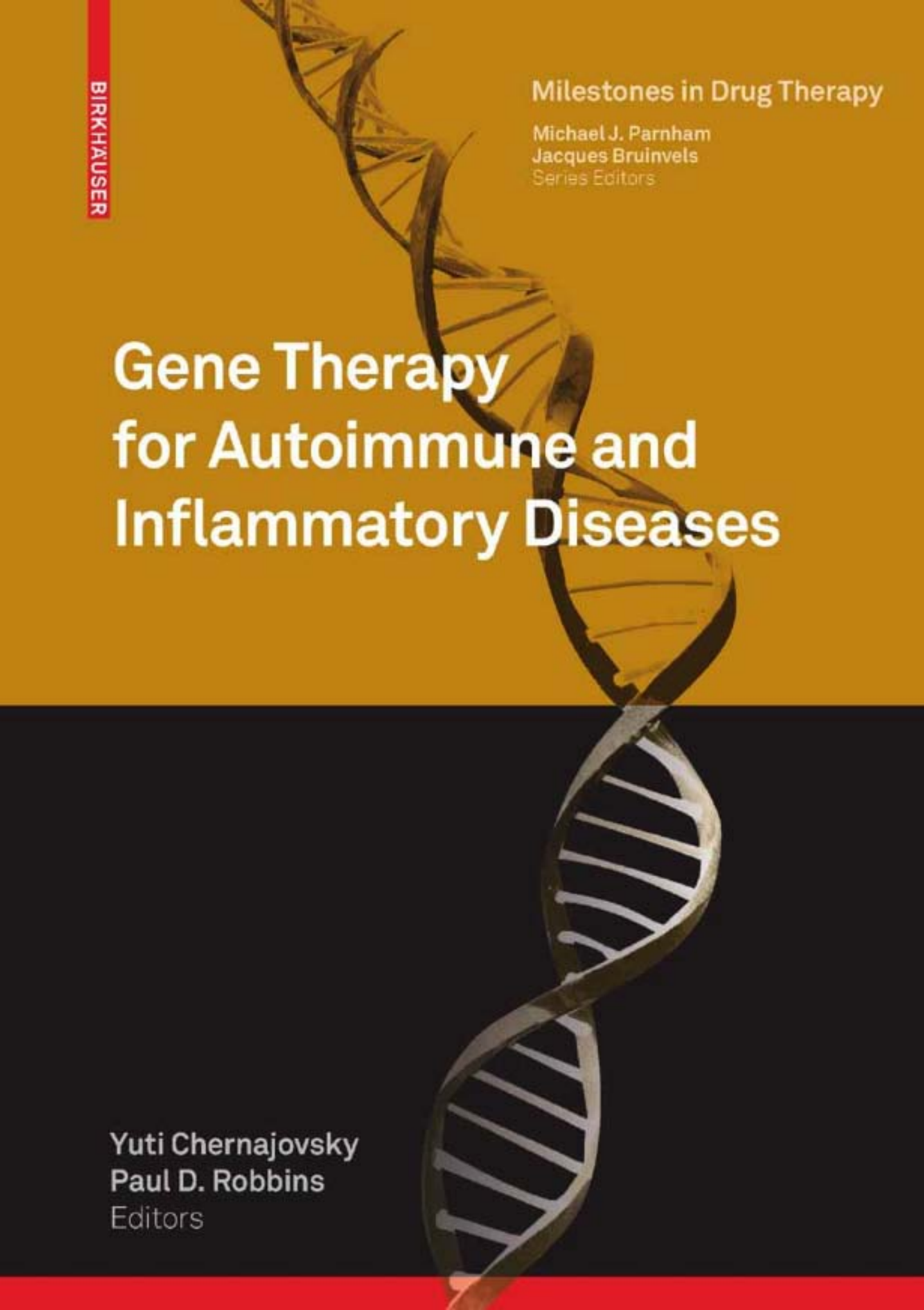
BIRKHÄUSER

Milestones in Drug Therapy

Michael J. Parnham
Jacques Bruinvels
Series Editors

Gene Therapy for Autoimmune and Inflammatory Diseases



Yuti Chernajovsky
Paul D. Robbins
Editors

BIRKHÄUSER

Milestones in Drug Therapy
MDT

Series Editors
Prof. Dr. Michael J. Parnham
Director of Science
MediMlijeko d.o.o.
HR-10000 Zagreb
Croatia

Prof. Dr. J. Bruinvels
Sweelincklaan 75
NL-3723 JC Bilthoven
The Netherlands

Gene Therapy for Autoimmune and Inflammatory Diseases

Edited by Yuti Chernajovsky and Paul D. Robbins

Birkhäuser

Library of Congress Control Number: 2010905304

ISBN: 978-3-0346-0164-1

P.O. Box 133, CH-4010 Basel, Switzerland
Part of Springer Science+Business Media
Printed on acid-free paper produced from chlorine-free pulp. TFC ∞

Printed in Germany
ISBN: 978-3-0346-0164-1 e-ISBN: 978-3-0346-0165-8

9 8 7 6 5 4 3 2 1 www. birkhauser.ch

Contents

List of Contributors

Florence Apparailly, Inserm, U 844, INM, Hôpital Saint Eloi, F-34295 Montpellier, France; e-mail: florence.apparailly@inserm.fr

Juliane Bremer, Institute of Neuropathology, Department of Pathology, University Hospital Zürich, Frauenklinikstrasse 26, 8091 Zürich, Switzerland; e-mail: juliane.bremer@usz.ch

Nicola Brunetti-Pierri, Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, 630E, Houston, TX 77030, USA; e-mail: nicolab@bcm.tmc.edu

Yuti Chernajovsky, Bone and Joint Research Unit, Barts and The London School of Medicine, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK; e-mail: y.chernajovsky@qmul.ac.uk

Anthony T. Cheung, enGene, Inc., 100-2386 East Mall, Vancouver, BC, Canada V6T 1Z3; e-mail: acheung@engeneinc.com

John A. Chiorini, Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, DHHS, Bethesda, MD 20892, USA, e-mail: jchiorin@mail.nih.gov

Christopher H. Evans, Center for Advanced Orthopaedic Studies, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA 02215; e-mail: cevans@bidmc.harvard.edu

Roberto Furlan, Clinical Neuroimmunology and Neuroimmunology Unit, Institute of Experimental Neurology, Department of Neuroscience, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy; e-mail: furlan.roberto@hsr.it

Alberto Gatta, Clinical Neuroimmunology and Neuroimmunology Unit, Institute of Experimental Neurology, Department of Neuroscience, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy; e-mail: a.gatta@studenti.hsr.it

Jeroen Geurts, Rheumatology Research and Advanced Therapeutics, Department of Rheumatology, Radboud University Nijmegen Medical Centre, Nijmegen Centre for Molecular Life Sciences, Nijmegen, 6525 GA, The Netherlands; e-mail: j.geurts@reuma.umcn.nl

Steven C. Ghivizzani, Department of Orthopaedics & Rehabilitation, University of Florida College of Medicine, Gainesville, Florida, USA 32610; e-mail: ghivisc@ortho.ufl.edu

Deborah R. Gill, Gene Medicine Group, Nuffield Department of Clinical Laboratory Sciences, Level 4, Academic block, John Radcliffe Hospital, Oxford OX3 9DU, UK; e-mail: deborah.gill@ndcls.ox.ac.uk

David J. Gould, Queen Mary University of London, Barts & The London School of Medicine and Dentistry, Bone & Joint Research Unit, William Harvey Research Institute, Charterhouse Square, London EC1M 6BQ; e-mail: d.j.gould@qmul.ac.uk

Matthew L. Hirsch, Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA

Stephen C. Hyde, Gene Medicine Group, Nuffield Department of Clinical Laboratory Sciences, Level 4, Academic block, John Radcliffe Hospital, Oxford OX3 9DU, UK; e-mail: steve.hyde@ndcls.ox.ac.uk

Ali S. Jawad, Department of Rheumatology, The Royal London Hospital, London, UK; e-mail: alismjawad1@hotmail.com

Christian Jorgensen, Inserm, U 844, INM, Hôpital Saint Eloi, F-34295 Mont - pellier, France; e-mail: christian.jorgensen@inserm.fr

Hans H. Jung, Department of Neurology, University Hospital Zürich, Frauen-klinikstrasse 26, 8091 Zürich, Switzerland; e-mail: hans.jung@usz.ch

Chengwen Li, Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA; e-mail: chengwen@med.unc.edu

Rizgar A. Mageed, Bone and Joint Research Unit, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK; e-mail: r.a.mageed@qmul.ac.uk

Chiara Maiorino, Clinical Neuroimmunology and Neuroimmunology Unit, Institute of Experimental Neurology, Department of Neuroscience, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy; e-mail: maiorino.chiara@hsr.it

Gianvito Martino, Clinical Neuroimmunology and Neuroimmunology Unit, Institute of Experimental Neurology, Department of Neuroscience, San Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy; e-mail: martino.gianvito@hsr.it

Philip Ng, Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, 630E, Houston, TX 77030, USA; e-mail: png@bcm.tmc.edu

Hirofumi Noguchi, Regenerative Research Islet Cell Transplant Program, Baylor All Saints Medical Center, Baylor Research Institute, 1400 8th Avenue, Fort Worth, TX 76104 USA; e-mail: hirofumn@baylorhealth.edu

Scott E. Plevy, University of North Carolina, Division of Gastroenterology and Hepatology, 103 Mason Farm Road, CB# 7032, 7341CMBRB, Chapel Hill, NC 27599, USA; e-mail: scott_plevy@med.unc.edu

Paul D. Robbins, Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh PA USA

Nienke Roescher, Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, DHHS, Bethesda, MD 20892, USA; e-mail: roeschern@mail.nih.gov

Francesca Ruffini, Clinical Neuroimmunology and Neuroimmunology Unit, Institute of Experimental Neurology, Department of Neuroscience, San

Raffaele Scientific Institute, Via Olgettina 58, 20132 Milan, Italy; e-mail: ruffini.francesca@hsr.it

Melanie A. Ruffner, University of Pittsburgh, Department of Bioengineering, 306 CNBIO, 300 Technology Drive, Pittsburgh, PA 15219, USA; e-mail: ruffner.melanie@medstudent.pitt.edu

R. Jude Samulski, Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA; e-mail: rjs@med.unc.edu

Maria C. Subang, Queen Mary University of London, Barts & The London School of Medicine and Dentistry, Bone & Joint Research Unit, William Harvey Research Institute, Charterhouse Square, London EC1M 6BQ; e-mail: m.c.subang@qmul.ac.uk

Stephanie G. Sumner-Jones, Gene Medicine Group, Nuffield Department of Clinical Laboratory Sciences, Level 4, Academic block, John Radcliffe Hospital, Oxford OX3 9DU, UK; e-mail: stephanie.sumner@ndcls.ox.ac.uk

Taher E. Taher, Bone and Joint Research Unit, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK; e-mail: t.e.i.taher@qmul.ac.uk

Paul P. Tak, Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, 1100 DD, Amsterdam, The Netherlands, e-mail: p.p.tak@amc.uva.nl

Ingo H. Tarner, Dpt. of Internal Medicine and Rheumatology, Justus-Liebig-University Giessen, Divison of Rheumatology, Clinical Immunology, Osteology and Physical Medicine, Kerckhoff-Klinik, Benekestrasse 2–8, 61231 Bad Nauheim, Germany; e-mail: Ingo.H.Tarner@innere.med.uni-giessen.de

Fons A.J. van de Loo, Rheumatology Research and Advanced Therapeutics, Department of Rheumatology, Radboud University Nijmegen Medical Centre, Nijmegen Centre for Molecular Life Sciences, Nijmegen, 6525 GA, The Netherlands; e-mail: a.vandeloo@reuma.umcn.nl

Wim B. van den Berg, Rheumatology Research and Advanced Therapeutics, Department of Rheumatology, Radboud University Nijmegen Medical Centre, Nijmegen Centre for Molecular Life Sciences, Nijmegen, 6525 GA, The Netherlands; e-mail: w.vandenberg@reuma.umcn.nl

Michael Weller, Department of Neurology, University Hospital Zürich, Frau - enklinikstrasse 26, 8091 Zürich, Switzerland; e-mail: michael.weller@usz.ch

Preface

In this monograph about gene therapy of autoimmune and inflammatory disorders we have gathered international experts and leaders from different fields to review the state of the art advances on topics ranging from disease entities to vectors and engineered cells.

The different approaches described in each chapter take into consideration the biomedical knowledge of these diseases and address the complexities of delivering long-term genetic interventions.

Gene therapy also serves as a testing ground for new therapeutic entities and helps provide proof of principle for their potential therapeutic role in animal models of disease. Scaling up from mice to men still remains an important hurdle not only from the quantitative point of view, but also for currently unknown and unexpected secondary effects of the vector or the transgene.

Some of these approaches have already been tested in the clinic, but much more needs to be done to understand the human conditions treated and the natural history of their pathology.

We are indebted to the secretarial assistance of Ms. Lin Wells (Bone and Joint Research Unit, London, UK) and the help of Hans Detlef Klüber for his help in getting this book published. We hope this book will be of interest to clinicians and scientists and inspiring to students of the subject who will use their own ingenuity and knowledge to further forward this discipline into clinical use.

Yuti Chernajovsky
London

Paul D. Robbins
Pittsburgh

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Gene therapy for arthritis

Florence Apparailly[1, 2, 3], Paul Peter Tak[4] and Christian Jorgensen[1, 2, 3]

[1] *Inserm, U 844, INM, Hôpital Saint Eloi, Montpellier, France*
[2] *Université Montpellier 1, UFR de Médecine, Montpellier, France*
[3] *CHU Lapeyronie, service Immuno-Rhumatologie, Montpellier, France*
[4] *Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

Abstract

Gene therapy has a potential for effective therapeutic intervention in rheumatoid arthritis (RA). Proof of concept has been demonstrated in animal models, either through local gene delivery to the joint space or through systemic gene delivery for immune intervention. This chapter reviews how certain clinical applications of gene therapy would be beneficial for RA patients and discusses the roadblocks that need to be solved for future clinical applications. Issues concerning vectors, promoters and the therapeutic genes are discussed and ideal features for safe, non toxic, efficient and regulated gene expression proposed. Finally, the results from preclinical studies and six clinical trials performed in RA patients are described.

Why gene therapy in arthritis?

During the last decade, development of targeted biotherapies, together with an improved timing and dosing of conventional therapy, have largely improved the outcome of established arthritis in many patients, but not all and still pose significant problems [1]. Current biotherapies target pro-inflammatory cytokines (for instance IL-6, TNF-α), co-stimulatory signals (CTLA4-Ig) or B (anti-CD20) lymphocytes [2] and are usually delivered in association with methotrexate (MTX). Presently, TNF-blocking agents are highly effective but still insufficient since partial remission can be reached in 60–70% of the RA patients treated, with only 20% improvement achieved, and the majority of these responders still have some actively inflamed joints [3, 4]. One major problem of the treatment with biologics is the need for ongoing therapy and flare when therapy is withdrawn. A growing body of evidence shows that these approaches are limited by loss of efficacy along the treatment, frequent relapses, and important adverse effects associated with the high systemic dosage of immune modulators needed to achieve therapeutic levels in the joints, including increased risk of serious infections, uncontrolled fevers and increased risk of malignancies or heart failure that can be life-threatening [5, 6]. A significant number of patients still do not respond at all to available biotherapies.

Thus, inconsistent and partial efficiency of current biotherapies support the need for the development of new therapeutic approaches that induce a prolonged remission with limited side effects, and poses great challenges to gene therapists [7].

A huge amount of work has investigated gene therapy in RA in the past decade, focusing on vector, promoter or target genes, the majority of which have been designed to test proof-of-principle in animal models of arthritis, and results convincingly support the fact that gene therapy can be an advantageous strategy in the treatment of inflammatory and destructive RA. Indeed, a gene therapy approach for the treatment of RA holds promise for joint specific targeting and long-term expression of anti-arthritic drugs, replacing the frequent administration of recombinant proteins [8]. Gene therapy is defined as an introduction of nucleic acids into a host cell for therapeutic purposes. It has thus the potential to stably deliver a gene product or multiple products in a target-specific and controllable manner. Since RA is publicly perceived as a non-life-threatening disease, specific aims for gene therapy in RA are requested, including the design of safe, efficient, long-life vectors, with minimal adverse effects. Indeed, all these prerequisites depend on multiple factors, including the mode of administration, the vector used, the therapeutic gene and the promoter encoded, as well as the immune status of the host. Reviewing the incredibly large amount of studies in the RA gene therapy field, we will try to delineate strategies that will be useful for clinical use in the near future.

Ex vivo strategy

This approach consists of engineering genetically modified cells *in vitro* that are subsequently injected directly into the affected joint. This procedure allows 1) controlling the quality of the injected material, especially when using a vector that might cause insertional mutagenesis, 2) sorting the transduced cells to be injected back, and 3) knowing precisely the level of transgene expression, in order to adapt the number of genetically modified cells to be injected to the clinically relevant level of transgene desired. Such approach has been achieved using genetically modified fibroblasts as well as immune cells, such as fibroblast-like synoviocytes, myoblasts, mesenchymal stromal cells, T cells and dendritic cells [9–14]. However, this procedure is laborious, costly and time consuming.

In vivo strategy

Although RA is a disorder affecting more than one joint and has systemic manifestations targeting other organs, among the possible routes, intra-articular administration is up-to-now the first most-favoured choice among gene therapy-based approaches for clear safety concerns. Indeed, local delivery of gene

therapy compounds is a promising approach to treat RA, with several potential advantages over systemic routes of therapy since the therapeutic agent is 1) directly injected within the affected joint, providing an easy targeting of the diseased tissue, 2) endogenously synthesised by the arthritic joint, allowing sustained suppression of inflammation while minimising dosage, and thus limiting systemic side effects that are associated with high and repeated dosages of the systemic approaches, and 3) easily accessible for injection, biopsy and even synovectomy, since joint space is routinely accessed in clinical practice for infiltration and tissue collection, and ultimately for orthopaedic surgery. The *in vivo* approach consists of the direct intra-articular injection of the genetic material. Because of the easy access to the joint space, local gene delivery has been achieved with a large panel of viral and non-viral vectors, mainly to target the synovium [15]. The obvious advantage is to obtain in one injection the production of the therapeutic agent directly by resident cells, with minimal manipulation of the targeted cells and spreading of the vector. It is a very simple medical act that does not involve heavy surgical procedures or anaesthesia and also allows treatment of as many joints as necessary. Independently of the vector and transgene used, its efficiency has been proved by many groups, in several different animal models of RA. Moreover, few groups reported the observation of a contra-lateral effect, i.e., an amelioration of the clinical features in the non-injected contra-lateral joint [16–18]. Although the cellular and molecular mechanisms underlying such an effect have not been completely unravelled, it might represent a way to treat several joints by injecting only once. For initial clinical trials, local therapy will be the reasonable approach to be followed, regarding the higher risk of systemic therapy. However, preclinical monitoring of the vector spreading and the immune response induction against the vector following intra-articular administration showed that the technique is not devoid of some breaches. These are major concerns that will need a close follow-up to avoid any setbacks for future application of gene therapy strategies. The ultimate goal of gene therapy will be the injection of a vector that targets a specific cell type.

Vectors for gene transfer in arthritis

One way to obtain a fair level of targeting is to use vectors that display restricted or specific tropisms. For gene transfer-based approaches, the main obstacle is indeed the choice of vector. Various gene transfer approaches have been explored to determine the most efficient for RA and are divided into two categories: viral and non-viral vectors.

Non-viral gene transfer methods used nucleic acids associated either with chemical (liposomes, polymeres) or mechanical (gene gun) means to increase transduction efficiency. One alternative to enhance nucleic acids' entrance into targeted tissue has been the use of *in vivo* electrotransfer (ET) [19, 20]. It is based on the same principle as for *in vitro* electroporation, i.e., applying

an electric field on each side of the target organ following nucleic acid injection (plasmid DNA, siRNA, ODN), and optimising parameters to each organ's characteristics. This technique has been validated for the muscle as systemic approach [21–25] and for the joint as local approach [26–28] overexpressing antagonists of pro-inflammatory agents or anti-inflammatory molecules. In a comparative study, direct gene transfer into arthritic joints using ET was less efficient to treat collagen-induced arthritis (CIA) in mice than intra-muscular ET [29]. In general, the major drawback of the ET approach is that the transgene expression is mostly silenced after one week and has to be performed under anaesthesia. Recent data have been obtained using hemocyanin-human TNF heterocomplex (hTNF kinoid) to induce an active anti-TNF immunotherapy [30, 31]. They showed that vaccination with the TNF kinoid provides the sustain expression of endogenously produced neutralising anti-hTNF antibodies, protecting hTNF transgenic mice against severe arthritis due to chronic TNF overproduction. The potential of the artificial chromosome expression system has also been explored for the treatment of RA [32]. Finally, conjugation with macromolecules such as cholesterol or PEG, or liposome formulations have been used to protect from enzymatic degradation, renal or macrophage-mediated clearance, and thus to enhance gene transfert efficacy. Our group has developed a synthetic vector based on the cationic liposome RPR209120/DOPE [33] to formulate therapeutic nucleic acids (plasmid DNA or siRNA) and demonstrated the potential of such anti-cytokine lipoplexes in the mouse CIA model following intravenous injection. Targeting TNF, IL-1, IL-6 or IL-18 using siRNA lipoplexes was very efficient in decreasing severity of established arthritis, both as individual and combined strategies [34, 35]. There are data suggesting that liposomes can be endocytosed by systemic monocytes entering the joints, and by joint macrophages [36]. Although liposome-based formulations could be envisaged for systemic non-viral anti-cytokine strategy in RA, pharmaco-toxicological studies need to be performed before planning any clinical trials. This strategy can also present a valuable tool for *in vivo* screening of new potential therapeutic targets. In conclusion, non-viral vectors allow local and systemic intervention, but their efficiency is reduced compared to viral vectors due to drug bioavailability issues, and most of the formulations tested are themselves inflammatory.

Concerning the viral strategy, several vectors are available to the scientists and the use of the vector of choice will most likely depend on its application. Most of the retroviral (RV) vectors used in clinical gene therapy trials were derived from the very well studied and characterised Moloney murine leukaemia oncoretrovirus (MMLV) [37]. They are small RNA viruses that replicate through a DNA intermediate and require cell division for infection and integration [38, 39]. Thus, they have only been used in *ex vivo* gene transfer approaches in which isolated cells can be propagated in culture, genetically modified after RV infection, and then implanted back into a recipient patient. Although synovium of inflamed joints produced higher RV-mediated

transduction efficiency than normal joints, human rheumatoid synovium does not contain enough proliferating cells to support efficient retroviral transduction. Direct injection of a RV vector encoding β-galactosidase into the engrafted human synovium of SCID mice resulted in less than 1% of transduced cells. *Ex vivo* infection could however reach 35% of synoviocytes in the presence of TNFα [40] or 50% when concentrating RV supernatants [41]. Few groups have also explored the possibility to use LV vectors from several species [42, 43] and showed that they transduce efficiently the non-dividing rheumatoid synovium, resulting in some clinical improvement in experimental arthritis models. However, the same potential problem of insertional mutagenesis exists as for the entire family of RV, their low titer of production is still an important technical limitation, and they are ethically very problematic for *in vivo* use. Local *in vivo* gene transfer has been achieved with recombinant AdV (adenoviral vectors), AAV (adeno-associated virus), HSV (Herpes simplex virus) and non-viral vectors using various animal models of RA. Since RA is a chronic inflammatory disorder, highly immunogenic vectors such as AdV vectors have been eliminated from the panel. Although the serotype 5 transduces very efficiently the rheumatoid synovium, allowing the rapid expression of therapeutic genes to high levels, AdV are highly immunogenic and thus only useful for transient transgene expression. To circumvent this main limitation, a few groups explored two tracks: the use of non-human AdV, such as canine AdV vectors [44], or the use of AdV from the 51 different human serotypes for which there is very few or no occurrence of a pre-existing humoural response in the human population [45]. Alternatively, targeted gene transfer to human synovium *ex vivo* explants and mouse CIA *in vivo* was efficiently increased with fiber-modified AdV [46]. Despite these interesting strategies, AdV are definitively not the gold standard approach for gene transfer in RA, but still very interesting experimental tools for proof-of-concept studies. Although interesting data have been generated using HSV for *in vivo* transfer of anti-arthritic genes to joints [47–49], they remain poorly studied in experimental models for RA. Indeed, these vectors showed high infectivity of joint tissues, can be produced at high titers and have a large packaging capacity, allowing the inclusion of multiple anti-arthritic genes. Few groups have also attempted to develop hybrid vectors that would represent the synthesis of positive features for each vector, but main obstacles remain the achievement of high titer viral batches. Finally, the capacity of 'gut-less' vectors has been evaluated *in vitro* on human RA-FLS, but nothing really came up to the *in vivo* validation, although they are less immunogenic.

One of the most promising viral vectors for human gene therapy that emerged over the last decade is the AAV. The biology of these parvoviruses, non-pathogenic and non-toxic in humans, has been extensively investigated, and methods of production, purification and titration for their clinical use highly improved their intrinsic limitations [50]. Recombinant (r)AAV vectors have been shown to direct efficient, prolonged and safe transgene expression in several tissues, with distinct tropism for each serotypes [51]. Cross-packaging of

serotypes allowed the transduction of a large panel of tissues and cell types [52, 53]. Moreover, physicochemical stability of rAAV facilitated storage and administration in the clinic. Currently, rAAV2 are used in a number of gene therapy clinical trials in haemophilia B, leucodystrophy, cystis fibrosis, LDL deficiency and RA [54–58]. First interest came from the capacity of the wild type vector to infect both dividing and non-dividing cells, as well as to stably integrate into a site-specific locus (q13.3) on chromosome 19 (AAVS1), conferring theoretical long-term and safe transgene expression. This property is lost when using rAAV and most of the transgene expression comes from episomal forms [59]. *In vitro*, most of the cell types found in the RA joints can be transduced by rAAV2, including RA-FLS, chondrocytes and macrophages [60–63]. Few studies used systemic injection of the rAAV vector, either intramuscularly [64, 65] or peri-articularly [66], and showed high and therapeutically efficient transgene expression, detectable in sera for at least 4 months. The feasibility of direct intra-articular gene transfer to rat and mouse arthritic joints has been well demonstrated [67–69]. Pattern of expression for AAV2 following local injection has been variable according to studies, from synovial lining cells [70], to synoviocytes and chondrocytes [61], muscle and synoviocytes [68, 71]. More importantly, in rat and mouse models of arthritis, the serotype 5 capsid mediated rapid, high and stable gene transfer of a reporter gene into joints injected [72, 73]. Transgene expression was already detectable 7 days after injection and lasted for at least 4 weeks in the AIA rat model [72], and at least 19 weeks in the mouse CIA model. The group of S. Ghivizzani reported that the rheumatic joints of horses were also efficiently transduced using rAAV5 [74]. Using two different TNF blocking agents (the TNFR1-mIgG1 fusion protein or a dimeric sTNFR2) as a proof of concept, two studies have demonstrated the feasible of rAAV5-mediated gene therapy in mouse CIA and rat AIA models of arthritis. When the anti-TNF molecule expression is under a strong constitutive promoter (CMV), the antagonist molecule was rapidly (within 2 weeks), highly and stably expressed for 9 weeks when delivered intra-articularly by a CMV-driven rAAV5 vector. This was associated with a decrease in arthritis incidence and severity in both animal models. More importantly, when the transgene was expressed under a NF-κB-responsive promoter inducible by inflammation; the clinical effect was associated with a transient expression of the anti-TNF molecule, only detectable during disease flares. These results suggest that the local rAAV5-mediated gene delivery of a disease-inducible therapeutic agent may be a key for successful treatment of RA by gene therapy [75, 76].

In conclusion, we are close to the ideal vector for *in vivo* delivery with the potential to confer high level, safe and persistent gene delivery. More recently, the rational design of AAV capsid mutants, and strategies such as the use of self-complementary vector genomes [77–79], has even more increased the potential of using AAVs for RA gene therapy.

The target genes

Among potential therapeutic genes for gene therapy in RA, several molecules have been validated in various animal models, and the already existing huge list of possible mediators or pathways to either over-express or inhibit is constantly growing [80–82]. Locally, the inflamed RA joint is highly infiltrated by immune cells such as T and B lymphocytes, and macrophages [83]. Furthermore, the numbers of effector cells like macrophages and fibroblast-like synoviocytes increase and the synovial membrane becomes hyperplastic, forming a tumour-like aggressive tissue called pannus, which invades cartilage and bone structures, leading to destruction of affected joints. Synovial fibroblasts play a central role in the chronic aspect of RA and are certainly the key cells to target since they integrate inflammatory signals in articular damage and produced themselves many of the detrimental pro-inflammatory and MMPs [84]. These processes are mediated by a number of cytokines (TNF-α, IL-1β, IL-6, IL-17, IFN-γ, etc.), chemokines (MCP-1, MCP-4, CCL18, etc.), cell adhesion molecules (ICAM-1, VCAM-1, etc.) and matrix metalloproteinases (MMP-3, -9, -13, etc.). There are pros and cons for the use of biologics targeting one or the other molecule [85] and current treatment strategies targeted against TNF-α, IL-1β and IL-6 are available for clinical practice [86]. Among them, the cytokines are certainly the most famous and oldest ones. Indeed, the key role of inflammation in RA prompted the first proof-of-concept studies for gene therapy approaches in RA to target TNF-α and IL-1β respectively in various animal models [16, 87–89]. There are many studies validating the well-established roles for inflammatory cytokines and MMPs in experimental arthritis, but due to their orchestrated cross talk [84], identification of other key molecules or regulators is critical to the development of new and more effective anti-arthritic drugs. Except from restoring the pro/anti-inflammatory cytokine balance, alternative strategies explored have been genetic synovectomy [90], targeting signaling pathways, angiogenesis inhibition, interfering with the extracellular matrix degradation process, or all together by targeting molecules that are key regulators with multipotent effects.

Specific promotion of synovial apoptosis has been explored by local delivery of AdV vector encoding TRAIL (TNF-related apoptosis-inducing ligand) [91], the tumour suppressor protein p53 [92], FADD (Fas-associated death domain protein) [93], PTEN, a negative regulator of PI 3-kinase/Akt signalling pathway involved in cell growth, proliferation and survival [94], or the TK (HSV thymidine kinase gene) in rhesus monkeys with CIA [95, 96]. All these approaches showed amelioration of arthritis symptoms associated with increased apoptosis in the synovium, decreased inflammatory cell infiltration, and stimulated new matrix synthesis by cartilage.

Angiogenesis is necessary for the development and maintenance of the pannus and the recruitment of infiltrating inflammatory cells associated with RA pathogenesis [97]. Local and systemic proof of concept studies have been successfully performed in experimental models of RA to validate the inhibition of

new synovial blood vessels formation by targeting the Tie2 function [98], the αvβ3 and αvβ5 integrins [99], the VEGF receptor [100], angiostatine [101], thrombospondin 1 and 2 [102, 103], and inhibitor of the urokinase plasminogen activator [104].

Matrix metalloproteinases (MMPs) are a major group of endopeptidases that resorb macromolecules of the extracellular matrix. They are essential for remodelling and wound repair in normal tissue as well as in pathological processes such as cartilage degradation and invasiveness of RA synovial fibroblasts observed in RA. Within rheumatoid joints, MMPs and their endogenous inhibitors TIMPs are produced by both macrophages and synoviocytes. In addition to their key role in matrix destruction, MMPs catalyse the cleavage of the transmembrane form of TNF, and thus play an important role in balancing inflammation. Since there is a broad-spectrum of synthetic inhibitors available, there are only few studies using gene therapy approaches to interfere with MMP activity. Retroviral gene transfer of ribozymes targeting MMP-1 [105] or antisense RNA targeting MT1-MMP [106] significantly reduced the invasiveness of RA synovium into the cartilage coimplanted in SCID mice [107]. AdV-mediated over-expression of TIMP-3 markedly reduced the invasiveness of RA synovium in the SCID mouse model [108] while MMPs inhibition through TIMP-1 gene transfer was not so effective [108, 109].

Another option is to target transcription factors that orchestrate many critical molecules in RA. One of the most widely studied is NF-κB, an ubiquitous and well-characterised protein responsible for the regulation of complex phenomena, with a pivotal role in controlling cell signalling under physiological and pathological conditions [110]. Among other functions, NF-κB is a critical element in the cross-talk between bone and the immune system [111], thus controlling many pathological processes implicated in RA through the expression of genes encoding the pro-inflammatory cytokines (e.g., IL-1, IL-2, IL-6, TNF-α, etc.), chemokines (e.g., IL-8, MIP-1α, MCP1, RANTES, eotaxin, etc.), adhesion molecules (e.g., ICAM, VCAM, E-selectin), inducible enzymes (COX-2 and iNOS), growth factors, some of the acute phase proteins, and immune receptors. Many gene therapy-based studies have demonstrated the relevance of targeting NF-κB as potential therapeutic approach in RA [69, 110, 112–114]. Indeed, inhibition of NF-κB was shown to be effective in reducing both inflammation and bone destruction in animal models of arthritis, through the alteration of osteoclastogenesis [115], synovial apoptosis [69], pro-inflammatory cytokines [116–118] and chondrocyte functions [119]. Another transcription factor, the activator protein 1 (AP-1) (Fos/Jun), was shown to be a potential target as synthetic inhibitor specifically decreased both downstream pro-inflammatory cytokines and MMPs production [120, 121].

An alternative approach would be to use vectors encoding anti-inflammatory cytokines that inhibit the production of pro-inflammatory cytokines. Anti-inflammatory cytokines showing a therapeutic effect in experimental arthritis include interferon-β (IFN-β), IL-4, IL-10 and IL-13. IFN-β reduces the secre-

tion of mediators of inflammation and destruction like IL-6, TNF-α, MMPs and prostaglandin E2, which are key players in the pathogenesis of RA [122]. IFN-β has in addition to anti-inflammatory properties an important role in bone homeostasis. Furthermore, IFN-β has anti-angiogenic properties, which could boost a therapeutic effect in RA. It has been shown that continuous IFN-β treatment, using daily IFN-β protein injections [122, 123] or *ex vivo* transduced cells secreting IFN-β [124] is very effective in CIA in both mice and Rhesus monkeys. Intra-articular IFN-β gene therapy has been tested in a number of preclinical studies in two different rat models of arthritis using either an AdV or an AAV5 vector. Local delivery of Ad or rAAV5 vectors expressing rat IFN-β after the onset of disease reduced paw swelling in both the treated and untreated, contralateral joint [125, 126]. Moreover, IFN-β treatment protected against joint destruction, which is a hallmark of RA. These results provide a strong rationale for IFN-β gene therapy as a novel therapeutic approach for arthritis.

Many other genes could be targeted. Among the most promising ones synoviolin, an E3 ubiquitin ligase, over-expressed in macrophages and synoviocytes under inflammation; TAK1 (Transforming growth factor-β activated kinase 1), a downstream mediator of IL-1, TNF and MMPs signal pathways, playing a central role in the regulation of catabolic events and inflammatory processes in RA; RANKL, a member of the TNF superfamily expressed by activated T cells and synovial fibroblasts, and playing a pivotal role in osteoclast differentiation [84]; and the adhesion molecule cadherin-11 that plays a key role in the formation of the hyperplastic synovial cells and in the determination of their behaviour in the RA pro-inflammatory and destructive tissue responses [127–129].

In conclusion, the future for gene therapy-based treatment of RA would be a personalised combination of transgenes targeting several pathways that dominate in the synovium of a particular recipient. This would most likely leads to an optimised disease treatment, allowing the physician to avoid picking the best treatment by trial and error.

Different promoter to provide safe and targeted gene expression

The control of transgene expression is optimal to prevent toxicity linked to the transgene and to ensure biological activity within a narrow therapeutic window. The ideal goal is to obtain a ligand-induced gene expression that is dose-dependent, specific, efficient, quickly on/off, and insignificant in basal conditions. It also implies that the regulated system relies on a ligand that is non-toxic and lipophilic, has a short half-life, is non-immunogenic by itself, and does not disturb the target tissue physiology. Many types of promoters can be used to achieve such a goal. Viral promoters such as CMV are strong promoters that might be silenced over time because of cellular toxicity triggered by high levels of transgene expression. Although cell-specific promoters restrict transgene

expression in the tissue of interest, they often provide weak levels of transgene expression. There are many natural promoters issued from mammals regulatory elements (steroid hormone responsive elements, acute-phase protein promoter, metallothionein promoter, heat shock promoters or interferon-inducible promoters) (for review [130]). They have the advantage to be activated by endogenous ligands that are non-toxic and immunologically inert; however they do not allow tight control of the therapeutic gene. Indeed, a basal level of inducer molecules will always be present in the body and may vary according to time of the day, patient's activity and health, providing a basal leakiness of transgene expression. Use of exogenous transactivator doses might induce toxicity and pleiotropic effects with undesired consequences and absence of selectivity. More importantly, the endogenous expression of the inducer prevents from stopping transgene expression in case of threat. Chimeric systems have been engineered to circumvent side effects. They are based on insect and mammalian nuclear hormone receptors and use either ecdysone or RU486 as inducer, on bacteria operon regulatory systems and use IPTG or tetracycline as inducers, or on chemically-induced human protein heterodimerisation using rapamycin as inducer. The use of disease-regulated promoters to drive restricted and fine-tuned transgene expression should indeed provide therapeutic levels of the molecule during flares, that are produced to avoid toxicity (excess) and inadequate production (no effect). One study tried to identify a promoter that is naturally activated by inflammation; several candidates were identified and the most interesting data were obtained using an hybrid interleukin-1 enhancer/interleukin-6 proximal promoter allowing achievement of efficacious local IL-4-based gene therapy under arthritic conditions [131]. Other groups successfully explored the use of an NF-κB-based promoter to drive transgene expression according to the level of joint inflammation [75, 76]. More recently, a COX-2 promoter element was used to efficiently drive *in vitro* the IL-4 transgene expression in transiently transfected articular chondrocytes only in response to inflammatory mediators (IL-1β and TNF-α) [132].

Finally, endogenous microRNA can be exploited to regulate transgene expression according to tissue and lineage. Indeed, sophisticated control of transgene expression was obtained with vectors that contain miRNA target sites [133]. Such vectors were shown to rapidly adjust transgene expression in response to changes in a specific miRNA expression, to sharply segregate transgene expression to a specific cell type through vector silencing in miRNA-expressing cells, without perturbing endogenous miRNA expression or regulation of natural targets. In conclusion, miRNA-regulated vectors represent an option for safer and more effective therapeutic applications.

Gene therapy just starts to reach a realistic clinical stage. Since it is definitively the only approach on the long run to solve the problem of long-term expression of the therapeutic genes, the improvement of such strategies largely depends on the generation of new hybrid vector systems allowing ideally cell-specific, long-term and controllable gene expression.

What have we learnt from preclinical studies?

Recent data showed that a modified capsid from the serotype 2, containing five amino acids from the serotype 1 capsid, was able to transduce primary synoviocytes and chondrocytes as efficiently as serotype 5 (poster presentation from Samulski et al. at the 5th GTARD, Seattle, US). This is a new interesting track that still requires to be investigated in term of immunogenicity, as for rAAV5. Although long-term expression can be achieved using rAAV5, the pre-existing immunity to wild type viruses remains an issue to be investigated in more detail. Indeed, recent findings in a clinical trial in which an AAV vector expressing coagulation factor IX was introduced into the liver of hemophilia B subjects highlighted a new issue previously not identified in animal studies, i.e., a capsid-specific immune responses in humans [134], underscoring the necessity to monitor these responses in future clinical trials.

Clinical experience of gene therapy in RA

Several clinical trials involving gene therapy in RA have been conducted around the world. The first trial has focused on safety and feasibility. This Phase I protocol conducted at the university of Pittsburgh was designed as an *ex vivo* strategy. Using gene transfer of a retrovirus encoding the IL-1 receptor antagonist (IL-1Ra) into autologous RA synovial fibroblasts (RASF), it was demonstrated that the therapeutic gene could be efficiently transferred in the joints of RA patients and successfully expressed [14]. After implantation of transduced RASF (10^6–10^7 cells/joint), the joints were removed for implant replacement 1 week later, justified by the advanced disease of selected patients. The injected cells were found to be localised at the lining layer of the synovium, as cell clusters expressing large amounts of IL-1Ra. Another potential concern in this study came from an adjacent control joint of one patient that received the highest number of transgenic cells (10^7 cells) and gave positive RT-PCR signal for IL-1Ra expression. It could be due to cross contamination at the time of injection or at the time of surgical resection, or due to migration of cells from the injected joint to other inflamed joints. In future trials, it would be optimal to insert a suicide gene to provide an additional level of safety. Unfortunately, none of the Phase I trials using such *ex vivo* approach were pursued to Phase II studies to establish efficacy, due to the inherent heavy logistic and practical limitations, as well as financial issues.

Three other Phase I and II clinical trials using non-viral gene therapy approaches were approved by FDA. They all aimed at evaluating the safety of the injection of non-viral vectors, i.e., a plasmid encoding the thymidine kinase of the herpes simplex virus injected intra-articularly (university of Michigan), an antisense RNA targeting the TNF after intravenous or subcutaneous delivery (Isis Pharmaceuticals, Carlsbad, US), and a NF-κB decoy oligodeoxynucleotide administered intra-articularly (Osaka university, Japan).

After treating 10 patients with no major improvement of the clinical end points, the last trial will explore the opportunity to increase doses of the NF-κB decoy ODN and insert chemical modifications for stabilisation.

The most recent gene therapy-based trial in RA was conducted by Targeted Genetics Corporation, a Seattle-based biotechnology company that is developing gene therapy products for the treatment of acquired and inherited diseases. The RA-specific gene therapy product is based on a recombinant serotype 2 adeno-associated virus (AAV2) derived vector that encodes a soluble form of the TNF receptor (TNFR: Fc) to block a key mediator of inflammation (tgACC94). The Phase I/II study was initiated in 2005 and designed to assess the safety and potential efficacy of different doses (10^{11}–10^{13} DRP/mL) of tgAAC94 administered directly to affected joints of subjects who have one or few inflamed joints and are not candidates for systemic TNF-antagonist therapy, or as a supplemental therapeutic for patients who do not fully respond to systemic anti-TNF biotherapy. By injecting the product directly into the joint, the hope has been to avoid the risks associated with systemic anti-TNF therapy, chiefly increased vulnerability to infection. The 127 subjects enrolled have received an initial dose of active drug or placebo into the knee, ankle, wrist, metacarpophalangeal or elbow, and 74 subjects out of the total 127 have received a second dose of active drug. Of those 74 subjects, 52 have received two doses of active drug. The first results of the dose-escalation study, 15 subjects with inflammatory arthritis received a single intra-articular injection of rAAV2-TNFR:Fc at 10^{10} particles per mL joint volume (n = 5) or 10^{11} (n = 6) or placebo (n = 4) into a knee (n = 14) or ankle (n = 1). Intra-articular injections of rAAV2-TNFR:Fc were well-tolerated with no major safety issues. Twelve weeks after injection, a 2-point decrease in swelling was noted in 2/11 subjects injected with rAAV2-TNFR:Fc, despite the TNFR:Fc protein was not detected Synovial fluid at the doses used [135]. The interim data indicated that tgAAC94 is well-tolerated for doses up to 5×10^{13} DRP. The most common adverse events noted were injection site reactions, seen in 10% of patients treated. However, two patients developed serious adverse events, and Jolee Mohr, a 36-year old woman, died in July 2007, few weeks after having the experimental drug injected into her right knee [136]. Autopsy findings and genomic analyses showed that death was a result of complications from an opportunistic infection, histoplasmosis dissemination, complicated by major internal bleeding, related to simultaneous adalimumab therapy and not related to the AAV injection. Although there were insufficient data available to determine if the patient may have had an immune response to the local injection of tgAAC94, no vector spreading could be detected outside the injected knee (<30 copies/microgram tissue). Independently of the gene therapy, Mohr was taking anti-TNFα for her arthritis, which increases the risk for this type of infection, and the contribution of tgAAC94 injections to the infection was considered as non significant, leading The US Food and Drug Administration to deliver administrative authorisation to pursue the trial.

Conclusion

Gene therapy holds great promise for the treatment of a wide variety of diseases, and in particular refractory RA. However, it is currently still early clinical development, and the FDA has not yet approved any human gene therapy product for commercial purpose. Six gene therapy trials in RA have been initiated since 1996 validating the concept. Knowledge from other gene therapy trials in unrelated disorders and from upstream research in animal models showed that intra-articular rAAV-mediated gene therapy combined with an inflammation-inducible promoter is the most promising approach, but it is important to put more efforts in safety studies on vector shedding, biodistribution and pharmacokinetics. Going forward, a very important aspect that is true for many AAV trials will be the need for immune suppression to prevent capside-specific immune-mediated responses.

References

1 Gabriel SE (2008) Tumor necrosis factor inhibition: A part of the solution or a part of the problem of heart failure in rheumatoid arthritis? *Arthritis Rheum* 58: 637–640
2 Apparailly F, Plence P, Noel D et al. (2004) Recent advances for cell/gene therapy in rheumatoid arthritis. *Curr Med Chem* 3: 119–138
3 Feldmann M, Maini SR (2008) Role of cytokines in rheumatoid arthritis: an education in pathophysiology and therapeutics. *Immunol Rev* 223: 7–19
4 Ghayur T, Banerjee S, Hugunin M et al. (1997) Caspase-1 processes IFN-gamma-inducing factor and regulates LPS-induced IFN-gamma production. *Nature* 386: 619–623
5 Bongartz T, Sutton AJ, Sweeting MJ et al. (2006) Anti-TNF antibody therapy in rheumatoid arthritis and the risk of serious infections and malignancies: systematic review and meta-analysis of rare harmful effects in randomized controlled trials. *Jama* 295: 2275–2285
6 Braun J, Kalden JR (2009) Biologics in the treatment of rheumatoid arthritis and ankylosing spondylitis. *Clin Exp Rheumatol* 27: S164–S167
7 Chernajovsky Y (2005) Gene therapy for arthritis – where do we stand? *Arthritis Res Ther* 7: 227–229
8 Vervoordeldonk MJ, Aalbers CJ, Tak PP (2008) Advances in local targeted gene therapy for arthritis: towards clinical reality. *Future Rheumatol* 3: 307–309
9 Bessis N, Chiocchia G, Kollias G et al. (1998) Modulation of proinflammatory cytokine production in tumour necrosis factor-alpha (TNF-alpha)-transgenic mice by treatment with cells engineered to secrete IL-4, IL-10 or IL-13. *Clin Exp Immunol* 111: 391–396
10 Kim SH, Kim S, Evans CH et al. (2001) Effective treatment of established murine collagen-induced arthritis by systemic administration of dendritic cells genetically modified to express IL-4. *J Immunol* 166: 3499–3505
11 Kim SH, Kim S, Oligino TJ et al. (2002) Effective treatment of established mouse collagen-induced arthritis by systemic administration of dendritic cells genetically modified to express FasL. *Mol Ther* 6: 584–590
12 Morita Y, Yang J, Gupta R et al. (2001) Dendritic cells genetically engineered to express IL-4 inhibit murine collagen-induced arthritis. *J Clin Invest* 107: 1275–1284
13 Annenkov A, Chernajovsky Y (2000) Engineering mouse T lymphocytes specific to type II collagen by transduction with a chimeric receptor consisting of a single chain Fv and TCR zeta. *Gene Ther* 7: 714–722
14 Evans CH, Robbins PD, Ghivizzani SC et al. (2005) Gene transfer to human joints: progress toward a gene therapy of arthritis. *Proc Natl Acad Sci U S A* 102: 8698–8703
15 Evans CH, Ghivizzani SC, Robbins PD (2006) Gene therapy for arthritis: what next? *Arthritis Rheum* 54: 1714–1729

16 Ghivizzani SC, Lechman ER, Kang R et al. (1998) Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to rabbit knees with experimental arthritis has local and distal anti-arthritic effects. *Proc Natl Acad Sci U S A* 95: 4613–4618
17 Kim SH, Lechman ER, Kim S et al. (2002) *Ex vivo* gene delivery of IL-1Ra and soluble TNF receptor confers a distal synergistic therapeutic effect in antigen-induced arthritis. *Mol Ther* 6: 591–600
18 Lechman ER, Keravala A, Nash J et al. (2003) The contralateral effect conferred by intra-articular adenovirus-mediated gene transfer of viral IL-10 is specific to the immunizing antigen. *Gene Ther* 10: 2029–2035
19 Mir LM (2000) Therapeutic perspectives of *in vivo* cell electropoermeabilization. *Bioelectrochemistry* 53: 1–10
20 Mir LM, Bureau MF, Gehl J et al. (1999) High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc Natl Acad Sci U S A* 96: 4262–4267
21 Bloquel C, Bessis N, Boissier MC et al. (2004) Gene therapy of collagen-induced arthritis by electrotransfer of human tumor necrosis factor-alpha soluble receptor I variants. *Hum Gene Ther* 15: 189–201
22 Bloquel C, Denys A, Boissier MC et al. (2007) Intra articular electrotransfer of plasmid encoding soluble TNF receptor variants in normal and arthritic mice. *J Gene Med* 9: 986–993
23 Kim JM, Ho SH, Hahn W et al. (2003) Electro-gene therapy of collagen-induced arthritis by using an expression plasmid for the soluble p75 tumor necrosis factor receptor-Fc fusion protein. *Gene Ther* 10: 1216–1224
24 Perez N, Plence P, Millet V et al. (2002) Tetracycline transcriptional silencer tightly controls transgene expression after *in vivo* intramuscular electrotransfer: application to interleukin 10 therapy in experimental arthritis. *Hum Gene Ther* 13: 2161–2172
25 Saidenberg-Kermanac'h N, Bessis N, Deleuze V et al. (2003) Efficacy of interleukin-10 gene electrotransfer into skeletal muscle in mice with collagen-induced arthritis. *J Gene Med* 5: 164–171
26 Grossin L, Cournil-Henrionnet C, Mir LM et al. (2003) Direct gene transfer into rat articular cartilage by *in vivo* electroporation. *Faseb J* 17: 829–835
27 Ohashi S, Kubo T, Kishida T et al. (2002) Successful genetic transduction *in vivo* into synovium by means of electroporation. *Biochem Biophys Res Commun* 293: 1530–1535
28 Schiffelers RM, Xu J, Storm G et al. (2005) Effects of treatment with small interfering RNA on joint inflammation in mice with collagen-induced arthritis. *Arthritis Rheum* 52: 1314–1318
29 Khoury M, Bigey P, Louis-Plence P et al. (2006) A comparative study on intra-articular *versus* systemic gene electrotransfer in experimental arthritis. *J Gene Med* 8: 1027–1036
30 Delavallee L, Le Buanec H, Bessis N et al. (2008) Early and long-lasting protection from arthritis in TNF{alpha} transgenic mice vaccinated against TNF{alpha}. *Ann Rheum Dis* 67: 1332–1338
31 Le Buanec H, Delavallee L, Bessis N et al. (2006) TNFalpha kinoid vaccination-induced neutralizing antibodies to TNFalpha protect mice from autologous TNFalpha-driven chronic and acute inflammation. *Proc Natl Acad Sci U S A* 103: 19442–19447
32 Adriaansen J, Vervoordeldonk MJ, Vanderbyl S et al. (2006) A novel approach for gene therapy: engraftment of fibroblasts containing the artificial chromosome expression system at the site of inflammation. *J Gene Med* 8: 63–71
33 Byk G, Dubertret C, Escriou V et al. (1998) Synthesis, activity, and structure – activity relationship studies of novel cationic lipids for DNA transfer. *J Med Chem* 41: 229–235
34 Khoury M, Escriou V, Courties G et al. (2008) Combined anti-cytokine siRNA lipoplexes efficiently suppress arthritis. *Arthritis Rheum* 58: 2356–2367
35 Khoury M, Louis-Plence P, Escriou V et al. (2006) Efficient new cationic liposome formulation for systemic delivery of small interfering RNA silencing tumor necrosis factor alpha in experimental arthritis. *Arthritis Rheum* 54: 1867–1877
36 Metselaar JM, van den Berg WB, Holthuysen AE et al. (2004) Liposomal targeting of glucocorticoids to synovial lining cells strongly increases therapeutic benefit in collagen type II arthritis. *Ann Rheum Dis* 63: 348–353
37 Buchschacher GL Jr, Wong-Staal F (2000) Development of lentiviral vectors for gene therapy for human diseases. *Blood* 95: 2499–2504
38 Kustikova OS, Baum C, Fehse B (2008) Retroviral integration site analysis in hematopoietic stem cells. *Methods Mol Biol* 430: 255–267
39 Thrasher AJ, Gaspar HB, Baum C et al. (2006) Gene therapy: X-SCID transgene leukaemogenic-

ity. *Nature* 443: E5–6; discussion E6–7
40 Jorgensen C, Demoly P, Noel D et al. (1997) Gene transfer to human rheumatoid synovial tissue engrafted in SCID mice [see comments]. *J Rheumatol* 24: 2076–2079
41 Yang S, Wu B, Mayton L et al. (2002) IL-1Ra and vIL-10 gene transfer using retroviral vectors ameliorates particle-associated inflammation in the murine air pouch model. *Inflamm Res* 51: 342–350
42 Gouze E, Pawliuk R, Gouze JN et al. (2003) Lentiviral-mediated gene delivery to synovium: potent intra-articular expression with amplification by inflammation. *Mol Ther* 7: 460–466
43 Lin YL, Noel D, Mettling C et al. (2004) Feline immunodeficiency virus vectors for efficient transduction of primary human synoviocytes: application to an original model of rheumatoid arthritis. *Hum Gene Ther* 15: 588–596
44 Kremer EJ, Boutin S, Chillon M et al. (2000) Canine adenovirus vectors: an alternative for adenovirus-mediated gene transfer. *J Virol* 74: 505–512
45 Seshidhar Reddy P, Ganesh S, Limbach MP et al. (2003) Development of adenovirus serotype 35 as a gene transfer vector. *Virology* 311: 384–393
46 Toh ML, Hong SS, van de Loo F et al. (2005) Enhancement of adenovirus-mediated gene delivery to rheumatoid arthritis synoviocytes and synovium by fiber modifications: role of arginine-glycine-aspartic acid (RGD)- and non-RGD-binding integrins. *J Immunol* 175: 7687–7698
47 Braz J, Beaufour C, Coutaux A et al. (2001) Therapeutic efficacy in experimental polyarthritis of viral-driven enkephalin overproduction in sensory neurons. *J Neurosci* 21: 7881–7888
48 Evans C, Goins WF, Schmidt MC et al. (1997) Progress in development of herpes simplex virus gene vectors for treatment of rheumatoid arthritis. *Adv Drug Deliv Rev* 27: 41–57
49 Oligino T, Ghivizzani S, Wolfe D et al. (1999) Intra-articular delivery of a herpes simplex virus IL-1Ra gene vector reduces inflammation in a rabbit model of arthritis. *Gene Ther* 6: 1713–1720
50 Grieger JC, Choi VW, Samulski RJ (2006) Production and characterization of adeno-associated viral vectors. *Nat Protoc* 1: 1412–1428
51 Coura Rdos S, Nardi NB (2007) The state of the art of adeno-associated virus-based vectors in gene therapy. *Virol J* 4: 99
52 Grimm D, Zhou S, Nakai H et al. (2003) Preclinical *in vivo* evaluation of pseudotyped adeno-associated virus vectors for liver gene therapy. *Blood* 102: 2412–2419
53 Rabinowitz JE, Rolling F, Li C et al. (2002) Cross-packaging of a single adeno-associated virus (AAV) type 2 vector genome into multiple AAV serotypes enables transduction with broad specificity. *J Virol* 76: 791–801
54 Arruda VR, Fields PA, Milner R et al. (2001) Lack of germline transmission of vector sequences following systemic administration of recombinant AAV-2 vector in males. *Mol Ther* 4: 586–592
55 Flotte TR (2004) Gene therapy progress and prospects: recombinant adeno-associated virus (rAAV) vectors. *Gene Ther* 11: 805–810
56 Janson C, McPhee S, Bilaniuk L et al. (2002) Clinical protocol. Gene therapy of Canavan disease: AAV-2 vector for neurosurgical delivery of aspartoacylase gene (ASPA) to the human brain. *Hum Gene Ther* 13: 1391–1412
57 Kay MA, Manno CS, Ragni MV et al. (2000) Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector. *Nat Genet* 24: 257–261
58 Manno CS, Pierce GF, Arruda VR et al. (2006) Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 12: 342–347
59 Wang J, Xie J, Lu H et al. (2007) Existence of transient functional double-stranded DNA intermediates during recombinant AAV transduction. *Proc Natl Acad Sci U S A* 104: 13104–13109
60 Arai Y, Kubo T, Fushiki S et al. (2000) Gene delivery to human chondrocytes by an adeno associated virus vector. *J Rheumatol* 27: 979–982
61 Goater J, Muller R, Kollias G et al. (2000) Empirical advantages of adeno associated viral vectors *in vivo* gene therapy for arthritis. *J Rheumatol* 27: 983–989
62 Madry H, Cucchiarini M, Terwilliger EF et al. (2003) Recombinant adeno-associated virus vectors efficiently and persistently transduce chondrocytes in normal and osteoarthritic human articular cartilage. *Hum Gene Ther* 14: 393–402
63 Veron P, Allo V, Riviere C et al. (2007) Major subsets of human dendritic cells are efficiently transduced by self-complementary adeno-associated virus vectors 1 and 2. *J Virol* 81: 5385–5394
64 Apparailly F, Millet V, Noel D et al. (2002) Tetracycline-inducible interleukin-10 gene transfer mediated by an adeno-associated virus: application to experimental arthritis. *Hum Gene Ther* 13: 1179–1188

65 Chan JM, Villarreal G, Jin WW et al. (2002) Intraarticular gene transfer of TNFR:Fc suppresses experimental arthritis with reduced systemic distribution of the gene product. *Mol Ther* 6: 727–736
66 Cottard V, Mulleman D, Bouille P et al. (2000) Adeno-associated virus-mediated delivery of IL-4 prevents collagen-induced arthritis. *Gene Ther* 7: 1930–1939
67 Pan RY, Chen SL, Xiao X et al. (2000) Therapy and prevention of arthritis by recombinant adeno-associated virus vector with delivery of interleukin-1 receptor antagonist. *Arthritis Rheum* 43: 289–297
68 Watanabe S, Imagawa T, Boivin GP et al. (2000) Adeno-associated virus mediates long-term gene transfer and delivery of chondroprotective IL-4 to murine synovium. *Mol Ther* 2: 147–152
69 Zhang HG, Huang N, Liu D et al. (2000) Gene therapy that inhibits nuclear translocation of nuclear factor kappaB results in tumor necrosis factor alpha-induced apoptosis of human synovial fibroblasts. *Arthritis Rheum* 43: 1094–1105
70 Pan RY, Xiao X, Chen SL et al. (1999) Disease-inducible transgene expression from a recombinant adeno-associated virus vector in a rat arthritis model. *J Virol* 73: 3410–3417
71 Zhang HG, Xie J, Yang P et al. (2000) Adeno-associated virus production of soluble tumor necrosis factor receptor neutralizes tumor necrosis factor alpha and reduces arthritis [In Process Citation]. *Hum Gene Ther* 11: 2431–2442
72 Adriaansen J, Tas SW, Klarenbeek PL et al. (2005) Enhanced gene transfer to arthritic joints using adeno-associated virus type 5: implications for intra-articular gene therapy. *Ann Rheum Dis* 64: 1677–1684
73 Apparailly F, Khoury M, Vervoordeldonk MJ et al. (2005) Adeno-associated virus pseudotype 5 vector improves gene transfer in arthritic joints. *Hum Gene Ther* 16: 426–434
74 Evans CH, Gouze E, Gouze JN et al. (2006) Gene therapeutic approaches-transfer *in vivo*. *Adv Drug Deliv Rev* 58: 243–258
75 Adriaansen J, Khoury M, de Cortie CJ et al. (2007) Reduction of arthritis following intra-articular administration of an adeno-associated virus serotype 5 expressing a disease-inducible TNF-blocking agent. *Ann Rheum Dis* 66: 1143–1150
76 Khoury M, Adriaansen J, Vervoordeldonk MJ et al. (2007) Inflammation-inducible anti-TNF gene expression mediated by intra-articular injection of serotype 5 adeno-associated virus reduces arthritis. *J Gene Med* 9: 596–604
77 DiPrimio N, Asokan A, Govindasamy L et al. (2008) Surface loop dynamics in adeno-associated virus capsid assembly. *J Virol* 82: 5178–5189
78 Li W, Asokan A, Wu Z et al. (2008) Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticles. *Mol Ther* 16: 1252–1260
79 Srivastava A (2008) Adeno-associated virus-mediated gene transfer. *J Cell Biochem* 105: 17–24
80 Adriaansen J, Vervoordeldonk MJ, Tak PP (2006) Gene therapy as a therapeutic approach for the treatment of rheumatoid arthritis: innovative vectors and therapeutic genes. *Rheumatology (Oxford)* 45: 656–668
81 Woods JM, Sitabkhan Y, Koch AE (2008) Gene therapy for rheumatoid arthritis: recent advances. *Curr Gene Ther* 8: 24–41
82 Russell ST, Hirsch R (2008) Gene therapy for arthritis. *Mol Rheumatol* 18: 2–14
83 Smeets TJ, Barg EC, Kraan MC et al. (2003) Analysis of the cell infiltrate and expression of pro-inflammatory cytokines and matrix metalloproteinases in arthroscopic synovial biopsies: comparison with synovial samples from patients with end stage, destructive rheumatoid arthritis. *Ann Rheum Dis* 62: 635–638
84 McInnes IB, Schett G (2007) Cytokines in the pathogenesis of rheumatoid arthritis. *Nat Rev Immunol* 7: 429–442
85 Okamoto H, Hoshi D, Kiire A et al. (2008) Molecular targets of rheumatoid arthritis. *Inflamm Allergy Drug Targets* 7: 53–66
86 Smolen JS, Redlich K, Zwerina J et al. (2005) Pro-inflammatory cytokines in rheumatoid arthritis: pathogenetic and therapeutic aspects. *Clin Rev Allergy Immunol* 28: 239–248
87 Joosten LA, Helsen MM, van de Loo FA et al. (2008) Anticytokine treatment of established type II collagen-induced arthritis in DBA/1 mice: a comparative study using anti-TNFalpha, anti-IL-1alpha/beta and IL-1Ra. *Arthritis Rheum* 58: S110–122
88 van de Loo FA, Joosten LA, van Lent PL et al. (1995) Role of interleukin-1, tumor necrosis factor alpha, and interleukin-6 in cartilage proteoglycan metabolism and destruction. Effect of *in situ* blocking in murine antigen- and zymosan-induced arthritis. *Arthritis Rheum* 38: 164–172

89 Lewthwaite J, Blake S, Hardingham T et al. (1995) Role of TNF alpha in the induction of antigen induced arthritis in the rabbit and the anti-arthritic effect of species specific TNF alpha neutralising monoclonal antibodies. *Ann Rheum Dis* 54: 366–374
90 Mountz JD, Zhang HG (2001) Regulation of apoptosis of synovial fibroblasts. *Curr Dir Autoimmun* 3: 216–239
91 Yao Q, Seol DW, Mi Z et al. (2006) Intra-articular injection of recombinant TRAIL induces synovial apoptosis and reduces inflammation in a rabbit knee model of arthritis. *Arthritis Res Ther* 8: R16
92 Yao Q, Wang S, Glorioso JC et al. (2001) Gene transfer of p53 to arthritic joints stimulates synovial apoptosis and inhibits inflammation. *Mol Ther* 3: 901–910
93 Kobayashi T, Okamoto K, Kobata T et al. (2000) Novel gene therapy for rheumatoid arthritis by FADD gene transfer: induction of apoptosis of rheumatoid synoviocytes but not chondrocytes. *Gene Ther* 7: 527–533
94 Wang CR, Shiau AL, Chen SY et al. (2008) Amelioration of collagen-induced arthritis in rats by adenovirus-mediated PTEN gene transfer. *Arthritis Rheum* 58: 1650–1656
95 Goossens PH, Schouten GJ, t Hart BA et al. (1999) Feasibility of adenovirus-mediated nonsurgical synovectomy in collagen-induced arthritis-affected rhesus monkeys. *Hum Gene Ther* 10: 1139–1149
96 Sant SM, Suarez TM, Moalli MR et al. (1998) Molecular lysis of synovial lining cells by *in vivo* herpes simplex virus-thymidine kinase gene transfer. *Hum Gene Ther* 9: 2735–2743
97 Colville-Nash PR, Scott DL (1992) Angiogenesis and rheumatoid arthritis: pathogenic and therapeutic implications. *Ann Rheum Dis* 51: 919–925
98 Chen Y, Donnelly E, Kobayashi H et al. (2005) Gene therapy targeting the Tie2 function ameliorates collagen-induced arthritis and protects against bone destruction. *Arthritis Rheum* 52: 1585–1594
99 Gerlag DM, Borges E, Tak PP et al. (2001) Suppression of murine collagen-induced arthritis by targeted apoptosis of synovial neovasculature. *Arthritis Res* 3: 357–361
100 Afuwape AO, Feldmann M, Paleolog EM (2003) Adenoviral delivery of soluble VEGF receptor 1 (sFlt-1) abrogates disease activity in murine collagen-induced arthritis. *Gene Ther* 10: 1950–1960
101 Kim JM, Ho SH, Park EJ et al. (2002) Angiostatin gene transfer as an effective treatment strategy in murine collagen-induced arthritis. *Arthritis Rheum* 46: 793–801
102 Jou IM, Shiau AL, Chen SY et al. (2005) Thrombospondin 1 as an effective gene therapeutic strategy in collagen-induced arthritis. *Arthritis Rheum* 52: 339–344
103 Park YW, Kang YM, Butterfield J et al. (2004) Thrombospondin 2 functions as an endogenous regulator of angiogenesis and inflammation in rheumatoid arthritis. *Am J Pathol* 165: 2087–2098
104 Apparailly F, Bouquet C, Millet V et al. (2002) Adenovirus-mediated gene transfer of urokinase plasminogen inhibitor inhibits angiogenesis in experimental arthritis. *Gene Ther* 9: 192–200
105 Rutkauskaite E, Zacharias W, Schedel J et al. (2004) Ribozymes that inhibit the production of matrix metalloproteinase 1 reduce the invasiveness of rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* 50: 1448–1456
106 Rutkauskaite E, Volkmer D, Shigeyama Y et al. (2005) Retroviral gene transfer of an antisense construct against membrane type 1 matrix metalloproteinase reduces the invasiveness of rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* 52: 2010–2014
107 Meinecke I, Rutkauskaite E, Cinski A et al. (2007) Gene transfer to synovial fibroblast: methods and evaluation in the SCID mouse model. *Methods Mol Med* 135: 393–412
108 van der Laan WH, Quax PH, Seemayer CA et al. (2003) Cartilage degradation and invasion by rheumatoid synovial fibroblasts is inhibited by gene transfer of TIMP-1 and TIMP-3. *Gene Ther* 10: 234–242
109 Apparailly F, Noel D, Millet V et al. (2001) Paradoxical effects of tissue inhibitor of metalloproteinases 1 gene transfer in collagen-induced arthritis. *Arthritis Rheum* 44: 1444–1454
110 Tak PP, Gerlag DM, Aupperle KR et al. (2001) Inhibitor of nuclear factor kappaB kinase beta is a key regulator of synovial inflammation. *Arthritis Rheum* 44: 1897–1907
111 Jimi E, Ghosh S (2005) Role of nuclear factor-kappaB in the immune system and bone. *Immunol Rev* 208: 80–87
112 Tas SW, Adriaansen J, Hajji N et al. (2006) Amelioration of arthritis by intraarticular dominant negative Ikk beta gene therapy using adeno-associated virus type 5. *Hum Gene Ther* 17: 821–832
113 Tomita T, Takano H, Tomita N et al. (2000) Transcription factor decoy for NFkappaB inhibits cytokine and adhesion molecule expressions in synovial cells derived from rheumatoid arthritis.

Rheumatology (Oxford) 39: 749–757
114 Tomita T, Takeuchi E, Tomita N et al. (1999) Suppressed severity of collagen-induced arthritis by *in vivo* transfection of nuclear factor kappaB decoy oligodeoxynucleotides as a gene therapy. *Arthritis Rheum* 42: 2532–2542
115 Kubota T, Hoshino M, Aoki K et al. (2007) NF-kappaB inhibitor dehydroxymethylepoxyquinomicin suppresses osteoclastogenesis and expression of NFATc1 in mouse arthritis without affecting expression of RANKL, osteoprotegerin or macrophage colony-stimulating factor. *Arthritis Res Ther* 9: R97
116 Campbell J, Ciesielski CJ, Hunt AE et al. (2004) A novel mechanism for TNF-alpha regulation by p38 MAPK: involvement of NF-kappa B with implications for therapy in rheumatoid arthritis. *J Immunol* 173: 6928–6937
117 Simmonds RE, Foxwell BM (2008) Signalling, inflammation and arthritis: NF-kappaB and its relevance to arthritis and inflammation. *Rheumatology (Oxford)* 47: 584–590
118 Tas SW, Hajji N, Stenvers DJ et al. (2006) Reduction of proinflammatory cytokine expression in the synovium by targeting IKKbeta *in vivo* in a rat model. *Arthritis Rheum* 54: 3716–3718
119 Lianxu C, Hongti J, Changlong Y (2006) NF-kappaBp65-specific siRNA inhibits expression of genes of COX-2, NOS-2 and MMP-9 in rat IL-1beta-induced and TNF-alpha-induced chondrocytes. *Osteoarthritis Cartilage* 14: 367–376
120 Aikawa Y, Morimoto K, Yamamoto T et al. (2008) Treatment of arthritis with a selective inhibitor of c-Fos/activator protein-1. *Nat Biotechnol* 26: 817–823
121 Fahmy RG, Waldman A, Zhang G et al. (2006) Suppression of vascular permeability and inflammation by targeting of the transcription factor c-Jun. *Nat Biotechnol* 24: 856–863
122 van Holten J, Reedquist K, Sattonet-Roche P et al. (2004) Treatment with recombinant interferon-beta reduces inflammation and slows cartilage destruction in the collagen-induced arthritis model of rheumatoid arthritis. *Arthritis Res Ther* 6: R239–249
123 Tak PP, Hart BA, Kraan MC et al. (1999) The effects of interferon beta treatment on arthritis. *Rheumatology (Oxford)* 38: 362–369
124 Triantaphyllopoulos KA, Williams RO, Tailor H et al. (1999) Amelioration of collagen-induced arthritis and suppression of interferon-gamma, interleukin-12, and tumor necrosis factor alpha production by interferon-beta gene therapy. *Arthritis Rheum* 42: 90–99
125 Adriaansen J, Fallaux FJ, de Cortie CJ et al. (2007) Local delivery of beta interferon using an adeno-associated virus type 5 effectively inhibits adjuvant arthritis in rats. *J Gen Virol* 88: 1717–1721
126 Adriaansen J, Kuhlman RR, Holten JV et al. (2006) Intraarticular interferon-beta gene therapy ameliorates adjuvant arthritis in rats. *Hum Gene Ther* 17: 985–996
127 Agarwal SK, Brenner MB (2006) Role of adhesion molecules in synovial inflammation. *Curr Opin Rheumatol* 18: 268–276
128 Kiener HP, Brenner MB (2005) Building the synovium: cadherin-11 mediates fibroblast-like synoviocyte cell-to-cell adhesion. *Arthritis Res Ther* 7: 49–54
129 Lee DM, Kiener HP, Agarwal SK et al. (2007) Cadherin-11 in synovial lining formation and pathology in arthritis. *Science* 315: 1006–1010
130 Yarranton GT (1992) Inducible vectors for expression in mammalian cells. *Curr Opin Biotechnol* 3: 506–511
131 Geurts J, Arntz OJ, Bennink MB et al. (2007) Application of a disease-regulated promoter is a safer mode of local IL-4 gene therapy for arthritis. *Gene Ther* 14: 1632–1638
132 Rachakonda PS, Rai MF, Schmidt MF (2008) Application of inflammation-responsive promoter for an *in vitro* arthritis model. *Arthritis Rheum* 58: 2088–2097
133 Brown BD, Gentner B, Cantore A et al. (2007) Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat Biotechnol* 25: 1457–1467
134 Mingozzi F, High KA (2007) Immune responses to AAV in clinical trials. *Curr Gene Ther* 7: 316–324
135 Mease PJ, Hobbs K, Chalmers A et al. (2009) Local delivery of a recombinant adeno-associated vector containing a tumor necrosis factor-{alpha} antagonist gene in inflammatory arthritis: a phase 1 dose-escalation safety and tolerability study. *Ann Rheum Dis* 68: 1247–1254
136 Williams DA (2007) RAC reviews serious adverse event associated with AAV therapy trial. *Mol Ther* 15: 2053–2054.

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Gene therapy for the treatment of inflammatory bowel disease

Melanie A. Ruffner[1], Scott E. Plevy[2] and Anthony T. Cheung[3]

[1] *University of Pittsburgh, Department of Bioengineering, Pittsburgh, PA, USA*
[2] *University of North Carolina, Division of Gastroenterology and Hepatology, Chapel Hill, NC, USA*
[3] *enGene, Inc., Vancouver, BC, Canada*

Abstract

Gene- and nucleic acid-based therapies for inflammatory bowel disease (IBD) have shown efficacy in animal models and are beginning to be utilized in clinical studies. Emerging research efforts are concentrating on the development of nonviral-based DNA delivery technologies that can be administered via oral and rectal routes to achieve therapeutic effects locally within the affected intestinal tissue. Although the majority of work is in preclinical development stages, some nucleic acid-based approaches for treating IBD have successfully progressed to early stages of clinical trials. In this chapter, we will focus on discussing these nucleic acid-based therapies as well as other potential new therapeutics for the treatment of IBD.

Inflammatory Bowel Disease

IBD is a common autoimmune disorder with unclear etiology. Patients with IBD experience a range of gastrointestinal (GI) symptoms, including diarrhea, abdominal pain, bloody stools, and malabsorption, often resulting in weight loss. There are also extra-intestinal manifestations such as joint, skin and eye disorders associated with IBD. Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of IBD. Both forms of IBD are chronic immunologically-mediated diseases of the bowel, which result in areas of tissue inflammation in the wall of the GI tract. Both are associated with a high morbidity and decreased quality of life [1].

The overall prevalence of IBD is about two cases per 1,000 people with more than 4 million persons worldwide inflicted by this disease, although some ethnic groups, such as Ashkenazi Jews have a particularly high incidence [2]. The peak incidence of diagnosis is between 10 and 40 years old, although diagnosis can occur at any age. In the United States alone there are approximately 1.5 million people affected by IBD [3]. The direct treatment cost and indirect costs of IBD in the US are estimated to be $6.3 and $3.6 billion annually, respectively [3, 4].

UC typically involves superficial, non-transmural ulceration of the colonic mucosa, and is restricted to the colon. Depending on the location of the affected area, patients can be classified as having proctitis (disease confined to the rectum), left-sided colitis (involving the sigmoid colon with or without involvement of the descending colon), or pancolitis (disease spreading across an exten-

sive part of the lower GI tract). About 40% of patients have proctitis, 30–40% have left-sided ulcerative colitis, and 20–30% have pancolitis. Due to the extensive ulceration of the rectum and colon, the chief symptom during flares of UC is bloody diarrhea.

The inflammation of CD, in contrast, can occur in confined regions anywhere along the GI tract. The terminal ileum is affected in up to 70% of patients. The lesions seen in CD are typically transmural in nature, affecting multiple layers or the full thickness of the GI tract. Histopathologically, Crohn's lesions sometimes reveal non-caseasting granulomas. Progressive inflammation in Crohn's disease can ultimately result in the formation of abscesses, fistulae, and strictures requiring surgical intervention. Presentation at diagnosis can range in appearance and severity, as different areas of the GI tract can be affected. Symptoms most commonly include abdominal pain, cramping and diarrhea, however, other symptoms such as nausea and vomiting, drainage due to fistula formation, as well as acute presentations can occur.

Pathogenesis of IBD

IBD develops in a genetically susceptible host under the influences of environmental factors that initiate and perpetuate an immune response leading to chronic mucosal inflammation. Approximately 40 IBD susceptibility loci in the human genome have been recently identified [5]. Among all the putative environmental factors, the one that has received the most attention is the role of the enteric microbiota in causing an inappropriate host immune response. This knowledge has led to the most widely accepted model of IBD pathogenesis, where in a genetically susceptible individual there is the loss of normal immunologic tolerance to the enteric microbiota.

Recent descriptions of genetic susceptibility loci in the human CD and UC highlight the importance of innate immune responses against the enteric microbiota in disease pathogenesis. The CD susceptibility genes *NOD2*, *ATG16L1*, and *IRGM* are involved in microbial recognition and eradication [5–7]. Furthermore, several susceptibility genes are part of the IL-12/23 pathway, an important link between innate and adaptive immunity.

Mechanisms by which *NOD2* mutations lead to disease development are still controversial, but highlight the importance of immune responses against the enteric microbiota. The NOD2 protein is an intracellular receptor expressed in monocytes/macrophages, dendritic cells and intestinal epithelial cells, and it is activated through binding of a bacterial product, muramyl dipeptide (MDP) [7]. MDP is a component of peptidoglycan, a major structure found in the Gram-positive bacterial cell wall. The activated NOD2 then triggers NF-κB signaling pathways, resulting in production of many pro-inflammatory cytokines. However, it is worth noting that despite elevated NF-κB activation in the intestinal mucosa of CD patients, innate immune cells with NOD2 variants have decreased NF-κB activation in response to MDP [8]. Recently, it has been pro-

posed that *NOD2* mutations in human macrophages may lead to defective expression of cytokine IL-10 [9]. Moreover, NOD2-deficient mice have higher susceptibility to infection by *Listeria monocytogens* given via the oral route, but not by systemic administration, suggesting that NOD2 plays a role in bacterial clearance in the gut [10]. This finding is consistent with decreased intestinal epithelial defensin expression in CD patients with *NOD2* mutations. Collectively, these results suggest that NOD2 participates in anti-bacterial immunity and impaired clearance of bacteria may be a primary defect in the pathogenesis of CD.

Genome wide screens have identified two other IBD susceptibility genes with immediate implications for macrophage mediated innate immunity, *IL23R* (IL-23 receptor) [11] and *ATG16L1* [12, 13]. IL-23 is produced by macrophages following activation by bacterial ligands and it functions to expand IL-17 producing helper T cells (Th17 cells). IL-23 has been shown to play an essential role in several murine colitis models [14–16]. However, functional consequences of the *IL23R* gene variations have not yet been reported. The *ATG16L1* gene is involved in autophagy. Autophagy was originally described as a cellular adaptation to cellular stress and nutrient deprivation; however, recent studies have implicated autophagy in the processing of intracellular bacteria such as *Mycobacterium* and *Streptococcus*. Functional knockdown of *ATG16L1* leads to defective autophagy in response to *Salmonella typhimurium* infection [17].

Recent research implicates the role of gut microbiota in prevalent human disease including obesity, diabetes, cancer and IBD. The complex and as of yet ill-defined gut microbiota can be viewed as a microbial organ system that contributes to a number of physiologic functions. Furthermore, microbes in the gut elicit protective functions against pathogens and modulate the host immune system. The importance of the microbiota in the initiation and perpetuation of IBD is most clearly demonstrated by studies in mouse models of IBD [18–21]. For example, colitis-prone, germ-free derived mice do not develop IBD until colonized with the enteric microbiota [18, 19]. The current state of knowledge reveals that the loss of immunologic tolerance to the intestinal microbiota is not global, but rather it appears that the innate and adaptive immune system are only reactive to specific bacteria or bacterial antigens. Therefore, interventions in IBD that can potentially restore tolerance to defined microbiota are a promising strategy for future IBD therapies. Undoubtedly, a thorough understanding of the complex interaction between the host and microbe is a requisite for building a more complete knowledge on the pathogenesis of IBD.

Treatment of IBD – current clinical standards

Both CD and UC are a significant burden to patients. Quality of life is significantly impacted during periods of active disease; and treatment and maintenance therapy are both extremely expensive. In a 2004 study of commercially insured US individuals, the mean expenditure for CD and UC was $8265 and

$5066 per patient-year, respectively. The three greatest direct expenditures of IBD were for medications, inpatient medical and surgical costs [3]. Expenditures were less in adults than in children, and equal between genders. Due to the chronic, relapsing and sometimes progressive nature of IBD, in many cases there are far-reaching effects on patients' lives beyond the cost of care. Pain, frequent diarrhea and other symptoms associated with IBD flares can result in absenteeism from school or work, as well as inability to participate in social and family functions [22]. Furthermore, a significant subset of patients may develop anxiety or depression while coping with the demands of living with IBD, and may benefit from psychotherapy in addition to proper management of their IBD [23].

Since IBD is a chronic disease, patients generally require lifelong treatment. Therapy for IBD can be divided into two main approaches: pharmacological and surgical therapy, which is required in up to 40% of cases of UC and 70% of cases of CD. Treatment for IBD depends on the severity of the disease. Each person experiences the disease differently, so treatment is prescribed accordingly.

Pharmacologic therapy

Current pharmacologic therapy for IBD is not curative, but instead focuses on inducing and maintaining clinical remission. Standard of care for IBD typically involves a multi-drug approach to induce remission, and then either mono- or multi-drug therapy to maintain remission once it has been achieved. A wide array of drugs with immunomodulatory properties are used for this purpose. The most commonly used drugs in the maintenance of UC remission are the aminosalicylates (e.g., 5-ASA, mesalamine), which act on the intestinal epithelium to mediate the release of anti-inflammatory mediators [24]. The aminosalicylates are typically administered locally, and depending on the extent of disease may be administered per the rectum as a suppository, foam or an enema. This inconvenient and messy dosing may limit patient compliance with aminosalicylate therapy, and more recently efforts have been made to increase the availability of more convenient oral and rectal dosing formulations [25].

Corticosteroid therapy is a primary treatment for moderate to severe relapses of CD and UC, due to their potent anti-inflammatory effects. They act via several mechanisms, including down-regulation of cytokine production, inhibition of NF-κB translocation, and suppression of arachidonic acid signaling [26]. However, these compounds exert many undesirable systemic side effects, including glucose intolerance, weight gain, cataract development, osteoporosis, immunosuppression and frequent infections [26]. Recent efforts have been focused on reducing these systemic side effects by reformulating certain poorly absorbed corticosteroids (e.g., budesonide) for localized delivery to the colon via enema or by mouth via extended-release tablets [27]. Furthermore, although effective in inducing response and remission, corticosteroids are ineffective in long-term maintenance of remission.

Thiopurines such as azathioprine and mercaptopurine are also used for induction and maintenance of CD and UC, particularly in patients who have required steroids to induce remission [28, 29]. Thiopurines function in part by modulating Rac1, thereby inducing T cell apoptosis [30]. IBD patients on thiopurines must be monitored for the development of leukopenia, which can lead to infections, and other potential side effects include GI intolerance, hepatotoxicity, pancreatitis and possibly lymphoma. Close monitoring of blood counts is necessary with the use of thiopurines.

Methotrexate is also used to induce remission or prevent relapse of CD [31]. Therapy with methotrexate and steroids has been shown to be more effective in inducing remission in CD with lower doses of steroids [32]. The use of methotrexate is limited by many undesirable side effects, such as headaches, nausea and vomiting, as well as more seriously by hepatitis and pneumonitis [33].

Medical therapy for IBD has been revolutionized by the introduction of biologic agents such as the TNF-α inhibitors, and is expected to evolve continuously as newer biological agents are introduced. Injectable biologics including anti-TNF-α antibodies and antibody fragments, such as infliximab (Remicade®), adalimumab (Humira®), and certolizumab pegol (Cimzia®) have been approved for the treatment of CD, whereas only infliximab has been approved for the treatment of UC. This class of biologics has recently shown significant advantages over conventional immunomodulators, as the efficacy is higher for patients who respond to the therapy. They are generally well tolerated, and relatively safe. The ACCENT-I trial demonstrated that maintenance infusions of infiximab every eight weeks was beneficial in maintaining clinical remission in CD patients who had responded to an initial first dose of infliximab [34]. Additionally, infliximab is the first agent shown to be beneficial in the treatment of fistulizing CD [35]. However, there are several serious systemic side effects associated with prolonged usages of these biologics, including development of tuberculosis, histoplasmosis, pneumonia, various infections and lymphomas [36]. Another limitation of this class of drug is the body's tendency to develop an immune reaction to the drug, leading to neutralization of its beneficial effects as well as infusion or injection site reactions. Notably, about 20–40% of IBD patients on infliximab develop loss of response to the drug [37]. Another disadvantage related to these medications is that they cannot be delivered orally, requiring patients to receive infusions or give themselves injections. Additionally, their cost is relatively high.

Natalizumab is an anti-α4 integrin monoclonal antibody that was approved in 2008 in the United States (US) for the treatment of CD and is approved in the US and European Union for the treatment of multiple sclerosis. Natalizumab exerts its effects by binding to the α4 subunits of integrins on T cells, which then inhibits the ability of T cells to bind to receptors such as VCAM-1 and MAdCAM-1 on the vasculature and extravasate into areas of inflammation [38]. In clinical trials, natalizumab was effective at inducing and maintaining remission in patients with moderate to severely active CD, and therefore may be especially beneficial to patients who do not respond to treat-

ment with steroids or anti-TNFα agents [39]. However, use of natalizumab has been associated with progressive multifocal leucoencephalopathy, a rare but potentially fatal demyelinating disease caused by reactivation of latent JC virus (a type of human polyomavirus, formerly known as papovavirus) reactivation [40]. Therefore, use of this agent requires careful evaluation of the risk-benefit ratio for the patient. Furthermore, in conjunction with the US FDA, distribution of natalizumab is restricted through a closed prescribing and distribution program that requires all prescribers, pharmacies and infusion centers to be specially registered in order to ensure proper monitoring of patients for adverse effects [38].

Surgical therapy

Surgery is a commonly used treatment option for IBD. In patients with severe disease, elective surgery can lead to improvement in quality of life. In UC, removal of the colon can be curative, but in CD removal of affected areas is not curative, as disease can recur throughout the GI tract.

It has been reported that approximately 25% to 40% of UC patients must eventually have their colons removed because of massive bleeding, severe illness, rupture of the colon, or risk of cancer. Surgical removal of the colon is commonly done if medical treatment fails, colonic dysplasia or colorectal cancer is detected, or if side effects of drugs used threaten the patient's health. Colectomy comprises patients quality of life and the procedure can lead to severe side effects, including fecal incontinence, pelvic sepsis and pouch failure [41]. About 70–90% of CD patients may require one surgery for removal of part of the colon or small bowel secondary to the complications of CD, such as the formation of strictures or fistulae. Conservative resection of the affected area is necessary, as recurrence is common and large proportion of patients will need a second operation, and some will require more than two resections [42]. Short-bowel syndrome, which is characterized by malnutrition, is a rare complication in patients following resection of the small intestine. CD patients with small-bowel syndrome must have their nutrition supplemented by parenteral nutrition, or supplied by total parenteral nutrition, which in turn carries a risk of sepsis [42]. In addition to surgical management of severe UC and CD, surgical care of IBD may also be necessary in acute situations. IBD may present acutely as toxic colitis, hemorrhage, perforation, intra-abdominal masses with abscesses or sepsis, or obstruction [43]. These acute surgical emergencies are rare, but life-threatening.

New approaches to the treatment of IBD

Given the success of anti-TNF-α biologics, it is perhaps not surprising that there is a growing interest in the development of specific biological therapies

targeted at the specific pathological pathways observed in IBD. There are numerous new approaches for the treatment of IBD currently under investigation, most of which are precisely targeted to address an aspect of the pathological process of IBD. Therapy targeted to the disease process could in theory offer increased efficacy and a reduction in the multi-system side effects that are common with the current pharmacologic therapy. However, as these are investigational therapies, their individual risk-benefit profiles need to be carefully determined. We broadly categorized current investigational treatments for IBD into the following five areas of therapeutic strategies.

Blocking pro-inflammatory cytokine activity

There is a growing popularity of novel antibody-based therapies for the treatment of IBD. One approach in the development of new therapies has been to block cytokine signaling necessary for the development and activation of T cells. Antibodies to block IL-12 family members via blocking the IL-12 and IL-23 shared p40 subunit [44], have been tried in preliminary clinical trials for CD and have demonstrated potential benefit. An anti-IL-6 receptor monoclonal antibody has shown promising results in mice by blocking T cell recruitment and increasing T cell apoptosis [45], and preliminary clinical studies have demonstrated safety in humans [46]. However, further work is needed to demonstrate efficacy for CD in large-scale clinical trials.

Anti-inflammatory cytokine treatment

Human IL-10 has long been considered as a candidate for treatment of IBD. IL-10 is expressed by T regulatory cells, and it acts to decrease T helper-cells activation and to inhibit the production of pro-inflammatory cytokines by macrophages. As an anti-inflammatory cytokine favoring the cellular mediated immune response (Th2 response), IL-10 has been shown to prevent onset of IBD in various mouse models. In addition, clinical trials with recombinant IL-10 protein delivered by intravenous and subcutaneous injection as a treatment for colitis were performed and demonstrated tolerable safety and some efficacy in Phase I and II clinical trials [47, 48]. However, Phase III clinical trials for IL-10 were halted due to lack of efficacy and potentially pro-inflammatory effects associated with patients receiving high dose IL-10 [49]. Upon reviewing the data, clinical trial studies indicated that only limited therapeutic advantage was achieved in colitis patients treated with IL-10 when the drug is administered systematically [50]. The therapeutic efficacy of recombinant IL-10 delivered by injection is limited, in part, by its poor bioavailability in the GI tissue and by the unwanted systemic side effects, including thrombocytopenia, anemia, and release of pro-inflammatory cytokines at higher systemic concentrations.

It is generally believed that the efficacy of IL-10 treatment could be improved by localized delivery of IL-10 protein to the gut while minimizing systemic exposure. However, IL-10 cannot be delivered orally as it would not be stable in the acidic and digestive enzyme-rich environment of the gut. There are numerous pre-clinical studies in animals demonstrating that a high level of IL-10 delivered locally with minimized systemic exposure can result in a strong anti-inflammatory response and prevent development of colitis [51–54]. Furthermore, a recent Phase I trial in Crohn's disease patients of a genetically modified *Lactococcus lactis* strain which expresses and secrete human IL-10 showed some improvement to disease activity [55]. It remains unclear if the IL-10 secreted by the transgenic bacteria in the intestinal lumen would cross the mucosal barrier sufficiently to activate its cellular targets situated in the submucosal layer. Although some pre-clinical studies in mouse models demonstrate that the IL-10-secreting *L. lactis* are actually taken up into the mucosal layer through the paracellular route, which may allow bacteria to produce IL-10 in the vicinity of responsive cells [56, 57].

T cell inhibition

Antibodies to proteins on T cells, such as CD4, CD3 or CD25, have been used in CD to attempt to selectively delete pathogenic T cells. In the case of CD4, little therapeutic benefit was seen, and patients experienced HIV-like side effects following therapy and trials were halted. Anti-CD3 (visilizumab) is thought to function by inducing selective apoptosis of activated T cells, and has shown beneficial therapeutic effects in Phase I trials [58]. In patients with steroid-refractory UC, 41% achieved clinical remission, and 44% achieved endoscopic remission. However, treatment was complicated by cytokine-release syndrome in 80% of patients at the lower dose and 100% of patients at the higher dose. Cytokine-release syndrome, in which antibody binding to the T cells results in release of preformed cytokine prior to T cell destruction, is characterized by fevers, rigors and hypotension.

Anti-CD25 (daclizumab, and basiliximab) is also of particular interest as the mechanism of steroid-refractory disease is postulated to be a function of IL-2 signaling in T cells, and blockade of CD25 (the IL-2 receptor on T cells) could aid in treatment of IBD, which has become resistant to treatment with steroids. Small studies of basiliximab in steroid-refractory UC patients suggest that it can induce remission in steroid refractory patients [59]. However, a Phase II trial of daclizumab in 159 patients with moderate UC demonstrated no therapeutic benefit compared to placebo [60]. Although anti-CD3 and anti-CD25 antibodies may have a niche role in the treatment of severe, steroid refractory IBD, more large-scale clinical studies are needed to prove their therapeutic efficacy in humans.

While these methods modify the number of pro-inflammatory T cell populations, other methods have been examined to modify the function of pro-

inflammatory T cells. For example, toralizumab, an anti-CD40L antibody, functions by blocking the CD40 ligand on T cells and making them unable to receive co-stimulatory signals from activated B cells in the intestinal lamina propria [61]. This agent helps to prevent activation of the T cells within the intestine, which has been effective in animal models of colitis and is currently being investigated in Phase II clinical trials [62]. Abatacept, a CTLA4-Ig fusion protein currently approved for use in rheumatoid arthritis, is also undergoing Phase II and III clinical trials in patients with active CD and UC. Abatacept has been shown to alter immune system function by a variety of mechanisms, including blocking B7 co-stimulation of T cells; binding to B7 molecules on dendritic cells; and activating indoleamine-2,3,-dioxygenase, which indirectly inhibits T cell function [63]. In murine models of colitis, abatacept treatment was able to suppress inflammatory cytokine production and inhibit disease progression [64].

Promoting intestinal repair

All of the strategies discussed previously focus on down-regulating the levels of inflammation within the intestine to minimize the area damaged by inflammation. However, some strategies have also been examined to accelerate the natural healing process of the intestinal tissue. In a preliminary clinical study in CD patients, growth hormone plus a high protein diet demonstrated beneficial effects compared to placebo in patients with disease severity ranging from remission to moderate disease [65]. It is believed that a portion of these effects is mediated through decreased intestinal permeability and increased intestinal protein turnover.

Glucagon-like peptide 2 (GLP-2) is a proglucagon peptide secreted by endocrine cells of the intestines in response to food ingestion. Administration of GLP-2 has been shown to be associated with decreased intestinal injury, bacterial adhesion and epithelial damage in various animal models for IBD [66]. Treatment with a human GLP-2 analog in mice with dextran sodium sulfate (DSS)-induced colitis demonstrated a reduced severity of disease [67]. Inhibiting dipeptidyl peptidase, which enzymatically breaks down endogenous GLP-2, has also been shown to partially ameliorate DSS-induced colitis in mice [68]. In 2009, the FDA allowed the beginning of a Phase I trial for ZP1848, a modified, dipeptidyl peptidase-resistant- GLP-2 agonist with a longer biological half-life, in CD patients.

Modifying the enteric microbiota

The manner in which the host immune system interacts with the endogenous intestinal microflora has been gathering a significant amount of attention in relation to the pathogenesis and therapy of IBD. There is a growing body of evi-

dence that certain components of the enteric microbiota may contribute to increased inflammation and disease activity within the intestine, whereas other strains of intestinal bacteria may be protective [69]. Significant differences have been demonstrated in the microbiota between IBD patients and healthy controls, with IBD patients having a reduction in the overall biodiversity of species [70].

One therapeutic approach to address this is to administer probiotics to IBD patients. Probiotics are living microorganisms which are administered orally. Many have been derived from cultured dairy products and consist of organisms such as *Lactobacillus* and *Biofidobacterium* species, among others [71]. While probiotic treatment involves the administration of living microorganisms, prebiotics are indigestible foodstuffs that are fermented by colonic bacteria. This drops the colonic pH, altering the growth of the endogenous microflora in a manner that promotes the growth of *Bifidobacteria*, *Lactobacilli*, non-pathologic *E. coli*, and decreasing Bacteroidaceae [71]. Prebiotic carbohydrates include lactulose, germinated barley foodstuff, fructo-oligosaccharides, and goat's milk oligosaccharides [72]. Approaches utilizing both pro- and prebiotics have shown therapeutic benefits for IBD in animal models and in clinical trials [73]. However, additional clinical trials are needed to determine whether these approaches are beneficial in patients as the majority of existing small studies have been negative.

The 'hygiene hypothesis' regarding the pathogenesis of IBD postulates that there is a higher incidence of IBD and other autoimmune disease in industrialized nations due to the decreased rate of childhood infections. It is believed that childhood infections with pathogens such as helminths may train the immune system, altering the immune response from a Th1- predominant to a Th2- predominant phenotype [74]. Investigation of the effects of helminth ova from *Trichuris suis,* which is not a human parasite and therefore theoretically cannot colonize humans, have shown promising results in both CD and UC patients [75, 76]. Although these results are promising, further work is required to demonstrate the long-term safety and efficacy of interventions altering the intestinal microbiota.

Applications of nucleic acid-based therapies in IBD treatment

Despite the addition of biologic therapy to the IBD treatment regimen, it remains a sobering fact that these treatments are still complicated by many undesirable side effects and reactivation of the disease. Although there are many promising new agents under development for the treatment of IBD, there are inherent limitations in the delivery of many of these medications. Gene- and nucleotide-based therapies have the potential to address some of these limitations.

One advantage of a gene therapy approach is the ability to deliver genes locally, achieving production and concentration of therapeutic protein in the intestinal tissue with negligible amounts in the general circulation, thereby elim-

inating potential systemic side effects. As discussed above, some therapeutic efficacy has been demonstrated in humans using low doses of injectable recombinant human IL-10. However, its effectiveness is limited by excessive systemic adverse drug effects at higher doses. It is also evident from these clinical studies that the pharmacokinetic profile of IL-10 after subcutaneous and intravenous administration shows a low volume of distribution (i.e., IL-10 is mostly confined to the blood compartment) and a relatively short half-life (1.5–3 h) [77–79]. Therefore, limited bioavailability of IL-10 at the gut tissue may explain the relatively low efficacy of injectable IL-10 treatment. Increasing the dose of subcutaneously delivered IL-10 is expected to result in higher plasma levels and increased IL-10 tissue levels. However, as mentioned earlier, systemic administration of IL-10 at higher doses triggered a pro-inflammatory response counteracting the local anti-inflammatory effect in the GI tract. This systemic pro-inflammatory effect may not occur if IL-10 could be concentrated locally in the gut by using gene transfer techniques to achieve local expression of IL-10 protein from the intestinal epithelial cells. Indeed, Lindsay et al. have demonstrated that rectal administration of a non-replicating adenoviral vector expressing murine IL-10 was able to induce clinical and histological remission in the IL-10 deficient mouse model for colitis [53]. While this demonstrates a compelling proof-of-principle, there remain concerns regarding the safety and commercial viability of viral vectors. Some of the limitations associated with the clinical use of viral vectors include: (1) endogenous virus recombination resulting in replication competent viruses; (2) host immunogenic reactions to viral particles or activation of pre-existing immunity towards the vector, which can lead to ineffective repeated dosing due to immune reaction to the viral vector; and (3) the high cost and limited scalability of viral vector manufacturing.

Non-viral methods for gene transfer to the intestine may be a better option for clinical gene therapy for IBD. However, very few studies exist looking at the use of these non-viral vector systems for delivery of therapeutic genes to treat IBD. Preclinical studies examining the use of chitosan as carrier of plasmid DNA to cells of the GI tract appear to be particularly promising, even though most of these studies are not related to treatment of IBD. Chitosan is derived from chitin, an abundant polysaccharide found in crustacean shells. The use of chitosan as a vehicle to deliver genes to eukaryotic cells has been evaluated for almost a decade. It has been demonstrated that this polymer can mediate gene delivery to various tissues *in vivo*. Notably, chitosan is non-toxic, biocompatible, biodegradable, mucoadhesive and positively charged, making it an excellent DNA carrier to cells in mucosal epithelium. Furthermore, due to its low toxicity and biocompatibility, chitosan has been widely used in the pharmaceutical industry as a drug carrier and as a biomaterial in various wound healing applications [80–83]. In addition to chitosan being safe for clinical use, chitosan-DNA nanoparticles can be easily manufactured at large scale with relatively low cost. It has also been shown that chitosan-DNA nanoparticles are amenable to lyophilization and reconstitution without sacrificing their efficiency in carrying genes into cells, thus giving this DNA vec-

tor system the potential to be administered in an oral or a pill formulation [84]. There have been demonstrations of successful transfection of mucosal cells in the duodenum, jejunum, ileum, and colon following oral administration of chitosan-DNA nanoparticles in mice [85]. For example, a sustained increase in TGF-β protein was detectable in mice given a gavage of a suspension containing chitosan-DNA nanoparticles carrying an expression plasmid for the TGF-β gene [86]. Notably, a significant amelioration of ovalbumin-induced food allergy symptoms in the treated mice was concomitantly observed. Similarly, Roy et al. have shown that oral delivery of chitosan-DNA nanoparticles expressing the dominant peanut allergen Arah 2 [87], led to protection from anaphylactic response in a murine model for peanut allergy. Other novel non-viral gene transfer systems are also being evaluated for delivery of DNA to gut mucosal cells for the treatment of IBD. Notably, a recent study by Bhavsar and Amiji showed that oral delivery of a novel microscapsules carrying a murine-IL-10 gene was therapeutically efficacious in a mouse model for colitis [88]. In this system, the expression plasmid for IL-10 was packaged in type B gelatin nanoparticles, which were further encapsulated by poly-ε-caprolactone to form microspheres. Following oral administration of these microspheres to mice with TNBS-induced colitis, suppression of inflammatory cytokine production in the colon was observed along with increased body weight and colon length. Taken together, these preliminary animal studies provide encouraging evidence that local delivery of the IL-10 gene to the gut by oral or intrarectal route could improve the therapeutic index of IL-10 in IBD by increasing its local concentrations in the affected tissue. Although current results in small animals are promising, these non-viral vectors require further testing in relevant large animal models to evaluate their feasibility and effectiveness to safely deliver a sufficient amount of gene to provide therapeutic levels of IL-10 expression in the gut, while maintaining a low enough systemic concentration to prevent side effects.

A successful gut-directed gene delivery platform also can be used to deliver other gene targets to gut mucosal cells for expression of various proteins to counteract the pro-inflammatory activities of the various cytokines associated with IBD. For instance, it has been shown that enema administration of adenovirus expressing a soluble murine IL-17 receptor-IgG fusion protein led to suppression of inflammation in the colon of mice with TNBS-induced colitis [89]. IL-17 is produced mainly by activated CD-4^+ T cells and activates numerous potent inflammatory mediators, including IL-6, nitric oxide synthase and the CXC chemokines IL-8 and granulocyte chemotactic protein-2 [90]. Over - expression of IL-17 has been observed in serum and intestinal mucosa from patients with IBD [91]. More recently, Sugimoto et al. demonstrated that direct injection of an IL-22 gene packaged in liposomes to the inflamed colon of TCRα knockout mice led to a rapid amelioration of local intestinal inflammation [92]. Interestingly, the tissue improvement in the inflamed colon of the treated mice was associated with enhanced mucous production. This local gene delivery approach further supports the promise of gene therapy as a therapeutic

strategy for IBD. Furthermore, in light of the validated effectiveness of anti-TNF-α in treating IBD, it would be worthwhile to explore the therapeutic benefits and efficacy of localized delivery of a gene encoding a secreted TNF-α inhibitor to the gut of patients with IBD. A similar gene therapy approach is currently being developed for inflammatory arthritis and has been proven to be tolerable in a Phase I clinical trial [93]. Gene delivery technologies can also be applied to concentrate delivery of various putative growth factors (e.g., GLP-2, growth hormone, IGF-1) to promote intestinal tissue healing in subjects with IBD. Ultimately, the progress and clinical success of using gene therapy to treat IBD fundamentally depend on the availability of effective and safe gene delivery systems for the GI tract.

Antisense oligonucleotides and small interference RNA (siRNA) can also be delivered in place of DNA in order to silence expression of certain pro-inflammatory genes associated with the pathogenesis of IBD. Alicaforsen, a 20-base pair phosphorothioate antisense oligodeoxynucleotide, designed to bind to a 3' untranslated region of human ICAM-1 mRNA, is being developed as a therapy for IBD. The recruitment of inflammatory cells to the inflamed site in the gut is mediated through the interaction between specific integrin receptors on the circulating leukocytes and its ligand, intercellular adhesion molecule-1 (ICAM-1), which is expressed in the vascular endotheleum. Although some efficacy was observed in early clinical trials, later stage placebo controlled trials failed to demonstrate efficacy of alicaforsen delivered by either subcutaneous or intravenous injections. Pharmacodynamic analysis revealed that the lack of therapeutic effects from subcutaneous or intravenous administration of alicaforsen was likely due to insufficient suppression of ICAM-1 expression in the gut vasculature. To improve exposure of the affected tissue to the drug, alicaforsen was reformulated for enema delivery. A small Phase II dose-ranging, double-blind and placebo-controlled clinical study was conducted in human subjects with mild to moderate left-sided ulcerative colitis to evaluate the efficacy of daily alicaforsen enema for a 6 week study period [94]. Interestingly, no improvement in mean Disease Activity Index (DAI) at the end of the treatment period was observed between the treatment and placebo group. However, a delayed and significant improvement in mean DAI was seen in alicaforsen-treated subjects at week 18 and 30 weeks. Although the mechanism for such a delayed response is unclear, the results suggest that if sufficient alicaforsen is delivered to the diseased tissue, it may exert prolonged disease altering effects.

siRNA is a novel class of investigational pharmaceutical compounds with potent and sequence-specific gene silencing properties. They are small double-stranded RNA ranging from 22–25 nucleotides in length. Once inside the cytoplasm of the cell, siRNA bind to Argonaut 2 (AGO2) and Dicer proteins to form the RNA-induced silencing complex (RISC), resulting in the cleavage of the sense strand of siRNA by AGO2. This activated RISC-siRNA complex then seeks out and hybridizes with complementary mRNA transcript, leading to its degradation and hence silencing of the target gene [95]. Due to the novelty of siRNA and the difficulties in delivering these small nucleotides to tar-

get cells, data on using siRNA in IBD therapy is scarce and preliminary at best. Peer et al. recently developed a targeted liposomal nanoparticle system to deliver cyclinD1-specific siRNA molecules to gut-infiltrating leukocytes [96]. Antibodies to β7 integrin were used to target the liposomal particles to gut-infiltrating leukocytes. In a DSS-induced model of colitis, systemic delivery of these nanoparticles reduced severity of colitis by suppressing leukocyte infiltration and decreasing expression of Th1-type cytokines such as IFN-γ, IL-12, and IL-2. Despite these promising preliminary observations, the widespread use of siRNA therapeutics for treating IBD requires further development of an efficient delivery system.

Recent discovery of the Toll-like receptors (TLRs) has opened a new nucleotide-based treatment option for IBD. TLRs play an important function in the activation of innate immunity response to microbial pathogens. They are expressed in a wide variety of immune and epithelial cells. TLR-9 is the predominant form of TLR expressed in colonic epithelial cells. It is an intracellular receptor found only within the endosomal compartment [97]. Unmethylated CpG motifs commonly found in microbial DNA are identified as natural ligands for TLR-9. Interestingly, Rachmilewitz et al. observed that the administration CpG-laden oligodeoxynucleotides (ODN) attenuates the degree of colonic inflammation in various models of experimental colitis [98]. In a more recent study, the same research team identified a specific class of CpG ODN that is effective in inhibiting pro-inflammatory cytokine production in colonic organ cultured collected from patients with UC [99]. These ODN represent a novel class of therapeutic compound for the treatment of IBD. In light of its mechanism of action, the efficacy of such a therapeutic approach may be dependent on the ability to deliver sufficient amounts of CpG ODN to gut cells at the inflamed site. Therefore, this therapy may only be used to treat IBD patients with inflamed tissues restricted to the lower colon, which can be easily accessed by intrarectal administration of the ODN. In addition, intrarectal delivery of an ODN decoy that binds to nuclear factor-κB (NF-κB) has been shown to result in considerable reduction of colitis pathology and local tissue levels of pro-inflammatory cytokines in various murine models of IBD [100]. NF-κB is a key transcriptional regulator of immune response in the GI tract of subjects with IBD.

Concluding remarks

Considerable progress has been made in recent years towards the development of effective nucleotide-based therapies for IBD that are more targeted and, thus, less toxic than current treatments. It is clear from preclinical and initial clinical studies that emerging technologies to deliver genes, oligonucleotides, and siRNA can be effectively applied to target the diseased gut tissue in subjects with IBD. However, the observed safety profile and efficacy of these novel treatments need to be further evaluated in the appropriate preclinical ani-

mal models and later in humans. Also, it remains to be seen to what extent the safety and efficacy profiles established in animal models will be translatable to the diverse human IBD patient population, whose intestinal flora composition, disease status, diets and previous treatment exposure are bound to have some impact on the efficiency of nucleotide delivery to the gut and ultimately on the treatment efficacy. Indeed, as is the case with current clinical therapies for IBD, it remains unlikely, given the heterogeneity of IBD, that a single treatment regimen will be efficacious in all cases. However, judging from current research progress, there should be little doubt that nucleotide-based therapies will eventually become an important option in the treatment arsenal available for controlling IBD and improving the quality of lives of many who suffer from this chronic, debilitating disease.

References

1 Casellas F, Lopez-Vivancos J, Vergara M, Malagelada J (1999) Impact of inflammatory bowel disease on health-related quality of life. *Dig Dis* 17: 208–218

2 Tukel T, Shalata A, Present D, Rachmilewitz D, Mayer L, Grant D, Risch N, Desnick RJ (2004) Crohn disease: frequency and nature of CARD15 mutations in Ashkenazi and Sephardi/Oriental Jewish families. *Am J Hum Genet* 74: 623–636

3 Kappelman MD, Rifas-Shiman SL, Porter CQ, Ollendorf DA, Sandler RS, Galanko JA, Finkelstein JA (2008) Direct health care costs of Crohn's disease and ulcerative colitis in US children and adults. *Gastroenterology*, 1907–1913

4 Longobardi T, Jacobs P, Bernstein CN (2003) Work losses related to inflammatory bowel disease in the United States: results from the National Health Interview Survey. *Am J Gastroenterol* 98: 1064–1072

5 Zhang H, Massey D, Tremelling M, Parkes M (2008) Genetics of inflammatory bowel disease: clues to pathogenesis. *Br Med Bull* 87: 17–30

6 Grant SF, Baldassano RN, Hakonarson H (2008) Classification of genetic profiles of Crohn's disease: a focus on the ATG16L1 gene. *Expert Rev Mol Diagn* 8: 199–207

7 Hugot JP (2006) CARD15/NOD2 mutations in Crohn's disease. *Ann N Y Acad Sci* 1072: 9–18

8 Barnich N, Aguirre JE, Reinecker HC, Xavier R, Podolsky DK (2005) Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-{kappa}B activation in muramyl dipeptide recognition. *J Cell Biol* 170: 21–26

9 Noguchi E, Homma Y, Kang X, Netea MG, Ma X (2009) A Crohn's disease-associated NOD2 mutation suppresses transcription of human IL10 by inhibiting activity of the nuclear ribonucleoprotein hnRNP-A1. *Nat Immunol* 10: 471–479

10 Kobayashi KS, Chamaillard M, Ogura Y, Henegariu O, Inohara N, Nunez G, Flavell RA (2005) Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. *Science* 307: 731–734

11 Duerr RH, Taylor KD, Brant SR, Rioux JD, Silverberg MS, Daly MJ, Steinhart AH, Abraham C, Regueiro M, Griffiths A et al. (2006) A genome-wide association study identifies IL23R as an inflammatory bowel disease gene. *Science* 314: 1461–1463. Epub 2006 Oct 1426.

12 Hampe J, Franke A, Rosenstiel P, Till A, Teuber M, Huse K, Albrecht M, Mayr G, De La Vega FM, Briggs J et al. (2007) A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet* 39: 207–211. Epub 2006 Dec 2031.

13 Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, Green T, Kuballa P, Barmada MM, Datta LW et al. (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 39: 596–604. Epub 2007 Apr 2015.

14 Izcue A, Hue S, Buonocore S, Arancibia-Carcamo CV, Ahern PP, Iwakura Y, Maloy KJ, Powrie F (2008) Interleukin-23 restrains regulatory T cell activity to drive T cell-dependent colitis. *Immunity* 28: 559–570

15 Elson CO, Cong Y, Weaver CT, Schoeb TR, McClanahan TK, Fick RB, Kastelein RA (2007) Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology* 132: 2359–2370
16 Becker C, Dornhoff H, Neufert C, Fantini MC, Wirtz S, Huebner S, Nikolaev A, Lehr HA, Murphy AJ, Valenzuela DM et al. (2006) Cutting edge: IL-23 cross-regulates IL-12 production in T cell-dependent experimental colitis. *J Immunol* 177: 2760–2764
17 Rioux JD, Xavier RJ, Taylor KD, Silverberg MS, Goyette P, Huett A, Green T, Kuballa P, Barmada MM, Datta LW et al. (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet* 39: 596–604
18 Kim SC, Tonkonogy SL, Albright CA, Tsang J, Balish EJ, Braun J, Huycke MM, Sartor RB (2005) Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. *Gastroenterology* 128: 891–906
19 Brimnes J, Reimann J, Nissen M, Claesson M (2001) Enteric bacterial antigens activate CD4(+) T cells from scid mice with inflammatory bowel disease. *Eur J Immunol* 31: 23–31
20 Ohkusa T, Yoshida T, Sato N, Watanabe S, Tajiri H, Okayasu I (2009) Commensal bacteria can enter colonic epithelial cells and induce proinflammatory cytokine secretion: a possible pathogenic mechanism of ulcerative colitis. *J Med Microbiol* 58: 535–545
21 Arrieta MC, Madsen K, Doyle J, Meddings J (2009) Reducing small intestinal permeability attenuates colitis in the IL10 gene-deficient mouse. *Gut* 58: 41–48
22 Ferry GD (1999) Quality of life in inflammatory bowel disease: background and definitions. *J Pediatr Gastroenterol Nutr* 28: S15–18
23 von Wietersheim J, Kessler H (2006) Psychotherapy with chronic inflammatory bowel disease patients: a review. *Inflamm Bowel Dis* 12: 1175–1184
24 Karagozian R, Burakoff R (2007) The role of mesalamine in the treatment of ulcerative colitis. *Ther Clin Risk Manag* 3: 893–903
25 Cohen RD (2006) Review article: evolutionary advances in the delivery of aminosalicylates for the treatment of ulcerative colitis. *Aliment Pharmacol Ther* 24: 465–474
26 Katz JA (2004) Treatment of inflammatory bowel disease with corticosteroids. *Gastroenterol Clin North Am* 33: 171–189, vii
27 Angelucci E, Malesci A, Danese S (2008) Budesonide: teaching an old dog new tricks for inflammatory bowel disease treatment. *Curr Med Chem* 15: 2527–2535
28 Sandborn W, Sutherland L, Pearson D, May G, Modigliani R, Prantera C (2000) Azathioprine or 6-mercaptopurine for inducing remission of Crohn's disease. *Cochrane Database Syst Rev*: CD000545
29 Pearson DC, May GR, Fick G, Sutherland LR (2000) Azathioprine for maintaining remission of Crohn's disease. *Cochrane Database Syst Rev*: CD000067
30 de Boer NK, van Bodegraven AA, Jharap B, de Graaf P, Mulder CJ (2007) Drug Insight: pharmacology and toxicity of thiopurine therapy in patients with IBD. *Nat Clin Pract Gastroenterol Hepatol* 4: 686–694
31 Alfadhli AA, McDonald JW, Feagan BG (2005) Methotrexate for induction of remission in refractory Crohn's disease. *Cochrane Database Syst Rev*: CD003459
32 Soon SY, Ansari A, Yaneza M, Raoof S, Hirst J, Sanderson JD (2004) Experience with the use of low-dose methotrexate for inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 16: 921–926
33 Fraser AG (2003) Methotrexate: first-line or second-line immunomodulator? *Eur J Gastroenterol Hepatol* 15: 225–231
34 Hanauer SB, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W et al. (2002) Maintenance infliximab for Crohn's disease: the ACCENT I randomised trial. *Lancet* 359: 1541–1549
35 Present DH, Rutgeerts P, Targan S, Hanauer SB, Mayer L, van Hogezand RA, Podolsky DK, Sands BE, Braakman T, DeWoody KL et al. (1999) Infliximab for the treatment of fistulas in patients with Crohn's disease. *N Engl J Med* 340: 1398–1405
36 Colombel JF, Loftus EV Jr, Tremaine WJ, Egan LJ, Harmsen WS, Schleck CD, Zinsmeister AR, Sandborn WJ (2004) The safety profile of infliximab in patients with Crohn's disease: the Mayo clinic experience in 500 patients. *Gastroenterology* 126: 19–31
37 Vermeire S, Van Assche G, Rutgeerts P (2009) Serum sickness, encephalitis and other complications of anti-cytokine therapy. *Best Pract Res Clin Gastroenterol* 23: 101–112
38 Baker DE (2007) Natalizumab: overview of its pharmacology and safety. *Rev Gastroenterol Disord* 7: 38–46

39 Targan SR, Feagan BG, Fedorak RN, Lashner BA, Panaccione R, Present DH, Spehlmann ME, Rutgeerts PJ, Tulassay Z, Volfova M et al. (2007) Natalizumab for the treatment of active Crohn's disease: results of the ENCORE Trial. *Gastroenterology* 132: 1672–1683
40 Calabrese LH, Molloy ES (2008) Progressive multifocal leucoencephalopathy in the rheumatic diseases: assessing the risks of biological immunosuppressive therapies. *Ann Rheum Dis* 67 Suppl 3: iii64–65
41 Baumgart DC, Sandborn WJ (2007) Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* 369: 1641–1657
42 Krupnick AS, Morris JB (2000) The long-term results of resection and multiple resections in Crohn's disease. *Semin Gastrointest Dis* 11: 41–51
43 Berg DF, Bahadursingh AM, Kaminski DL, Longo WE (2002) Acute surgical emergencies in inflammatory bowel disease. *Am J Surg* 184: 45–51
44 Ding C, Xu J, Li J (2008) ABT-874, a fully human monoclonal anti-IL-12/IL-23 antibody for the potential treatment of autoimmune diseases. *Curr Opin Investig Drugs* 9: 515–522
45 Ito H, Hirotani T, Yamamoto M, Ogawa H, Kishimoto T (2002) Anti-IL-6 receptor monoclonal antibody inhibits leukocyte recruitment and promotes T-cell apoptosis in a murine model of Crohn's disease. *J Gastroenterol* 37 Suppl 14: 56–61
46 Ito H (2005) Treatment of Crohn's disease with anti-IL-6 receptor antibody. *J Gastroenterol* 40 Suppl 16: 32–34
47 Fedorak RN, Gangl A, Elson CO, Rutgeerts P, Schreiber S, Wild G, Hanauer SB, Kilian A, Cohard M, LeBeaut A et al. (2000) Recombinant human interleukin 10 in the treatment of patients with mild to moderately active Crohn's disease. The Interleukin 10 Inflammatory Bowel Disease Cooperative Study Group. *Gastroenterology* 119: 1473–1482
48 van Deventer SJ, Elson CO, Fedorak RN (1997) Multiple doses of intravenous interleukin 10 in steroid-refractory Crohn's disease. Crohn's Disease Study Group. *Gastroenterology* 113: 383–389
49 Tilg H, van Montfrans C, van den Ende A, Kaser A, van Deventer SJ, Schreiber S, Gregor M, Ludwiczek O, Rutgeerts P, Gasche C et al. (2002) Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon gamma. *Gut* 50: 191–195
50 Schreiber S, Fedorak RN, Nielsen OH, Wild G, Williams CN, Nikolaus S, Jacyna M, Lashner BA, Gangl A, Rutgeerts P et al. (2000) Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. Crohn's Disease IL-10 Cooperative Study Group. *Gastroenterology* 119: 1461–1472
51 Nakase H, Okazaki K, Tabata Y, Ozeki M, Watanabe N, Ohana M, Uose S, Uchida K, Nishi T, Mastuura M et al. (2002) New cytokine delivery system using gelatin microspheres containing interleukin-10 for experimental inflammatory bowel disease. *J Pharmacol Exp Ther* 301: 59–65
52 Lindsay JO, Sandison A, Cohen P, Brennan FM, Hodgson HJ (2004) IL-10 gene therapy is therapeutic for dextran sodium sulfate-induced murine colitis. *Dig Dis Sci* 49: 1327–1334
53 Lindsay JO, Ciesielski CJ, Scheinin T, Brennan FM, Hodgson HJ (2003) Local delivery of adenoviral vectors encoding murine interleukin 10 induces colonic interleukin 10 production and is therapeutic for murine colitis. *Gut* 52: 363–369
54 Lindsay J, Van Montfrans C, Brennan F, Van Deventer S, Drillenburg P, Hodgson H, Te Velde A, Sol Rodriguez Pena M (2002) IL-10 gene therapy prevents TNBS-induced colitis. *Gene Ther* 9: 1715–1721
55 Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, van Deventer SJ, Neirynck S, Peppelenbosch MP, Steidler L (2006) A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. *Clin Gastroenterol Hepatol* 4: 754–759
56 Waeytens A, Ferdinande L, Neirynck S, Rottiers P, De Vos M, Steidler L, Cuvelier CA (2008) Paracellular entry of interleukin-10 producing Lactococcus lactis in inflamed intestinal mucosa in mice. *Inflamm Bowel Dis* 14: 471–479
57 Steidler L, Hans W, Schotte L, Neirynck S, Obermeier F, Falk W, Fiers W, Remaut E (2000) Treatment of murine colitis by Lactococcus lactis secreting interleukin-10. *Science* 289: 1352–1355
58 Plevy S, Salzberg B, Van Assche G, Regueiro M, Hommes D, Sandborn W, Hanauer S, Targan S, Mayer L, Mahadevan U et al. (2007) A phase I study of visilizumab, a humanized anti-CD3 monoclonal antibody, in severe steroid-refractory ulcerative colitis. *Gastroenterology* 133: 1414–1422
59 Creed TJ, Probert CS, Norman MN, Moorghen M, Shepherd NA, Hearing SD, Dayan CM (2006) Basiliximab for the treatment of steroid-resistant ulcerative colitis: further experience in moderate and severe disease. *Aliment Pharmacol Ther* 23: 1435–1442

60 Van Assche G, Sandborn WJ, Feagan BG, Salzberg BA, Silvers D, Monroe PS, Pandak WM, Anderson FH, Valentine JF, Wild GE et al. (2006) Daclizumab, a humanised monoclonal antibody to the interleukin 2 receptor (CD25), for the treatment of moderately to severely active ulcerative colitis: a randomised, double blind, placebo controlled, dose ranging trial. *Gut* 55: 1568–1574
61 Polese L, Angriman I, Scarpa M, Norberto L, Sturniolo GC, Cecchetto A, Ruffolo C, D'Amico DF (2003) Role of CD40 and B7 costimulators in inflammatory bowel diseases. *Acta Biomed* 74 Suppl 2: 65–70
62 Liu Z, Geboes K, Colpaert S, Overbergh L, Mathieu C, Heremans H, de Boer M, Boon L, D'Haens G, Rutgeerts P et al. (2000) Prevention of experimental colitis in SCID mice reconstituted with CD45RBhigh CD4+ T cells by blocking the CD40–CD154 interactions. *J Immunol* 164: 6005–6014
63 Alegre ML, Fallarino F (2006) Mechanisms of CTLA-4-Ig in tolerance induction. *Curr Pharm Des* 12: 149–160
64 Davenport CM, McAdams HA, Kou J, Mascioli K, Eichman C, Healy L, Peterson J, Murphy S, Coppola D, Truneh A (2002) Inhibition of pro-inflammatory cytokine generation by CTLA4-Ig in the skin and colon of mice adoptively transplanted with CD45RBhi CD4+ T cells correlates with suppression of psoriasis and colitis. *Int Immunopharmacol* 2: 653–672
65 Slonim AE, Bulone L, Damore MB, Goldberg T, Wingertzahn MA, McKinley MJ (2000) A preliminary study of growth hormone therapy for Crohn's disease. *N Engl J Med* 342: 1633–1637
66 Drucker DJ (2002) Biological actions and therapeutic potential of the glucagon-like peptides. *Gastroenterology* 122: 531–544
67 Drucker DJ, Yusta B, Boushey RP, DeForest L, Brubaker PL (1999) Human [Gly2]GLP-2 reduces the severity of colonic injury in a murine model of experimental colitis. *Am J Physiol* 276: G79–91
68 Yazbeck R, Howarth GS, Geier MS, Demuth HU, Abbott CA (2008) Inhibiting dipeptidyl peptidase activity partially ameliorates colitis in mice. *Front Biosci* 13: 6850–6858
69 Bengmark S (2007) Bioecological control of inflammatory bowel disease. *Clin Nutr* 26: 169–181
70 Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Folsch UR, Timmis KN, Schreiber S (2004) Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. *Gut* 53: 685–693
71 Damaskos D, Kolios G (2008) Probiotics and prebiotics in inflammatory bowel disease: microflora 'on the scope'. *Br J Clin Pharmacol* 65: 453–467
72 Looijer-van Langen MA, Dieleman LA (2009) Prebiotics in chronic intestinal inflammation. *Inflamm Bowel Dis* 15: 454–462
73 Hedin C, Whelan K, Lindsay JO (2007) Evidence for the use of probiotics and prebiotics in inflammatory bowel disease: a review of clinical trials. *Proc Nutr Soc* 66: 307–315
74 Moreels TG, Pelckmans PA (2005) Gastrointestinal parasites: potential therapy for refractory inflammatory bowel diseases. *Inflamm Bowel Dis* 11: 178–184
75 Summers RW, Elliott DE, Urban JF Jr, Thompson RA, Weinstock JV (2005) Trichuris suis therapy for active ulcerative colitis: a randomized controlled trial. *Gastroenterology* 128: 825–832
76 Summers RW, Elliott DE, Qadir K, Urban JF Jr, Thompson R, Weinstock JV (2003) Trichuris suis seems to be safe and possibly effective in the treatment of inflammatory bowel disease. *Am J Gastroenterol* 98: 2034–2041
77 Radwanski E, Chakraborty A, Van Wart S, Huhn RD, Cutler DL, Affrime MB, Jusko WJ (1998) Pharmacokinetics and leukocyte responses of recombinant human interleukin-10. *Pharm Res* 15: 1895–1901
78 Huhn RD, Radwanski E, Gallo J, Affrime MB, Sabo R, Gonyo G, Monge A, Cutler DL (1997) Pharmacodynamics of subcutaneous recombinant human interleukin-10 in healthy volunteers. *Clin Pharmacol Ther* 62: 171–180
79 Huhn RD, Radwanski E, O'Connell SM, Sturgill MG, Clarke L, Cody RP, Affrime MB, Cutler DL (1996) Pharmacokinetics and immunomodulatory properties of intravenously administered recombinant human interleukin-10 in healthy volunteers. *Blood* 87: 699–705
80 Davis SS, Illum L (2003) Absorption enhancers for nasal drug delivery. *Clin Pharmacokinet* 42: 1107–1128
81 Dyer AM, Hinchcliffe M, Watts P, Castile J, Jabbal-Gill I, Nankervis R, Smith A, Illum L (2002) Nasal delivery of insulin using novel chitosan based formulations: a comparative study in two animal models between simple chitosan formulations and chitosan nanoparticles. *Pharm Res* 19: 998–1008
82 Song J, Suh CH, Park YB, Lee SH, Yoo NC, Lee JD, Kim KH, Lee SK (2001) A phase I/IIa study

on intra-articular injection of holmium-166-chitosan complex for the treatment of knee synovitis of rheumatoid arthritis. *Eur J Nucl Med* 28: 489–497
83 Wedmore I, McManus JG, Pusateri AE, Holcomb JB (2006) A special report on the chitosan-based hemostatic dressing: experience in current combat operations. *J Trauma* 60: 655–658
84 Mao HQ, Roy K, Troung-Le VL, Janes KA, Lin KY, Wang Y, August JT, Leong KW (2001) Chitosan-DNA nanoparticles as gene carriers: synthesis, characterization and transfection efficiency. *J Control Release* 70: 399–421
85 Zheng F, Shi XW, Yang GF, Gong LL, Yuan HY, Cui YJ, Wang Y, Du YM, Li Y (2007) Chitosan nanoparticle as gene therapy vector via gastrointestinal mucosa administration: results of an *in vitro* and *in vivo* study. *Life Sci* 80: 388–396
86 Li F, Wang L, Jin XM, Yan CH, Jiang S, Shen XM (2009) The immunologic effect of TGF-beta1 chitosan nanoparticle plasmids on ovalbumin-induced allergic BALB/c mice. *Immunobiology* 214: 87–99
87 Roy K, Mao HQ, Huang SK, Leong KW (1999) Oral gene delivery with chitosan – DNA nanoparticles generates immunologic protection in a murine model of peanut allergy. *Nat Med* 5: 387–391
88 Bhavsar MD, Amiji MM (2008) Oral IL-10 gene delivery in a microsphere-based formulation for local transfection and therapeutic efficacy in inflammatory bowel disease. *Gene Ther* 15: 1200–1209
89 Zhang Z, Zheng M, Bindas J, Schwarzenberger P, Kolls JK (2006) Critical role of IL-17 receptor signaling in acute TNBS-induced colitis. *Inflamm Bowel Dis* 12: 382–388
90 Kolls JK, Linden A (2004) Interleukin-17 family members and inflammation. *Immunity* 21: 467–476
91 Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, Bamba T, Fujiyama Y (2003) Increased expression of interleukin 17 in inflammatory bowel disease. *Gut* 52: 65–70
92 Sugimoto K, Ogawa A, Mizoguchi E, Shimomura Y, Andoh A, Bhan AK, Blumberg RS, Xavier RJ, Mizoguchi A (2008) IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. *J Clin Invest* 118: 534–544
93 Mease PJ, Hobbs K, Chalmers A, El-Gabalawy H, Bookman A, Keystone E, Furst DE, Anklesaria P, Heald AE (2009) Local delivery of a recombinant adeno-associated vector containing a tumor necrosis factor-{alpha} antagonist gene in inflammatory arthritis: a phase 1 dose-escalation safety and tolerability study. *Ann Rheum Dis* 68: 1247–1254
94 van Deventer SJ, Wedel MK, Baker BF, Xia S, Chuang E, Miner PB Jr, (2006) A phase II dose ranging, double-blind, placebo-controlled study of alicaforsen enema in subjects with acute exacerbation of mild to moderate left-sided ulcerative colitis. *Aliment Pharmacol Ther* 23: 1415–1425
95 Gilmore IR, Fox SP, Hollins AJ, Akhtar S (2006) Delivery strategies for siRNA-mediated gene silencing. *Curr Drug Deliv* 3: 147–155
96 Peer D, Park EJ, Morishita Y, Carman CV, Shimaoka M (2008) Systemic leukocyte-directed siRNA delivery revealing cyclin D1 as an anti-inflammatory target. *Science* 319: 627–630
97 Pedersen G, Andresen L, Matthiessen MW, Rask-Madsen J, Brynskov J (2005) Expression of Toll-like receptor 9 and response to bacterial CpG oligodeoxynucleotides in human intestinal epithelium. *Clin Exp Immunol* 141: 298–306
98 Rachmilewitz D, Karmeli F, Takabayashi K, Hayashi T, Leider-Trejo L, Lee J, Leoni LM, Raz E (2002) Immunostimulatory DNA ameliorates experimental and spontaneous murine colitis. *Gastroenterology* 122: 1428–1441
99 Rachmilewitz D, Karmeli F, Shteingart S, Lee J, Takabayashi K, Raz E (2006) Immunostimulatory oligonucleotides inhibit colonic proinflammatory cytokine production in ulcerative colitis. *Inflamm Bowel Dis* 12: 339–345
100 De Vry CG, Prasad S, Komuves L, Lorenzana C, Parham C, Le T, Adda S, Hoffman J, Kahoud N, Garlapati R et al. (2007) Non-viral delivery of nuclear factor-kappaB decoy ameliorates murine inflammatory bowel disease and restores tissue homeostasis. *Gut* 56: 524–533

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Gene therapy for diabetes

Hirofumi Noguchi

Regenerative Research Islet Cell Transplant Program, Baylor All Saints Medical Center, Baylor Research Institute, Fort Worth, TX, USA

Abstract

Diabetes mellitus is a devastating disease and the World Health Organization expects that the number of diabetic patients will increase to 300 million by the year 2025. Intensive glycemic control with insulin therapy to both Type 1 and Type 2 diabetic patients can reduce the risk of diabetic complications, but also increase the incidence of hypoglycemia. Many studies have shown the possibility of gene therapy for treatment of diabetes. Gene therapy can be successfully applied to treat Type 1 diabetes (T1D) and to facilitate the replacement of insulin-producing cells by islet transplantation, by differentiation of stem/progenitor cells, or by reversible immortalization in rodent models. Here we review potential approaches for the development of gene therapies for diabetes.

Introduction

Type 1 diabetes (T1D) results from autoimmune-mediated destruction of insulin-producing β cells in the islets of Langerhans of the pancreas. Failure in self tolerance towards β cells involves a series of complex events that are governed by environmental and genetic factors. T1D is initiated and/or progresses when genetically predisposed individuals encounter an environmental insult [1–4]. More than 20 chromosomal loci contribute to Type 1 diabetes susceptibility, with the most prominent genes identified to date encoding specific alleles of major histocompatibility complex (MHC) [2, 5–11], insulin [12], and CTLA-4 [13, 14]. Environmental agents influencing T1D susceptibility remain largely undefined, although microbial infections and diet have been implicated [1–4]. The major effectors of β cell destruction are $CD4^+$ and $CD8^+$ T cells that appear to be reactive to antigens expressed by β cells, although other immune cells, including B cells, NKT cells, dendritic cells (DCs), and macrophages are also involved in the development of diabetes [1–4]. Moreover, a reduced frequency and/or aberrant function of regulatory T cells expressing CD4, CD25, and FoxP3, have been reported in diabetic patients [15–17].

Many studies have showed that intensive glycemic control with insulin therapy to both Type 1 and Type 2 diabetic patients can reduce the risk of diabetic complications, but is also associated with an increased incidence of hypoglycemia [18–20]. It seems logical that replacement of the islet tissue itself, either by transplanting a pancreatic organ or by transplanting purified pancre-

atic islets, offers a better approach than simply replacing insulin that has been lost. Islet allotransplantation can achieve insulin independence in Type 1 diabetic patients without major surgery [21, 22]. Despite the promise offered by this approach, logistical hurdles necessitate a comprehensive strategy aimed at different molecular and cellular determinants of the autoimmune pathology of T1D. Moreover, the clinical benefit of this protocol can be provided only to a small minority of patients and is not permanent. Current isolation techniques usually recover fewer than half the islets from a given pancreas, necessitating islet transplantation from two or more donors to achieve euglycemia [21, 22]. Nonetheless, the promising results afforded by islet transplantation, coupled with the shortage of cadaver pancreases relative to the potential demand, have lent strong impetus to the search for new sources of insulin-producing cells. Here we review possibilities for the development of gene therapy for diabetes, especially Type 1 diabetes.

Approaches for treating Type 1 diabetes by gene transfer

Prevention of autoimmunity by gene therapy

The etiopathogenesis of T1D is most comprehensively understood in rodent models, especially the non-obese diabetic (NOD) and biobreeding (BB) rat strains [2, 5–11, 23]. Immunopathologically, these strains exhibit progressive infiltration of leukocytes around and inside the islets, which is associated with the eradication of almost all β cells. The immune-mediated processes of β cell destruction in T1D are highly dependent on the MHC [2, 5], at least in the NOD mouse and the BB rat models. Transgenic NOD mice with different MHC Class I and Class II alleles do not develop diabetes, indicating that antigen presentation is an important determinant of disease onset or development [6, 7]. Based on this observation, one attractive hypothesis has been that T1D is primarily due to failure of negative selection of autoreactive T cells, either in the thymus or in the periphery, or to a breakdown in tolerance to β cell-specific antigen. If T1D is the result of a failure to delete lymphocytes in the thymus that would normally recognize β cell antigen, it is reasonable to propose that thymic overexpression of putative autoantigens in early life, before the onset of Type 1 diabetes in individuals deemed at high risk by genetic screening criteria, could prevent the disease. This line of reasoning was initially adopted in studying diabetes in the NOD mouse as well as in the BB rat. In NOD mice, the expression of a proinsulin transgene controlled by the MHC Class II promoter, resulting in intrathymic expression of insulin, was able to prevent diabetes [6]. This outcome was also achieved in BB rats as well as in young NOD mice by intrathymic injection of islet extracts [8–11]. Intrathymic injection of insulin B-chain or the 65 kD variant of glutamic acid decarboxylase (GAD) into young NOD mice also was able to suppress the onset of T1D [23]. These data suggest that thymic overexpression of islet autoantigens, such

as proinsulin and GAD, before the onset of Type 1 diabetes in individuals could prevent the disease.

Another useful approach could be to mobilize dendritic cells *in vivo* by vaccine-based methods. The direct injection of viral vectors encoding immunoregulatory transgenes has achieved prolonged delay in diabetes incidence in the NOD mouse [24, 25] and follow-up to some of these approaches indicates the augmentation of regulatory T cell subsets [26–29].

Islet transplantation with immunoregulatory gene transfection

Islet allotransplantation can achieve insulin independence in Type 1 diabetic patients without major surgery. However, the requirement and side effects of pharmacological immunosuppressives and the reliance on cadaveric donors are significant impediments to widespread implementation. Studies in rodent models of allogeneic islet transplantation in the presence or absence of autoimmune diabetes indicate that islets engineered to express immunoregulatory molecules exhibit considerable refractoriness to rejection and, in certain examples, long-term survival. Gene transfer of cytotoxic T lymphocyte-associated protein 4 (CTLA-4) with an immunoglobulin (Ig)-G1 Fc protein (CTLA-4Ig) to islets *in vitro* followed by transplantation has shown that its local expression can result in prolongation of allogeneic as well as xenogeneic graft survival [30–32]. The CD28 molecule is located at the surface of $CD4^+$ T cells and delivers a costimulatory signal. CTLA4-Ig prevents the interaction between CD28–CD80/86. In a nonhuman primate model, the combination of the following drugs: LEA29Y (an engineered CTLA4-Ig molecule with increased CD86 and CD80 binding activity), sirolimus, and the anti-IL-2R regimen has significantly prolonged islet allograft survival [33]. The cotransplantation of allogeneic murine islets with syngeneic myoblasts engineered to express CTLA-4Ig led to prolongation of islet survival [34]. The CD154 molecule is also located at the surface of $CD4^+$ T cells and delivers a costimulatory signal. The costimulatory blocking agent, anti-CD154 which prevents the CD40–CD154 interaction, has reached the preclinical stage. Islet transplantation with the use of anti-CD154 monotherapy consistently allows for allogeneic islet engraftment and long-term insulin independence in this highly relevant preclinical model [35]. From these studies, the concept of maintenance therapy with costimulatory blocking agents has emerged as a valid strategy for clinical islet transplantation. A clinical trial utilizing humanized anti-CD154 mAb in recipients of solitary islet transplants commenced in 1999; however, it was reported that unusual thromboembolic complications occurred in kidney transplant recipients receiving mAb in a concurrent trial [36, 37]. To circumvent this potential complication, an Emory University group developed a chimeric antibody targeting CD40 as an alternative to CD154. Anti-CD40 combined with LEA29Y dramatically facilitates long-term islet allograft survival [38]. Therefore, gene transfer of anti-CD40 to islets *in vitro* followed by transplantation could prolong islet allograft survival.

Induction of insulin-producing cells from stem/progenitor cells by gene transfection

The success achieved over the last few years with islet transplantation suggest that diabetes can be cured by the replenishment of deficient β cells. These observations are proof of concept and have intensified interest in treating diabetes or other diseases not only by cell transplantation, but also by stem cells. It has been shown that overexpression of embryonic transcription factors, such as PDX-1, Ngn3, BETA2/NeuroD, Pax4, and/or MafA in stem cells could efficiently induce their differentiation into insulin-expressing cells. Overexpression of PDX-1 induced insulin expression in pancreatic ductal cells [39, 40] or the liver [41, 42], and improved the glucose tolerance of streptozotocin-induced diabetic mice [41, 42]. When the expression of Ngn3 is directed ectopically into the embryonic epithelium, pancreas precursor cells develop prematurely and exclusively into glucagon-producing cells [43, 44]. Overexpression of Ngn3 in ductal progenitor cells induced differentiation into insulin-producing cells [45]. Adenoviral-mediated introduction of BETA2/NeuroD induced β cell neogenesis in the liver and reversed diabetes in mice with betacellulin gene therapy [46] or PDX-1/VP16 gene therapy [42]. The constitutive expression of Pax4 leads to the formation of islet-like spheroid structures that produce increased levels of insulin [47]. MafA overexpression, concomitant with PDX-1 and NeuroD, markedly increased insulin gene expression in the liver and dramatically ameliorated glucose tolerance in diabetic mice [48]. Recently, it was demonstrated that adenoviral-mediated gene delivery induced *in vivo* reprogramming of adult pancreatic exocrine cells to β cells. A specific combination of three transcription factors (PDX-1, Ngn3, and MafA) reprogrammed differentiated pancreatic exocrine cells in adult mice into cells that closely resembled β cells. The induced β cells are indistinguishable from endogenous islet β cells in size, shape and ultrastructure. They express genes essential for beta-cell function and can ameliorate hyperglycemia by remodeling local vasculature and secreting insulin [49].

Reversible immortalization of pancreatic β cells by gene transfer and site-specific recombination

A strategy called reversible immortalization has been used to temporarily 'immortalize' primary cells in order to obtain populations of primary cells that actively divide *in vitro* without entering senescence [50]. The strategy involves introducing a construct containing immortalizing genes into primary cells, expanding the cells in culture, and finally efficiently removing the immortalizing genes using the Cre/LoxP system. The construct contains the simian virus 40 gene encoding the large tumor antigen (SV40Tag) and/or human telomerase reverse transcriptase (hTERT). Our group reported the transformation of human primary β cells with immortalizing genes of SV40Tag and

hTERT [51]. Our reversibly immortalized pancreatic β cell clone (NAKT-15) secreted insulin in response to glucose stimulation and non-glucose secretagogues, resulting in the expression of proteins characteristic of β cells such as PDX-1 and secretory granule proteins such as chromogranin A and synaptophysin. NAKT-15 cells did not senesce after more than 50 passages in culture and could be continuously expanded. Transplantation into diabetic mice of NAKT-15 cells reverted by Cre protein resulted in perfect control of blood glucose within 2 weeks. The mice remained normoglycemic for longer than 30 weeks. No tumors were noted on necropsy of transplanted mice.

Reversible immortalization of human pancreatic β cells by lentivector-mediated transfer of a specific gene [52] or by regulatory elements of the bacterial tetracycline (tet) operon for conditional expression of SV40Tag oncoprotein in transgenic mouse β cells [53] were also reported. One advantage of these immortalized cells is that further genetic manipulations, such as inserting genes for immunosuppressive proteins, should be relatively easy compared to primary β cells because the immortalized cells divide actively. The establishment of reversibly-immortalized pancreatic β cells is one step toward overcoming the limitation of transplanting primary pancreatic β cells to control diabetes.

Conclusions

Diabetes gene therapy appears promising in light of experiments showing that it can result in prevention of autoimmunity, overcome the need for immunosuppression after islet transplantation, and obtaining larger populations of pancreatic β cells. Both *in vivo* and *in vitro* approaches have promise, and further investigation of the safety of gene therapy is needed. Major advances have recently been made in protein transduction technology where novel proteins and peptides can be delivered into cells [39, 54–56]. The protein transduction system has low toxicity and a high delivery yield. The use of this system opens up interesting therapeutic possibilities. We have developed an immunosuppressive agent for islet transplantation using protein transduction technology [54]. We and other groups have shown that protein transduction of PDX-1, BETA2/NeuroD, and TAT-Ngn3 into stem/progenitor cells induces insulin gene expression [39, 55, 56], suggesting that protein transduction domain-mediated delivery of PDX-1, BETA2/NeuroD, and/or TAT-Ngn3 could be a safe and valuable strategy for facilitating the differentiation of ductal progenitor cells into insulin-producing cells without requiring gene transfer technology. Protein transduction has some disadvantages compared to gene transduction including transient expression and lower efficiency. Gene therapy with safer vectors/strategies and protein therapy could be much better than the systemic effects and toxicities which are associated with many drugs in use today.

Acknowledgements
The author wishes to thank Dr. Steven Phillips for his careful reading and editing of this manuscript. This work was supported in part by the All Saints Health Foundation.

References

1 Anderson MS, Bluestone JA (2005) The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 23: 447–485
2 Bach JF (1994) Insulin-dependent diabetes mellitus as an autoimmune disease. *Endocr Rev* 15: 516–542
3 Eisenbarth GS (2004) Prediction of type 1 diabetes: the natural history of the prediabetic period. *Adv Exp Med Biol* 552: 268–290
4 Tisch R, McDevitt H (1996) Insulin-dependent diabetes mellitus. *Cell* 85: 291–297
5 Eisenbarth GS (1993) Molecular aspects of the etiology of type I diabetes mellitus. *J Diabetes Complications* 7: 142–150
6 French MB, Allison J, Cram DS, Thomas HE, Dempsey-Collier M, Silva A, Georgiou HM, Kay TW, Harrison LC, Lew AM (1997) Transgenic expression of mouse proinsulin II prevents diabetes in nonobese diabetic mice. *Diabetes* 46: 34–39
7 Miyazaki T, Matsuda Y, Toyonaga T, Miyazaki J, Yazaki Y, Yamamura K (1992) Prevention of autoimmune insulitis in nonobese diabetic mice by expression of major histocompatibility complex class I Ld molecules. *Proc Natl Acad Sci U S A* 89: 9519–9523
8 Posselt AM, Naji A, Roark JH, Markmann JF, Barker CF (1991) Intrathymic islet transplantation in the spontaneously diabetic BB rat. *Ann Surg* 214: 363–371
9 Posselt AM, Barker CF, Friedman AL, Naji A (1992) Prevention of autoimmune diabetes in the BB rat by intrathymic islet transplantation at birth. *Science* 256: 1321–1324
10 Koevary SB, Blomberg M (1992) Prevention of diabetes in BB/Wor rats by intrathymic islet injection. *J Clin Invest* 89: 512–516
11 Charlton B, Taylor-Edwards C, Tisch R, Fathman CG (1994) Prevention of diabetes and insulitis by neonatal intrathymic islet administration in NOD mice. *J Autoimmun* 7: 549–560
12 Awata T, Kawasaki E, Ikegami H, Kobayashi T, Maruyama T, Nakanishi K, Shimada A, Iizuka H, Kurihara S, Osaki M, Uga M, Kawabata Y, Tanaka S, Kanazawa Y, Katayama S (2007) Insulin gene/IDDM2 locus in Japanese type 1 diabetes: contribution of class I alleles and influence of class I subdivision in susceptibility to type 1 diabetes. *J Clin Endocrinol Metab* 92: 1791–1795
13 Hill NJ, Lyons PA, Armitage N, Todd JA, Wicker LS, Peterson LB (2000) NOD Idd5 locus controls insulitis and diabetes and overlaps the orthologous CTLA4/IDDM12 and NRAMP1 loci in humans. *Diabetes* 49: 1744–1747
14 Ueda H, Howson JM, Esposito L, Heward J, Snook H, Chamberlain G, Rainbow DB, Hunter KM, Smith AN, Di Genova G et al. (2003) Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. *Nature* 423: 506–511
15 Arif S, Tree TI, Astill TP, Tremble JM, Bishop AJ, Dayan CM, Roep BO, Peakman M (2004) Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. *J Clin Invest* 113: 451–463
16 Brusko TM, Wasserfall CH, Clare-Salzler MJ, Schatz DA, Atkinson MA (2005) Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes. *Diabetes* 54: 1407–1414
17 Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI (2005) Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. *Diabetes* 54: 92–99
18 The Diabetes Control and Complications Trial Research Group (1993) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N Engl J Med* 329: 977–986
19 UK Prospective Diabetes Study (UKPDS) Group (1998) Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet* 352: 837–853
20 Ohkubo Y, Kishikawa H, Araki E, Miyata T, Isami S, Motoyoshi S, Kojima Y, Furuyoshi N, Shichiri M (1995) Intensive insulin therapy prevents the progression of diabetic microvascular complications in Japanese patients with non-insulin-dependent diabetes mellitus: a randomized prospective 6-year study. *Diabetes Res Clin Pract* 28: 103–117
21 Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV (2000) Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med* 343: 230–238
22 Noguchi H, Iwanaga Y, Okitsu T, Nagata H, Yonekawa Y, Matsumoto S (2006) Evaluation of islet transplantation from non-heart beating donors. *Am J Transplant* 6: 2476–2482

23 Cetkovic-Cvrlje M, Gerling IC, Muir A, Atkinson MA, Elliott JF, Leiter EH (1997) Retardation or acceleration of diabetes in NOD/Lt mice mediated by intrathymic administration of candidate beta-cell antigens. *Diabetes* 46: 1975–1982
24 Prud'homme GJ, Chang Y (1999) Prevention of autoimmune diabetes by intramuscular gene therapy with a nonviral vector encoding an interferon-gamma receptor/IgG1 fusion protein. *Gene Ther* 6: 771–777
25 Piccirillo CA, Chang Y, Prud'homme GJ (1998) TGF-beta1 somatic gene therapy prevents autoimmune disease in nonobese diabetic mice. *J Immunol* 161: 3950–3956
26 Morse MA, Deng Y, Coleman D, Hull S, Kitrell-Fisher E, Nair S, Schlom J, Ryback ME, Lyerly HK (1999) A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. *Clin Cancer Res* 5: 1331–1338
27 Heiser A, Maurice MA, Yancey DR, Coleman DM, Dahm P, Vieweg J (2001) Human dendritic cells transfected with renal tumor RNA stimulate polyclonal T-cell responses against antigens expressed by primary and metastatic tumors. *Cancer Res* 61: 3388–3393
28 Dhodapkar MV, Steinman RM, Krasovsky J, Munz C, Bhardwaj N (2001) Antigen-specific inhibition of effector T cell function in humans after injection of immature dendritic cells. *J Exp Med* 193: 233–238
29 Prud'homme GJ (2003) Prevention of autoimmune diabetes by DNA vaccination. *Expert Rev Vaccines* 2: 533–540
30 Lenschow DJ, Zeng Y, Thistlethwaite JR, Montag A, Brady W, Gibson MG, Linsley PS, Bluestone JA (1992) Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science* 257: 789–792
31 Guo Z, Mital D, Mo YY, Tian Y, Shen J, Chong AS, Foster P, Sankary H, McChesney L, Jensik SC, Williams JW (1998) Effect of gene gun-mediated CTLA4IG and Fas ligand gene transfection on concordant xenogeneic islet graft rejection. *Transplant Proc* 30: 589
32 Gainer AL, Korbutt GS, Rajotte RV, Warnock GL, Elliott JF (1997) Expression of CTLA4-Ig by biolistically transfected mouse islets promotes islet allograft survival. *Transplantation* 63: 1017–1021
33 Adams AB, Shirasugi N, Durham MM, Strobert E, Anderson D, Rees P, Cowan S, Xu H, Blinder Y, Cheung M et al. CP (2002) Calcineurin inhibitor-free CD28 blockade-based protocol protects allogeneic islets in nonhuman primates. *Diabetes* 51: 265–270
34 Chahine AA, Yu M, McKernan MM, Stoeckert C, Lau HT (1995) Immunomodulation of pancreatic islet allografts in mice with CTLA4Ig secreting muscle cells. *Transplantation* 59: 1313–1318
35 Kenyon NS, Chatzipetrou M, Masetti M, Ranuncoli A, Oliveira M, Wagner JL, Kirk AD, Harlan DM, Burkly LC, Ricordi C (1999) Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. *Proc Natl Acad Sci U S A* 96: 8132–8137
36 Kawai T, Andrews D, Colvin RB, Sachs DH and Cosimi AB (2000) Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* 6: 114
37 Buhler L, Alwayn IP, Appel JZ 3rd, Robson SC and Cooper DK (2001) Anti-CD154 monoclonal antibody and thromboembolism. *Transplantation* 71: 491
38 Adams AB, Shirasugi N, Jones TR, Durham MM, Strobert EA, Cowan S, Rees P, Hendrix R, Price K, Kenyon NS et al. (2005) Development of a chimeric anti-CD40 monoclonal antibody that synergizes with LEA29Y to prolong islet allograft survival. *J Immunol* 174: 542–550
39 Noguchi H, Kaneto H, Weir GC, Bonner-Weir S (2003) PDX-1 protein containing its own antennapedia-like protein transduction domain can transduce pancreatic duct and islet cells. *Diabetes* 52: 1732–1737
40 Taniguchi H, Yamato E, Tashiro F, Ikegami H, Ogihara T, Miyazaki J (2003) beta-cell neogenesis induced by adenovirus-mediated gene delivery of transcription factor pdx-1 into mouse pancreas. *Gene Ther* 10: 15–23
41 Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A (2000) Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. *Nat Med* 6: 568–572
42 Kaneto H, Nakatani Y, Miyatsuka T, Matsuoka TA, Matsuhisa M, Hori M, Yamasaki Y (2005) PDX-1/VP16 fusion protein, together with NeuroD or Ngn3, markedly induces insulin gene transcription and ameliorates glucose tolerance. *Diabetes* 54: 1009–1022
43 Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendahl U, Edlund H (1999) Notch signalling controls pancreatic cell differentiation. *Nature* 400: 877–881

44 Schwitzgebel VM, Scheel DW, Conners JR, Kalamaras J, Lee JE, Anderson DJ, Sussel L, Johnson JD, German MS (2000) Expression of neurogenin3 reveals an islet cell precursor population in the pancreas. *Development* 127: 3533–3542
45 Heremans Y, Van De Casteele M, in't Veld P, Gradwohl G, Serup P, Madsen O, Pipeleers D, Heimberg H (2002) Recapitulation of embryonic neuroendocrine differentiation in adult human pancreatic duct cells expressing neurogenin 3. *J Cell Biol* 159: 303–312
46 Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, Chan L (2003) NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. *Nat Med* 9: 596–603
47 Blyszczuk P, Czyz J, Kania G, Wagner M, Roll U, St-Onge L, Wobus AM (2003) Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells. *Proc Natl Acad Sci U S A* 100: 998–1003.
48 Kaneto H, Matsuoka TA, Nakatani Y, Miyatsuka T, Matsuhisa M, Hori M, Yamasaki Y (2005) A crucial role of MafA as a novel therapeutic target for diabetes. *J Biol Chem* 280: 15047–15052
49 Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA (2008) *In vivo* reprogramming of adult pancreatic exocrine cells to beta-cells. *Nature* 455: 627–632
50 Westerman KA, Leboulch P (1996) Reversible immortalization of mammalian cells mediated by retroviral transfer and site-specific recombination. *Proc Natl Acad Sci U S A* 93: 8971–8976
51 Narushima M, Kobayashi N, Okitsu T, Tanaka Y, Li SA, Chen Y, Miki A, Tanaka K, Nakaji S, Takei K et al. (2005) A human beta-cell line for transplantation therapy to control type 1 diabetes. *Nat Biotechnol* 23: 1274–1282
52 Salmon P, Oberholzer J, Occhiodoro T, Morel P, Lou J, Trono D (2000) Reversible immortalization of human primary cells by lentivector-mediated transfer of specific genes. *Mol Ther* 2: 404–414
53 Milo-Landesman D, Surana M, Berkovich I, Compagni A, Christofori G, Fleischer N, Efrat S (2001) Correction of hyperglycemia in diabetic mice transplanted with reversibly immortalized pancreatic beta cells controlled by the tet-on regulatory system. *Cell Transplant* 10: 645–650
54 Noguchi H, Matsushita M, Okitsu T, Moriwaki A, Tomizawa K, Kang S, Li ST, Kobayashi N, Matsumoto S, Tanaka K et al. (2004) A new cell-permeable peptide allows successful allogeneic islet transplantation in mice. *Nat Med* 10: 305–309
55 Noguchi H, Bonner-Weir S, Wei FY, Matsushita M, Matsumoto S (2005) BETA2/NeuroD protein can be transduced into cells due to an arginine- and lysine-rich sequence. *Diabetes* 54: 2859–2866
56 Dominguez-Bendala J, Klein D, Ribeiro M, Ricordi C, Inverardi L, Pastori R, Edlund H (2005) TAT-mediated neurogenin 3 protein transduction stimulates pancreatic endocrine differentiation *in vitro*. *Diabetes* 54: 720–726

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Gene therapy for cystic fibrosis lung disease

Stephanie G. Sumner-Jones, Deborah R. Gill and Stephen C. Hyde

Gene Medicine Group, Nuffield Department of Clinical Laboratory Sciences, John Radcliffe Hospital, Oxford, UK
United Kingdom Cystic Fibrosis Gene Therapy Consortium

Abstract

Cystic fibrosis (CF) is characterised by respiratory and pancreatic deficiencies that stem from the loss of fully functional CFTR (CF transmembrane conductance regulator) at the membrane of epithelial cells. Current treatment modalities aim to delay the deterioration in lung function, which is mostly responsible for the relatively short life expectancy of CF sufferers; however none have so far successfully dealt with the underlying molecular defect. Novel pharmacological approaches to ameliorate the lack of active CFTR in respiratory epithelial cells are beginning to address more of the pathophysiological defects caused by CFTR mutations. However, CFTR gene replacement by gene therapy remains the most likely option for addressing the basic defects, including ion transport and inflammatory functions of CFTR. In this chapter, we will review the latest preclinical and clinical advances in pharmacotherapy and gene therapy for CF lung disease.

Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder among Caucasians. It is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene identified by positional cloning in 1989 [1–3], which encodes a chloride (Cl^-) channel expressed in the epithelia of many tissues. The gene encodes a single polypeptide chain of 1480 amino acids, with a predicted molecular weight of around 168 kDa [1]. The CFTR protein is embedded in the apical membrane of epithelial cells and is made up of distinct structural domains, including two membrane spanning domains (MSDs), two nucleotide binding domains (NBD) containing conserved motifs for ATP binding and hydrolysis, and a regulatory domain (R) [4]. The CFTR protein, a member of the ABC family of proteins, normally functions as a cAMP-activated Cl^- channel [5], and has been shown to interact with other ion channels and transporters, such as the amiloride-sensitive epithelial sodium channel, ENaC (recently reviewed by Berdiev et al. [6]) and the outwardly rectifying chloride channel (ORCC) (reviewed by Kunzelmann, [7]).

CFTR mutations

The Cystic Fibrosis Mutation Database (http://www.genet.sickkids.on.ca) currently lists 1604 mutations located throughout the gene and affecting all domains of the protein. The most common mutation ΔF508, is found in 70% of disease alleles [2], and is caused by a deletion of three consecutive base pairs, resulting in the loss of a phenylalanine (F508) [1]. Mutations in the CFTR gene are grouped into five classes, based on their effect on CFTR protein expression and/or function: Class I–III mutations commonly cause severe disease phenotypes, Class IV and V mutations tend to be associated with milder disease, although not systematically for lung disease which can be highly variable even within identical genetic backgrounds [8, 9].

Pathophysiology of CF lung disease

Mutations in CFTR disrupt transport in the epithelium of several tissues, which results in the production of abnormally thick, sticky mucus. The main cause of morbidity in CF is lung disease [10], with deterioration of lung function and pulmonary failure being the cause of death for the majority (>90%) of patients. The production of thick, sticky mucus in the lumen of the lung impedes mucociliary clearance [11], a consequence of which is chronic inflammation and recurrent bacterial infections (typically *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex), in a self-perpetuating cycle that leads to the progressive destruction of lung tissue [10]. In severe cases, *P. aeruginosa* can form antibiotic resistant biofilms in the lumen of the airways, the presence of which correlates with a decline in lung function [12].

Airway epithelial cells (AEC) are covered in air surface liquid (ASL), made up of a mucus layer that traps potentially harmful particulate matter that is inhaled, and the periciliary liquid (PCL) layer. The PCL provides a less viscous layer for the cilia to beat and remove the mucus (containing the trapped particles) from the airways by mucociliary clearance. The PCL also acts as a lubricant between the mucus layer and the mucins tethered to the cell surface to facilitate cough clearance [13, 14]. Finally PCL contains antimicrobial peptides and proteins (e.g., defensins, lysozyme, lactoferrin and anti-microbial surfactant proteins) to fight pathogens [15]. In CF these processes are disrupted, causing dysregulation of liquid movement, and lung infection and inflammation [16].

The low volume hypothesis to explain CF lung disease

The low volume hypothesis [17], also know as the ‘isotonic volume transport/mucus clearance’ theory, implicates reduced ASL volume as the initiating event in CF lung pathology. The airway epithelium is thought to regulate ASL

volume so that the height of the PCL layer is approximately the same as that of the extended cilia on the cell surface of the epithelium (around 7 μm) [18], allowing them to beat efficiently. In CF airways, the ASL volume and hence PCL height is decreased, making the mucus layer sticky and harder to move [18]. The resulting flattening of the cilia prevents them from beating [19], causing mucus to adhere to cells [14]. Together these events lead to defective mucociliary clearance, initiating the chronic cycle of infection and inflammation characteristic of CF lungs (reviewed by Boucher, 2007 [20]).

There is now a significant amount of evidence to support the low volume hypothesis in primary human cell culture and mouse models. Most recently, accelerated Na^+ absorption, leading to a decrease in PCL height and reduced mucociliary clearance was demonstrated *in vivo* in the ENaCβ over-expressing mouse (reviewed by Mall, 2008 [21]) and in primary air-liquid interface (ALI) cultures from CF patients [18, 19].

The recent availability of the ENaCβ over-expressing mouse, with its lung pathology that closely mirrors that of CF patients, has provided a powerful tool for understanding the links between CFTR deficiencies and the complex pathophysiology of CF. For example sterile inflammation is also observed in these mice [22], supporting the hypothesis that inflammation can occur independently of infection.

Pharmacological approaches to treat CF lung disease

Pharmacological processes that restore effective ASL height and mucociliary clearance in CF patients could be targeted to upregulate CFTR activity, modify alternative channels (ENaC, Calcium-activated Cl^- channel (CaCC)), or rehydrate the ASL with hyperosmotic agents such as inhaled mannitol and hypertonic saline [23, 24]. Current approaches to pharmacological correction of CFTR include: 1) drugs that increase the level of CFTR protein synthesised, 2) CFTR correctors to increase trafficking out of the ER, and 3) CFTR potentiators that correct gating defects of CFTR at the membrane (Fig. 1).

Drugs that increase CFTR protein levels

Several strategies to increase the amount of CFTR at the cell surface have been investigated, including increasing overall transcription levels with butyrate [25] or sodium 4-phenylbutyrate (4-PBA) [26], although neither has had an impact in clinical trials. Several drugs that promote read-through of nonsense stop codons have been shown to produce full-length CFTR, including aminoglycoside antibiotics (e.g., gentamicin) [27, 28] or the recently developed PTC124 [29]. The latter appears capable of improving electrophysiological features of nasal epithelium and several clinically relevant outcome measures (FEV1, FVC, circulating neutrophils) [30–32]. Interestingly it has been

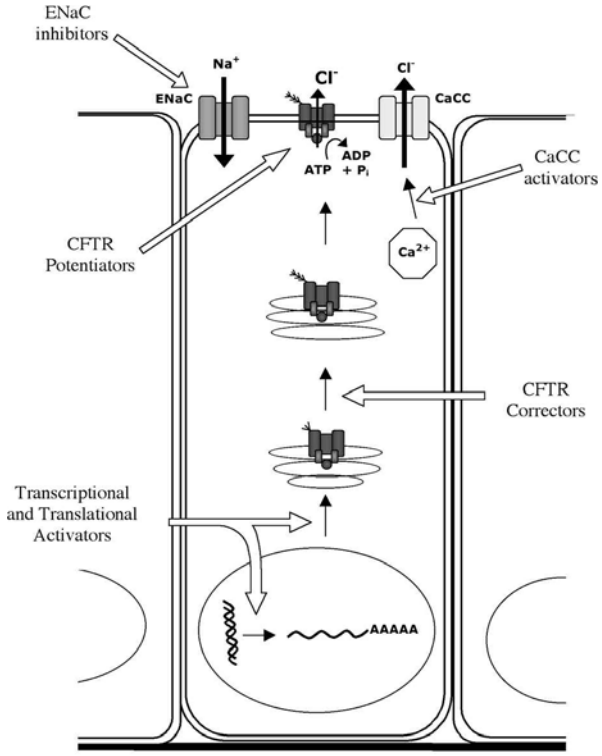


Figure 1. Schematic representation of sites of action for pharmacological correction of CFTR deficiency. Pharmacological drugs that are being investigated for the correction of CFTR deficiency may act by increasing the level of mRNA by enhancing transcription or translation ('Transcriptional and Translational Activators'), correcting the trafficking defect through the endoplasmic reticulum ('CFTR Correctors'), increasing activity of CFTR at the membrane ('CFTR Potentiators'), or regulating other ion channels such as Epithelial Na^+ Channel (ENaC) ('ENaC inhibitors') or Ca^{2+}-activated Cl channel (CaCC). Sites of action of these drugs in an airway epithelial cell are indicated by the white arrows, relative to CFTR biosynthesis and ATP-dependent channel activity, and relative to the pathways for activation and inhibition of CaCC and ENaC respectively.

reported that a drug such as PTC124 may not be effective in all cases of premature stop codons, due to exonic skipping which removes the early stop codon from the mature mRNA [33].

CFTR correctors, drugs to increase trafficking of CFTR

Enhancing trafficking of CFTR using chemical chaperones can increase CFTR levels in the membrane in preclinical studies [34–36]. Recent efforts to identify more CFTR-specific correctors have involved high-throughput screening [37–40]. The phosphodiesterase-5 (PDE-5) inhibitor, sildenafil, shows promis-

ing correction of the CF defect *in vitro* [41] and is currently in Phase I/II clinical studies expected to conclude in 2010. KM1160, a much more potent analogue of sildenafil, is in early stages of development [42]. A different corrector, Miglustat, functions through inhibition of α-1,2-glucosidase, thus preventing rapid degradation of ΔF508 in the ER, and has shown promise in multiple preclinical studies [43–47]. Miglustat is now in a Phase II trial (Actelion). VX-809 is a ΔF508 CFTR corrector that was discovered through a collaborative programme, between Vertex and Cystic Fibrosis Foundation Therapeutics Inc. It recently entered a Phase II safety/efficacy study [48]. Results of both these studies are eagerly awaited.

CFTR potentiators, drugs that increase conductance at the apical membrane

Other potential drug therapies have been based on increasing the activation of CFTR, by increasing the level of cyclic nucleotides in the cell using phosphodiesterase (PDE) inhibitors such as milrinone [49, 50], or by direct activation with curcumin [51], genistein or 8-cyclopentyl-1,3-dipropylxanthine (CPX) [52]. The most promising advances involve Vertex compound VX-770 [53], a drug that increases the open probability and Cl^- conductance of CFTR; it has completed a Phase IIa trial [54] and entered the FDA registration programme in 2009.

Modulation of other ion channels

Upregulation of CaCC (Ca^{2+}-activated Cl^- channel) by Denufosol and Moli1901 (Lancovutide®) was recently demonstrated to be a safe and tolerable way of inducing some Cl^- transport via non-CFTR channels [55–58]. Further evaluation of efficacy in CF patients for both drugs is ongoing.

Alternatively, ENaC activity could be inhibited in order to decrease the elevated Na^+ absorption seen in CF airways. The Parion compound 552-02 [59] is a recent improvement on the ENaC inhibitor, amiloride, and has already entered clinical trials, including one for CF, which is currently under way.

Inhibition of serine proteases can prevent activation of ENaC, but most candidates have not yet proceeded beyond preclinical studies [60]. A recent small-molecule inhibitor of proteases involved in ENaC regulation, Camostat, was tested with some success in sheep aerosol studies, improving mucociliary clearance for several hours after administration [61]. Partial correction (75% towards normal) of the Na^{2+} transport defect in the nose of CF patients was reported [62], confirming that this is a promising candidate for evaluation in the clinic for CF lung disease.

Although these data are encouraging, the majority of these new drug treatments have only modest ability to correct the CF defect, do not act on all

CFTR mutations, and even if successful, may only benefit a small proportion of patients depending on their genotype. It is also likely that the pharmacological approaches discussed here could require use in combination with each other for optimal correction of the CF ion transport defects, for example a corrector with a potentiator, and simultaneously adjusting ENaC activity with a third component; this may limit their use in the clinic and will certainly take time to evaluate in trials.

Gene therapy clinical trials

The basic concept of gene therapy involves introducing a gene into target cells to prevent or slow the progression of a disease. CF is a good candidate for this technology as it is primarily caused by mutations in a single gene, a normal copy of which could be delivered to patients via topical delivery to the lung, without invasive techniques or surgery. Moreover, a gene complementation approach would directly target the cause of the disease and could correct many aspects of the complex lung pathology. A single therapy to treat the underlying defect could greatly reduce the high therapeutic burden that CF patients currently have to endure. In addition, one therapy might be suitable to treat subjects with a wide variety of mutations, meaning that a single treatment strategy could be relevant to all patients. Proof of principle for both viral and non-viral *CFTR* gene transfer was quickly established in CF patients [63] and to date, 25 trials have been completed involving approximately 450 CF individuals (see Griesenbach, 2009 [64]).

Adenovirus and Adeno-Associated Virus

Two DNA viral vectors, adenovirus (Ad) and adeno-associated virus (AAV), have been evaluated in CF gene therapy clinical trials. Trials with Ad vectors have been disappointing, compared with preclinical studies, both in terms of persistence of gene expression and the level of gene transfer in the human airways. Gutless adenovirus (also referred to as helper-dependent Ad), in which all of the viral genome is removed (apart from the inverted terminal repeats (ITRs) and the viral packaging sequence) [65] have shown extended duration of transgene expression, with reduced toxicity and immunogenicity in mice compared with previous generations of Ad [66], but re-administration remains problematic due to the presence of viral capsid proteins [67].

An alternative viral vector that has been investigated in CF clinical studies is the non-pathogenic AAV. Phase I and II clinical trials administering a single dose of AAV2 expressing CFTR to the nose [68–71] and lungs [72, 73] of CF patients were deemed safe and resulted in consistent detection of vector-derived DNA for between 30 days and 10 weeks after delivery. CFTR mRNA was very rarely detected in the trials, although two studies reported transient

correction of the Cl^- conductance defect in the nose for up to 2 weeks after delivery [69, 70]. Treatment did not result in any detectable clinical benefit in lung function [71] and neutralising antibodies against the vector were detected in the serum [72, 73]. Two trials to re-administer the virus have been performed, with a similar lack of clinical benefit [74, 75]. Once again, this has been disappointing in comparison with preclinical studies in mice in particular, but is partly attributable to a paucity of AAV2 receptors on the apical membrane of human cells [76].

Thus, alternative serotypes with potentially improved tropism for airway epithelial cells are being investigated. AAV5 and AAV6 appear to transduce airway epithelial cells more efficiently than AAV2 [77, 78], and up to 90% transduction efficiency was recently reported in mouse airways with AAV6 using the hybrid chicken β-actin/rabbit β globin promoter/intron with the human CMV immediate early enhancer (CAG) [79].

The lack of efficiency in the repeat administration clinical studies [74, 75] is in keeping with some preclinical studies showing an inability to re-administer AAV2 and AAV5 in airways of animals, unless genotypes were switched for re-administration [80–83]. Interestingly, it has been demonstrated that AAV9 serotype virus could be re-administered successfully to murine airways 1 month after the initial dose [84].

In addition to difficulties with tropism and immune responses, the utility of AAV for CF has been hampered by the limited packaging capacity of most rAAV vectors (<5 kb). Advances in this field have included development of miniCFTR genes that may be packaged more efficiently [85–88], including safety studies in non-human primates [89] and functional studies in mice [90], and the discovery that AAV2/5 could in fact package large genomes (up to 8.9 kb) with a reasonably yield compared with other rAAV pseudotypes (1 to 4 and 7 to 9) [91]. Together with evidence suggesting that AAV vectors may be able to target progenitor cells of the mouse lung [92], thus avoiding the need for repeat administration, this work continues to make incremental improvements.

Negative strand RNA viruses

The murine parainfluenza virus type 1 [or Sendai virus (SeV)], the human respiratory syncytial virus (RSV) and the human parainfluenza virus type 3 (PIV3) are negative strand RNA viruses whose life cycle is completed in the cytoplasm. They have all been shown to transfect AECs efficiently via the apical membrane [93, 94], and express functional CFTR channels *in vivo* [95], but elicit an immune response that currently inhibits repeated administration [96]. Although such viruses may be useful for acute diseases that require only transient gene expression, in the context of CF their use is for now restricted to preclinical proof of principle studies, until the immunological barriers to repeat administration can be resolved.

Lentiviruses

Lentiviruses are retroviruses that transduce non-dividing cells including terminally differentiated AECs. The viral dsDNA genome stably integrates into the genome of transduced cells after its RNA has been reverse transcribed, so expression is likely to last for the lifetime of the cell (approximately 17 months for AECs, [97]). VSVG-pseudotyped HIV-derived lentivirus carrying the CFTR gene transiently and partially corrected the Cl^- defect in CF knockout mouse nose for up to 46 days, although pre-treatment with the tight junction opener lysophosphatidylcholine was necessary [98]. There have been attempts to improve the tropism of lentivirus by pseudotyping with envelope glycoproteins from the filoviruses Ebola or Marburg [99], GP64 of baculovirus [100], the spike envelope glycoprotein of the SARS virus [101] and the F and HN proteins of SeV [102]. This latter vector, F/HN-SIV, was able to transduce polarised epithelial cells from both the apical and basolateral side and importantly, murine AEC *in vivo* without the need for pre-conditioning, with gene expression *in vivo* persisting for at least 17 months, i.e., the lifetime of AECs [97, 103]. This is consistent with gene expression for up to 12 months in mouse nose with GP64-FIV [100]. As with other viruses, it will be important that researchers address the challenge of multiple repeat administrations without loss of efficacy, or find ways of targeting stem cell populations of the airways, to treat the chronic aspects of CF lung diseases.

Non-viral vectors for CF gene therapy

The need for effective long-term repeated administration to treat CF lung disease had led to the investigation of non-viral vectors, which take the form of circular plasmid DNA (pDNA) delivered to cells as naked pDNA in diluents such as PBS, saline or water, or complexed with agents such as lipids or polycations as protection from extracellular degradation and to aid cellular entry. The relative lack of efficiency compared with viral vectors is counterbalanced by reduced safety concerns regarding integration, more flexible and easier production methodology, extended storage and an unlimited packaging capacity [104, 105].

Non-viral Phase I clinical trials to deliver pDNA expressing CFTR began in the mid 1990s, with a variety of cationic liposome formulations delivered to the nose and/or lungs of CF patients. In general, gene transfer was well tolerated and evidence of *CFTR* gene transfer (as measured by vector-specific mRNA or CFTR-mediated chloride transport) has been established in some, but not all, studies (reviewed in Rosenecker et al., 2006 [106]).

One side effect of lipid formulations has been the transient mild flu-like symptoms reported by Ruiz and Alton in the lung trials of GL67:DOPE:DMPE-PEG/pDNA [107, 108]. This inflammation may be related to the stimulation of the Toll-like receptor 9 by bacterially-derived CpG dinucleotides in the formu-

lation [109, 110]. In a bid to reduce this response, the UK CFGT Consortium has generated a CpG-free pDNA [111], for CF clinical trials now under way. The deletion of CpG motifs is one of the latest aspects of plasmid development programmes in the field of CF gene therapy. A common feature of previous trials has been the transient nature of any correction that was measured. Persistence of expression has been improved in preclinical studies by swapping viral promoters such as CMV (cytomegalovirus) for human promoters including UbC (polyubiquitin C) and EF-1α (elongation factor 1-α) [112, 113]. Similarly specificity has been improved in mice using the human cytokeratin (K18) and FOXJ1 promoters which both directed epithelial cell-specific transgene expression in mice [114, 115]. Clinical trial data will confirm whether the modifications to the DNA construct have improved the duration and tolerability of gene transfer in the nose and lungs of CF patients. Table 1 summarises the features of recent non-viral vector developments in the context of airway gene therapy.

Cationic polymers as mediators for CF plasmid DNA gene therapy

A particular polycation that has shown promise for lung gene therapy is polyethylenimine (PEI), the most commonly used forms of which are 25 kDa branched PEI and 22 kDa linear PEI. Although not used in lung gene therapy clinical trials to date, PEI/pDNA complexes have led to successful gene delivery in a clinically relevant model of aerosol gene delivery [116], and work towards improving the formulation by concentrating the particles has shown promising results in murine airway studies [117]. Following comparison of over 25 different non-viral formulations, concentrated 25 kDa PEI has been selected as a ‘Wave 2’ product by the UK CFGT Consortium. Additional improvements may also come from the field of integrases, with the recent demonstration that mice treated with integrase-encoding and reporter constructs complexed with PEI expressed the reporter protein longer than those treated with a non-integrase-encoding construct [118].

The only cationic polymers used in clinical trials for CF gene therapy to date have been compacted DNA nanoparticles, which consist of a single molecule of pDNA compacted with a 30-mer lysine polymer covalently linked to polyethylene glycol (PEG) [119]. The advantage of this gene transfer agent is the identification of its receptor for cell uptake, nucleolin [120] and nuclear translocation bypassing the endosomal pathway. A Phase I study resulted in detection of vector-derived DNA 3 days after dosing in nasal epithelium of CF patients, and partial to complete correction of the Cl^- transport defect in some patients [121].

Modifier genes and future directions

It is clear that the genotype of CF patients does not entirely predict the course of disease, particularly the rate of decline in lung function. A number of stud-

Table 1. Features of promoter/enhancer combinations for improved lung gene expression using plasmid DNA constructs

	Construct description	Model	Feature	Ref.
pCUBI	1.1 kb I/E hCMV enh, human ubiquitin B prom/intr	Lung instillation in mice	Duration for over 12 weeks vs pCF1 (4 weeks) (hCMV I/E enh/prom and hybrid int)	[112]
pUbLux	1.2 kb of human polyubiquitin C prom, exon 1, int 1 and exon 2 sequences	Lung instillation in mice	>26 weeks vs viral enh/prom constructs: pCIKLux (hCMV I/E enh/prom and hybrid int), pRIKLux (hCMV IE enh, RSV 3' LTR prom) and pSIKLux (SV40 enh/early prom.) (<7 days, ±14 d, ±14d)	[113]
pEFLux	1.3 kb of hEFI gene, including prom, exon 1, intr A and exon 2 sequences	Lung instillation in mice	>4 weeks vs viral enh/prom constructs as above	[113]
pG4-hCEFI	CpG-free I/E hCMV enh, hEF1 prom. to exon 2 fragment (from pEFLux)	Lung instillation in mice	Stable expression to >56 days, and no inflammatory response, compared with CpG-rich pG1-CMV (=pCIKLux) (<14d duration, high inflammatory response)	[111]
FoxJ1	1 kb of FoxJ1 genomic region and mouse transthyretin (TTR) gene intron	Transgenic mice	Tissue and cell specificity: ciliated cells of tracheal, bronchial and nasal epithelium	[115]
K18mLacZ	2.2 kb of K18 5' promoter proximal flanking sequence, KRT18 promoter, and first intron/enhancer with mutated cryptic splice site	Transgenic mice	Cell specificity: restricted to the epithelium lining major airways, and submucosal glands in the lungs	[114]

Abbreviations: enh, enhancer; prom, promoter; int, intron; I/E hCMV, immediate/early human cytomegalovirus; hEFI, human elongation factor 1α; K18/KRT18, cytokeratin 18; LTR, long terminal repeat

ies have attempted to identify modifier genes for different aspects of CF, as reviewed by Collaco and Cutting (2008) [122]. Lead candidates include inflammatory mediators and cytokines [122], and most recently IFRD1 [123], as well as gene polymorphisms that affect the response to bacterial infection (beta-defensins [124, 125], 8.1 ancestral MHC haplotype [126] and IL10 [127, 128]). In a disease such as CF where many factors influence the course of disease, with different clinical parameters to take into account (lung function, bacterial burden, inflammation), the identification of significant modifier genes will require large population studies. Ultimately this could provide new targets for anti-inflammatory drugs or gene silencing by RNAi strategies [129] to ameliorate disease.

Indeed it is likely that gene silencing therapies, including shRNA in pDNA constructs, will be evaluated that are not directly based on modifier genes, but on the general pathophysiology of CF. For example, a reduction in the transcription factor nuclear factor kappa B (NFκB), which regulates many pro-inflammatory cytokines and plays a central role in the exaggerated innate immune response in CF [130], or the ER-membrane protein BAP31, involved in blocking misfolded ΔF508 CFTR [131], may be beneficial. With further improvements in non-viral gene transfer, gene silencing may become a realistic treatment option.

Conclusion

No single, novel, therapeutic approach to CF treatment has yet shown sufficient promise to stand out in the field. However as our understanding of the molecular processes in the CF lung deepens with better preclinical models to evaluate them, it is becoming clearer that early and broad intervention will be necessary to prevent the multifaceted defects that accompany CFTR mutations. Pharmacological approaches are heading into clinical trials at a regular pace, with some preclinical studies showing correction of several major defects. Although the challenges of finding a safe and effective formulation permit only slow progress, gene therapy still provides a great opportunity for an ‘all-round’ therapeutic intervention.

Acknowledgements
We thank Dr Hazel Painter and Anna Lawton for helping with the design of the figure. Work in our laboratory is funded by a grant from the UK Cystic Fibrosis Trust to the UK CF Gene Therapy Consortium (www.cfgenetherapy.org.uk).

References

1 Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL et al. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* 245: 1066–1073

2 Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science* 245: 1073–1080
3 Rommens JM, Zengerling-Lentes S, Kerem B, Melmer G, Buchwald M, Tsui LC (1989) Physical localization of two DNA markers closely linked to the cystic fibrosis locus by pulsed-field gel electrophoresis. *Am J Hum Genet* 45: 932–941
4 Hyde SC, Emsley P, Hartshorn MJ, Mimmack MM, Gileadi U, Pearce SR, Gallagher MP, Gill DR, Hubbard RE, Higgins CF (1990) Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. *Nature* 346: 362–365
5 Sheppard DN, Welsh MJ (1999) Structure and function of the CFTR chloride channel. *Physiol Rev* 79: S23–45
6 Berdiev BK, Qadri YJ, Benos DJ (2009) Assessment of the CFTR and ENaC association. *Mol Biosyst* 5: 123–127
7 Kunzelmann K (2001) CFTR: interacting with everything? *News Physiol Sci* 16: 167–170
8 Rowntree RK, Harris A (2003) The phenotypic consequences of CFTR mutations. *Ann Hum Genet* 67: 471–485
9 Zielenski J (2000) Genotype and phenotype in cystic fibrosis. *Respiration* 67: 117–133
10 Gibson RL, Burns JL, Ramsey BW (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. *Am J Respir Crit Care Med* 168: 918–951
11 Robinson M, Bye PT (2002) Mucociliary clearance in cystic fibrosis. *Pediatr Pulmonol* 33: 293–306
12 Pier GB (2000) Role of the cystic fibrosis transmembrane conductance regulator in innate immunity to Pseudomonas aeruginosa infections. *Proc Natl Acad Sci USA* 97: 8822–8828
13 Wanner A, Salathe M, O'Riordan TG (1996) Mucociliary clearance in the airways. *Am J Respir Crit Care Med* 154: 1868–1902
14 Knowles MR, Boucher RC (2002) Mucus clearance as a primary innate defense mechanism for mammalian airways. *J Clin Invest* 109: 571–577
15 Aarbiou J, Rabe KF, Hiemstra PS (2002) Role of defensins in inflammatory lung disease. *Ann Med* 34: 96–101
16 Chambers LA, Rollins BM, Tarran R (2007) Liquid movement across the surface epithelium of large airways. *Respir Physiol Neurobiol* 159: 256–270
17 Boucher RC (1994) Human airway ion transport. Part one. *Am J Respir Crit Care Med* 150: 271–281
18 Matsui H, Grubb BR, Tarran R, Randell SH, Gatzy JT, Davis CW, Boucher RC (1998) Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease. *Cell* 95: 1005–1015
19 Tarran R, Grubb BR, Parsons D, Picher M, Hirsh AJ, Davis CW, Boucher RC (2001) The CF salt controversy: *in vivo* observations and therapeutic approaches. *Mol Cell* 8: 149–158
20 Boucher RC (2007) Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu Rev Med* 58: 157–170
21 Mall MA (2008) Role of cilia, mucus, and airway surface liquid in mucociliary dysfunction: lessons from mouse models. *J Aerosol Med Pulm Drug Deliv* 21: 13–24
22 Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC (2004) Increased airway epithelial Na^+ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med* 10: 487–493
23 Daviskas E, Anderson SD (2006) Hyperosmolar agents and clearance of mucus in the diseased airway. *J Aerosol Med* 19: 100–109
24 Clunes MT, Boucher RC (2008) Front-runners for pharmacotherapeutic correction of the airway ion transport defect in cystic fibrosis. *Curr Opin Pharmacol* 8: 292–299
25 Cheng SH, Fang SL, Zabner J, Marshall J, Piraino S, Schiavi SC, Jefferson DM, Welsh MJ, Smith AE (1995) Functional activation of the cystic fibrosis trafficking mutant delta F508-CFTR by overexpression. *Am J Physiol* 268: L615–624
26 Zeitlin PL, Diener-West M, Rubenstein RC, Boyle MP, Lee CK, Brass-Ernst L (2002) Evidence of CFTR function in cystic fibrosis after systemic administration of 4-phenylbutyrate. *Mol Ther* 6: 119–126
27 Wilschanski M, Yahav Y, Yaacov Y, Blau H, Bentur L, Rivlin J, Aviram M, Bdolah-Abram T, Bebok Z, Shushi L et al. (2003) Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. *N Engl J Med* 349: 1433–1441
28 Sermet-Gaudelus I, Renouil M, Fajac A, Bidou L, Parbaille B, Pierrot S, Davy N, Bismuth E, Reinert P, Lenoir G et al. (2007) *In vitro* prediction of stop-codon suppression by intravenous gen-

tamicin in patients with cystic fibrosis: a pilot study. *BMC Med* 5: 5
29 Welch EM, Barton ER, Zhuo J, Tomizawa Y, Friesen WJ, Trifillis P, Paushkin S, Patel M, Trotta CR, Hwang S et al. (2007) PTC124 targets genetic disorders caused by nonsense mutations. *Nature* 447: 87–91
30 Kerem E, Hirawat S, Armoni S, Yaakov Y, Shoseyov D, Cohen M, Nissim-Rafinia M, Blau H, Rivlin J, Aviram M et al. (2008) Effectiveness of PTC124 treatment of cystic fibrosis caused by nonsense mutations: a prospective phase II trial. *Lancet* 372: 719–727
31 Sermet-Gaudelus I, De Boeck K, Casimir G, Leal T, Vermeulen F, Mogenet A, Roussel D, Hage P, Hanssens L, Constantine S et al. (2008) Children with nonsense-mutation-mediated cystic fibrosis respond to investigational treatment with PTC124. *Pediatric Pulmonology* Suppl. 31: 313
32 Kerem E, Yaakov Y, Armoni S, Pugatsch T, Shoseyov D, Cohen M, Blau H, Hirawat S, Reha A, Elfring G et al. (2008) PTC124 induces time-dependent improvements in Chloride conductance and clinical parameters in patients with nonsense-mutation-mediated cystic fibrosis. *Pediatric Pulmonology* Suppl. 31: 294
33 Aznarez I, Zielenski J, Rommens JM, Blencowe BJ, Tsui LC (2007) Exon skipping through the creation of a putative exonic splicing silencer as a consequence of the cystic fibrosis mutation R553X. *J Med Genet* 44: 341–346
34 Egan ME, Glockner-Pagel J, Ambrose C, Cahill PA, Pappoe L, Balamuth N, Cho E, Canny S, Wagner CA, Geibel J et al. (2002) Calcium-pump inhibitors induce functional surface expression of Delta F508-CFTR protein in cystic fibrosis epithelial cells. *Nat Med* 8: 485–492
35 Rubenstein RC, Egan ME, Zeitlin PL (1997) *In vitro* pharmacologic restoration of CFTR-mediated chloride transport with sodium 4-phenylbutyrate in cystic fibrosis epithelial cells containing delta F508-CFTR. *J Clin Invest* 100: 2457–2465
36 Rubenstein RC, Zeitlin PL (1998) A pilot clinical trial of oral sodium 4-phenylbutyrate (Buphenyl) in deltaF508-homozygous cystic fibrosis patients: partial restoration of nasal epithelial CFTR function. *Am J Respir Crit Care Med* 157: 484–490
37 Pedemonte N, Lukacs GL, Du K, Caci E, Zegarra-Moran O, Galietta LJ, Verkman AS (2005) Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. *J Clin Invest* 115: 2564–2571
38 Van Goor F, Straley KS, Cao D, Gonzalez J, Hadida S, Hazlewood A, Joubran J, Knapp T, Makings LR, Miller M et al. (2006) Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. *Am J Physiol Lung Cell Mol Physiol* 290: L1117–1130
39 Wang Y, Bartlett MC, Loo TW, Clarke DM (2006) Specific rescue of cystic fibrosis transmembrane conductance regulator processing mutants using pharmacological chaperones. *Mol Pharmacol* 70: 297–302
40 Wang Y, Loo TW, Bartlett MC, Clarke DM (2007) Correctors promote maturation of cystic fibrosis transmembrane conductance regulator (CFTR)-processing mutants by binding to the protein. *J Biol Chem* 282: 33247–33251
41 Dormer RL, Harris CM, Clark Z, Pereira MM, Doull IJ, Norez C, Becq F, McPherson MA (2005) Sildenafil (Viagra) corrects DeltaF508-CFTR location in nasal epithelial cells from patients with cystic fibrosis. *Thorax* 60: 55–59
42 Robert R, Carlile GW, Pavel C, Liu N, Anjos SM, Liao J, Luo Y, Zhang D, Thomas DY, Hanrahan JW (2008) Structural analog of sildenafil identified as a novel corrector of the F508del-CFTR trafficking defect. *Mol Pharmacol* 73: 478–489
43 Norez C, Noel S, Wilke M, Bijvelds M, Jorna H, Melin P, DeJonge H, Becq F (2006) Rescue of functional delF508-CFTR channels in cystic fibrosis epithelial cells by the alpha-glucosidase inhibitor miglustat. *FEBS Lett* 580: 2081–2086
44 Antigny F, Norez C, Becq F, Vandebrouck C (2008) Calcium homeostasis is abnormal in cystic fibrosis airway epithelial cells but is normalized after rescue of F508del-CFTR. *Cell Calcium* 43: 175–183
45 Noel S, Wilke M, Bot AG, De Jonge HR, Becq F (2008) Parallel improvement of sodium and chloride transport defects by miglustat (n-butyldeoxynojyrimicin) in cystic fibrosis epithelial cells. *J Pharmacol Exp Ther* 325: 1016–1023
46 Dechecchi MC, Nicolis E, Norez C, Bezzerri V, Borgatti M, Mancini I, Rizzotti P, Ribeiro CM, Gambari R, Becq F et al. (2008) Anti-inflammatory effect of miglustat in bronchial epithelial cells. *J Cyst Fibros* 7: 555–565
47 Norez C, Antigny F, Noel S, Vandebrouck C, Becq F (2009) A CF respiratory epithelial cell chron-

ically treated by miglustat acquires a non-CF like phenotype. *Am J Respir Cell Mol Biol* ePub ahead of print: Jan 8
48 Vertex (2009) Trial to enrol approximately 90 patients with the F508del CFTR mutation. *Business Wire*
49 Kelley TJ, al-Nakkash L, Drumm ML (1995) CFTR-mediated chloride permeability is regulated by type III phosphodiesterases in airway epithelial cells. *Am J Respir Cell Mol Biol* 13: 657–664
50 Smith SN, Middleton PG, Chadwick S, Jaffe A, Bush KA, Rolleston S, Farley R, Delaney SJ, Wainwright B, Geddes DM et al. (1999) The *in vivo* effects of milrinone on the airways of cystic fibrosis mice and human subjects. *Am J Respir Cell Mol Biol* 20: 129–134
51 Mall M, Kunzelmann K (2005) Correction of the CF defect by curcumin: hypes and disappointments. *Bioessays* 27: 9–13
52 Andersson C, Roomans GM (2000) Activation of deltaF508 CFTR in a cystic fibrosis respiratory epithelial cell line by 4-phenylbutyrate, genistein and CPX. *Eur Respir J* 15: 937–941
53 Van Goor F, Hadida S, Grootenhuis PD (2007) VX-770, a potent, selective, and orally bioavailable potentiator of CFTR gating. *Pediatric Pulmonology* Suppl. 30: 289
54 Accurso F, Rowe SM, Durie PR, Konstan MW, Dunitz J, Hornick DB, Sagel SD, Boyle MP, Uluer AZ, Upadhyay D et al. (2008) Interim results of phase 2a of VX-770 to evaluate safety, pharmacokinetics, and biomarkers of CFTR activity in cystic fibrosis subjects with G551D. *Pediatric Pulmonology* Suppl. 31: 295
55 Deterding RR, Lavange LM, Engels JM, Mathews DW, Coquillette SJ, Brody AS, Millard SP, Ramsey BW (2007) Phase 2 randomized safety and efficacy trial of nebulized denufosol tetrasodium in cystic fibrosis. *Am J Respir Crit Care Med* 176: 362–369
56 Kellerman D, Rossi Mospan A, Engels J, Schaberg A, Gorden J, Smiley L (2008) Denufosol: a review of studies with inhaled P2Y(2) agonists that led to Phase 3. *Pulm Pharmacol Ther* 21: 600–607
57 Grasemann H, Stehling F, Brunar H, Widmann R, Laliberte TW, Molina L, Doring G, Ratjen F (2007) Inhalation of Moli1901 in patients with cystic fibrosis. *Chest* 131: 1461–1466
58 Zeitlin PL, Boyle MP, Guggino WB, Molina L (2004) A phase I trial of intranasal Moli1901 for cystic fibrosis. *Chest* 125: 143–149
59 Hirsh AJ, Zhang J, Zamurs A, Fleegle J, Thelin WR, Caldwell RA, Sabater JR, Abraham WM, Donowitz M, Cha B et al. (2008) Pharmacological properties of N-(3,5-diamino-6-chloropyrazine-2-carbonyl)-N'-4-[4-(2,3-dihydroxypropoxy) phenyl]butyl-guanidine methanesulfonate (552-02), a novel epithelial sodium channel blocker with potential clinical efficacy for cystic fibrosis lung disease. *J Pharmacol Exp Ther* 325: 77–88
60 Planes C, Caughey GH (2007) Regulation of the epithelial Na^+ channel by peptidases. *Curr Top Dev Biol* 78: 23–46
61 Coote K, Atherton-Watson HC, Sugar R, Young A, MacKenzie-Beevor A, Gosling M, Bhalay G, Bloomfield G, Dunstan A, Bridges RJ et al. (2009) Camostat attenuates airway epithelial sodium channel function *in vivo* through the inhibition of a channel-activating protease. *J Pharmacol Exp Ther* 329: 764–774
62 Rowe SM, Reeves G, Young H, Fulton JC, Sthanam M, Abbi S, Roberts A, Renard D, Harris JL, Danahay H et al. (2008) Correction of sodium transport with nasal administration of the prostasin inhibitor QAU145 in CF subjects. *Pediatric Pulmonology* Suppl. 31: 295
63 Zabner J, Couture LA, Gregory RJ, Graham SM, Smith AE, Welsh MJ (1993) Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. *Cell* 75: 207–216
64 Griesenbach U, Alton EW (2009) Gene transfer to the lung: lessons learned from more than 2 decades of CF gene therapy. *Adv Drug Deliv Rev* 61: 128–139
65 Kochanek S, Clemens PR, Mitani K, Chen HH, Chan S, Caskey CT (1996) A new adenoviral vector: Replacement of all viral coding sequences with 28 kb of DNA independently expressing both full-length dystrophin and beta-galactosidase. *Proc Natl Acad Sci U S A* 93: 5731–5736
66 Palmer DJ, Ng P (2005) Helper-dependent adenoviral vectors for gene therapy. *Hum Gene Ther* 16: 1–16
67 Hiatt PW, Brunetti-Pierri N, McConnell R, Palmer D, Grove N, Finegold M, Beaudet A, Ng P (2008) Pulmonary transduction in nonhuman primates by HDAd: Duration of transgene expression and vector re-administration. *Pediatric Pulmonology* Suppl. 31: 297
68 Flotte T, Carter B, Conrad C, Guggino W, Reynolds T, Rosenstein B, Taylor G, Walden S, Wetzel R (1996) A phase I study of an adeno-associated virus-CFTR gene vector in adult CF patients with

mild lung disease. *Hum Gene Ther* 7: 1145–1159
69 Wagner JA, Messner AH, Moran ML, Daifuku R, Kouyama K, Desch JK, Manley S, Norbash AM, Conrad CK, Friborg S et al. (1999) Safety and biological efficacy of an adeno-associated virus vector-cystic fibrosis transmembrane regulator (AAV-CFTR) in the cystic fibrosis maxillary sinus. *Laryngoscope* 109: 266–274
70 Wagner JA, Reynolds T, Moran ML, Moss RB, Wine JJ, Flotte TR, Gardner P (1998) Efficient and persistent gene transfer of AAV-CFTR in maxillary sinus. *Lancet* 351: 1702–1703
71 Wagner JA, Nepomuceno IB, Messner AH, Moran ML, Batson EP, Dimiceli S, Brown BW, Desch JK, Norbash AM, Conrad CK et al. (2002) A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. *Hum Gene Ther* 13: 1349–1359
72 Aitken ML, Moss RB, Waltz DA, Dovey ME, Tonelli MR, McNamara SC, Gibson RL, Ramsey BW, Carter BJ, Reynolds TC (2001) A Phase I study of aerosolized administration of tgAAVCF to cystic fibrosis subjects with mild lung disease. *Hum Gene Ther* 12: 1907–1916
73 Flotte TR, Zeitlin PL, Reynolds TC, Heald AE, Pedersen P, Beck S, Conrad CK, Brass-Ernst L, Humphries M, Sullivan K et al. (2003) Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: a two-part clinical study. *Hum Gene Ther* 14: 1079–1088
74 Moss RB, Milla C, Colombo J, Accurso F, Zeitlin PL, Clancy JP, Spencer LT, Pilewski J, Waltz DA, Dorkin HL et al. (2007) Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: a randomized placebo-controlled phase 2B trial. *Hum Gene Ther* 18: 726–732
75 Moss RB, Rodman D, Spencer LT, Aitken ML, Zeitlin PL, Waltz D, Milla C, Brody AS, Clancy JP, Ramsey B et al. (2004) Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest* 125: 509–521
76 Summerford C, Samulski RJ (1998) Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 72: 1438–1445
77 Halbert CL, Allen JM, Miller AD (2001) Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. *J Virol* 75: 6615–6624
78 Zabner J, Seiler M, Walters R, Kotin RM, Fulgeras W, Davidson BL, Chiorini JA (2000) Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. *J Virol* 74: 3852–3858
79 Halbert CL, Lam SL, Miller AD (2007) High-efficiency promoter-dependent transduction by adeno-associated virus type 6 vectors in mouse lung. *Hum Gene Ther* 18: 344–354
80 Halbert CL, Standaert TA, Aitken ML, Alexander IE, Russell DW, Miller AD (1997) Transduction by adeno-associated virus vectors in the rabbit airway: efficiency, persistence, and readministration. *J Virol* 71: 5932–5941
81 Halbert CL, Standaert TA, Wilson CB, Miller AD (1998) Successful readministration of adeno-associated virus vectors to the mouse lung requires transient immunosuppression during the initial exposure. *J Virol* 72: 9795–9805
82 Halbert CL, Rutledge EA, Allen JM, Russell DW, Miller AD (2000) Repeat transduction in the mouse lung by using adeno-associated virus vectors with different serotypes. *J Virol* 74: 1524–1532
83 Sumner-Jones SG, Gill DR, Hyde SC (2007) Lack of repeat transduction by rAAV5/5 vectors in the mouse airway. *J Virol* 81: 12360–12367
84 Limberis MP, Wilson JM (2006) Adeno-associated virus serotype 9 vectors transduce murine alveolar and nasal epithelia and can be readministered. *Proc Natl Acad Sci USA* 103: 12993–12998
85 Ostedgaard LS, Randak C, Rokhlina T, Karp P, Vermeer D, Ashbourne Excoffon KJ, Welsh MJ (2003) Effects of C-terminal deletions on cystic fibrosis transmembrane conductance regulator function in cystic fibrosis airway epithelia. *Proc Natl Acad Sci USA* 100: 1937–1942
86 Ostedgaard LS, Rokhlina T, Karp PH, Lashmit P, Afione S, Schmidt M, Zabner J, Stinski MF, Chiorini JA, Welsh MJ (2005) A shortened adeno-associated virus expression cassette for CFTR gene transfer to cystic fibrosis airway epithelia. *Proc Natl Acad Sci USA* 102: 2952–2957
87 Ostedgaard LS, Zabner J, Vermeer DW, Rokhlina T, Karp PH, Stecenko AA, Randak C, Welsh MJ (2002) CFTR with a partially deleted R domain corrects the cystic fibrosis chloride transport defect in human airway epithelia *in vitro* and in mouse nasal mucosa *in vivo*. *Proc Natl Acad Sci USA* 99: 3093–3098

88 Zhang L, Wang D, Fischer H, Fan PD, Widdicombe JH, Kan YW, Dong JY (1998) Efficient expression of CFTR function with adeno-associated virus vectors that carry shortened CFTR genes. *Proc Natl Acad Sci USA* 95: 10158–10163
89 Fischer AC, Smith CI, Cebotaru L, Zhang X, Askin FB, Wright J, Guggino SE, Adams RJ, Flotte T, Guggino WB (2007) Expression of a truncated cystic fibrosis transmembrane conductance regulator with an AAV5-pseudotyped vector in primates. *Mol Ther* 15: 756–763
90 Mueller C, Torrez D, Braag S, Martino A, Clarke T, Campbell-Thompson M, Flotte TR (2008) Partial correction of the CFTR-dependent ABPA mouse model with recombinant adeno-associated virus gene transfer of truncated CFTR gene. *J Gene Med* 10: 51–60
91 Allocca M, Doria M, Petrillo M, Colella P, Garcia-Hoyos M, Gibbs D, Kim SR, Maguire A, Rex TS, Di Vicino U et al. (2008) Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invest* 118: 1955–1964
92 Liu X, Luo M, Guo C, Yan Z, Wang Y, Lei-Butters DC, Engelhardt JF (2009) Analysis of adeno-associated virus progenitor cell transduction in mouse lung. *Mol Ther* 17: 285–293
93 Ferrari S, Griesenbach U, Shiraki-Iida T, Shu T, Hironaka T, Hou X, Williams J, Zhu J, Jeffery PK, Geddes DM et al. (2004) A defective nontransmissible recombinant Sendai virus mediates efficient gene transfer to airway epithelium *in vivo*. *Gene Ther* 11: 1659–1664
94 Zhang L, Bukreyev A, Thompson CI, Watson B, Peeples ME, Collins PL, Pickles RJ (2005) Infection of ciliated cells by human parainfluenza virus type 3 in an *in vitro* model of human airway epithelium. *J Virol* 79: 1113–1124
95 Ferrari S, Griesenbach U, Iida A, Farley R, Wright AM, Zhu J, Munkonge FM, Smith SN, You J, Ban H et al. (2007) Sendai virus-mediated CFTR gene transfer to the airway epithelium. *Gene Ther* 14: 1371–1379
96 Griesenbach U, Geddes DM, Alton EW (2006) Gene therapy progress and prospects: cystic fibrosis. *Gene Ther* 13: 1061–1067
97 Rawlins EL, Hogan BL (2008) Ciliated epithelial cell lifespan in the mouse trachea and lung. *Am J Physiol Lung Cell Mol Physiol* 295: L231–234
98 Limberis M, Anson DS, Fuller M, Parsons DW (2002) Recovery of airway cystic fibrosis transmembrane conductance regulator function in mice with cystic fibrosis after single-dose lentivirus-mediated gene transfer. *Hum Gene Ther* 13: 1961–1970
99 Sinn PL, Hickey MA, Staber PD, Dylla DE, Jeffers SA, Davidson BL, Sanders DA, McCray PB Jr, (2003) Lentivirus vectors pseudotyped with filoviral envelope glycoproteins transduce airway epithelia from the apical surface independently of folate receptor alpha. *J Virol* 77: 5902–5910
100 Sinn PL, Burnight ER, Hickey MA, Blissard GW, McCray PB Jr, (2005) Persistent gene expression in mouse nasal epithelia following feline immunodeficiency virus-based vector gene transfer. *J Virol* 79: 12818–12827
101 Kobinger GP, Limberis MP, Somanathan S, Schumer G, Bell P, Wilson JM (2007) Human immunodeficiency viral vector pseudotyped with the spike envelope of severe acute respiratory syndrome coronavirus transduces human airway epithelial cells and dendritic cells. *Hum Gene Ther* 18: 413–422
102 Kobayashi M, Iida A, Ueda Y, Hasegawa M (2003) Pseudotyped lentivirus vectors derived from simian immunodeficiency virus SIVagm with envelope glycoproteins from paramyxovirus. *J Virol* 77: 2607–2614
103 Mitomo K, Griesenbach U, Inoue M, Washizawa K, Fujiawa S, Iwasaki H, Somerton L, Farley R, Singh C, Hasegawa M et al. (2007) Potential airway stem/progenitor cell integration after transduction with a lentiviral vector pseudo-typed with Sendai virus enveolope proteins. *Pediatric Pulmonology* Suppl. 30
104 Nishikawa M, Hashida M (2002) Nonviral approaches satisfying various requirements for effective *in vivo* gene therapy. *Biol Pharm Bull* 25: 275–283
105 Ratko TA, Cummings JP, Blebea J, Matuszewski KA (2003) Clinical gene therapy for nonmalignant disease. *Am J Med* 115: 560–569
106 Rosenecker J, Huth S, Rudolph C (2006) Gene therapy for cystic fibrosis lung disease: current status and future perspectives. *Curr Opin Mol Ther* 8: 439–445
107 Alton EW, Stern M, Farley R, Jaffe A, Chadwick SL, Phillips J, Davies J, Smith SN, Browning J, Davies MG et al. (1999) Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial. *Lancet* 353: 947–954
108 Ruiz FE, Clancy JP, Perricone MA, Bebok Z, Hong JS, Cheng SH, Meeker DP, Young KR, Schoumacher RA, Weatherly MR et al. (2001) A clinical inflammatory syndrome attributable to

aerosolized lipid-DNA administration in cystic fibrosis. *Hum Gene Ther* 12: 751–761
109 Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K et al. (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* 408: 740–745
110 Scheule RK (2000) The role of CpG motifs in immunostimulation and gene therapy. *Adv Drug Deliv Rev* 44: 119–134
111 Hyde SC, Pringle IA, Abdullah S, Lawton AE, Davies LA, Varathalingam A, Nunez-Alonso G, Green AM, Bazzani RP, Sumner-Jones SG et al. (2008) CpG-free plasmids confer reduced inflammation and sustained pulmonary gene expression. *Nat Biotechnol* 26: 549–551
112 Yew NS, Przybylska M, Ziegler RJ, Liu D, Cheng SH (2001) High and sustained transgene expression *in vivo* from plasmid vectors containing a hybrid ubiquitin promoter. *Mol Ther* 4: 75–82
113 Gill DR, Smyth SE, Goddard CA, Pringle IA, Higgins CF, Colledge WH, Hyde SC (2001) Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1alpha promoter. *Gene Ther* 8: 1539–1546
114 Chow Y-H, Plumb J, Wen Y, Steer B, Lu Z, Hu J (2000) Targeting transgene expression to airway epithelia and submucosal glands, prominent sites of human CFTR expression. *Mol Ther* 2: 359–367
115 Ostrowski LE, Hutchins JR, Zakel K, O'Neal WK (2003) Targeting expression of a transgene to the airway surface epithelium using a ciliated cell-specific promoter. *Mol Ther* 8: 637–645
116 McLachlan G, Baker A, Tennant P, Gordon C, Vrettou C, Renwick L, Blundell R, Cheng SH, Scheule RK, Davies L et al. (2007) Optimizing aerosol gene delivery and expression in the ovine lung. *Mol Ther* 15: 348–354
117 Davies LA, McLachlan G, Sumner-Jones SG, Ferguson D, Baker A, Tennant P, Gordon C, Vrettou C, Baker E, Zhu J et al. (2008) Enhanced lung gene expression after aerosol delivery of concentrated pDNA/PEI complexes. *Mol Ther* 16: 1283–1290
118 Aneja MK, Imker R, Rudolph C (2007) Phage phiC31 integrase-mediated genomic integration and long-term gene expression in the lung after nonviral gene delivery. *J Gene Med* 9: 967–975
119 Liu G, Li D, Pasumarthy MK, Kowalczyk TH, Gedeon CR, Hyatt SL, Payne JM, Miller TJ, Brunovskis P, Fink TL et al. (2003) Nanoparticles of compacted DNA transfect postmitotic cells. *J Biol Chem* 278: 32578–32586
120 Chen X, Kube DM, Cooper MJ, Davis PB (2008) Cell surface nucleolin serves as receptor for DNA nanoparticles composed of pegylated polylysine and DNA. *Mol Ther* 16: 333–342
121 Konstan MW, Davis PB, Wagener JS, Hilliard KA, Stern RC, Milgram LJ, Kowalczyk TH, Hyatt SL, Fink TL, Gedeon CR et al. (2004) Compacted DNA nanoparticles administered to the nasal mucosa of cystic fibrosis subjects are safe and demonstrate partial to complete cystic fibrosis transmembrane regulator reconstitution. *Hum Gene Ther* 15: 1255–1269
122 Collaco JM, Cutting GR (2008) Update on gene modifiers in cystic fibrosis. *Curr Opin Pulm Med* 14: 559–566
123 Gu Y, Harley IT, Henderson LB, Aronow BJ, Vietor I, Huber LA, Harley JB, Kilpatrick JR, Langefeld CD, Williams AH et al. (2009) Identification of IFRD1 as a modifier gene for cystic fibrosis lung disease. *Nature* 458: 1039–1042
124 Hollox EJ, Davies J, Griesenbach U, Burgess J, Alton EW, Armour JA (2005) Beta-defensin genomic copy number is not a modifier locus for cystic fibrosis. *J Negat Results Biomed* 4: 9
125 Tesse R, Cardinale F, Santostasi T, Polizzi A, Manca A, Mappa L, Iacoviello G, De Robertis F, Logrillo VP, Armenio L (2008) Association of beta-defensin-1 gene polymorphisms with Pseudomonas aeruginosa airway colonization in cystic fibrosis. *Genes Immun* 9: 57–60
126 Laki J, Laki I, Nemeth K, Ujhelyi R, Bede O, Endreffy E, Bolbas K, Gyurkovits K, Csiszer E, Solyom E et al. (2006) The 8.1 ancestral MHC haplotype is associated with delayed onset of colonization in cystic fibrosis. *Int Immunol* 18: 1585–1590
127 Corvol H, Boelle PY, Brouard J, Knauer N, Chadelat K, Henrion-Caude A, Flamant C, Muselet-Charlier C, Boule M, Fauroux B et al. (2008) Genetic variations in inflammatory mediators influence lung disease progression in cystic fibrosis. *Pediatr Pulmonol* 43: 1224–1232
128 Tesse R, Cardinale F, Santostasi T, Polizzi A, Mappa L, Manca A, De Robertis F, Silecchia O, Armenio L (2008) Association of interleukin-10 gene haplotypes with Pseudomonas aeruginosa airway colonization in cystic fibrosis. *J Cyst Fibros* 7: 329–332
129 Nichols DP, Konstan MW, Chmiel JF (2008) Anti-inflammatory therapies for cystic fibrosis-related lung disease. *Clin Rev Allergy Immunol* 35: 135–153
130 Vij N, Mazur S, Zeitlin PL (2009) CFTR is a negative regulator of NFkB mediated innate

immune response. *PLoS ONE* 4: e4464
131 Lambert G, Becker B, Schreiber R, Boucherot A, Reth M, Kunzelmann K (2001) Control of cystic fibrosis transmembrane conductance regulator expression by BAP31. *J Biol Chem* 276: 20340–20345

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Gene therapy of multiple sclerosis

Roberto Furlan, Chiara Maiorino, Alberto Gatta, Francesca Ruffini and Gianvito Martino

Clinical Neuroimmunology and Neuroimmunology Unit, Institute of Experimental Neurology, Department of Neuroscience, San Raffaele Scientific Institute, Milan, Italy

Abstract

Multiple sclerosis (MS) constitutes a difficult challenge for the design of innovative therapies: the aetiology is unknown, the pathogenesis only partially understood, and the whole process is multi-focal, chronic, and occurring beyond anatomical barriers, making the delivery of potentially therapeutic molecules difficult. Gene therapy, thus, constitutes a realistic alternative to ensure prolonged, and site-specific delivery of therapies. Recent advancements in the comprehension of the immunopathological processes leading to central nervous system inflammation, and the development of new gene therapy tools, such as RNA-interference, are rapidly leading to a large array of possibilities of intervention, documented in the present review in its animal model, experimental autoimmune encephalomyelitis (EAE). Since progressive forms of MS remain orphan of efficient therapies, the field is open for less conventional interventions such as gene therapy.

Introduction

Autoimmune diseases are the result of an aberrant immune response against a self component of the organism. Their aetiology is commonly unknown, although environmental and genetic factors contribute to pathogenesis. Indeed, a genome-wide screening conducted in multiple sclerosis (MS) patients [1], and recently confirmed in an independent cohort [2], has identified IL-2 and IL-7 receptor genes as associated to susceptibility to the disease.

Autoimmune disorders have an estimated total incidence of 90 newly diagnosed cases per 100,000 people. Prevalence rates range from less than 5 per 100,000 (e.g., uveitis) to more than 1,000 per 100,000 (e.g., rheumatoid arthritis). Almost all autoimmune diseases disproportionally affect women [3]. The target of an autoimmune disease can be organ specific, for example the insulin-secreting pancreatic β-cells in Type 1 diabetes mellitus (IDDM), cartilage and bone in rheumatoid arthritis (RA), central nervous system (CNS) myelin in multiple sclerosis (MS), and gut epithelium in inflammatory bowel disease (IBD), or non-organ specific, as in the case of systemic lupus erythematosus (SLE) and Sjögren's syndrome [4]. The causes of autoimmune diseases remain largely unknown however potential mechanisms that trigger the aberrant autoimmune response have been proposed: (i) disruption of cell or tissue barrier with the consequent release of sequestered self antigen and access of T cell

to privileged immunological sanctuary (e.g., the eye in some forms of uveitis); (ii) infection of antigen presenting cells (APC) with induction of their co-stimulatory activity in the presence only of a self-antigen (e.g., effect of adjuvants in induction of EAE); (iii) binding of pathogen to self protein where pathogen acts as carrier to allow anti-self response (e.g., interstitial nephritis); (iv) molecular mimicry where a self-antigen similar to a pathogen-derived antigen becomes a wrong target for T cell or cross-reactive antibodies (e.g., diabetes, multiple sclerosis); and, (v) polyclonal activation of auto-reactive T cell for a pathogen super-antigen (e.g., rheumatoid arthritis) [5]. All the pathologies that are classified under the family of autoimmune diseases share some clinical and immunological features: (i) as mentioned above, women are more susceptible than men, with the important exception of Type 1 diabetes; (ii) disease course is usually unpredictable – from benign cases to malignant overaggressive situations; and (iii) an immunological mechanism sustained by Th1/Th17-polarised $CD4^+$ cells is thought to be responsible for auto-aggressive reaction with the triggering event related to the hypotheses mentioned above [6].

Current available therapies do not control the evolution of autoimmune diseases in a satisfactory way. Therapeutic strategies are restricted to immunosuppressive, anti-inflammatory and/or palliative therapies that can not completely preserve the patients' quality of life. Thus the development of new approaches, such as gene therapy, to treat this kind of diseases is of high priority. Gene therapy has been so far widely applied to experimental models of autoimmune disorders. Even if the number of clinical trials is increasing during the years, to our knowledge, there are three Phase I trials for rheumatoid arthritis and completion of one Phase II trial for multiple sclerosis has recently been reported [7]. In this study, naked plasmid DNA encoding full length MBP was used to vaccinate relapsing-remitting MS patients with the aim to cause immune tolerance. Although not significant, an encouraging reduction of new MRI enhancing lesions has been reported [7].

Thus, gene therapy may represent a valid therapeutic strategy for autoimmune diseases for the following reasons: (i) these disorders have an unpredictable course, but usually lead to a chronic phase that may be targeted by gene therapy due to its ability to provide continuous release of the desired gene-product for a long period of time; (ii) gene therapy holds the potential to reach directly the target organ of the autoimmune reaction reducing side effects in non-affected sites; and, (iii) gene therapy may be used to modify, in an almost permanent way, crucial pathogenic mechanisms or to re-establish immune homeostasis.

Among autoimmune disorders, multiple sclerosis offers two more challenges: (i) it is the result of the pathologic interaction between the most complex systems in the human body: the nervous and the immune systems; (ii) the inflammatory reaction occurs beyond anatomic barriers such as the blood brain barrier.

EAE is an immune-mediated demyelinating disease of the central nervous system used as animal model for neuroinflammatory diseases, such as multiple

sclerosis. EAE is mostly induced in rodents by immunisation with whole homogenised CNS tissue, with myelin proteins such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG) or myelin proteolipid protein (PLP), or with peptides representing their encephalitogenic epitopes [8].

Several reviews have addressed in the past the topic of gene therapy of EAE and multiple sclerosis [6, 9, 10]. Improvement of gene therapy tools, leading to new vectors (i.e., the success of lentiviral vectors), the possibility to control expression by inducible promoters, the ability to suppress gene expression, topics that are covered by specific chapters, allow for implemented or completely new gene therapy approaches. We will, thus, focus here on the most recent developments concerning gene therapy approaches in EAE, especially considering the targeted pathways (Tab. 1).

Modulation of T cells by cytokines

In the animal models of autoimmune disorders, the most frequently delivered molecules by gene therapy are by far cytokines, chemokines and their receptors because they still represent the most plausible target therapy. This is due to the general understanding of the basic immunological mechanism sustaining the pathogenesis of these diseases. The aim of gene therapy, in this context, is to reduce the pro-inflammatory process by switching the T helper type 1 (Th1) lymphocytes arm of the immune response towards an anti-inflammatory ambient with the prevailing presence of T helper type 2 (Th2) lymphocytes-mediated immune responses. Recent studies have taken in the picture two more T cell populations: IL-17 producing T (Th17) cells and regulatory T (Treg) cells [11]. For this reason, new gene therapy approaches have been designed to modulate the functions of these newly discovered sub-populations of T cells in inflammation and neurodegeneration.

Modulation of the pathogenic immune process can, in principle, be achieved in different ways: (i) direct inhibition of pro-inflammatory cytokines and chemokines; (ii) immune deviation towards a Th2 anti-inflammatory profile; (iii) modulation of co-stimulation; and (iv) induction of central antigen-specific tolerance.

As mentioned above, cytokines and chemokines are still the most frequently used molecules to interfere with the adaptive immune system, but also decoy receptors and newly synthesised immunotoxins will be discussed.

Interferon-β

Beta-interferon (IFN-β) is a cytokine with potent immunomodulatory properties and is among the few approved drugs for MS. Administration by subcutaneous or intramuscular injection every other day, or weekly, clearly raise concerns of patient compliance, while gene therapy might provide long-lasting

Table 1. Recent gene therapy approaches in EAE

Therapeutic gene	Gene vector	References
Modulation of T cells by cytokines		
IFN-β	Naked DNA	[12]
	Naked DNA	[13]
	Naked DNA	[14]
IL-4	Adenovirus	[15]
	HSV-1	[17, 18]
	Naked DNA	[19]
IL-10	HSV-1	[17]
	Naked DNA	[21]
TGF-β	Retrovirus	[23]
	SFV	[22]
IL-25	Adenovirus	[25]
IL-1ra	HSV-1	[27]
7ND	Naked DNA	[28]
CXCR3	Naked DNA	[29]
CCR2	Naked DNA	[29]
CXCL10	Immunotoxin	[35]
CCL5	Immunotoxin	[39]
IL-18 binding protein	Immunotoxin	[39]
	Retrovirus	[40]
Induction of tolerance		
PLP	Mini-gene	[42, 44]
MBP	Mini-gene	[42]
MOG35-55-IgG	Retrovirus	[46]
Innate immune system		
Trem-2	Transduced microglia	[50]
Angiogenesis		
sFlt1 (antiVEGF)	Adenovirus	[57]
Antioxidative Therapy		
SOD2 ribozyme	AAV	[62]
EC-SOD	AAV	[68]
CAT	AAV	[61]
Gene and stem cell therapy		
IFN-β	Retrovirus in mesenchimal s.c.	[62]
BDNF	Retrovirus in mesenchimal s.c.	[64,65]
Olig-2	Transfected neural s.c.	[67]

release through a single injection. Indeed, systemic expression of murine IFN-β following intramuscular delivery of plasmid DNA to the hind limb of mice, is effective in reducing the clinical manifestations of disease in EAE mice [12, 13]. Jaini et al. compared the therapeutic efficacies of electroporation (EP)-mediated intramuscular IFN-β gene transfer with repeated alternate-

day injections of recombinant IFN-β after the onset of relapsing-remitting EAE. Results showed that a single optimised EP-mediated intramuscular administration of IFN-β expressing plasmid provides long-term expression of interferon-inducible genes [14]. To optimise the effectiveness, IFN-β transgene protein production should parallel the course of the disease. For this reason, the gene for IFN-β was placed under the control of an inflammation-responsive (NF-κB) promoter.

Interleukin-4

Interleukin-4 (IL-4) is a cytokine that induces differentiation of naive helper T cells (Th0 cells) to Th2 cells. Upon activation by IL-4 via the interleukin-4 receptor (IL4R), Th2 cells subsequently produce additional IL-4. IL-4 has other biological roles, such as the stimulation of activated B cell and T cell proliferation, and the differentiation of $CD4^+$ T cells into Th2 cells. IL-4 is one of the most widely used cytokines to treat experimental models of autoimmune disorders. The aim of this treatment is to shift the T cell response towards the Th2 arm to control the degeneration produced by the autoreactive attack. It has been successfully administered in EAE through different viral vectors. Butti et al. showed that a single administration of an IL-4 helper adenoviral vector (HD-Ad) into the cerebrospinal fluid (CSF) circulation of immunocompetent mice [15], allowed persistent transduction of neuroepithelial cells and up to 5 months central nervous system (CNS) IL-4 transgene expression without toxicity [16] or immunogenicity [17]. Broberg et al. using herpes simplex virus type 1 (HSV-1) derived vectors, proved that the direct effect of IL-4 on EAE development is more therapeutically relevant than the down-regulation of Th1 type cytokines [18]. This group also designed studies to investigate for routes alternative to intracranial injection to deliver HSV-1-derived vectors to the CNS of mice using reporter genes, IL-4, and interleukin-10 (IL-10) as transgenes. Results indicate that intranasal infection is the most efficient way to spread molecules to the CNS, after intracranial injection; viruses did not grow in cultures from brain samples, but the viral DNA persisted 21 days post-infection; viral replication occurred mainly on days 4 and 7 post-infection in trigeminal ganglia and, to a lower extent, in the brain where the vectors spread to the midbrain more efficiently than to other brain areas [19]. Ho et al. also tried to determine the optimal delivery route of cytokine DNA plasmids leading to significant beneficial effects both in early and late treatments of EAE. They directly compared the effects of intrasplenic (i.s.) and intramuscular (i.m.) electro-transfer of IL-4 DNA in a rat EAE model. Histological analysis showed that spinal cord inflammation was considerably reduced using the i.s. delivery route. Their results provide the first demonstration that i.s. electro-transfer of IL-4 DNA is more effective both in the prevention and modulation of EAE than i.m. transfer and that i.s. electro-gene transfer may represent a new approach to cytokine therapy in autoimmune diseases [20].

Interleukin-10

Interleukin-10 (IL-10) has been also extensively used in gene therapy protocols for autoimmunity. IL-10 is an anti-inflammatory cytokine, primarily produced by monocytes, Th2 lymphocytes and $CD4^+CD25^+Foxp3^+$ regulatory T cells. It has pleiotropic effects in immune-regulation and inflammation. It downregulates the expression of Th1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. It also enhances B cell survival, proliferation, and antibody production. It is capable of inhibiting synthesis of pro-inflammatory cytokines like IFN-γ, IL-2, IL-3 and TNF-α [21]. Anti-inflammatory IL-10 gene therapy to treat the autoimmune neuroinflammation induces beneficial behavioural and histopathological changes. This therapy can reverse inflammation-induced paralysis, decrease disease associated reduction in sensitivity to touch, and suppress CNS glial activation associated with disease progression [22].

Th17-Treg cytokines family

The cytokine playing a central role in the differentiation path that can lead to either $Foxp3^+$ suppressor or $IL\text{-}17^+$ effector T cells is transforming growth factor (TGF)-β [11]. When TGF-β signalling prevails, suppressor T_{regs} are generated, while in combination with IL-6 or IL-4, TGF-β induces the differentiation of Th17 and Th9 cells, apparently playing a crucial role in initiation of autoimmune processes [11]. Vähä-Koskela et al. constructed and characterised CNS-homing gene delivery vectors based on non-virulent Semliki Forest virus (SFV) expressing TGF-β1. Intraperitoneal administration of this vector significantly reduced disease severity suggesting that immunomodulation by neurotropic viral vectors may offer a promising treatment strategy for autoimmune CNS disorders [23]. In a different study, TGF-β1 has been used to downregulate EAE: Zargarova et al. retrovirally transduced primary skin fibroblasts *ex vivo* with TGF-β1 and then intraperitoneally administered the transduced cells during the priming phase of EAE. They observed a significant reduction in mortality *versus* control EAE mice treated with non-transduced fibroblasts [24].

IL-25 is a cytokine that belongs to the IL-17 cytokine family and is secreted by Th2 cells and mast cells. It induces the production of other cytokines, including IL-4, IL-5 and IL-13 in multiple tissues, which stimulate the expansion of eosinophils [25]. IL-25 adenoviral delivery in the CNS at several time points during relapsing-remitting or chronic EAE completely suppressed disease. This treatment was associated with elevated production of IL-13, which is required for suppression of Th17 responses by direct inhibition of IL-23, IL-1β, and IL-6 expression in activated dendritic cells [26].

Decoy receptors

One different strategy to interfere with the immune process is to inhibit selected signalling pathways. This is usually achieved through delivery of molecules that can antagonistically bind to cytokine or cytokines receptors and eventually reduce or switch off their activity. The most extensively studied target, to this respect, is interleukin-1 (IL-1), the most relevant primary proinflammatory cytokine. IL-1 receptor antagonist (IL-1ra) is a naturally occurring IL-1 antagonist that binds on the cell surface to the IL-1 receptor (IL1R), thus preventing IL-1 from signalling to that cell [27]. CNS-specific IL-1ra gene therapy with HSV-1 vector, however, was shown to be therapeutically efficient only if delivered before disease onset, while ineffective on established EAE [28].

Gene therapy has been used also to reduce migration of immune cells involved in the detrimental phase of the autoimmune disorders. Park et al. studied the differential effects of the delivery of a dominant-negative variant of CCL2 (7ND) by gene therapy on acute, biphasic and chronic EAE: in acute EAE and in the first attack of biphasic EAE, this therapy, especially effective in inhibiting migration of macrophages, was most ineffective because pathogenic T cells are mainly involved in lesion formation; in contrast, during the relapse of biphasic EAE and during chronic EAE, macrophages play a major role in the disease process and 7ND delivery by gene therapy was more effective [29]. A similar strategy has been attempted by intramuscular administration of decoy chemokine receptors, i.e., plasmids encoding the binding sites of CXCR3 and CCR2. This treatment showed to be effective in suppressing the development of relapses during chronic-relapsing EAE [30].

Immunotoxins

Immunotoxins are hybrid proteins consisting of a targeting moiety and a toxin moiety [31, 32]. Immunotoxins aim to target specific cells and kill them. In the last few years this new class of molecules have been used in several gene therapy approaches.

A truncated diphtheria toxin (DT), DT390, has been used to construct recombinant immunotoxins, in order to eliminate the non-specific binding of full length DT as well as to bypass the inhibitory effect of pre-existing anti-DT antibodies in human blood [31]. Substitution of the DT binding domain with ligands that bind to specific cell surface receptor can target the toxin to cell subpopulations and result in specific killing [33, 34]. CNS-infiltrating $CXCR3^+$ T cells are increased in EAE and MS, and the expression of its ligand CXCL10 was also detected in demyelinating brain lesions [35]. Mice intramuscularly injected with the eukaryotik expression plasmid SRα coding for the CXCL10-specific immunotoxin DT390-IP-10 showed a delayed onset of EAE and milder symptoms. Immunohistochemical staining confirmed significantly reduced

infiltrating CXCR3$^+$ cells in the inflammatory lesions of CNS from immunotoxin treated mice [36].

Regulated on activation normal T cells expressed and secreted (RANTES) protein, also known as chemokine (C-C motif) ligand 5 (CCL-5), is a chemotactic cytokine for T cells, eosinophils, and basophils [37]. Mice treated with DT390-RANTES-Srα develop a milder EAE with less CCR5$^+$-infiltrating cells in the CNS as compared to control mice [38]. Interleukin-18 (IL-18), an antigen-presenting cells (APCs)-derived protein, works together with IL-12 to induce cell-mediated immunity [39]. Gene delivery of cationic liposome-embedded DT390-IL-18-SRα in EAE mice caused a delayed onset of disease with decreased symptoms, possibly due to a reduction of infiltrating inflammatory cells into the brain [40]. Intrathecal injection of naked plasmid DNA encoding for the same gene yielded similar results [41].

Induction of tolerance

Central tolerance is the mechanism by which maturing T and B cells, reactive to self antigens, are deleted. Peripheral tolerance, on the other hand, occurs through anergy induction in T cells seeing their antigen in the absence of proper co-stimuli [42]. In order to ameliorate or arrest the course of EAE by induction of tolerance, a gene therapy approach in which animals are continuously exposed to low levels of proteolipid protein (PLP) antigen in absence of a co-stimulatory signal is usually used. To provide this therapeutic antigen-specific signal, mice are subcutaneously (s.c.) injected in the neck/shoulder area with syngeneic fibroblast cells retrovirally transduced with PLP 101–157 or myelin basic protein (MBP) 89–101. Treatments with both antigens expressed in syngeneic fibroblast cells were successful [43, 44]. Furthermore, cytokine analyses of brain and spinal cord lymphocytes demonstrated that the treatment induces an anti-inflammatory Th2 profile, indicating that this antigen specific therapy acts by a cytokine-induced pathway [44]. Also injection of PLP and MPB transduced bone marrow stem cells yielded positive results [45]. In a different study, retrovirally transduced B cells expressing MBP have been shown to suppress EAE induced by passive transfer of activated T cells, not only in naive but also in primed animals [46]. This group recently also showed that B cell expressing a MOG35-55-IgG fusion protein can delay the onset and/or decrease the severity of the disease in MOG and MBP-induced EAE mouse models as well as lead to a reduction in antibody titre to target CNS antigens [47]. While these approaches are very effective in an experimental setting, were the autoantigen is defined *a priori*, it is very difficult to envisage their efficient transfer to humans, were antigen-specific therapies have not been developed even in autoimmune disorders where the antigen is known (i.e., myasthenia gravis). The only human gene therapy for MS had the aim to obtain immune tolerance towards MBP and yielded very limited effects, but other MBP-directed therapies in humans have determined exacerbation of the

disease [48], raising a safety issue of these kind of approaches in human patients.

Innate immune system

Microglial triggering receptor expressed on myeloid cells-2 (TREM-2) stimulates phagocytosis and downregulates inflammatory signals in microglia [49]. Clearance of cellular debris and resolution of inflammation are important for recovery and repair in neuroinflammatory diseases [50]. In 2007, Takahashi et al. showed that TREM2-transduced myeloid cells applied intravenously migrated into the inflammatory spinal cord lesions of EAE-diseased mice, showed increased lysosomal and phagocytic activity, cleared degenerated myelin, and created an anti-inflammatory cytokine milieu within the CNS [51]. Taken together these results suggest that TREM-2 mediate nervous tissue debris clearance and facilitate recovery in EAE mice.

Angiogenesis

Angiogenesis is a highly orchestrated process involving the sprouting of new capillary-like structures from existing vasculature that mature into a system of new blood vessels, resulting in neovascularisation [52]. The angiogenic process, triggered and modified by a number of factors, including cytokines, chemokines, growth factors, and matrix metalloproteinases (MMPs), is central to many pathophysiologic conditions, such as tumour growth, diabetic retinopathy, and rheumatoid arthritis (RA) [53, 54] and thus is an interesting and promising therapeutic target for autoimmunity. Vascular Endothelial Growth Factor (VEGF) has important roles in endothelial cell proliferation, vascular permeability and angiogenesis in a variety of inflammatory lesions. VEGF expression was found to be associated with inflammatory cells in lesions of both MS patients and animals with acute EAE [55, 56]. A currently known selective and specific inhibitor of VEGF is a soluble form of the Flt-1 VEGF receptor (sFlt-1) [57]. Zhu et al. recently demonstrated that sFlt-1(1–3) (the first tree ectodomain of sFlt-1) gene transfer into the brain inhibits autoimmune inflammation in the CNS and ameliorates the severity of EAE in Dark Agouti (DA) rats [58].

Antioxidative therapy

Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, nitric oxide, and peroxynitrite are mediators of demyelination and disruption of the blood brain barrier (BBB) in EAE [59, 60]. Several gene therapy approaches are developed to reduce the action of ROS in different pathways. Cellular

defenses against ROS include catalase (CAT) and superoxide dismutase (SOD). SOD dismutes superoxide to hydrogen peroxide (H_2O_2), and CAT detoxifies the H_2O_2 to H_2O and O_2. rAAV-mediated delivery of extracellular-SOD (EC-SOD) and CAT provides long-lasting suppression against neuronal and axonal loss associated with permanent visual disability in the EAE DBA/1J mouse model [61, 62].

Stem cell mediated gene therapy

Since the very beginning of gene therapy for experimental autoimmune diseases, and EAE in particular, genetically modified cells have been used as Trojan horses to deliver their therapeutic product to the target organ. It is only since the discovery of the therapeutic potential of stem cells, however, that this strategy has been used in the attempt to combine the two approaches. We already described the use of myeloid precursors to deliver a tolerogenic signal [45], or to induce a 'protective' phenotype to microglial cells [51]. On the other hand, Makar and colleagues transduced mesenchimal stem cells, known for their immunomodulatory properties and their ability to target inflammatory sites, to deliver IFN-β to EAE mice [63], or to induce release of BDNF, trying to combine immunomodulation and pro-regenerative signals [64, 65]. Finally, to increase the regenerative potential of neural precursor cells (NPCs), that only account for a small fraction of remyelinating oligodendrocytes when transplanted in EAE [66], NPCs have been transduced with olig-2 to foster their differentiation in myelin-forming cells and tested in a model of focal demyelination [67]. Exploiting the potential of the combination of gene and stem cell therapy will certainly be further pursued in the near future, in the attempt to address both inflammation and brain repair mechanisms.

Conclusions

Gene therapy is under scrutiny in human trials because of the occurrence of severe side effects. The improvement of the gene therapy tools, however, and the recent advancement of knowledge on pathogenic mechanisms leading to CNS autoimmunity, will soon lead to renewed interest in a therapeutic approach that holds incomparable flexibility, ability to target multi-focal, chronic diseases such as multiple sclerosis, and is potentially able to immediately translate the discovery of new therapeutic targets to the bedside.

References

1 Hafler DA, Compston A, Sawcer S, Lander ES, Daly MJ, De Jager PL, de Bakker PI, Gabriel SB, Mirel DB, Ivinson AJ et al. (2007) Risk alleles for multiple sclerosis identified by a genomewide study. *N Engl J Med* 357: 851–862

2 Australia and New Zealand Multiple Sclerosis Genetics Consortium (2009) Genome-wide associ-

ation study identifies new multiple sclerosis susceptibility loci on chromosomes 12 and 20. *Nat Genet* 41: 824–828
3 Cooper GS, Stroehla BC (2003) The epidemiology of autoimmune diseases. *Autoimmunity reviews* 2: 119–125
4 Steinman L (2004) Immune therapy for autoimmune diseases. *Science* 305: 212–216
5 Janeway CA, Travers P, Walport M, Shlomchik MJ (2005) Autoimmunity and transplantation. In: CA Janeway, P Travers, M Walport, MJ Shlomchik (eds) *Immunobiology*, 5th edition. Taylor & Francis Group, New York, 501–551
6 Furlan R, Butti E, Pluchino S, Martino G (2004) Gene therapy for autoimmune diseases. *Curr Opin Mol Ther* 6: 525–536
7 Garren H, Robinson WH, Krasulova E, Havrdova E, Nadj C, Selmaj K, Losy J, Nadj I, Radue EW, Kidd BA et al. (2008) Phase 2 trial of a DNA vaccine encoding myelin basic protein for multiple sclerosis. *Ann Neurol* 63: 611–620
8 Furlan R, Cuomo C, Martino G (2009) Animal models of multiple sclerosis. *Methods Mol Biol* 6: 157–173
9 Baker D, Hankey DJ (2003) Gene therapy in autoimmune, demyelinating disease of the central nervous system. *Gene Ther* 10: 844–853
10 Chernajovsky Y, Gould DJ, Podhajcer OL (2004) Gene therapy for autoimmune diseases: quo vadis? *Nat Rev Immunol* 4: 800–811
11 Awasthi A, Murugaiyan G, Kuchroo VK (2008) Interplay between effector Th17 and regulatory T cells. *J Clin Immunol* 28: 660–670
12 Schaefer C, Hidalgo TR, Cashion L, Petry H, Brooks A, Szymanski P, Qian HS, Gross C, Wang P, Liu P et al. (2006) Gene-based delivery of IFN-beta is efficacious in a murine model of experimental allergic encephalomyelitis. *J Interferon Cytokine Res* 26: 449–454
13 Harkins RN, Szymanski P, Petry H, Brooks A, Qian HS, Schaefer C, Kretschmer PJ, Orme A, Wang P, Rubanyi GM et al. (2008) Regulated expression of the interferon-beta gene in mice. *Gene Ther* 15: 1–11
14 Jaini R, Hannaman D, Johnson JM, Bernard RM, Altuntas CZ, Delasalas MM, Kesaraju P, Luxembourg A, Evans CF, Tuohy VK (2006) Gene-based intramuscular interferon-beta therapy for experimental autoimmune encephalomyelitis. *Mol Ther* 14: 416–422
15 Martino G, Furlan R, Comi G, Adorini L (2001) The ependymal route to the CNS: an emerging gene-therapy approach for MS. *Trends Immunol* 22: 483–490
16 Butti E, Bergami A, Recchia A, Brambilla E, Del Carro U, Amadio S, Cattalini A, Esposito M, Stornaiuolo A, Comi G et al. (2008) IL4 gene delivery to the CNS recruits regulatory T cells and induces clinical recovery in mouse models of multiple sclerosis. *Gene Ther* 15: 504–515
17 Butti E, Bergami A, Recchia A, Brambilla E, Franciotta D, Cattalini A, Stornaiuolo A, Lachapelle F, Comi G, Mavilio F et al. (2008) Absence of an intrathecal immune reaction to a helper-dependent adenoviral vector delivered into the cerebrospinal fluid of non-human primates. *Gene Ther* 15: 233–238
18 Broberg EK, Salmi AA, Hukkanen V (2004) IL-4 is the key regulator in herpes simplex virus-based gene therapy of BALB/c experimental autoimmune encephalomyelitis. *Neurosci Lett* 364: 173–178
19 Broberg EK, Peltoniemi J, Nygårdas M, Vahlberg T, Röyttä M, Hukkanen V (2004) Spread and replication of and immune response to gamma134.5-negative herpes simplex virus type 1 vectors in BALB/c mice. *J Virol* 78: 13139–13152
20 Ho SH, Lee HJ, Kim DS, Jeong JG, Kim S, Yu SS, Jin Z, Kim S, Kim JM (2006) Intrasplenic electro-transfer of IL-4 encoding plasmid DNA efficiently inhibits rat experimental allergic encephalomyelitis. *Biochem Biophys Res Commun* 343: 816–824
21 Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19: 683–765
22 Sloane E, Ledeboer A, Seibert W, Coats B, van Strien M, Maier SF, Johnson KW, Chavez R, Watkins LR, Leinwand L et al. (2009) Anti-inflammatory cytokine gene therapy decreases sensory and motor dysfunction in experimental Multiple Sclerosis: MOG-EAE behavioral and anatomical symptom treatment with cytokine gene therapy. *Brain Behav Immun* 23: 92–100
23 Vähä-Koskela MJ, Kuusinen TI, Holmlund-Hampf JC, Furu PT, Heikkilä JE, Hinkkanen AE (2007) Semliki Forest virus vectors expressing transforming growth factor beta inhibit experimental autoimmune encephalomyelitis in Balb/c mice. *Biochem Biophys Res Commun* 355: 776–781

24 Zargarova T, Kulakova O, Prassolov V, Zharmukhamedova T, Tsyganova V, Turobov V, Ivanov D, Parfenov M, Sudomoina M, Chernajovsky Y et al. (2004) Prevention of experimental autoimmune encephalomyelitis in DA rats by grafting primary skin fibroblasts engineered to express transforming growth factor-beta1. *Clin Exp Immunol* 137: 313–319
25 Lee J, Ho WH, Maruoka M, Corpuz RT, Baldwin DT, Foster JS, Goddard AD, Yansura DG, Vandlen RL, Wood WI et al. (2001) IL-17E, a novel proinflammatory ligand for the IL-17 receptor homolog IL-17Rh1. *J Biol Chem* 276: 1660–1664
26 Kleinschek MA, Owyang AM, Joyce-Shaikh B, Langrish CL, Chen Y, Gorman DM, Blumenschein WM, McClanahan T, Brombacher F, Hurst SD et al. (2007) IL-25 regulates Th17 function in autoimmune inflammation. *J Exp Med* 204: 161–170
27 Dinarello CA (2002) The IL-1 family and inflammatory diseases. *Clin Exp Rheumatol* 20: S1–13
28 Furlan R, Bergami A, Brambilla E, Butti E, De Simoni MG, Campagnoli M, Marconi P, Comi G, Martino G (2007) HSV-1-mediated IL-1 receptor antagonist gene therapy ameliorates MOG(35–55)-induced experimental autoimmune encephalomyelitis in C57BL/6 mice. *Gene Ther* 14: 93–98
29 Park L, Lee E, Lee S, Lim M, Hong H, Shin G, Park Y (2008) TGFbeta plasmid construction and delivery for the prevention of type 1 diabetes. *Ann N Y Acad Sci* 1150: 177–182
30 Matsumo Y, Sakuma H, Miyakoshi A, Tsukada Y, Kohyama K, Park IK, Tanuma N (2005) Characterization of relapsing autoimmune encephalomyelitis and its treatment with decoy chemokine receptor genes. *J Neuroimmunol* 170: 49–61
31 Thompson J, Hu H, Scharff J, Neville DM (1995) An anti-CD3 single-chain immunotoxin with a truncated diphtheria toxin avoids inhibition by pre-existing antibodies in human blood. *J Biol Chem* 270: 28037–28041
32 Williams DP, Snider CE, Strom TB, Murphy JR (1990) Structure/function analysis of interleukin-2-toxin (DAB486-IL-2). Fragment B sequences required for the delivery of fragment A to the cytosol of target cells. *J Biol Chem* 265: 11885–11889
33 Arora N, Masood R, Zheng T, Cai J, Smith DL, Gill PS (1999) Vascular endothelial growth factor chimeric toxin is highly active against endothelial cells. *Cancer Res* 59: 183–188
34 Thompson J, Stavrou S, Weetall M, Hexham JM, Digan ME, Wang Z, Woo JH, Yu Y, Mathias A, Liu YY et al. (2001) Improved binding of a bivalent single-chain immunotoxin results in increased efficacy for *in vivo* T-cell depletion. *Protein Eng* 14: 1035–1041
35 Balashov KE, Rottman JB, Weiner HL, Hancock WW (1999) CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1alpha and IP-10 are expressed in demyelinating brain lesions. *Proc Natl Acad Sci USA* 96: 6873–6878
36 Chen W, Li H, Jia Y, Lv M, Li M, Feng P, Hu H, Zhang L (2007) *In vivo* administration of plasmid DNA encoding recombinant immunotoxin DT390-IP-10 attenuates experimental autoimmune encephalomyelitis. *J Autoimmun* 28: 30–40
37 Donlon TA, Krensky AM, Wallace MR, Collins FS, Lovett M, Clayberger C (1990) Localization of a human T-cell-specific gene, RANTES (D17S136E), to chromosome 17q11.2-q12. *Genomics* 6: 548–553
38 Jia Y, Li H, Chen W, Li M, Lv M, Feng P, Hu H, Zhang L (2006) Prevention of murine experimental autoimmune encephalomyelitis by *in vivo* expression of a novel recombinant immunotoxin DT390-RANTES. *Gene Ther* 13: 1351–1359
39 Okamura H, Tsutsi H, Komatsu T, Yutsudo M, Hakura A, Tanimoto T, Torigoe K, Okura T, Nukada Y, Hattori K (1995) Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 378: 88–91
40 Jia J, Li H, Tai S, Lv M, Liao M, Yang Z, Zhang B, Zhou B, Zhang G, Zhang L (2008) Construction and preliminary investigation of a plasmid containing a novel immunotoxin DT390-IL-18 gene for the prevention of murine experimental autoimmune encephalomyelitis. *DNA Cell Biol* 27: 279–285
41 Schif-Zuck S, Westermann J, Netzer N, Zohar Y, Meiron M, Wildbaum G, Karin N (2005) Targeted overexpression of IL-18 binding protein at the central nervous system overrides flexibility in functional polarization of antigen-specific Th2 cells. *J Immunol* 174: 4307–4315
42 Sprent J, Kishimoto H (2001) The thymus and central tolerance. *Philos Trans R Soc Lond, B, Biol Sci* 356: 609–616
43 Louie KA, Weiner LP, Du J, Kochounian HH, Fling SP, Wei W, McMillan M (2005) Cell-based gene therapy experiments in murine experimental autoimmune encephalomyelitis. *Gene Ther* 12: 1145–1153
44 Weiner LP, Louie KA, Atalla LR, Kochounian HH, Du J, Wei W, Hinton DR, Gordon EM,

Anderson WF, McMillan M (2004) Gene therapy in a murine model for clinical application to multiple sclerosis. *Ann Neurol* 55: 390–399
45 Xu B, Haviernik P, Wolfraim LA, Bunting KD, Scott DW (2006) Bone marrow transplantation combined with gene therapy to induce antigen-specific tolerance and ameliorate EAE. *Mol Ther* 13: 42–48
46 Melo ME, Qian J, El-Amine M, Agarwal RK, Soukhareva N, Kang Y, Scott DW (2002) Gene transfer of Ig-fusion proteins into B cells prevents and treats autoimmune diseases. *J Immunol* 168: 4788–4795
47 Xu B, Scott DW (2004) A novel retroviral gene therapy approach to inhibit specific antibody production and suppress experimental autoimmune encephalomyelitis induced by MOG and MBP. *Clin Immunol* 111: 47–52
48 Bielekova B, Goodwin B, Richert N, Cortese I, Kondo T, Afshar G, Gran B, Eaton J, Antel J, Frank JA et al. (2000) Encephalitogenic potential of the myelin basic protein peptide (amino acids 83–99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med* 6: 1167–1175
49 Takahashi K, Rochford CD, Neumann H (2005) Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J Exp Med* 201: 647–657
50 Carson MJ (2002) Microglia as liaisons between the immune and central nervous systems: functional implications for multiple sclerosis. *Glia* 40: 218–231
51 Takahashi K, Prinz M, Stagi M, Chechneva O, Neumann H (2007) TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS Med* 4: e124
52 Carmeliet P (2003) Angiogenesis in health and disease. *Nat Med* 9: 653–660
53 Folkman J (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1: 27–31
54 Koch AE, Halloran MM, Haskell CJ, Shah MR, Polverini PJ (1995) Angiogenesis mediated by soluble forms of E-selectin and vascular cell adhesion molecule-1. *Nature* 376: 517–519
55 Kirk SL, Karlik SJ (2003) VEGF and vascular changes in chronic neuroinflammation. *J Autoimmun* 21: 353–363
56 Su JJ, Osoegawa M, Matsuoka T, Minohara M, Tanaka M, Ishizu T, Mihara F, Taniwaki T, Kira J (2006) Upregulation of vascular growth factors in multiple sclerosis: correlation with MRI findings. *J Neurol Sci* 243: 21–30
57 Kendall RL, Wang G, Thomas KA (1996) Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. *Biochem Biophys Res Commun* 226: 324–328
58 Zhu C, Hu XQ, Xiong ZJ, Lu ZQ, Zhou GY, Wang DJ (2008) Adenoviral delivery of soluble VEGF receptor 1 (sFlt-1) inhibits experimental autoimmune encephalomyelitis in dark Agouti (DA) rats. *Life Sci* 83: 404–412
59 Honegger CG, Krenger W, Langemann H (1989) Measurement of free radical scavengers in the spinal cord of rats with experimental autoimmune encephalomyelitis. *Neurosci Lett* 98: 327–332
60 Ruuls SR, Van Der Linden S, Sontrop K, Huitinga I, Dijkstra CD (1996) Aggravation of experimental allergic encephalomyelitis (EAE) by administration of nitric oxide (NO) synthase inhibitors. *Clin Exp Immunol* 103: 467–474
61 Qi X, Hauswirth WW, Guy J (2007) Dual gene therapy with extracellular superoxide dismutase and catalase attenuates experimental optic neuritis. *Mol Vis* 13: 1–11
62 Qi X, Sun L, Lewin AS, Hauswirth WW, Guy J (2007) Long-term suppression of neurodegeneration in chronic experimental optic neuritis: antioxidant gene therapy. *Investigative Ophthalmology & Visual Science* 48: 5360–5370
63 Makar TK, Trisler D, Bever CT, Goolsby JE, Sura KT, Balasubramanian S, Sultana S, Patel N, Ford D, Singh IS et al. (2008) Stem cell based delivery of IFN-beta reduces relapses in experimental autoimmune encephalomyelitis. *J Neuroimmunol* 196: 67–81
64 Makar TK, Bever CT, Singh IS, Royal W, Sahu SN, Sura TP, Sultana S, Sura KT, Patel N, Dhib-Jalbut S et al. (2009) Brain-derived neurotrophic factor gene delivery in an animal model of multiple sclerosis using bone marrow stem cells as a vehicle. *J Neuroimmunol* 210: 40–51
65 Makar TK, Trisler D, Sura KT, Sultana S, Patel N, Bever CT (2008) Brain derived neurotrophic factor treatment reduces inflammation and apoptosis in experimental allergic encephalomyelitis. *J Neurol Sci* 270: 70–76
66 Pluchino S, Quattrini A, Brambilla E, Gritti A, Salani G, Dina G, Galli R, Del Carro U, Amadio

S, Bergami A et al. (2003) Injection of adult neurospheres induces recovery in a chronic model of multiple sclerosis. *Nature* 422: 688–694

67 Copray S, Balasubramaniyan V, Levenga J, de Bruijn J, Liem R, Boddeke E (2006) Olig2 overexpression induces the *in vitro* differentiation of neural stem cells into mature oligodendrocytes. *Stem Cells* 24: 1001–1010

68 Qi X, Lewin AS, Sun L, Hauswirth WW, Guy J (2007) Suppression of mitochondrial oxidative stress provides long-term neuroprotection in experimental optic neuritis. *Investigative Ophthalmology & Visual Science* 48: 681–691

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Gene therapy for myositis

Hans H. Jung[1], Juliane Bremer[2] and Michael Weller[1]

[1] *Department of Neurology, University Hospital Zurich, Zürich, Switzerland*
[2] *Institute of Neuropathology, Department of Pathology, University Hospital Zürich, Switzerland*

Abstract

The inflammatory myopathies, polymyositis (PM), dermatomyositis (DM) and inclusion body myositis (IBM), lead to moderate to severe muscle weakness and are characterised by the presence of endomysial inflammation. Each entity has unique clinical, immunopathological and histological characteristics which are associated with different responses to therapies and prognosis. In DM and PM, first-line treatment options include oral corticosteroids, other immunosuppressant drugs, and intravenous immunoglobulins. Patients with IBM, by contrast, usually show a poor or no response to immunomodulatory treatments. Patients with IBM and non-responding patients with PM and DM are candidates for alternative treatment options and experimental therapies including gene therapy. The genetic treatment of inflammatory muscle disorders could involve at least two different strategies: first, to ectopically express local immune modulatory, notably immunosuppressive molecules which would limit inflammation and autoimmunity more effectively than systemic immunosuppressive treatment; second, strategies to promote the repair or allow for the replacement of damaged muscle might be envisaged. Immunosuppressive molecules might include HLA-G, a non-classical major histocompatibility (MHC) Class I molecule, or other cell surface molecules which negatively modulate immune effector cell function. Muscle regeneration might be promoted by myotrophic factors including utrophin or insulin-like growth factors. In addition, cell-based therapies using stem cells or myoblasts might have a therapeutic potential in neuromuscular disorders.

Introduction

The inflammatory myopathies include three distinct major entities: polymyositis (PM), dermatomyositis (DM) and inclusion body myositis (IBM) [1]. Although the common hallmarks of all three conditions are moderate to severe muscle weakness and the presence of endomysial inflammation they have distinct clinical features and immunopathological and histological characteristics. In addition, the response to immunomodulatory treatments differs significantly between these disorders, namely in IBM compared to PM and DM.

PM has usually a subacute onset and predominantly affects proximal muscles. Initial symptoms are often unspecific and there are no extramuscular features which may interfere with an early diagnosis [2]. The common age of onset is in the forth or fifth decade. DM also develops subacutely and preferentially affects proximal muscles. The characteristic skin manifestations may accompany or precede muscle weakness and include an erythema in sun-exposed areas, in particular face and eyelids, often together with a periorbital

oedema, but also of extensor joint surfaces [2]. DM has a biphasic age distribution including a juvenile type with an age range between four and twelve years and adult form with usual onset in the fifth or sixth decade. PM as well as DM may arise as an idiopathic autoimmune condition but also in association with other autoimmune disorders such as scleroderma, systemic lupus erythematosus or mixed connective tissue disease.

IBM, on the other hand, usually has a insidious onset and a slow progression, and may affect both the proximal and the distal muscles, often with an asymmetrical pattern and predominantly involving quadriceps, foot extensors and finger flexors [3]. IBM is the most common muscle disease in patients above the age of 50 years and, inversely to PM and DM, more males than females are affected. In contrast to PM and DM, IBM may result in significant weakness and atrophy, and usually does not respond to corticosteroids or immunosuppressive agents.

In DM, a humoral immunopathology leading to an injury of endothelial cells and muscle fibres by a chronic intracellular overproduction of interferon type 1-inducible proteins has been postulated. PM and IBM share common immunopathological features consisting of upregulation of MHC-1 Class I antigen on the sarcolemma of muscle cells and T cell-mediated cytotoxicity [2, 4]. In addition, a degenerative process in IBM has to be postulated since there are amyloid deposits within vacuolated muscle fibres showing an immunoreaction to various neurodegeneration-related proteins including amyloid precursor protein (APP), presenilin I, apolipoprotein E and phosphorylated tau.

Diagnosis

The diagnosis of myositis is based on the combination of determination of serum creatine kinase (CK), electromyography (EMG) and muscle biopsy. Although serum CK may be elevated in all three forms of myositis, it can be normal or only slightly elevated in DM and IBM [2, 3]. The EMG may show a myopathic pattern with short duration, polyphasic motor unit potentials as well as spontaneous activity such as fibrillations and positive sharp waves [5]. The electrodiagnostic alterations are usually most pronounced in DM and PM and may be discrete or lacking in IBM but also in long-lasting, slowly progressive PM and DM.

Although the common histological hallmark of inflammatory myopathies is mononuclear endomysial inflammation, the different forms of myositis have characteristic pathological features (Figs 1 and 2) [6]. The pathology of DM is characterised by a perifascicular lesion pattern. The inflammatory cells are predominantly located perivascularly and perimysially, although some may be endomysially (Fig. 1) [6]. Histological findings include perifascicular atrophy, rare areas of apparent infarction, and focal loss of myofibrils [6]. In PM and IBM, by contrast, the inflammatory cells, mainly consisting of $CD8^+$ T cells are predominantly located in the endomysial parenchyma, and may invade

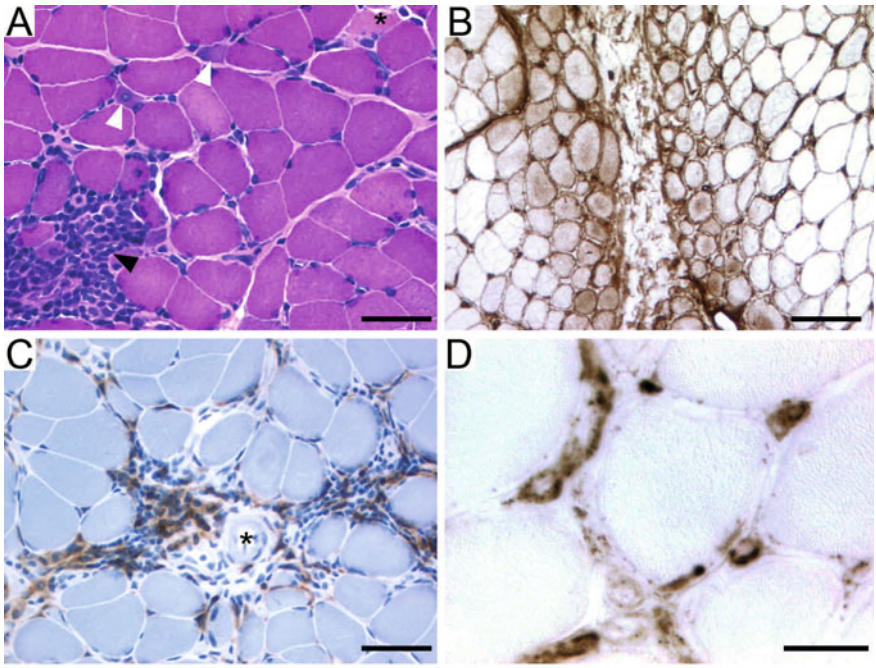


Figure 1. Histopathology of DM. A: H&E staining shows lymphocytic infiltrates in the endomysium (black arrowhead), necrotic muscle fibres (star), and basophilic regenerating fibres (white arrowhead). Scale bar = 50 µm. B: Immunohistochemistry for MHC Class I antigen shows upregulation on the sarcolemma of muscle cells especially on perifascicular atrophic myofibres reflecting the classical distribution pattern in dermatomyositis (→ perifascicular atrophy). Scale bar = 100 µm. C: As revealed by immunohistochemistry for CD4, inflammatory infiltrates consist mainly of $CD4^+$ cells, including T helper cells, besides the $CD20^+$ B cell population (not shown). Perivascular localisation of inflammatory infiltrates is a common finding. A blood vessel is marked with a star. Scale bar = 50 µm. D: Immunohistochemistry shows upregulation of membrane attack complex (MAC, C5b-9) on endomysial capillaries that normally lack MAC expression. Scale bar = 30 µm.

non-necrotic muscle fibres. Furthermore, muscle fibres in PM and IBM show sarcolemmal expression of MHC Class I antigen (Fig. 2) [6]. IBM is in addition characterised by cytoplasmic and intranuclear inclusions containing beta amyloid and several other proteins including phosphorylated Tau. Somatic mitochondrial mutations (mtDNA) are another common finding in IBM, resulting in segmental loss of cytochrome c oxidase (COX) activity in muscle fibres, and ragged-red fibres [6].

Immunopathogenesis

Possible targets for gene therapy may arise from the knowledge of the pathogenetic basis of inflammatory myopathies. In DM, it has been proposed that

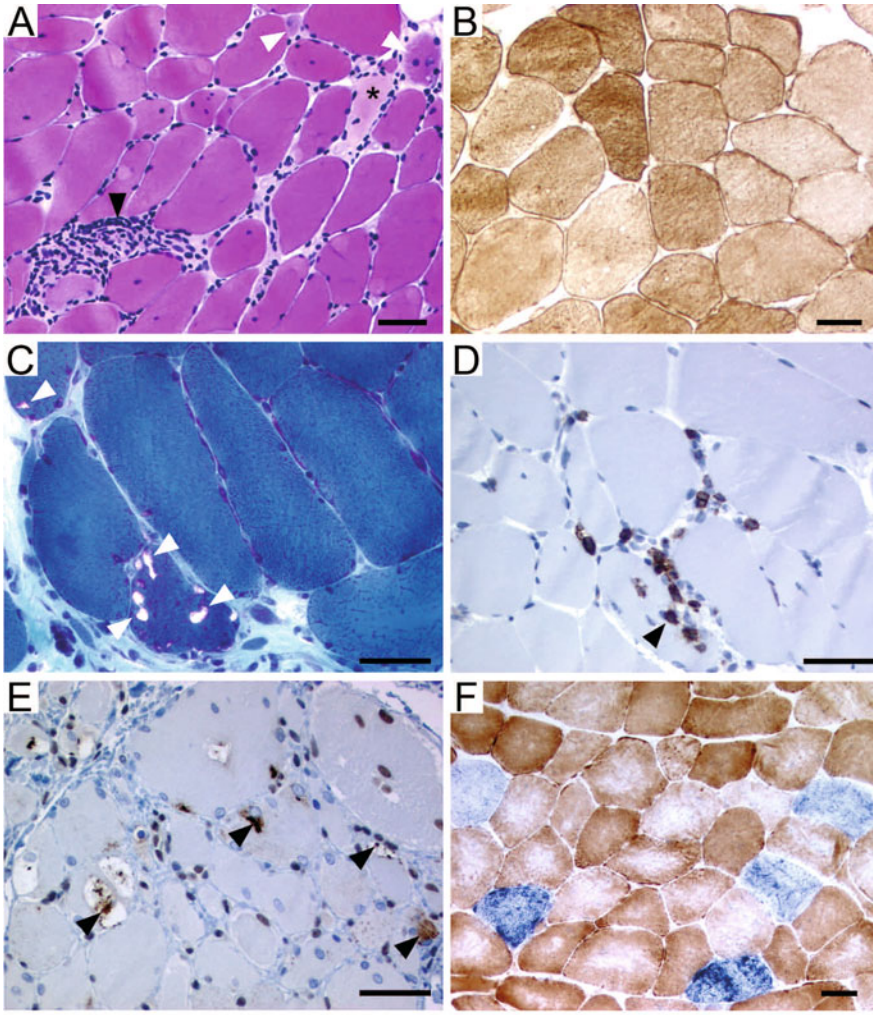


Figure 2. Histopathology of PM and IBM. A: H&E staining shows lymphocytic infiltrates in the endomysium (black arrowhead), necrotic muscle fibres (star), and basophilic regenerating fibres (white arrowhead) in a case of PM. B: Upregulation of MHC Class I antigen on the sarcolemma of muscle fibres in PM in a random distribution. C: Gomori trichrome staining shows rimmed vacuoles (white arrowheads) in IBM. D: $CD8^+$ T cells in the endomysium in IBM. Some $CD8^+$ T cells invade a non-necrotic muscle fibre (black arrowhead). E: Protein aggregates in IBM are positive for TAR DNA-binding protein-43 (TDP43) in immunohistochemistry (black arrowheads). F: Combined enzyme histochemistry for cytochrome oxidase (COX; brown reaction product) followed by succinic dehydrogenases (SDH; blue reaction product). COX-negative fibres in IBM are SDH-positive and therefore appear blue. All scale bars = 50 μm.

autoantibodies are directed against the endothelium and cause vascular injury leading to ischemic damage of muscle fibres [2]. However, distinct pathogenic autoantibodies to a specific endothelial antigen have not been identified yet and

there is no direct evidence for muscle fibre ischemia [7]. Microarray studies in adult DM have demonstrated a high differential expression of gene transcripts belonging to a common pathway of genes induced by interferon-α and interferon-β he so-called type 1 interferons, but not the type 2 interferon-γ [8, 9]. Type 1 interferons in DM are possibly produced by intramuscular and skin plasmacytoid dendritic cells (pDC) [9]. In addition, pDCs also act as antigen presenting cells for T helper [10] and T regulatory cells [11], and stimulate B cell development into plasma cells [12]. Therefore, endothelial cells and myofibres may be injured in DM by the chronic intracellular overproduction of interferon type 1-inducible proteins [13]. It remains unclear why perifascicular myofibres are preferentially vulnerable and express interferon type 1-inducible proteins. pDC interferon type 1 production is mediated through Toll-like receptor-dependent pathways [14]. Since viral DNA and RNA are known to activate pDC, the presence of a chronic viral infection has been discussed. However, no viral genetic material from a range of viral species has been detected in DM or other inflammatory myopathies [15].

In PM and IBM, $CD8^+$ T cells surrounding and invading MHC Class I antigen-expressing, non-necrotic muscle fibres are the primary effector cells mediating muscle fibre injury [2, 4]. Muscle fibres physiologically do not express MHC Class I antigens. In IBM and PM, however, MHC Class I antigens including HLA-G are ubiquitously expressed even on muscle fibres without histological signs of inflammation [16–18]. In other chronic myopathies or dystrophies, MHC Class I antigen upregulation is not present or only found in degenerating or regenerating muscle fibres. In PM and IBM, the chronic upregulation of MHC Class I antigens may promote gene transcription of cytokines including interleukins (IL) 1, 2, 5, 10, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β as well as several chemokines such as monocyte chemoattractant protein-1a, macrophage inflammatory protein-1a, monokine induced by gamma interferon and interferon-gamma-inducible protein-10 thus contributing to T cell activation and persistence of the inflammation [2, 19, 20]. Various chemokine receptors on the endothelium and inflammatory cells are upregulated as well, enhancing the local immune response. In addition, adhesion molecules and their ligands such as vascular cell adhesion molecule, intracellular adhesion molecule, integrins and metalloproteinases are overexpressed and facilitate the migration of lymphocytes toward the muscle fibres [19–22].

Although IBM shows considerable resistance to conventional immunotherapies, it shares many immunopathological features with PM [23]. Several observations support the crucial role of immunopathogenesis of IBM. The inflammation is often more pronounced in early disease stages whereas the vacuolar changes become more prominent later in the course of disease [24]. Muscle fibres act as antigen-presenting cells, with upregulation of MHC Class I antigens and there is a clonal expansion of invasive $CD8^+$ T cells [3]. In addition, an increased expression of cytokines and chemokines including IL1, interferon γ, TGF-β, and TNF-α has been demonstrated [19, 21]. Microarray studies showed abundant immunoglobulin transcripts in IBM-affected muscles

[8]. Finally, an association of IBM with autoantibodies and autoimmune diseases as well as the autoimmune 8 · 1 MHC ancestral haplotype (B8-DR3-DR52-DQ2) has been observed [25, 26].

An additional degenerative process in IBM has to be postulated due to the presence of vacuoles in muscle fibres which are not invaded by T cells and pronounced deposits of beta amyloid within these vacuolated muscle fibres showing an immunoreaction to various neurodegeneration-related proteins including amyloid precursor protein (APP), presenilin I, apolipoprotein E and phosphorylated tau [27]. It remains unclear whether these deposits directly contribute to disease pathogenesis or are secondary phenomena. It has been postulated that accumulation of the amyloid-beta peptide, which is cleaved by proteolysis from APP, is an early pathologic event in both Alzheimer disease and IBM. Skeletal muscle specific overexpression of beta-APP in transgenic mice resulted in an intracellular immunoreactivity to beta-APP in muscle fibres and the development of some histopathologic and clinical features comparable to IBM. However, the inflammatory infiltrates mainly consisted of granulocytes rather than of lymphocytes as in IBM [28]. In such a transgenic mouse model of IBM with increased expression of beta-APP, administration of lipopolysaccharide (LPS) led to acute and chronic inflammation, concomitantly increased levels of phosphorylated tau and beta-amyloid via a glycogen synthase kinase 3-beta (GSK3B)-dependent pathway, as well as an exacerbation of motor decline [29]. These data support an important role of inflammation in IBM.

On the other hand, aggregation of these neurodegeneration-related proteins seems to be not specific for sporadic IBM and may be observed in several other myopathies, such as X-linked Emery-Dreifuss muscular dystrophy, myofibrillar myopathies or dysferlinopathies and might represent therefore an unspecific stress response of muscle fibres [23]. In addition, the relative resistance of IBM to conventional immunotherapies is also found in other immune disorders such as primary progressive multiple sclerosis, where autoimmune and degenerative features coexist from the onset of the disease.

Conventional therapies

In DM and PM, treatment options of first choice are oral or intravenous corticosteroids [30, 31]. Since not all patients respond to these standard treatments and many develop side effects, immunosuppressant drugs such as methotrexate, azathioprine or cyclophosphamide are given as second line agents in combination with corticosteroids [30, 31]. If this combination is not sufficient, intravenous immunoglobulins or additional immunosuppressant drugs such as cyclosporine A or mycophenolate are alternative treatment options. About 25% of patients with DM and PM, however, do not response to this escalating treatment regime and continue to suffer clinical progression or relapses. In addition, patients with IBM usually show a poor or no response to standard

treatments including corticosteroids, immunosuppressant drugs or intravenous immunoglobulins [3, 30, 31].

These non-responding patients are candidates for alternative treatment options and experimental therapies. New immune therapies directed toward cytokine modulation, immune cell migration, or modification of certain immune cells subsets, notably B and T cells, are currently being in preclinical studies. Some of these approaches may qualify as genetic therapies.

Gene therapy

Gene therapy may be defined as a group of therapeutic approaches which are based on the use of genetic information as part of the overall therapeutic concept. It can aim to replace mutated or deleted DNA in monogenetic hereditary disorders. In addition, gene therapy may use DNA to express a therapeutic transgene to obtain certain desirable effects in distinct tissues, e.g., local immunosuppression in inflamed muscle. The ectopic expression of therapeutic gene products can be achieved in stem cells or differentiated, post-mitotic cells using various vehicles, most commonly liposomes or viral vectors, in particular adenoviruses, recombinant adenovirus-associated viruses (AAV), herpes simplex type 1 viruses, lentiviruses or plasmids. In inflammatory muscle disorders, these strategies might involve the local expression of immune modulatory molecules thereby limiting inflammation as well as the expression of myotrophic factors, in particular in IBM.

In muscle disorders, gene therapy has been first evaluated as a treatment of Duchenne muscular dystrophy (DMD). Several strategies have emphasised either introduction of a functional, recombinant version of the dystrophin gene, functional parts of the gene, so-called micro-dystrophins, or exon skipping by modification of dystrophin pre-mRNA splicing using antisense oligonucleotides or delivery of small interfering RNAs for RNA inhibition [32–34]. Delivery of the vectors by intramuscular injection transfected only a small region of muscle tissue around the injection site and was not suitable for this generalised muscle disorder. Therefore, alternative routes of administration including local intra-arterial or systemic intravenous ways as well as several strategies enhancing transfection efficiency including cationic lipid formulations, electroporation, pressurised isolated-limb perfusion or microbubbles and ultrasound were proposed [35–37]. Gene replacement mediated by several types of vectors as well as exon skipping techniques evoked a clinical effect in various DMD models, but were not successful in human studies [34, 38]. In addition to the replacement of dystrophins or parts of it using mini-dystrophin cassettes for the restoration of normal dystrophin function, the expression or upregulation of other structural muscle proteins has been discussed as a therapeutic strategy in DMD. For example, upregulation of utrophin partially rescued the dystrophic phenotypes in transgenic murine models, but not in human patients [39].

Besides insufficient efficacy for clinical use, problems with safety may arise depending on the vector used. Adenovirus vectors may induce a viral immune response, high transduction levels are mainly achieved only in regenerating muscle, and there is a limited persistence of transgene expression. In herpes simplex vectors, viral toxicity may represent an additional problem. Plasmid vectors are relatively safe and allow a simple engineering. However, transfection requires efficient techniques which are not yet applicable in a clinical setting.

In addition to the classical gene therapies, cell-based therapies including myoblasts or stem cells might offer therapeutic perspectives in muscle diseases. Myoblast transfer therapy (MTT) was the first cellular gene therapy proposed for DMD [40]. The clinical benefit of MTT, however, was minimal and there was evidence for considerable immunological reactions [41–43]. Stem cells with myogenic potential have been described in various populations, including bone marrow, circulating blood, adult skeletal muscle or foetal myogenic cells. Clinical implications might come closer in myocardial infarction where the injection of autologous myoblasts showed that circumscribed muscle regions can be successfully targeted if the damage to this muscle is not too profound [44]. A similar strategy might be useful for focal muscular dystrophies such as oculo-pharyngeal muscular dystrophy or facio-scapulo-humeral muscular dystrophy [45, 46]. In generalised muscular disorders involving large muscles, such as DMD, however, MTT seems less feasible and other strategies such as exon-skipping or circulating stem cells might be more promising.

Gene therapy in inflammatory muscle disorders

The genetic treatment of inflammatory muscle disorders could involve at least two different strategies: first, to ectopically express local immune modulatory, notably immunosuppressive molecules which would limit inflammation and autoimmunity more effectively than systemic immunosuppressive treatment; and second, strategies to promote the repair or allow for the replacement of damaged muscle may become feasible. Immunosuppressive molecules possibly suitable for gene therapy include HLA-G, a non-classical MHC Class I molecule, or other cell surface molecules which negatively modulate immune effector cell function [18]. Muscle regeneration might be promoted by several myotrophic factors including utrophin and insulin-like growth factor (IGF) [47].

Targets for gene therapy

One candidate molecule to induce tolerance and prevent further immune-mediated muscle damage is HLA-G, a non-classical MHC Class I molecule with highly limited tissue distribution which has been attributed immune regulatory functions. HLA-G is expressed in inflamed muscle as demonstrated *in vivo* and by cultured myoblasts *in vitro* [48]. HLA-G has been proposed to play an

important role in human muscle immunobiology [49]. It leads to a downregulation of primary as well as secondary allogeneic immune responses by interacting with virtually all cellular cytotoxic immune effectors. Small numbers of HLA-G-expressing cells are required to convey significant immune inhibitory effects *in vitro*. Therefore, HLA-G represents a promising anti-inflammatory principle which might be possibly applicable for gene therapy in inflammatory myopathies [18].

Another possible target for gene therapy in inflammatory myopathies is derived from a rare form of hereditary inclusion body myopathy (HIBM; OMIM 600737). This autosomal-recessive disorder was initially found in Jews originating from Middle Eastern countries and rarely in non-Jewish families from India, USA and the Bahamas. The patients suffering from HIBM share the typical muscle pathology with sporadic IBM including rimmed vacuoles and filamentous inclusions. Recently, mutations in the UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE) gene, the key enzyme in the biosynthetic pathway of sialic acid, have been identified to cause HIBM [50]. Subsequent studies demonstrated that the pathogenic GNE mutations result in impaired enzymatic activity. However, the overall sialylation of muscle cells was not equally affected, suggesting additional pathogenic mechanisms in HIBM [51]. As a consequence, no simple enzyme replacement therapy or gene transfer will be feasible in this rare hereditary condition as well as in sporadic IBM.

Additional clues for effective gene therapy in inflammatory muscle diseases might arise from microarray analyses of inflammatory myopathies [52]. Patients with DM and sporadic IBM have a high expression of immunoglobulin, adhesion molecules, chemokines and cytokine genes. In the muscle tissue of DM patients with clinical improvement in response to intravenous immunoglobulins, more than 2,000 genes were significantly downregulated and there was also an upregulation of different chemokines and several immunoglobulin-related genes whereas in IBM and muscle of DM patients with absent therapeutic response, the majority of these genes remained unchanged, including genes for IL-22, cell adhesion molecules, complement factor C1q, and several structural protein genes. These data indicate that a subset of the immunoregulatory or structural muscle genes modulated by intravenous immunoglobulin therapy is connected with a clinical response in DM. These modulated genes might represent possible targets for gene therapeutic approaches in inflammatory muscle disease.

Conclusion and outlook

Gene therapies have the potential for a promising therapy of inflammatory muscle disorders which are not responding to standard immunomodulating treatments, in particular in IBM. However, several technical and biological difficulties hamper the long-term expression of transgenes and thereby the feasi-

bility of this treatment. In addition, gene therapy might be limited by possible immunogenicity and cytotoxicity of viral vectors. Specifically adapted strategies are warranted to overcome these difficulties in the future.

References

1 Dalakas MC (1991) Polymyositis, dermatomyositis and inclusion-body myositis. *N Engl J Med* 325: 1487–1498.
2 Dalakas MC, Hohlfeld R (2003) Polymyositis and dermatomyositis. *Lancet* 362: 971–982.
3 Needham M, Mastaglia FL (2007) Inclusion body myositis: current pathogenetic concepts and diagnostic and therapeutic approaches. *Lancet Neurol* 6: 620–631.
4 Wiendl H, Hohlfeld R, Kieseier BC (2005) Immunobiology of muscle: advances in understanding an immunological microenvironment. *Trends Immunol* 26: 373–380.
5 Gold R, Dalakas MC, Toyka KV (2003) Immunotherapy in autoimmune neuromuscular disorders. *Lancet Neurol* 2: 22–32.
6 Hewer E, Goebel HH (2008) Myopathology of non-infectious inflammatory myopathies – the current status. *Pathol Res Pract* 204: 609–623.
7 Greenberg SA, Amato AA (2004) Uncertainties in the pathogenesis of adult dermatomyositis. *Curr Opin Neurol* 17: 359–364.
8 Greenberg SA, Sanoudou D, Haslett JN, Kohane IS, Kunkel LM, Beggs AH, Amato AA (2002) Molecular profiles of inflammatory myopathies. *Neurology* 59: 1170–1182.
9 Greenberg SA, Pinkus JL, Pinkus GS, Burleson T, Sanoudou D, Tawil R, Barohn RJ, Saperstein DS, Briemberg HR, Ericsson M et al. (2005) Interferon-alpha/beta-mediated innate immune mechanisms in dermatomyositis. *Ann Neurol* 57: 664–678.
10 Boonstra A, Asselin-Paturel C, Gilliet M, Crain C, Trinchieri G, Liu YJ, O'Garra A (2003) Flexibility of mouse classical and plasmacytoid-derived dendritic cells in directing T helper type 1 and 2 cell development: dependency on antigen dose and differential toll-like receptor ligation. *J Exp Med* 197: 101–109.
11 Nestle FO, Conrad C, Tun-Kyi A, Homey B, Gombert M, Boyman O, Burg G, Liu YJ, Gilliet M (2005) Plasmacytoid predendritic cells initiate psoriasis through interferon-alpha production. *J Exp Med* 202: 135–143.
12 Jego G, Pascual V, Palucka AK, Banchereau J (2005) Dendritic cells control B cell growth and differentiation. *Curr Dir Autoimmun* 8: 124–139.
13 Greenberg SA (2007) Proposed immunologic models of the inflammatory myopathies and potential therapeutic implications. *Neurology* 69: 2008–2019.
14 Fuchsberger M, Hochrein H, O'Keeffe M (2005) Activation of plasmacytoid dendritic cells. *Immunol Cell Biol* 83: 571–577.
15 Leff RL, Love LA, Miller FW, Greenberg SJ, Klein EA, Dalakas MC, Plotz PH (1993) Viruses in idiopathic inflammatory myopathies: absence of candidate viral genomes in muscle. *Lancet* 339: 1192–1195.
16 Karpati G, Pouliot Y, Carpenter S (1988) Expression of immunoreactive major histocompatibility complex products in human skeletal muscles. *Ann Neurol* 23: 64–72.
17 Emslie-Smith AM, Arahata K, Engel AG (1989) Major histocompatibility complex class I antigen expression, immunolocalization of interferon subtypes, and T cell-mediated cytotoxicity in myopathies. *Hum Pathol* 20: 224–231.
18 Wiendl H, Mitsdoerffer M, Hofmeister V, Wischhusen J, Weiss EH, Dichgans J, Lochmuller H, Hohlfeld R, Melms A, Weller M (2003) The non-classical MHC molecule HLA-G protects human muscle cells from immune-mediated lysis: implications for myoblast transplantation and gene therapy. *Brain* 126: 176–185.
19 Lundberg I, Brengman JM, Engel AG (1995) Analysis of cytokine expression in muscle in inflammatory myopathies, Duchenne dystrophy, and non-weak controls. *J Neuroimmunol* 63: 9–16.
20 De Bleecker JL, De Paepe B, Vanwalleghem IE, Schroder JM (2002) Differential expression of chemokines in inflammatory myopathies. *Neurology* 58: 1779–1785.
21 Figarella-Branger D, Civatte M, Bartoli C, Pellissier JF (2003) Cytokines, chemokines, and cell adhesion molecules in inflammatory myopathies. *Muscle Nerve* 28: 659–682.

22 Choi YC, Dalakas MC (2000) Expression of matrix metalloproteinases in the muscle of patients with inflammatory myopathies. *Neurology* 54: 65–71.
23 Dalakas MC (2006) Therapeutic targets in patients with inflammatory myopathies: present approaches and a look to the future. *Neuromuscul Disord* 16: 223–236.
24 Dalakas MC (2002) Muscle biopsy findings in inflammatory myopathies. *Rheum Dis Clin North Am* 28: 779–798, vi.
25 Badrising UA, Schreuder GM, Giphart MJ, Geleijns K, Verschuuren JJ, Wintzen AR, Maat-Schieman ML, van Doorn P, van Engelen BG, Faber CG et al. (2004) Associations with autoimmune disorders and HLA class I and II antigens in inclusion body myositis. *Neurology* 63: 2396–2398.
26 Price P, Witt C, Allcock R, Sayer D, Garlepp M, Kok CC, French M, Mallal S, Christiansen F (1999) The genetic basis for the association of the 8.1 ancestral haplotype (A1, B8, DR3) with multiple immunopathological diseases. *Immunol Rev* 167: 257–274.
27 Askanas V, Engel WK (1998) Sporadic inclusion-body myositis and hereditary inclusion-body myopathies: current concepts of diagnosis and pathogenesis. *Curr Opin Rheumatol* 10: 530–542.
28 Sugarman MC, Yamasaki TR, Oddo S, Echegoyen JC, Murphy MP, Golde TE, Jannatipour M, Leissring MA, LaFerla FM (2002) Inclusion body myositis-like phenotype induced by transgenic overexpression of beta APP in skeletal muscle. *Proc Natl Acad Sci USA* 99: 6334–6339.
29 Kitazawa M, Trinh DN, LaFerla FM (2008) Inflammation induces tau pathology in inclusion body myositis model via glycogen synthase kinase-3beta. *Ann Neurol* 64: 15–24.
30 Wiendl H (2008) Idiopathic inflammatory myopathies: current and future therapeutic options. *Neurotherapeutics* 5: 548–557.
31 Dalakas MC (2003) Therapeutic approaches in patients with inflammatory myopathies. *Semin Neurol* 23: 199–206.
32 Dominski Z, Kole R (1993) Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proc Natl Acad Sci USA* 90: 8673–8677.
33 van Deutekom JC, van Ommen GJ (2003) Advances in Duchenne muscular dystrophy gene therapy. *Nat Rev Genet* 4: 774–783.
34 Arnett AL, Chamberlain JR, Chamberlain JS (2009) Therapy for neuromuscular disorders. *Curr Opin Genet Dev* 19: 290–297.
35 Murakami T, Nishi T, Kimura E, Goto T, Maeda Y, Ushio Y, Uchino M, Sunada Y (2003) Full-length dystrophin cDNA transfer into skeletal muscle of adult mdx mice by electroporation. *Muscle Nerve* 27: 237–241.
36 Lu QL, Mann CJ, Lou F, Bou-Gharios G, Morris GE, Xue SA, Fletcher S, Partridge TA, Wilton SD (2003) Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. *Nat Med* 9: 1009–1014.
37 Gollins H, McMahon J, Wells KE, Wells DJ (2003) High-efficiency plasmid gene transfer into dystrophic muscle. *Gene Ther* 10: 504–512.
38 van Vliet L, de Winter CL, van Deutekom JC, van Ommen GJ, Aartsma-Rus A (2008) Assessment of the feasibility of exon 45–55 multiexon skipping for Duchenne muscular dystrophy. *BMC Med Genet* 9: 105.
39 Tinsley J, Deconinck N, Fisher R, Kahn D, Phelps S, Gillis JM, Davies K (1998) Expression of full-length utrophin prevents muscular dystrophy in mdx mice. *Nat Med* 4: 1441–1444.
40 Partridge TA, Morgan JE, Coulton GR, Hoffman EP, Kunkel LM (1989) Conversion of mdx myofibres from dystrophin-negative to -positive by injection of normal myoblasts. *Nature* 337: 176–179.
41 Gussoni E, Pavlath GK, Lanctot AM, Sharma KR, Miller RG, Steinman L, Blau HM (1992) Normal dystrophin transcripts detected in Duchenne muscular dystrophy patients after myoblast transplantation. *Nature* 356: 435–438.
42 Mendell JR, Kissel JT, Amato AA, King W, Signore L, Prior TW, Sahenk Z, Benson S, McAndrew PE, Rice R et al. (1995) Myoblast transfer in the treatment of Duchenne's muscular dystrophy. *N Engl J Med* 333: 832–838.
43 Gussoni E, Blau HM, Kunkel LM (1997) The fate of individual myoblasts after transplantation into muscles of DMD patients. *Nat Med* 3: 970–977.
44 Laflamme MA, Murry CE (2005) Regenerating the heart. *Nat Biotechnol* 23: 845–856.
45 Mouly V, Aamiri A, Périé S, Mamchaoui K, Barani A, Bigot A, Bouazza B, François V, Furling D, Jacquemin V et al. (2005) Myoblast transfer therapy: is there any light at the end of the tunnel? *Acta Myol* 24: 128–133.

46 Périé S, Mamchaoui K, Mouly V, Blot S, Bouazza B, Thornell LE, St Guily JL, Butler-Browne G (2006) Premature proliferative arrest of cricopharyngeal myoblasts in oculo-pharyngeal muscular dystrophy: Therapeutic perspectives of autologous myoblast transplantation. *Neuromuscul Disord* 16: 770–781.
47 Singleton JR, Feldman EL (2001) Insulin-like growth factor-I in muscle metabolism and myotherapies. *Neurobiol Dis* 8: 541–554.
48 Wiendl H, Behrens L, Maier S, Johnson MA, Weiss EH, Hohlfeld R (2000) Muscle fibers in inflammatory myopathies and cultured myoblasts express the nonclassical major histocompatibility antigen HLA-G. *Ann Neurol* 48: 679–684.
49 Hohlfeld R, Engel AG (1994) The immunobiology of muscle. *Immunol Today* 15: 269–274.
50 Eisenberg I, Avidan N, Potikha T, Hochner H, Chen M, Olender T, Barash M, Shemesh M, Sadeh M, Grabov-Nardini G et al. (2001) The UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase gene is mutated in recessive hereditary inclusion body myopathy. *Nat Genet* 29: 83–87.
51 Salama I, Hinderlich S, Shlomai Z, Eisenberg I, Krause S, Yarema K, Argov Z, Lochmuller H, Reutter W, Dabby R et al. (2005) No overall hyposialylation in hereditary inclusion body myopathy myoblasts carrying the homozygous M712T GNE mutation. *Biochem Biophys Res Commun* 328: 221–226.
52 Raju R, Dalakas MC (2005) Gene expression profile in the muscles of patients with inflammatory myopathies: effect of therapy with IVIg and biological validation of clinically relevant genes. *Brain* 128: 1887–1896.

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Gene therapy for osteoarthritis

Steven C. Ghivizzani[1] and Christopher H. Evans[2]

[1] *Department of Orthopaedics & Rehabilitation, University of Florida College of Medicine, Gainesville, Florida, USA*
[2] *Center for Advanced Orthopaedic Studies, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts, USA*

Abstract

Osteoarthritis (OA) is an incurable, chronic, painful, debilitating joint disease characterized primarily by the gradual erosion of protective articular cartilage. Although joint damage contributes to the onset of OA, the continued synthesis of inflammatory cytokines, such as interleukin-1, by synovial cells and chondrocytes is thought to drive the progression of disease. Numerous naturally occurring proteins have been identified with the potential to block inflammatory processes, or alternatively, stimulate the repair of damaged cartilage. Problems lie in the lack of effective methods of delivery, as most proteins have limited half-lives *in vivo*. Through the use of gene therapy applications, exogenous transgenes can be delivered to cells and tissues of arthritic joints, thereby redirecting their biology for sustained, local synthesis of therapeutic transgene products. A wide variety of transgenes and methods of delivery are currently under investigation for their capacity to block ongoing inflammation or stimulate repair and regeneration of cartilage tissues in OA.

Introduction: osteoarthritis a disease of eroding cartilage

Osteoarthritis (OA), the most common form of arthritis, is a chronic, painful, degenerative joint disease that affects millions of people worldwide. Some estimates place the incidence as high as 8% of the population with annual societal costs in the US alone exceeding 100 billion dollars [1, 2]. The pathogenesis of OA is marked by gradual degeneration of the articular cartilage in affected joints, accompanied by sclerosis of subchondral bone, marginal osteophytes and soft tissue inflammation. The presenting clinical symptoms are typically joint pain, stiffness and loss of mobility, which can progress to complete joint failure. Although OA can occur in any joint, it primarily affects the knees, hips, hands and feet [3].

Distinct from rheumatoid (RA) arthritis, which is considered an inflammatory autoimmune condition, the onset of OA is often associated with a prior joint injury, either from acute damage from trauma or sports injury, or from long-term over-loading. Injuries involving ligament and meniscal tears often cause malalignment of the joint and lead to cartilage degradation from biomechanical instability [4–7]. Other risk factors include genetic predisposition, advancing age and obesity [8]. Indeed OA affects approximately 60% of men

and 70% of women over the age of 65, and the obese have more than a four-fold greater risk of developing OA of the knee [9, 10]. Over the coming decades, the incidence of OA is expected to steadily increase as the average age of the population rises, and the prevalence of obesity in Western society continues to escalate.

Articular cartilage, when healthy, serves to protect the articulating surfaces of the long bones from the forces of movement and locomotion. Early OA appears grossly as local splitting and fibrillation across the cartilage surface, but gradually progresses to full thickness erosions with denuded bone in late stages [2, 11]. The end result is painful bone-on-bone articulation and loss of joint function. Under normal circumstances articular cartilage is a highly durable tissue that can provide a lifetime of nearly friction-free movement. It is primarily composed of a uniformly dense, hydrated, extracellular matrix that is both avascular and aneural. The load-bearing demands placed on the tissue preclude its permeation with blood vessels and nerves, which would compromise its architecture and protective properties. However, because cartilage lacks innervation, injuries often go undetected, enabling activities associated with the injury to continue and thereby worsen the tissue damage. Further, since there is no blood supply, reparative cells and protein factors are not released into the site of injury, as would normally occur in vascular tissues; thus, when cartilage is injured the damage often remains permanently.

Current approaches to treatment

The patient burden of OA is exacerbated by the inadequacies of present treatments. Early to moderate OA is typically addressed with palliative measures, such as weight loss, physical therapy and non-steroidal anti-inflammatory drugs (NSAIDs) as indicated in Table 1, adapted from the American College of Rheumatology's '*Guidelines for the medical management of osteoarthritis*' [12]. While behavioral modification can slow the progression of disease, current pharmacologic approaches to treatment of OA are directed toward the management of joint pain, but have little impact on the degenerative process [3].

Table 1. Medical management of patients with osteoarthritis of the knee

Non-pharmacologic therapy	Pharmacologic therapy
Patient education	Intra-articular steroid injections (knee)
Health professional social support	Non-opioid analgesics (e.g., acetaminophen)
Weight loss (if over weight)	Topical analgesics (e.g., capsaicin and methylsalicylate creams)
Physical therapy	Non-steroidal anti-inflammatory drugs
Occupational therapy	Opioid analgesics (e.g., propoxyphene, codeine, oxycodone)

In the many cases where these approaches fail to control the symptoms and progression of OA, surgical intervention is often indicated [13]. Arthroscopic lavage and debridement may provide symptomatic relief, although two major studies have cast doubt on this [14, 15]. An osteotomy is sometimes performed to realign the forces in the knee joint, so that load is now borne by areas of intact cartilage [16]. This measure can provide relief for several years until the newly weight-bearing articular cartilage erodes and symptoms reappear. In general, though, osteotomy is viewed as a tactic intended to delay the surgical implantation of a prosthetic knee joint. The failure of existing treatments to control OA satisfactorily can be gauged from the fact that over 300,000 knee and 200,000 hip arthroplasties are performed in the United States each year for OA. Such statistics help explain the increasing popularity of over-the-counter agents, such as chondroitin sulfate, glucosamine and other 'nutraceuticals' touted in the lay-press as beneficial for OA and cartilage synthesis [17]. Unfortunately the benefit of these agents has not held up to rigorous clinical testing [18, 19].

Currently, there is an immediate and pressing need for the development of therapies that will improve the clinical outcome of patients with mild-to-moderate OA. These are individuals whose disease interferes with their quality of life, but who do not yet require total joint replacement surgery. Although the goal is to cure OA, a therapy that improved clinical symptoms and significantly postponed the need for total joint replacement would represent an enormous advance [20].

Articular cartilage matrix composition and OA

For the cartilage to function optimally, maintenance of the proper composition of the matrix is essential. Distinct from most other connective tissues, which are largely composed of collagen type I, articular cartilage primarily contains collagen type II. Its other main components are proteoglycans, water and a population of chondrocytes that reside in the matrix at low density. The collagen II fibers combine with collagen types IX and XI to form a lattice that provides shape and tensile strength to the cartilage matrix, and serve to hold and constrain the high proteoglycan content [21]. Aggrecan, the major proteoglycan, is attached to long hyaluronic acid polymers, forming complex 'bottle-brush' configurations that serve to imbibe the water and restrict its flow through the matrix [22, 23]. This provides for the cushioning effects and elasticity of the cartilage during joint loading. The role of the chondrocytes is to both monitor and maintain the structural integrity of the cartilage matrix. Variations in the composition and density of the constituent matrix molecules can reduce its protective qualities and leave it vulnerable to damage.

Chondrocytes in adult cartilage typically have low metabolic activity and slowly digest and re-synthesize the cartilage matrix. The rate and balance of these activities is dictated by a variety of external cues, such as mechanical stimulation from joint articulation, the level of breakdown components of the

matrix, and autocrine and paracrine stimulation from a variety of cytokines and growth factors. The sum of these external signals directs the metabolic status of the chondrocyte and its respective synthetic and degradative activities [3]. Under optimal conditions, the anabolic and catabolic activities of the chondrocyte remain in equilibrium, such that the structural integrity and protective capacity of the matrix are maintained, and the volume of the cartilage remains constant. Situations that disrupt this equilibrium in favor of catabolic activity [24], or that contribute to chondrocyte apoptosis or senescence [25], result in a net reduction in cartilage volume and integrity, which contributes to the onset and progression of OA. The abnormal behavior of the chondrocyte in OA is illustrated by the gradual fibrillation of the cartilage, and increasing alterations in the quantity, distribution and composition of the matrix proteins [24].

Since the chondrocytes are responsible for building the cartilage matrix and maintaining its vitality, for pharmacologic treatment approaches to have disease-modifying capacity, they must effectively target the biology of these cells. Because OA is a disease that typically affects only a small number of joints in any individual, a difficult challenge for pharmacologic treatments is to achieve and maintain beneficial doses of drug specifically within the diseased tissues. Despite continued attempts to package drugs in carriers with the capacity to selectively target joints or diseased joint tissues, none has proven to be effective. Thus, most drugs for OA are administered systemically and interact with tissues throughout the body or are administered locally via intra-articular injection.

Mediators of disease in OA

Although excessive and/or traumatic joint loading are considered initiating factors for OA, the specific biomechanical disturbances responsible for maintaining the pathologic biology of the OA chondrocyte are not completely understood. Biochemical analyses indicate that many of the downstream effects can be attributed to the enhanced production of primary inflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), by synovial cells and chondrocytes. This supports the view that OA is a slowly progressing, local inflammatory disease rather than simply a process of mechanical wear and tear of ageing cartilage [26].

Of the inflammatory cytokines, IL-1 is widely thought to occupy a predominant role in OA pathogenesis. Unlike articular cartilage recovered from normal human joints without disease, cartilage recovered from OA knee joints at the time of total knee replacement actively synthesizes IL-1 [27]. *In vivo*, transection of the canine anterior cruciate ligament elevates the synthesis of IL-1 by the synovium and articular chondrocytes, and the knee joints predictably develop pathologies of OA [28–31].

Local overproduction of IL-1 serves to stimulate the synthesis and release of many of the biological mediators of disease in OA. These include a variety of proteases, such as matrix metalloproteinases (MMPs) -1, -3, -8 and -13 which

target the collagen lattice [24, 26, 32, 33], and A Disintegrin And Metalloproteinases with Thrombospondin motifs (ADAMTS) -4 and -5 (aggrecanases-1 and -2) that digest the proteoglycans [22, 23, 34]. In addition to stimulating the production of degradative enzymes, IL-1 has a pronounced suppressive effect on chondrocyte proteoglycan and collagen II synthesis. IL-1 also stimulates chondrocytes to produce other mediators of OA, including phospholipase A_2, cyclooxygenase-2 (COX-2), nitric oxide (NO) and prostaglandin E_2 (PGE_2) as well as numerous inflammatory cytokines and chemokines [24, 35–39].

Gene therapy approaches

Although local gene therapy was initially conceived as a method to treat RA [40], its application appears much better suited to OA [41]. In contrast to RA, a systemic, polyarthritic, autoimmune, condition, OA typically occurs in only one or a limited number of joints and has no known systemic component. Therefore the genetic treatment of all individual affected joints is technically and ethically feasible, perhaps even multiple times, if necessary.

Despite considerable progress in the study of cartilage biology over the last two decades, there is insufficient knowledge of the specific genetic determinants that cause or predispose one to OA to allow serious consideration of gene correction or gene replacement approaches. Forms of OA caused by single gene defects are rare, and tend to be dominant negatives. Although there is a strong genetic contribution to OA, this occurs as multiple polymorphisms occurring in many different genes [42–45] Moreover, strategies that target mutations and polymorphisms would probably need to be applied at an early age – likely decades in advance of the onset of any symptoms. Thus, as with most gene-based therapies for musculoskeletal disorders, those being developed for the treatment of OA are devised to overcome deficiencies with conventional drug delivery to enable sustained, production of anti-arthritic gene products specifically within diseased joint tissues.

In the simplest embodiment of this concept, a recombinant vector carrying an anti-arthritic gene under independent transcriptional control, is injected directly into the joint space to modify receptive cells *in situ*. The molecular machinery of the genetically altered cells is thereby redirected toward the overproduction of the transgene products. These most frequently take the form of secreted proteins, which are released into the synovial fluid to interact with tissues throughout the joint [46, 47]. *Ex vivo* approaches can also be employed where the target cells are genetically modified in culture and then are injected into the joint space where they attach and engraft into the synovial lining and express the transgene product [48]. Variations can include *ex vivo* modification of cells, which are then incorporated into biocompatible support matrices in tissue engineering applications to repair damaged cartilage.

When designing gene therapy strategies for OA, the chondrocytes emerge as highly desirable targets. In monolayer culture, chondrocytes have been

shown to be amenable to genetic modification with a variety of vector systems, including transfection with plasmid DNAs [49] and viral transduction with adenovirus [50, 51], adeno-associated virus [52], retrovirus [53] and lentivirus [54]. In contrast to findings in culture, efficient genetic modification of these cells *in situ* has not been possible with existing technologies, as the dense cartilage matrix surrounding the cells effectively excludes most vector systems. Detailed mapping studies have shown that the majority of vectors are incapable of penetrating even the most superficial cartilage layer to infect resident chondrocyte populations [55]. Although there are isolated reports that adeno-associated virus (AAV), being a significantly smaller particle than other well-characterized viral vector systems can enter the cartilage matrix [52], this has not been conclusively demonstrated.

Regardless of whether certain vectors are capable of interacting with a limited number of chondrocytes *in situ*, following direct injection into the joint they have unrestricted access to the large populations of fibroblastic cells in the synovial lining and capsule. Thus strategies involving direct intra-articular injection must account for the fact that typically >99% of gene transfer will occur in the cells of the synovium and joint capsule [55]. This is critical, since many gene products that may have beneficial stimulatory or anabolic properties in cartilage can generate undesirable side effects in neighboring tissues.

The majority of strategies explored to date have been designed around secreted proteins; however, therapeutic transgene products may also take the form of bioactive RNAs that inactivate or cleave messenger RNAs encoding proteins that mediate disease processes. The area of RNA interference has gained considerable momentum in the last several years, and its use would eliminate concerns about immune responses to transgenic protein [56, 57]. However, there are distinct limitations with regard to siRNA for treating chronic joint disease. The first lies with the fact that the intra-articular administration of siRNA molecules will only have transient effects and thus, will be of little use over extended periods. Therefore, to enable continuous synthesis of the siRNA within the target cells, vectors containing siRNA expression cassettes must be used [58], and a high percentage of effector cells must be genetically modified for the strategy to elicit a potent biological response. Conversely, with the use of a secreted transgene product, a limited number of cells can be modified to secrete large quantities of the gene product into the synovial fluid allowing cells throughout the joint tissues can be affected.

In considering the types of genes that might be used to treat OA, two complementary therapeutic strategies are currently being pursued. The first is *chondroprotection*, and involves gene delivery of proteins that block the deleterious effects of specific cytokines, inflammatory mediators and degradative enzymes. The second strategy is directed toward *cartilage repair or regeneration* through stimulation of chondrocytes and/or mesenchymal stem cells (MSCs) using cDNAs for various anabolic, proliferative or chondrogenic agents. Proof of concept of both approaches has been demonstrated in several publications.

Chondroprotection: inhibition of IL-1

Since IL-1 sits atop of the inflammatory cascade in OA, agents that inhibit its actions hold great promise as therapeutic agents in its treatment. Unfortunately, traditional pharmacologic approaches have failed to produce clinically useful molecules that effectively inhibit this cytokine. Biology, however, offers two promising naturally-occurring candidates: IL-1Ra and the soluble IL-1 type II receptor (sIL-1RII) [59, 60]. IL-1Ra functions as a competitive inhibitor; by binding to the type I IL-1 signaling receptor, it blocks the binding of IL-1. Unlike IL-1, IL-1Ra fails to recruit the IL-1R accessory protein (IL-1-AcP) to the complex and thus does not activate a transmembrane signal. The sIL-1RII molecule in contrast, binds and neutralizes IL-1 directly. Despite their differing modes of action, the two molecules are approximately equipotent in blocking the actions of IL-1. Our group and others have selected IL-1Ra for study in the context of gene therapy for OA because it is smaller, and the recombinant protein is biologically well characterized and is currently in clinical use for RA [61, 62].

IL-1Ra is available in recombinant form as the drug anakinra (Kineret^{TM}) for the treatment of RA, but must be self-administered at a dose of 100 mg daily by subcutaneous injection [63]. While anakinra was highly effective against experimental RA in rats that were continuously infused with the protein, the clinical response in humans to daily injections has been marginal [64]. Clinical studies of anakinra for OA following a single intra-articular injection have been similarly disappointing and have demonstrated no significant improvement. However, in the recent study by Chevalier et al. [65], although there was no lasting effect on the clinical parameters of OA after injection of Kineret, there was an improvement in the pain score at day 4; this was not evident at week 4. This supports the proposition that the relative lack of clinical efficacy of IL-1Ra can be attributed to its brief intra-articular half-life of less than 1 h [66]. Further, it is neither feasible nor safe to administer IL-1Ra by frequent intra-articular injection. The systemic application of IL-1Ra has been associated with adverse side effects primarily from injection site reactions, and to a lesser degree from infections and neutropenia [67]. Thus, while IL-1Ra has promising activities there is no conventional method of delivery that can provide sustained therapeutic levels intra-articularly [66].

Despite its questionable benefit in treating arthritis as a recombinant protein, IL-1Ra is well suited to local gene therapy applications. Primary attractions are is its safety profile [67] and ease of application. IL-1Ra has no agonist or immunomodulatory effects, and is already approved for clinical use as a recombinant protein. Sustained intra-articular synthesis of IL-1Ra as a transgene product readily overcomes its short *in vivo* half-life, and it does not require sensitive transcriptional regulation. IL-1Ra simply needs to be expressed at beneficial levels intra-articularly; over-production is not known to provoke an adverse response. Therefore, the IL-1Ra cDNA can be administered using existing regulatory sequences, including the well-characterized CMV or E1α promoters that provide high level, constitutive expression [55].

The efficacy of local IL-1Ra gene delivery for OA has been demonstrated in preclinical studies in a variety of animal models. In rabbits with an MCL transection and medial meniscectomy OA model, delivery of the cDNA for human IL-1Ra, *ex vivo* by retrovirally transduced synovial fibroblasts or by direct injection of recombinant adenovirus was found to protect against matrix degradation and loss of proteoglycans [68, 69]. A similar study involving *ex vivo* IL-1Ra delivery to canine joints 2 days after induction of an ACL transection model, was reported to suppress early degenerative changes in the articular cartilage of the tibial plateau and femoral condyles [70]. A further study used adenovirus to transfer equine IL-1Ra cDNA into the joints of horses in an osteochondral fragment model of OA and showed improvement in joint lameness, and protection of the cartilage from erosion associated with the model [71]. Improving lameness is an important finding, because it suggests a reduction in pain, as hinted by data from the trial of Chevallier et al. [65]. This indicates that IL-1Ra gene therapy could have effects on both clinical symptoms and the underlying pathophysiology of the disease. Drugs that achieve the latter would be disease modifying; at present, there are no disease modifying drugs for OA. Building from these findings, a Phase I safety trial involving AAV-mediated delivery of IL-1Ra into the knee joints of those with OA is currently awaiting final FDA approval.

Following these promising results, additional studies have found that the protective effects of IL-1Ra can be augmented by co-delivery of other anti-inflammatory cDNAs with complementing activities. For example, local delivery of the cDNA for IL-10, an anti-inflammatory cytokine [69], or a soluble type I TNF receptor [68], together with IL-1Ra to the joints of rabbits with experimental OA were found to provide greater inhibition of cartilage degradation than with IL-1Ra alone.

Other chondroprotective approaches being pursued involve the adenoviral-mediated delivery of the cDNA for kallistatin, a secreted protein that blocks inflammation, angiogenesis and cellular apoptosis. Originally found to have benefit in an RA model [72], local kallistatin gene transfer was recently found to have a protective effect on cartilage in rats with an ACL transection model of OA, when co-administered with hyaluronic acid [73]. Related strategies include the local overexpression of TIMP-1 to inhibit IL-1-induced MMP activity [74], and the gene delivery of glucose-6 fructose amido transferase (GFAT; the rate limiting enzyme in the hexosamine pathway), which is intended to increase the synthesis and release of glucosamine into the joint [75].

Cartilage repair and regeneration

Since OA causes the breakdown and loss of articular cartilage, delivery of gene-based therapeutics that could help to rebuild lost or damaged cartilage has the potential to reverse the course of disease. In these approaches, the sustained transgenic expression of stimulatory proteins can be used to drive chondrocytes

toward proliferation and enhanced matrix synthesis and/or to direct the differentiation of progenitor cells toward the chondrogenic phenotype. Many of the gene products with potential in these applications were identified in studies of embryonic skeletal development [76]. Among these, the most potent are members of the transforming growth factor (TGF) superfamily, including TGF-β1 [77], and certain bone morphogenetic proteins (BMPs) [78–80] and growth and differentiation factors (GDFs) [81, 82]. Other proteins with promising activity include fibroblast growth factor 2 (FGF2) [83] and insulin-like growth factor-1 (IGF-1) [84]. The methods being explored to deliver these gene products are technically diverse and range from simple intra-articular injection of recombinant vectors to complex tissue engineering strategies.

As with chondroprotection, vectors or genetically modified cells injected directly into the joint space provide transgene expression that emanates primarily from the synovium and capsular tissues. The transgene products then diffuse through the synovial fluid and cartilage matrix to stimulate the chondrocytes. The utility of this straightforward approach has been demonstrated with the use of muscle-derived stem cells (MDSCs) retrovirally modified to express BMP-4. Delivery of these cells to the joints of rats with a chemically induced model of OA was found to stimulate measurable repair of the damaged cartilage; this effect was enhanced by co-delivery of the cDNA for Flt-1, which encodes a soluble protein that inhibits angiogenesis [85, 86]. It has also been reported that intra-articular injection of allogenic chondrocytes genetically modified to express TGF-β1 enhanced cartilage repair in rabbits [87, 88] – a procedure that has been advanced into Phase I clinical testing in Korea and the US.

Unfortunately, many protein factors that stimulate cartilage repair via anabolic and proliferative pathways also have the potential to generate undesirable, often dramatic, side effects in collateral, non-cartilaginous tissues following *in vivo* gene transfer to joints. For example, intra-articular delivery of adenovirus containing the cDNA for TGF-β1 induces an extraordinarily potent fibrotic response in the synovium and capsule [89]. Systemic pathologies such as pulmonary fibrosis and even death occurred when TGF-β1 was expressed at high levels. Expression of TGF-β1 and BMP-2, but not IGF-1, has also been shown to induce the formation of osteophytes, and ectopic cartilage and bone formation [89, 90]. Thus, anabolic strategies should be approached with considerable caution. Rather than widespread release of the gene products throughout the joint tissues, these molecules are best applied when expression is restricted to the confines of the defect, limiting exposure to non-damaged regions to the greatest possible extent.

Tissue engineering: *Ex vivo* delivery of genetically modified chondrocytes

Since cartilage has little capacity for regeneration, it is widely thought that restoration of the articulating surfaces or repair of lesions of significant size

will require the surgical delivery of space-filling cellular material, to both resynthesize the missing cartilage matrix and continue to maintain it over time. The implantation of unmodified cells has proved less than satisfactory and generally gives rise to the formation of fibrocartilage repair tissue. Genetic modification of the cells prior to implantation could be used to provide sustained production of chondrogenic factors within the healing lesion to stimulate the implanted cells to maintain the articular cartilage phenotype and may result in better quality repair tissue [91].

The cell types used most frequently for these applications are chondrocytes and mesenchymal progenitor cells [92]. Chondrocytes seem a logical choice as they are indigenous to articular cartilage and, thus, are pre-programmed to perform the appropriate cellular functions. Isolation of autologous cells, however, requires invasive harvest of healthy, non-weight-bearing articular cartilage of which there is a very limited supply. While it is possible to expand chondrocyte populations in culture, they typically dedifferentiate with passage [93]. Despite these drawbacks, autologous chondrocyte transplantation for cartilage repair is already in clinical practice [92], and the additional procedures for genetic modification of the cells could be readily incorporated into existing clinical practices and infrastructure.

As mentioned previously, chondrocytes can be genetically modified with most vector systems, viral and non-viral, and in response to expression of certain growth factor transgenes show increased synthesis of cartilage matrix proteins [51, 94]. Following implantation into cartilage defects, transgene expression has been shown to persist for several weeks or more. Moreover, chondrocytes modified to express proteins such as BMP-7 [95], IGF-1 [96] or FGF-2 [97] and implanted in various hydrogel matrices show enhanced repair of full thickness osteochondral defects in the joints of horses and rabbits. The repair tissue typically shows improved morphology and higher content of collagen type II and proteoglycans relative to controls.

Tissue engineering: genetically modified mesenchymal progenitors

In contrast to the rarity and inaccessibility of chondrocytes, mesenchymal stem cells (MSCs) are readily isolated from most tissues, and have the capacity to differentiate into cells of most mesenchymal lineages, including chondrocytes [98, 99]. These cells have advantages in that they are plentiful and can be expanded in culture without loss of multipotency. However, they must be successfully induced to differentiate into chondrocytes, and then maintain that phenotype over time, which adds layers of complexity to their use. Numerous growth factors have been found to stimulate chondrogenesis in these cells, but a clear consensus of the most suitable has not been established [98].

A wide variety of potentially beneficial activities have been ascribed to MSCs, but despite reports to the contrary, they are common fibroblastic cells and do not have the intrinsic capacity to seek out cartilaginous lesions and

selectively adhere to and repair the damaged regions. Indeed, their behavior is like any other fibroblastic cell suspension following intra-articular injection; their ensuing distribution is dictated by the fluid and mechanical forces associated with articulation. Thus, injected cells most frequently nest in the recesses of the joint capsule. Furthermore, it is our experience that MSCs do not confer significant immunosuppressive activities when used for local *ex vivo* gene delivery, as allogenic MSCs and autologous MSCs expressing foreign transgenes are eliminated from the joint tissues by the cellular immune system within 10–20 days (Ghivizzani and Evans, unpublished observations).

The potential value of gene delivery for directing chondrogenesis of mesenchymal progenitor cells has been demonstrated in several high-density aggregate systems, modeled after cell condensation during embryonal chondrogenesis [76]. For example, Palmer et al. showed that following adenoviral-mediated delivery of the cDNAs for BMP-2 or TGF-β1 to primary MSCs in monolayer, the cells would express the gene products in a vector/dose related manner. Upon seeding the cells into aggregate culture the modified cells could provide effective autocrine and paracrine stimulation to effect chondrogenic differentiation in 2–3 weeks as indicated by the chondrocytic phenotype of the cells and robust synthesis of extracellular matrix enriched for collagen type II and proteoglycans [100]. Similar results have been reported with gene transfer of BMP-4 [101] and -13 [102], as well as GDF-5 [103] and with the chondrogenic transcription factor Sox 9 [104].

Several published reports have examined the capacity of gene-modified MSCs to enhance cartilage repair in animal models. Most involve the seeding of the genetically altered cells onto a biocompatible scaffold or matrix followed by implantation into full thickness or osteochondral defects. As with chondrocytes, MSCs express exogenous transgenes for several weeks following implantation. Expression of genes such as BMP-2 [105], TGF-β1 [106] and IGF-1 [105] have been shown to stimulate the implanted cells to generate repair tissue of articular cartilage phenotype in rats and rabbits.

Although the specific methods used in each of these reports vary considerably, from the source of the cells, the growth factor genes, the vectors used to deliver the cDNAs, as well as the methods of implantation, there is a consensus in that gene delivery and local expression of certain genes can provide a positive influence on the rate and quality of articular cartilage repair tissue generated [41]. Among the various publications, though, no method has emerged that is clearly superior; further, the ability of the repair tissues to maintain the articular cartilage phenotype over time has not been adequately addressed. While these findings represent important first steps toward clinical translation, significant work remains to optimize the effectiveness and safety of gene transfer for cartilage repair.

Persistence of intra-articular transgene expression

Gene therapy for chronic joint disease, such as OA, is based on the concept of sustained delivery of therapeutic gene products to diseased joint tissues. Ideally, the treatment would provide long-term, perhaps even life-long, benefit. As described previously, intra-articular gene delivery strategies have historically targeted the synovium. This tissue covers almost all the interior surfaces of the joint capsule and is populated predominantly by fibroblasts and macrophage-like cells, whose number and proportion vary depending on the disease condition. With respect to arthritis gene therapy, the vast majority of research has focused on testing vectors for efficiency of gene transfer to synovium and on screening cDNAs for their therapeutic efficacy in animal models. Although development of gene-based treatments for RA has progressed into at least three Phase I clinical trials [107], the duration for which synovial lining cells could support the expression of exogenous transgenes has not been frequently investigated. The consensus among published studies was that transgenic expression persisted for only 2–3 weeks. Until recently it had not been determined if exogenous transgenes could be functionally expressed in the synovium for a sufficient length of time to justify the use of gene-based therapies for chronic joint diseases.

To address this important point, using normal and immunocompromised rats, Gouze et al. [55] examined the capacity of various cell types in joint tissues to maintain and express exogenous transgenes following direct intra-articular gene delivery using lentivirus or adenovirus. It was found that transgenic expression could persist for the lifetime of the animal but required precise immunological compatibility between the vector, transgene product and host [55, 108]. Persistence was not dependent on vector integration or promoter origin (viral *versus* eukaryotic). Two phenotypically distinct sub-populations of genetically modified cells were identified within the joint: i) transient cells, with a half-life of a few weeks and ii) stable cells that reside in the joint tissues indefinitely. Contrary to the prevailing assumption, the transient sub-population was composed almost exclusively of synovial fibroblasts, indicating that the synovium is not an appropriate tissue upon which to base a long-term therapy. Fibroblasts in the ligaments, tendons and capsule emerged as the primary source of therapeutic transgene expression after the first few weeks post-vector delivery (Fig. 1). These studies showed that the biology of the cells in the joint can support a therapeutic platform based on sustained expression of an exogenous transgene, and that cell turnover and immune reactivity are the key determinants in achieving sustained transgenic expression intra-articularly [55].

Self-complementary adeno-associated virus for gene delivery in OA

Although functional transgenic expression has been reported intra-articularly with several viral vector systems [108–114], to this point, none has proven to

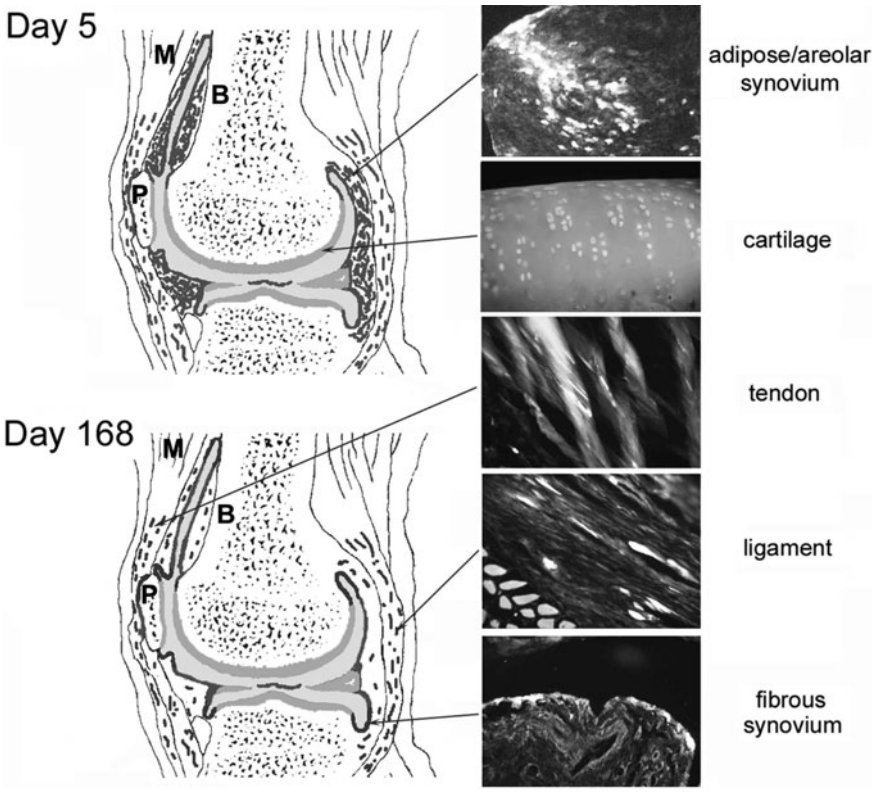


Figure 1. Fibroblasts resident in fibrous articular tissues support stable expression of exogenous transgenes. Following intra-articular injection of a lentiviral vector containing the cDNA for green fluorescent protein (LV-GFP) or adenovirus with GFP (Ad.GFP) into the knees of nude rats, groups of animals were sacrificed at days 5 and 168. The knee joints and surrounding tissues were harvested intact, decalcified and processed for histology. For each joint, the approximate positions of fluorescent cells identified in serial, sagittal whole-knee sections were tabulated in gray on knee joint diagrams similar to that shown on the left. The diagrams shown are representative of the results observed with both viruses at the respective times. On the right, images are shown characteristic of the appearance of the GFP+ cells in tissue sections at the different times (20× magnification). Lines indicate the approximate regions represented by the tissue sections. The numbers of GFP+ cells in the synovium and subsynovium were reduced dramatically at day 168. The density and distribution of GFP+ cells in the tendon, ligament and fibrous synovium were largely unchanged over the duration of the experiment. No fluorescent cells were seen in the articular cartilage with either virus at any time point. B = bone, M = muscle, and P = patella.

be ideally suited for treatment of chronic disease in humans. Adenovirus and herpes simplex virus are both capable of highly efficient gene transfer, but usable forms of these vectors contain and express native viral proteins that cause transduced cells to be eliminated by immune-mediated cytotoxicity. This limits therapeutic gene expression to only a few weeks. High-capacity (gutted) adenoviral vectors have been generated in which all viral coding regions have

been eliminated, but recombinants are difficult to generate, and the system is not well-suited for large-scale virus production [115–117] (see chapter by Brunetti-Pierri and Ng) for further details on gutted adenoviral vectors). Lentiviral vectors [118] also enable very high levels of gene transfer intra-articularly [108], but random integration risks insertional mutagenesis. Furthermore, the best-developed systems are based on HIV-1, which raises additional safety concerns.

Over the last several years, vectors derived from adeno-associated virus (AAV) have emerged as the most favorable viral systems for use in human clinical applications [119]. This is largely based on their safety profile [120] and capacity to infect a wide variety of cell types [121]. In this regard, recombinant AAV offers many advantages for the treatment of chronic joint diseases. The wild type virus is not associated with any pathologic human condition. The recombinant form does not integrate into the genome of the target cell with significant frequency [122] and does not contain native viral coding sequences, which reduces the immunogenicity of the transduced cell [123]. AAV vectors can infect both dividing and quiescent cells, and persistent transgenic expression *in vivo* has been observed in many animal models [119, 124].

The AAV vector is comprised of an ~5000 nucleotide single-stranded DNA genome packaged in a small (20–30 nm), non-enveloped icosahedral particle by three capsid proteins (viral proteins; VPs1–3). The only *cis*-elements on the vector DNA are 145 nucleotide-long inverted terminal repeats (ITRs) that fold to form unique terminal hairpins [121]. While the simple structure of the vector may have safety advantages, it can be also be an impediment to transduction. This process involves attachment of the virus to specific cell-surface receptors, endocytosis and intracellular transport in early and late endosomes, endosomal escape, entry into the nucleus and uncoating, and finally the conversion of the single DNA strand into a duplex which can be recognized by the nuclear transcriptional proteins [122, 125, 126]. Infection is largely passive and highly dependent upon the presence of capsid-specific cell-surface receptors and the endogenous biological activity of the specific target cell. Because of this, the efficiency of AAV gene transfer is widely variable among cell types.

Several groups have shown that certain stimuli, such as UV radiation which increases the production of endogenous DNA repair and synthesis proteins, can significantly enhance intra-articular transgene expression from conventional AAV vectors [113, 127, 128]. This result indicates that second strand DNA synthesis is rate-limiting in AAV transduction of joint tissues. Accordingly, AAV vectors that are self-complementary (sc) (i.e., double stranded, containing both + and – DNA strands) overcome limitations imposed by conventional single stranded vectors [129, 130]. Indeed, we have found that scAAV vectors provide >20-fold increase of gene expression with a rapid onset in synovial and capsular cells *in vitro* and *in vivo* in the joints of experimental animals [131]. scAAV-mediated transgene expression in the knees of rabbits was sufficient to mediate therapeutic responses in these animals.

scAAV vectors can be produced either by generation of vector plasmids that are ~half-genome sized combined with selective purification of the infectious double stranded form [130], or through the use of half-genome sized vector plasmids containing a mutation in one of the terminal resolution sequences of the AAV ITRs [129]. Both strategies generate + and – strand viral genomes that are covalently linked at one terminal repeat. Because the AAV genomes of sc-vectors are half wild type size (~2.5 kb) the resulting 2× viral construct (~5 kb) can be packaged into the normal AAV capsid. This adaptation of the AAV vector now provides temporal transduction and expression profiles comparable to that achieved with recombinant adenovirus. This, combined with a favorable safety profile may finally provide a viral vector delivery system appropriate for clinical use in treating arthritic conditions (for more details on AAV, see chapter by Li, Hirsch and Samulski).

Future directions

Much like development of gene therapies for RA, the published literature describes an ever-increasing number of genes whose transfer produces beneficial effects in small animal models of OA. Further, the emergence of scAAV vectors provides a system for safe and efficient *in vivo* gene delivery to joint tissues. A major frustration, however, is the lack of momentum (or obvious funding mechanisms) to push these findings into clinical trials. Certainly undertaking human testing of a gene-based therapy for OA is a daunting enterprise. Clinical trials are exorbitantly expensive, requiring, in addition to preclinical efficacy data, supporting safety data (typically generated at an independent commercial laboratory); production and validation of a clinical grade vector; administrative staff to support navigation of regulatory bodies, and finally clinical staff and facilities costs to implement the actual study. Furthermore, the conservative, staged progression of human trials can delay determination of clinical efficacy of a specific therapy until Phase II. Unfortunately, by the time a gene therapy trial has progressed into the clinic, the vector technology is frequently obsolete. Nevertheless, one clinical trial for OA is underway in Korea and the US, and another is under discussion with the FDA. While human gene therapy for OA is inching along the clinical trials pathway, the field of veterinary medicine offers additional, more expeditious alternatives.

Given the potential stigma associated with delivery of a genetic medicine for a common non-life threatening condition, diseases such as OA with close naturally-occurring equivalents in the veterinary world offer economical and immediate opportunities to establish the efficacy of gene- and cell-based therapies in actual disease, and on a relevant scale without the risk or expense associated with conducting human clinical trials. For example, OA arises frequently in racehorses from excessive, rapid and repetitive loading [132]. As in humans, equine OA is characterized clinically by persistent pain and dysfunc-

tion of the affected joint. The pathogenesis is similarly marked by progressive deterioration of the articular cartilage, subchondral bone sclerosis, marginal osteophytes and soft tissue inflammation. Detailed analyses point to identical mediators in both human and equine OA whereby local overproduction of inflammatory cytokines, primarily IL-1, leads to production of NO, PGE_2 and degradative enzymes including MMPs and aggrecanases, that promote cartilage matrix degeneration [133].

In the case of equine OA, the size of the joints most commonly affected are equivalent in size to human knees. Moreover, the horse can readily perform controlled exercise, allowing analysis of joint function, and current clinical treatment and diagnostic modalities of OA are similar in humans and horses. The equine joint offers the rare chance to study both gene and cell-based therapies in actual arthritis, rather than in contrived, rapidly induced models of questionable validity. It is highly likely that a track record of safety and efficacy in the veterinary arena would not only facilitate the development of gene therapies for human OA, but also lead to novel gene-based veterinary medicines.

Acknowledgements
This work was supported in part by grants AR051085, AR052809, AR058776 to CHE and AR048566 to SCG from the National Institute of Arthritis Musculoskeletal and Skin Diseases.

References

1 Dagenais S, Garbedian S, Wai EK (2009) Systematic review of the prevalence of radiographic primary hip osteoarthritis. *Clin Orthop Relat Res* 467: 623–637
2 Hunter DJ, Felson DT (2006) Osteoarthritis. *BMJ* 332: 639–642
3 Felson DT (2009) Developments in the clinical understanding of osteoarthritis. *Arthritis Res Ther* 11: 203
4 Felson DT (2004) An update on the pathogenesis and epidemiology of osteoarthritis. *Radiol Clin North Am* 42: 1–9, v
5 Roos H, Adalberth T, Dahlberg L, Lohmander LS (1995) Osteoarthritis of the knee after injury to the anterior cruciate ligament or meniscus: the influence of time and age. *Osteoarthritis Cartilage* 3: 261–267
6 Lohmander LS, Ostenberg A, Englund M, Roos H (2004) High prevalence of knee osteoarthritis, pain, and functional limitations in female soccer players twelve years after anterior cruciate ligament injury. *Arthritis Rheum* 50: 3145–3152
7 Englund M, Roos EM, Lohmander LS (2003) Impact of type of meniscal tear on radiographic and symptomatic knee osteoarthritis: a sixteen-year follow up of meniscectomy with matched controls. *Arthritis Rheum* 48: 2178–2187
8 Felson DT (2004) Risk factors for osteoarthritis: understanding joint vulnerability. *Clin Orthop Relat Res* S16–21
9 Messier SP, Loeser RF, Miller GD, Morgan TM, Rejeski WJ, Sevick MA, Ettinger WH Jr, Pahor M, Williamson JD (2004) Exercise and dietary weight loss in overweight and obese older adults with knee osteoarthritis: the Arthritis, Diet, and Activity Promotion Trial. *Arthritis Rheum* 50: 1501–1510
10 Christensen R, Astrup A, Bliddal H (2005) Weight loss: the treatment of choice for knee osteoarthritis? A randomized trial. *Osteoarthritis Cartilage* 13: 20–27
11 Smith RL (1999) Degradative enzymes in osteoarthritis. *Front Biosci* 4: D704–712
12 Hochberg MC, Altman RD, Brandt KD, Clark BM, Dieppe PA, Griffin MR, Moskowitz RW, Schnitzer TJ (1995) Guidelines for the medical management of osteoarthritis. Part II.

Osteoarthritis of the knee. American College of Rheumatology. *Arthritis Rheum* 38: 1541–1546
13 Gunther KP (2001) Surgical approaches for osteoarthritis. *Best Pract Res Clin Rheumatol* 15: 627–643
14 Kirkley A, Birmingham TB, Litchfield RB, Giffin JR, Willits KR, Wong CJ, Feagan BG, Donner A, Griffin SH, D'Ascanio LM et al. (2008) A randomized trial of arthroscopic surgery for osteoarthritis of the knee. *N Engl J Med* 359: 1097–1107
15 Moseley JB, O'Malley K, Petersen NJ, Menke TJ, Brody BA, Kuykendall DH, Hollingsworth JC, Ashton CM, Wray NP (2002) A controlled trial of arthroscopic surgery for osteoarthritis of the knee. *N Engl J Med* 347: 81–88
16 Bert JM, Gasser SI (2002) Approach to the osteoarthritic knee in the aging athlete: debridement to osteotomy. *Arthroscopy* 18: 107–110
17 Deal CL, Moskowitz RW (1999) Nutraceuticals as therapeutic agents in osteoarthritis. The role of glucosamine, chondroitin sulfate, and collagen hydrolysate. *Rheum Dis Clin North Am* 25: 379–395
18 Clegg DO, Reda DJ, Harris CL, Klein MA, O'Dell JR, Hooper MM, Bradley JD, Bingham CO 3rd, Weisman MH, Jackson CG et al. (2006) Glucosamine, chondroitin sulfate, and the two in combination for painful knee osteoarthritis. *N Engl J Med* 354: 795–808
19 Sawitzke AD, Shi H, Finco MF, Dunlop DD, Bingham CO 3rd, Harris CL, Singer NG, Bradley JD, Silver D, Jackson CG et al. (2008) The effect of glucosamine and/or chondroitin sulfate on the progression of knee osteoarthritis: a report from the glucosamine/chondroitin arthritis intervention trial. *Arthritis Rheum* 58: 3183–3191
20 Evans CH, Gouze JN, Gouze E, Robbins PD, Ghivizzani SC (2004) Osteoarthritis gene therapy. *Gene Ther* 11: 379–389
21 Eyre DR, Weis MA, Wu JJ (2006) Articular cartilage collagen: an irreplaceable framework? *Eur Cell Mater* 12: 57–63
22 Arner EC (2002) Aggrecanase-mediated cartilage degradation. *Curr Opin Pharmacol* 2: 322–329
23 Huang K, Wu LD (2008) Aggrecanase and aggrecan degradation in osteoarthritis: a review. *J Int Med Res* 36: 1149–1160
24 Goldring MB, Otero M, Tsuchimochi K, Ijiri K, Li Y (2008) Defining the roles of inflammatory and anabolic cytokines in cartilage metabolism. *Ann Rheum Dis* 67 Suppl 3: iii75–82
25 Aigner T, Kim HA, Roach HI (2004) Apoptosis in osteoarthritis. *Rheum Dis Clin North Am* 30: 639–653, xi
26 Pelletier JP, Martel-Pelletier J, Abramson SB (2001) Osteoarthritis, an inflammatory disease: potential implication for the selection of new therapeutic targets. *Arthritis Rheum* 44: 1237–1247
27 Attur MG, Patel IR, Patel RN, Abramson SB, Amin AR (1998) Autocrine production of IL-1 beta by human osteoarthritis-affected cartilage and differential regulation of endogenous nitric oxide, IL-6, prostaglandin E2, and IL-8. *Proc Assoc Am Physicians* 110: 65–72
28 Fernandes JC, Martel-Pelletier J, Pelletier JP (2002) The role of cytokines in osteoarthritis pathophysiology. *Biorheology* 39: 237–246
29 Pelletier JP, Faure MP, DiBattista JA, Wilhelm S, Visco D, Martel-Pelletier J (1993) Coordinate synthesis of stromelysin, interleukin-1, and oncogene proteins in experimental osteoarthritis. An immunohistochemical study. *Am J Pathol* 142: 95–105
30 Pond MJ, Nuki G (1973) Experimentally-induced osteoarthritis in the dog. *Ann Rheum Dis* 32: 387–388
31 Sadouk MB, Pelletier JP, Tardif G, Kiansa K, Cloutier JM, Martel-Pelletier J (1995) Human synovial fibroblasts coexpress IL-1 receptor type I and type II mRNA. The increased level of the IL-1 receptor in osteoarthritic cells is related to an increased level of the type I receptor. *Lab Invest* 73: 347–355
32 Burrage PS, Mix KS, Brinckerhoff CE (2006) Matrix metalloproteinases: role in arthritis. *Front Biosci* 11: 529–543
33 Murphy G, Nagase H (2008) Reappraising metalloproteinases in rheumatoid arthritis and osteoarthritis: destruction or repair? *Nat Clin Pract Rheumatol* 4: 128–135
34 Tortorella MD, Malfait AM, Deccico C, Arner E (2001) The role of ADAM-TS4 (aggrecanase-1) and ADAM-TS5 (aggrecanase-2) in a model of cartilage degradation. *Osteoarthritis Cartilage* 9: 539–552
35 Gouze JN, Gouze E, Popp MP, Bush ML, Dacanay EA, Kay JD, Levings PP, Patel KR, Saran JP, Watson RS et al. (2006) Exogenous glucosamine globally protects chondrocytes from the arthritogenic effects of IL-1beta. *Arthritis Res Ther* 8: R173

36 Hubbard JR, Steinberg JJ, Bednar MS, Sledge CB (1988) Effect of purified human interleukin-1 on cartilage degradation. *J Orthop Res* 6: 180–187
37 Mengshol JA, Vincenti MP, Coon CI, Barchowsky A, Brinckerhoff CE (2000) Interleukin-1 induction of collagenase 3 (matrix metalloproteinase 13) gene expression in chondrocytes requires p38, c-Jun N-terminal kinase, and nuclear factor kappaB: differential regulation of collagenase 1 and collagenase 3. *Arthritis Rheum* 43: 801–811
38 Stadler J, Stefanovic-Racic M, Billiar TR, Curran RD, McIntyre LA, Georgescu HI, Simmons RL, Evans CH (1991) Articular chondrocytes synthesize nitric oxide in response to cytokines and lipopolysaccharide. *J Immunol* 147: 3915–3920
39 Geng Y, Blanco FJ, Cornelisson M, Lotz M (1995) Regulation of cyclooxygenase-2 expression in normal human articular chondrocytes. *J Immunol* 155: 796–801
40 Evans CH, Ghivizzani SC, Kang R, Muzzonigro T, Wasko MC, Herndon JH, Robbins PD (1999) Gene therapy for rheumatic diseases. *Arthritis Rheum* 42: 1–16
41 Evans CH, Ghivizzani SC, Robbins PD (2009) Orthopedic gene therapy in 2008. *Mol Ther* 17: 231–244
42 Valdes AM, Loughlin J, Oene MV, Chapman K, Surdulescu GL, Doherty M, Spector TD (2007) Sex and ethnic differences in the association of ASPN, CALM1, COL2A1, COMP, and FRZB with genetic susceptibility to osteoarthritis of the knee. *Arthritis Rheum* 56: 137–146
43 Valdes AM, Van Oene M, Hart DJ, Surdulescu GL, Loughlin J, Doherty M, Spector TD (2006) Reproducible genetic associations between candidate genes and clinical knee osteoarthritis in men and women. *Arthritis Rheum* 54: 533–539
44 Loughlin J (2005) Polymorphism in signal transduction is a major route through which osteoarthritis susceptibility is acting. *Curr Opin Rheumatol* 17: 629–633
45 Ikegawa S (2007) New gene associations in osteoarthritis: what do they provide, and where are we going? *Curr Opin Rheumatol* 19: 429–434
46 Ghivizzani SC, Oligino TJ, Glorioso JC, Robbins PD, Evans CH (2001) Direct gene delivery strategies for the treatment of rheumatoid arthritis. *Drug Discov Today* 6: 259–267
47 Evans CH, Gouze E, Gouze JN, Robbins PD, Ghivizzani SC (2006) Gene therapeutic approaches-transfer *in vivo*. *Adv Drug Deliv Rev* 58: 243–258
48 Bandara G, Mueller GM, Galea-Lauri J, Tindal MH, Georgescu HI, Suchanek MK, Hung GL, Glorioso JC, Robbins PD, Evans CH (1993) Intraarticular expression of biologically active interleukin 1-receptor-antagonist protein by *ex vivo* gene transfer. *Proc Natl Acad Sci USA* 90: 10764–10768
49 Madry H, Trippel SB (2000) Efficient lipid-mediated gene transfer to articular chondrocytes. *Gene Ther* 7: 286–291
50 Baragi VM, Renkiewicz RR, Jordan H, Bonadio J, Hartman JW, Roessler BJ (1995) Transplantation of transduced chondrocytes protects articular cartilage from interleukin 1-induced extracellular matrix degradation. *J Clin Invest* 96: 2454–2460
51 Smith P, Shuler FD, Georgescu HI, Ghivizzani SC, Johnstone B, Niyibizi C, Robbins PD, Evans CH (2000) Genetic enhancement of matrix synthesis by articular chondrocytes: comparison of different growth factor genes in the presence and absence of interleukin-1. *Arthritis Rheum* 43: 1156–1164
52 Madry H, Cucchiarini M, Terwilliger EF, Trippel SB (2003) Recombinant adeno-associated virus vectors efficiently and persistently transduce chondrocytes in normal and osteoarthritic human articular cartilage. *Hum Gene Ther* 14: 393–402
53 Kang R, Marui T, Ghivizzani SC, Nita IM, Georgescu HI, Suh JK, Robbins PD, Evans CH (1997) *Ex vivo* gene transfer to chondrocytes in full-thickness articular cartilage defects: a feasibility study. *Osteoarthritis Cartilage* 5: 139–143
54 Gouze E, Pawliuk R, Pilapil C, Gouze JN, Fleet C, Palmer GD, Evans CH, Leboulch P, Ghivizzani SC (2002) *In vivo* gene delivery to synovium by lentiviral vectors. *Mol Ther* 5: 397–404
55 Gouze E, Gouze JN, Palmer GD, Pilapil C, Evans CH, Ghivizzani SC (2007) Transgene persistence and cell turnover in the diarthrodial joint: implications for gene therapy of chronic joint diseases. *Mol Ther* 15: 1114–1120
56 Khoury M, Jorgensen C, Apparailly F (2007) RNAi in arthritis: prospects of a future antisense therapy in inflammation. *Curr Opin Mol Ther* 9: 483–489
57 Khoury M, Escriou V, Courties G, Galy A, Yao R, Largeau C, Scherman D, Jorgensen C, Apparailly F (2008) Efficient suppression of murine arthritis by combined anticytokine small interfering RNA lipoplexes. *Arthritis Rheum* 58: 2356–2367

58 Chen LX, Lin L, Wang HJ, Wei XL, Fu X, Zhang JY, Yu CL (2008) Suppression of early experimental osteoarthritis by *in vivo* delivery of the adenoviral vector-mediated NF-kappaBp65-specific siRNA. *Osteoarthritis Cartilage* 16: 174–184
59 Arend WP, Gabay C (2000) Physiologic role of interleukin-1 receptor antagonist. *Arthritis Res* 2: 245–248
60 Attur MG, Dave MN, Leung MY, Cipolletta C, Meseck M, Woo SL, Amin AR (2002) Functional genomic analysis of type II IL-1beta decoy receptor: potential for gene therapy in human arthritis and inflammation. *J Immunol* 168: 2001–2010
61 Bresnihan B (2002) Preventing joint damage as the best measure of biologic drug therapy. *J Rheumatol Suppl* 65: 39–43
62 Cohen SB, Woolley JM, Chan W (2003) Interleukin 1 receptor antagonist anakinra improves functional status in patients with rheumatoid arthritis. *J Rheumatol* 30: 225–231
63 Kary S, Burmester GR (2003) Anakinra: the first interleukin-1 inhibitor in the treatment of rheumatoid arthritis. *Int J Clin Pract* 57: 231–234
64 Burls A, Jobanputra P (2004) The trials of anakinra. *Lancet* 364: 827–828
65 Chevalier X, Goupille P, Beaulieu AD, Burch FX, Bensen WG, Conrozier T, Loeuille D, Kivitz AJ, Silver D, Appleton BE (2009) Intraarticular injection of anakinra in osteoarthritis of the knee: a multicenter, randomized, double-blind, placebo-controlled study. *Arthritis Rheum* 61: 344–352
66 Martel-Pelletier J, Pelletier JP (2009) Osteoarthritis: A single injection of anakinra for treating knee OA? *Nat Rev Rheumatol* 5: 363–364
67 Fleischmann RM, Tesser J, Schiff MH, Schechtman J, Burmester GR, Bennett R, Modafferi D, Zhou L, Bell D, Appleton B (2006) Safety of extended treatment with anakinra in patients with rheumatoid arthritis. *Ann Rheum Dis* 65: 1006–1012
68 Wang HJ, Yu CL, Kishi H, Motoki K, Mao ZB, Muraguchi A (2006) Suppression of experimental osteoarthritis by adenovirus-mediated double gene transfer. *Chin Med J (Engl)* 119: 1365–1373
69 Zhang X, Mao Z, Yu C (2004) Suppression of early experimental osteoarthritis by gene transfer of interleukin-1 receptor antagonist and interleukin-10. *J Orthop Res* 22: 742–750
70 Pelletier JP, Caron JP, Evans C, Robbins PD, Georgescu HI, Jovanovic D, Fernandes JC, Martel-Pelletier J (1997) *In vivo* suppression of early experimental osteoarthritis by interleukin-1 receptor antagonist using gene therapy. *Arthritis Rheum* 40: 1012–1019
71 Frisbie DD, Ghivizzani SC, Robbins PD, Evans CH, McIlwraith CW (2002) Treatment of experimental equine osteoarthritis by *in vivo* delivery of the equine interleukin-1 receptor antagonist gene. *Gene Ther* 9: 12–20
72 Wang CR, Chen SY, Shiau AL, Wu CL, Jou IM, Chao L, Chao J (2007) Upregulation of kallistatin expression in rheumatoid joints. *J Rheumatol* 34: 2171–2176
73 Hsieh J, Shen PC, Shiau AL, Wu CL, Lee CH, Jou IM, Wang CR, Chao J, Chao L, Teo ML (2008) Adenovirus-mediated kallistatin gene transfer ameliorates disease progression in a rat model of osteoarthritis induced by anterior cruciate ligament transection. *Hum Gene Ther* in press
74 Kafienah W, Al-Fayez F, Hollander AP, Barker MD (2003) Inhibition of cartilage degradation: a combined tissue engineering and gene therapy approach. *Arthritis Rheum* 48: 709–718
75 Gouze JN, Gouze E, Palmer GD, Kaneto H, Ghivizzani SC, Grodzinsky AJ, Evans CH (2004) Adenovirus-mediated gene transfer of glutamine: fructose-6-phosphate amidotransferase antagonizes the effects of interleukin-1beta on rat chondrocytes. *Osteoarthritis Cartilage* 12: 217–224
76 Tuan RS (2004) Biology of developmental and regenerative skeletogenesis. *Clin Orthop Relat Res* S105–117
77 Blaney Davidson EN, van der Kraan PM, van den Berg WB (2007) TGF-beta and osteoarthritis. *Osteoarthritis Cartilage* 15: 597–604
78 Seeherman H, Wozney JM (2005) Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. *Cytokine Growth Factor Rev* 16: 329–345
79 Chubinskaya S, Hurtig M, Rueger DC (2007) OP-1/BMP-7 in cartilage repair. *Int Orthop* 31: 773–781
80 Miljkovic ND, Cooper GM, Marra KG (2008) Chondrogenesis, bone morphogenetic protein-4 and mesenchymal stem cells. *Osteoarthritis Cartilage* 16: 1121–1130
81 Edwards CJ, Francis-West PH (2001) Bone morphogenetic proteins in the development and healing of synovial joints. *Semin Arthritis Rheum* 31: 33–42
82 Mikic B (2004) Multiple effects of GDF-5 deficiency on skeletal tissues: implications for therapeutic bioengineering. *Ann Biomed Eng* 32: 466–476
83 Naski MC, Ornitz DM (1998) FGF signaling in skeletal development. *Front Biosci* 3: d781–794

84 Schmidt MB, Chen EH, Lynch SE (2006) A review of the effects of insulin-like growth factor and platelet derived growth factor on *in vivo* cartilage healing and repair. *Osteoarthritis Cartilage* 14: 403–412
85 Kubo S, Cooper GM, Matsumoto T, Phillippi JA, Corsi KA, Usas A, Li G, Fu FH, Huard J (2009) Blocking vascular endothelial growth factor with soluble Flt-1 improves the chondrogenic potential of mouse skeletal muscle-derived stem cells. *Arthritis Rheum* 60: 155–165
86 Matsumoto T, Cooper GM, Gharaibeh B, Meszaros LB, Li G, Usas A, Fu FH, Huard J (2009) Cartilage repair in a rat model of osteoarthritis through intraarticular transplantation of muscle-derived stem cells expressing bone morphogenetic protein 4 and soluble Flt-1. *Arthritis Rheum* 60: 1390–1405
87 Song SU, Cha YD, Han JU, Oh IS, Choi KB, Yi Y, Hyun JP, Lee HY, Chi GF, Lim CL et al. (2005) Hyaline cartilage regeneration using mixed human chondrocytes and transforming growth factor-beta1- producing chondrocytes. *Tissue Eng* 11: 1516–1526
88 Song SU, Hong YJ, Oh IS, Yi Y, Choi KB, Lee JW, Park KW, Han JU, Suh JK, Lee KH (2004) Regeneration of hyaline articular cartilage with irradiated transforming growth factor beta1-producing fibroblasts. *Tissue Eng* 10: 665–672
89 Mi Z, Ghivizzani SC, Lechman E, Glorioso JC, Evans CH, Robbins PD (2003) Adverse effects of adenovirus-mediated gene transfer of human transforming growth factor beta 1 into rabbit knees. *Arthritis Res Ther* 5: R132–139
90 Gelse K, Jiang QJ, Aigner T, Ritter T, Wagner K, Poschl E, von der Mark K, Schneider H (2001) Fibroblast-mediated delivery of growth factor complementary DNA into mouse joints induces chondrogenesis but avoids the disadvantages of direct viral gene transfer. *Arthritis Rheum* 44: 1943–1953
91 Trippel SB, Ghivizzani SC, Nixon AJ (2004) Gene-based approaches for the repair of articular cartilage. *Gene Ther* 11: 351–359
92 Richter W (2007) Cell-based cartilage repair: illusion or solution for osteoarthritis. *Curr Opin Rheumatol* 19: 451–456
93 Lefebvre V, Peeters-Joris C, Vaes G (1990) Production of collagens, collagenase and collagenase inhibitor during the dedifferentiation of articular chondrocytes by serial subcultures. *Biochim Biophys Acta* 1051: 266–275
94 Shuler FD, Georgescu HI, Niyibizi C, Studer RK, Mi Z, Johnstone B, Robbins RD, Evans CH (2000) Increased matrix synthesis following adenoviral transfer of a transforming growth factor beta1 gene into articular chondrocytes. *J Orthop Res* 18: 585–592
95 Hidaka C, Goodrich LR, Chen CT, Warren RF, Crystal RG, Nixon AJ (2003) Acceleration of cartilage repair by genetically modified chondrocytes over expressing bone morphogenetic protein-7. *J Orthop Res* 21: 573–583
96 Goodrich LR, Hidaka C, Robbins PD, Evans CH, Nixon AJ (2007) Genetic modification of chondrocytes with insulin-like growth factor-1 enhances cartilage healing in an equine model. *J Bone Joint Surg Br* 89: 672–685
97 Kaul G, Cucchiarini M, Arntzen D, Zurakowski D, Menger MD, Kohn D, Trippel SB, Madry H (2006) Local stimulation of articular cartilage repair by transplantation of encapsulated chondrocytes overexpressing human fibroblast growth factor 2 (FGF-2) *in vivo*. *J Gene Med* 8: 100–111
98 Chen FH, Tuan RS (2008) Mesenchymal stem cells in arthritic diseases. *Arthritis Res Ther* 10: 223
99 Stoddart MJ, Grad S, Eglin D, Alini M (2009) Cells and biomaterials in cartilage tissue engineering. *Regen Med* 4: 81–98
100 Palmer GD, Steinert A, Pascher A, Gouze E, Gouze JN, Betz O, Johnstone B, Evans CH, Ghivizzani SC (2005) Gene-induced chondrogenesis of primary mesenchymal stem cells *in vitro*. *Mol Ther* 12: 219–228
101 Steinert AF, Proffen B, Kunz M, Hendrich C, Ghivizzani SC, Noth U, Rethwilm A, Eulert J, Evans CH (2009) Hypertrophy is induced during the *in vitro* chondrogenic differentiation of human mesenchymal stem cells by bone morphogenetic protein-2 and bone morphogenetic protein-4 gene transfer. *Arthritis Res Ther* 11: R148
102 Nochi H, Sung JH, Lou J, Adkisson HD, Maloney WJ, Hruska KA (2004) Adenovirus mediated BMP-13 gene transfer induces chondrogenic differentiation of murine mesenchymal progenitor cells. *J Bone Miner Res* 19: 111–122
103 Feng G, Wan Y, Balian G, Laurencin CT, Li X (2008) Adenovirus-mediated expression of growth and differentiation factor-5 promotes chondrogenesis of adipose stem cells. *Growth Factors* 26:

132–142
104 Tsuchiya H, Kitoh H, Sugiura F, Ishiguro N (2003) Chondrogenesis enhanced by overexpression of sox9 gene in mouse bone marrow-derived mesenchymal stem cells. *Biochem Biophys Res Commun* 301: 338–343
105 Gelse K, von der Mark K, Aigner T, Park J, Schneider H (2003) Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells. *Arthritis Rheum* 48: 430–441
106 Pagnotto MR, Wang Z, Karpie JC, Ferretti M, Xiao X, Chu CR (2007) Adeno-associated viral gene transfer of transforming growth factor-beta1 to human mesenchymal stem cells improves cartilage repair. *Gene Ther* 14: 804–813
107 Evans CH, Robbins PD, Ghivizzani SC, Wasko MC, Tomaino MM, Kang R, Muzzonigro TA, Vogt M, Elder EM, Whiteside TL et al. (2005) Gene transfer to human joints: progress toward a gene therapy of arthritis. *Proc Natl Acad Sci USA* 102: 8698–8703
108 Gouze E, Pawliuk R, Gouze JN, Pilapil C, Fleet C, Palmer GD, Evans CH, Leboulch P, Ghivizzani SC (2003) Lentiviral-mediated gene delivery to synovium: potent intra-articular expression with amplification by inflammation. *Mol Ther* 7: 460–466
109 Adriaansen J, Khoury M, de Cortie CJ, Fallaux FJ, Bigey P, Scherman D, Gould DJ, Chernajovsky Y, Apparailly F, Jorgensen C et al. (2007) Reduction of arthritis following intra-articular administration of an adeno-associated virus serotype 5 expressing a disease-inducible TNF-blocking agent. *Ann Rheum Dis* 66: 1143–1150
110 Apparailly F, Khoury M, Vervoordeldonk MJ, Adriaansen J, Gicquel E, Perez N, Riviere C, Louis-Plence P, Noel D, Danos O et al. (2005) Adeno-associated virus pseudotype 5 vector improves gene transfer in arthritic joints. *Hum Gene Ther* 16: 426–434
111 Cottard V, Mulleman D, Bouille P, Mezzina M, Boissier MC, Bessis N (2000) Adeno-associated virus-mediated delivery of IL-4 prevents collagen-induced arthritis. *Gene Ther* 7: 1930–1939
112 Ghivizzani SC, Lechman ER, Kang R, Tio C, Kolls J, Evans CH, Robbins PD (1998) Direct adenovirus-mediated gene transfer of interleukin 1 and tumor necrosis factor alpha soluble receptors to rabbit knees with experimental arthritis has local and distal anti-arthritic effects. *Proc Natl Acad Sci USA* 95: 4613–4618
113 Goater J, Muller R, Kollias G, Firestein GS, Sanz I, O'Keefe RJ, Schwarz EM (2000) Empirical advantages of adeno associated viral vectors *in vivo* gene therapy for arthritis. *J Rheumatol* 27: 983–989
114 Oligino T, Ghivizzani S, Wolfe D, Lechman E, Krisky D, Mi Z, Evans C, Robbins P, Glorioso J (1999) Intra-articular delivery of a herpes simplex virus IL-1Ra gene vector reduces inflammation in a rabbit model of arthritis. *Gene Ther* 6: 1713–1720
115 Goncalves MA, de Vries AA (2006) Adenovirus: from foe to friend. *Rev Med Virol* 16: 167–186
116 Imperiale MJ, Kochanek S (2004) Adenovirus vectors: biology, design, and production. *Curr Top Microbiol Immunol* 273: 335–357
117 Kamen A, Henry O (2004) Development and optimization of an adenovirus production process. *J Gene Med* 6 Suppl 1: S184–192
118 Naldini L, Verma IM (2000) Lentiviral vectors. *Adv Virus Res* 55: 599–609
119 Kwon I, Schaffer DV (2008) Designer gene delivery vectors: molecular engineering and evolution of adeno-associated viral vectors for enhanced gene transfer. *Pharm Res* 25: 489–499
120 Erles K, Sebokova P, Schlehofer JR (1999) Update on the prevalence of serum antibodies (IgG and IgM) to adeno-associated virus (AAV). *J Med Virol* 59: 406–411
121 Wu Z, Asokan A, Samulski RJ (2006) Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther* 14: 316–327
122 Choi VW, McCarty DM, Samulski RJ (2005) AAV hybrid serotypes: improved vectors for gene delivery. *Curr Gene Ther* 5: 299–310
123 Samulski RJ, Chang LS, Shenk T (1987) A recombinant plasmid from which an infectious adeno-associated virus genome can be excised *in vitro* and its use to study viral replication. *J Virol* 61: 3096–3101
124 Zaiss AK, Muruve DA (2005) Immune responses to adeno-associated virus vectors. *Curr Gene Ther* 5: 323–331
125 Ding W, Zhang L, Yan Z, Engelhardt JF (2005) Intracellular trafficking of adeno-associated viral vectors. *Gene Ther* 12: 873–880
126 Sanlioglu S, Monick MM, Luleci G, Hunninghake GW, Engelhardt JF (2001) Rate limiting steps of AAV transduction and implications for human gene therapy. *Curr Gene Ther* 1: 137–147
127 Katakura S, Jennings K, Watanabe S, Adachi E, Thornton S, Gao GP, Wilson JM, Burstein H,

Trapnell B, Hirsch R (2004) Recombinant adeno-associated virus preferentially transduces human, compared to mouse, synovium: implications for arthritis therapy. *Mod Rheumatol* 14: 18–24

128 Ulrich-Vinther M, Duch MR, Soballe K, O'Keefe RJ, Schwarz EM, Pedersen FS (2004) *In vivo* gene delivery to articular chondrocytes mediated by an adeno-associated virus vector. *J Orthop Res* 22: 726–734

129 McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ (2003) Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo*. *Gene Ther* 10: 2112–2118

130 McCarty DM, Monahan PE, Samulski RJ (2001) Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther* 8: 1248–1254

131 Kay JD, Gouze E, Oligino TJ, Gouze JN, Watson RS, Levings PP, Bush ML, Dacanay A, Nickerson DM, Robbins PD et al. (2009) Intra-articular gene delivery and expression of interleukin-1Ra mediated by self-complementary adeno-associated virus. *J Gene Med* 11: 605–614

132 Goodrich LR, Nixon AJ (2006) Medical treatment of osteoarthritis in the horse – a review. *Vet J* 171: 51–69

133 McIlwraith CW (2005) Arthroscopy to gene therapy – 30 years of looking in joints. In: *51st Annual Convention of the American Association of Equine Practitioners – AAEP, 2005- Seattle, WA, USA*. American Association of Equine Practitioners, p. 2613

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Gene therapy: Sjögren's syndrome

Nienke Roescher[1, 2], Paul Peter Tak[2] and John A. Chiorini[1]

[1] *Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, DHHS, Bethesda, MD, USA*
[2] *Division of Clinical Immunology and Rheumatology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands*

Abstract

Sjögrens syndrome (SS) is characterized by inflammation and dysfunction of the secretory organs. In the majority of patients the salivary and lachrymal glands are predominantly affected, although systemic symptoms are common. The pathogenesis of the disease is not well understood and to date there is no universally effective therapy available. The development of gene therapy and in particular local gene therapy applied to the salivary glands may be effective in the disease. Animal studies have shown that treatment with immunomodulatory molecules like interleukin 10 or vasoactive intestinal peptide can influence salivary function positively while changing the local inflammatory environment. Future research will have to show whether this approach is feasible in humans.

Sjögren's syndrome

Sjögren's syndrome (SS) is a systemic chronic inflammatory autoimmune disease predominantly affecting the lachrymal and salivary glands. Patients complain of dry eyes and dry mouth (sicca symptoms), the latter leading to pain, discomfort, dental caries and infection of the mouth with opportunistic pathogens like *Candida albicans*. SS is often accompanied by systemic symptoms, such as Raynaud's phenomenon, arthritis, fatigue and vasculitis. Women are nine times more likely to be affected than men and the estimated prevalence is 0.5% for the general population [1]. The diagnosis is based on objective and subjective criteria of dryness of the secretory glands, inflammation of the salivary gland and the presence of auto-antibodies in the serum. It is termed primary SS (pSS) in the absence of an underlying disease, and secondary SS (sSS) when related to other autoimmune disease like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [2]. The pathogenesis and pathophysiology of SS are poorly understood. A number of human leukocyte antigen (HLA) subtypes are associated with SS [3] and viral and other environmental factors together with these genetics are all thought to play a role in the initiation and progression of the disease [4].

Chronic inflammation is central to SS and this is reflected by an imbalance of pro-inflammatory cytokines over anti-inflammatory cytokines. Overex-

pression of numerous pro-inflammatory cytokines in the blood, salivary glands and the saliva has been shown in several studies. The most consistently upregulated cytokines are interferon γ (IFNγ), IFNα, Interleukin (IL) 12, IL18, IL6, and B-Cell activating factor (BAFF). The presence of other cytokines like IL1β and IL17 may also play a role in the pathogenesis of SS, but data on these are incomplete. Concomitantly, IL4, an important anti-inflammatory cytokine, and Transforming Growth Factor β (TGFβ) are downregulated. In contrast, the anti-inflammatory cytokine IL10 is highly expressed in SS patients compared to healthy controls and may contribute to B cell activation and auto-antibody production (reviewed in [5]).

Histologically, SS is characterized by localized infiltrates (foci) of mononuclear cells in the salivary glands. The degree of infiltration observed ranges from mild to severe and can be accompanied by glandular atrophy and fibrosis. Foci are generally comprised of T cells (80%) and B cells (20%) and in a subgroup of patients these foci are organized to resemble germinal centers [6]. SS patients are at a higher risk than the normal population of developing non-Hodgkin's lymphoma in the exocrine glands [7, 8]. A possible mechanism for this may be chronic cytokine-driven stimulation of the infiltrating B and/or T cells in these germinal centers leading to lymphoma genesis [9].

The salivary gland in SS

The salivary gland is primarily composed of two cell types: Epithelial (ductal) cells and acinar (secretory) cells. Acinar cells are the only cells in the gland that produce saliva, they also produce more than 80% of the proteins found in saliva. The fluid secreted by acinar cells is modified by the ducts during its passage to the mouth. NaCl is reabsorbed from the secreted fluid whereas K^+ and HCO_3- are added by the ductal cells in addition to the secretion of proteins like growth factors and IgA (reviewed in [10]. In SS both acinar and ductal cells are thought to be involved in the pathogenesis of the disease. Acinar cells loose their ability to secrete fluid, due to destruction by inflammation, altered cell signaling or aberrations in the neurological signal that drives secretion. The ductal epithelial cells also play an important role in SS. Ductal cells can function as antigen presenting cells in the gland [11] and express co-stimulatory molecules like CD40 [12], CD80 and CD86 [13] indicating an active role in inflammation. Moreover, histologically most infiltrates are found around the ducts, thus SS is also known as autoimmune epithelitis [14]. Figure 1 shows the major processes involved in SS.

The cause of sicca symptoms experienced by SS patients can be directly due to the destruction or dysfunction of parenchymal tissue of the secretory organs by inflammation in some patients. Recently recognized dysfunction without overt infiltration in a significant number of patients has lead to the hypothesis that the dryness of the gland may be caused by a mechanism independent of or in addition to destruction [15, 16]. The type 3 muscarinic receptor (M3R),

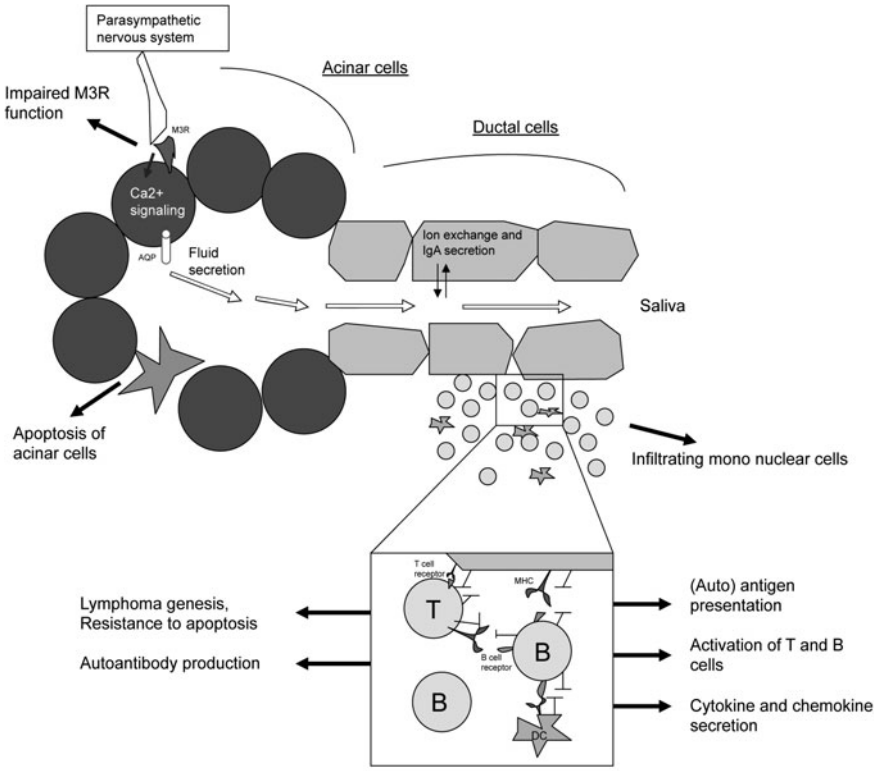


Figure 1. Simplified schematic of fluid secretion in the salivary gland and the processes involved in Sjögren's syndrome (SS). Upon stimulation of the Muscarinic type 3 receptor (M3R) and via Ca^{2+} signaling, fluid is secreted by the acinar cells through the aquaporins (AQP). The fluid (open arrows) passes through the ducts into the oral cavity while epithelial cells modify its content. In SS reduced saliva can be the result of many processes interfering with the secretory capacity of the gland (black arrows). The infiltrating B and T cells interacting with each other through their respective receptors in interaction with the MHC-peptide complex and the epithelial cells and dendritic cells (DC) are shown in the enlarged area (the co-stimulatory molecules are depicted as black T's). Each of the processes involved in SS can potentially be targeted with gene therapy. (IgA, immunoglobulin type A; B, B cell; T, T cell)

which is part of the parasympathetic nervous system, is the main neurological receptor involved in saliva secretion in murine models as shown by knockout studies [17]. It is also thought to be the main receptor involved in human saliva secretion. There is cumulating evidence that serum of a subgroup of patients interferes with the function of the M3R *in vitro* [18, 19]. The details of this interaction are still unclear. One mechanism could be that auto-antibodies against the M3R are involved, but data on the detection of these antibodies are conflicting [20, 21].

Current treatment for SS

To date there is no universally effective therapy for SS. Treatment is based on symptom relief and the prevention and treatment of complications. Patients are advised to take extra oral care and undergo regular dental check ups. For the sicca symptoms, artificial tears, punctal plugs and artificial saliva can be applied. Some patients receive symptomatic relief from secretagogues like cimeveline and pilocarpine [22]. The use of immunosuppressants has been unsatisfactory; these drugs can improve systemic conditions associated with the disease but the drugs have little effect on the sicca symptoms [23–26]. Most cytokine-directed therapies have also proved ineffective in SS, even though these therapies have been applied successfully in the treatment of other rheumatoid diseases. For instance, blocking the pro-inflammatory cytokine tumor necrosis factor α (TNFα) in RA, an autoimmune disease with many similarities to SS, has lead to greatly improved disease control with an acceptable safety profile [27]. However, unlike RA, the use of TNFα blockers did not lead to improved saliva or tear production in clinical trials with SS patients [28]. Another recently investigated therapy targets B cells. Rituximab (RTX), an anti-CD20 antibody that depletes $CD20^+$ B cells, was first approved for treatment of patients with relapsed or refractory lymphoma [29] and has quickly revolutionized the treatment of B cell lymphomas. To date RTX has been used not only in malignancies but also has been successfully used in the treatment of autoimmune diseases like RA [30] and SLE [31]. RTX in patients with SS resulted in depletion of peripheral B cell without having an effect on the numbers of natural killer cells, T helper cells and cytotoxic T cells. Furthermore, it was shown to have some beneficial effect on parameters like fatigue, vasculitis, and arthralgias with minimal side effects, some studies also reported beneficial effects on objective and subjective sicca symptoms [32–34]. However, since most studies were open label and the overall patient numbers were low [35], it is unclear to date whether RTX is a good therapy for SS.

Gene therapy for SS

In summary, several treatments that are effective in other autoimmune inflammatory diseases failed in SS. There are many reasons for this failure: First, it is possible that these drugs are aimed at the wrong target. The inhibition of TNFα in SS patients for instance was shown to lead to paradoxical upregulation of TNFα [36], and also IFNα and BAFF were shown to be upregulated after treatment [37]. Second, it is possible that these drugs reached suboptimal dose levels in the affected organs due to poor penetration of the drug. Third, it is possible that the drug was not given for a sufficient period of time or too late in the course of the disease to elicit a response. SS patients often suffer from symptoms long before they are diagnosed [1]. A therapy therefore may need to be applied long term to reverse damage that has accumulated over several

years. As a therapeutic approach, gene therapy may offer the possibility to address some of these concerns.

The use of local gene therapy

Since the salivary gland is heavily involved in the pathogenesis of SS, locally applied therapy to the salivary gland is very attractive and has a number of advantages over systemic therapy. First, the salivary glands can be easily reached by retrograde cannulation of the orifices of the salivary ducts in the mouth (as is routinely performed in scintigraphy of the salivary glands). This technique can be used not only to introduce anti-inflammatory small molecules directly to the gland but can also deliver vectors able to direct the expression of immunomodulatory proteins or the gland physiology in a non-invasive manner. Second, the entire salivary gland can be targeted since the luminal membrane of both the acinar and ductal cells within the gland is exposed. Third, because of their natural secretory activity, the salivary glands are an excellent organ to locally achieve a high level of the drugs compared to systemic administration. Fourth, long-term expression is possible from the salivary gland due to the relatively low turnover of the epithelial cells in the gland. Fifth, systemic exposure to the drugs can be limited by treating the salivary glands only, possibly leading to fewer side effects.

Experience with local gene therapy

Several studies have recently been conducted to address the utility of gene therapy in treating salivary gland dysfunction and inflammation in animal models of SS (Tab. 1). The Non-Obese Diabetic (NOD) mouse is the most widely used model to study the spontaneous development and treatment of SS. Classically, this mouse is studied for diabetes. From the age of 10 weeks these

Table 1. Gene therapy in NOD mice

Animal model	Gene and vector	Results	Ref.
NOD	IL10 AAV2	Prevents loss of salivary flow, reduced number of foci in the salivary gland	[43]
NOD	VIP AAV2	Prevents loss of salivary flow, no effects on infiltrates	[45]
NOD	sTNFR1:IgG1 AAV2	Reduced salivary flow over time, reduction in pro-inflammatory cytokines, upregulation of systemic pro-inflammatory cytokines	[46]

Abbreviations: NOD, non-obese diabetic mouse; IL10, interleukin 10: AAV, adeno associated virus; VIP, vasoactive intestinal peptide; sTNFR1:IgG1; soluble Tumor Necrosis Factor receptor 1

mice spontaneously develop insulin-dependent diabetes preceded by autoimmune insulitis of the pancreas [38]. Independently of this disease [39], but around the same age, the mice can also spontaneously develop autoimmune exocrinopathy of the salivary glands with gender-dependent loss in gland activity, monocytic infiltration of the salivary and lachrymal glands. With age, NOD mice also develop auto-antibodies against the nuclear antigens Ro and La. However this SS-like phenotype is unstable and the factors that contribute to the disease are still largely unknown [40]. One of the first studies to test the local application of gene therapy to the salivary gland used a viral vector encoding the anti-inflammatory cytokine IL10. IL10 was shown to be beneficial in a number of preclinical models of autoimmune diseases [41, 42]. IL10 was administered locally to the salivary glands of NOD mice by retrograde cannulation of the salivary gland ducts, or intramuscular injection in the quadriceps muscle using a recombinant adeno associated viral type 2 (AAV2) vector. The vector was given at 8 weeks, before onset of the disease, and at 16 weeks, at a more progressive stage of the disease. At 20 weeks, the mice treated with IL10 directly into the salivary gland showed significantly higher salivary flow in the early and late treatment group compared with the intramuscularly or control vector treatment groups [43].

Similarly, positive results have also been observed using an AAV vector encoding the neuropeptide vasoactive intestinal peptide (VIP). This peptide has a broad range of functions. It is a neurotransmitter causing vasodilatation, but is also known to be involved in immune tolerance and is a potent suppressor of a variety of pro-inflammatory cytokines [44]. NOD mice were treated with a single dose of 10^{10} viral particles containing the VIP gene per salivary gland. At the age of 16 weeks, 8 weeks after the treatment, VIP treated mice showed no loss of salivary flow compared to the control group. There was no difference in focus score or apoptotic index between the different groups, but there was significantly less IL2, IL12, TNFα, and IL10 present in glandular extracts, in contrast to serum levels, where no difference was observed [45]. Treatment of NOD mice with both the IL10 and the VIP constructs did not lead to noticeable side effects; the mice appeared to be healthy during treatment.

In contrast, local application of TNF inhibitors did not improve inflammation and dysfunction of the salivary glands in NOD mice. This supports the lack of efficacy seen in previous studies in humans when systemic treatment was studied. Salivary gland-directed gene therapy with a soluble TNF receptor (TNFR1:IgG) in NOD mice decreased salivary gland activity. Moreover, treatment decreased some pro-inflammatory cytokines locally in the gland, while TGF-β was upregulated. In plasma however, the opposite was observed, with upregulated pro-inflammatory cytokines and downregulated TGF-β levels [46]. The reason for the failure of this treatment in mice is not completely understood, but could be due to circulating receptor-complex formation [27] or underlying mutations in the TNF pathway as have been described in humans and mice [47, 48]. This observation further emphasizes the negative effect of

this class of drug on salivary gland function and suggests that other molecules should be explored as targets for therapeutic intervention in SS.

Future therapeutic targets

Although the studies with IL10 and VIP are promising, other potential therapeutics should be explored not only as possible future drugs but also to better understand the underlying pathophysiology associated with SS. Novel therapeutics in SS can be divided in an immunological and a non-immunological group. Examples of immunological targets include molecules that can interfere with the proliferation and activation of B and T cells or affect chemotaxis of lymphocytes, macrophages and dendritic cells. Non-immunological therapies comprise of molecules that enhance fluid secretion, for instance the introduction of water channels like aquaporins (AQP). Similarly, therapies that enhance the sensitivity of salivary and lachrymal glands to neuro-stimulatory signals could be explored as a therapeutic approach to treatment of SS.

Immunological targets

Cytokines: IFNα, IFNγ and IL12

Many pro-inflammatory cytokines are upregulated in SS and blocking one or more of these cytokines may result in reduced inflammation. Some of these cytokines are also involved in secretory dysfunction making them even more interesting candidates for treatment. IFNα and IFNγ together with IL12, are all closely related in function and are all overexpressed in SS patients [49–51]. In addition, the majority of cytokines and transcription factors that are overexpressed in patients are IFN inducible [52–56], a profile that has been referred to as 'the Interferon Signature' [53]. IFNγ is also highly expressed in individuals with sicca symptoms who do not have any histological inflammatory markers in the salivary gland [51]. Moreover, prolonged treatment of the human salivary gland cell line (HSG) with IFNγ in the presence or absence of TNFα leads to a persistent depletion of intracellular Ca^{2+} stores (important for signal transduction leading to fluid secretion), and thus to an exhausted response system [57]. These two observations suggest that IFNs can affect the secretory capacity of the glands. Since IFNγ also reduces the growth of HSG in a concentration dependent way *in vitro* [58, 59], interfering with the interferon system, for instance by introducing a soluble receptor may reduce sicca symptoms, local damage and inflammation in the salivary glands of SS patients.

Chemokines

Chemokines are chemotactic cytokines that are very important in orchestrating mobilizing and, to a lesser degree, regulating homeostasis of a wide range of

hematopoietic cells. The role of chemokines in many autoimmune disease like RA [2, 60, 61] and autoimmune thyroiditis [62] is well established and there is evidence they play an important role in SS as well. Chemokines are important for the homing of T and B cells to the gland and for the survival of malignant B cells [63]. The chemokines CXCL9 and CXCL10 for instance, were found to be significantly upregulated in SS salivary glands, but not in normal salivary glands. These chemokines were predominantly expressed in ductal epithelium in close proximity to lymphoid infiltrates. Moreover high titers of CXCL9 and CXCL10 were produced after patient-derived SG cells were treated with IFNγ *in vitro* [64]. Upregulation of these chemokines is known to attract the IFNγ producing plasmacytoid DC [55], and B cells [65]. Interfering with such a system may be useful in reducing inflammation in the gland by blocking chemotaxis of lymphocytes and dendritic cells, and may also reduce the risk of developing lymphoma.

The co-stimulatory pathway

The co-stimulatory pathway has been shown to play a role in many autoimmune diseases. In studies on RA [66, 67] and psoriasis [68, 69] the principle of blocking co-stimulatory pathways as a treatment has been shown to be safe and effective. In short, to activate B and T cells, simultaneous binding of co-stimulatory molecules is required in addition to the binding of the T or B cell receptor with the peptide-major histocompatibility complex (MHC). In the absence of the co-stimulatory signal, binding of the T or B cell receptor to the peptide-MHC can lead to tolerance and anergy [70]. Co-stimulatory signals are bi-directional and can lead to activation of B cells, upregulation of adhesion molecules, class switching and enhancement of $CD4^+$ and $CD8^+$ effector functions as well as many other pro-immune activities. One of the co-stimulatory pathways known to be involved in SS is the CD40 interaction with CD40 ligand (CD40L/CD154) [12, 64, 71]. In NOD mice, blocking the CD40–CD40L interaction around 4 weeks of age prevents the onset of insulitis and diabetes by inhibiting the development and chemotaxis of pathogenic Th1 cells to the islets of Langerhans in the pancreas [72], the effect on the SS phenotype has not been studied yet. Blocking these pathways in SS may promote tolerance over autoimmunity by reducing auto-reactive T cells, moreover it may affect the activation of B cells and the differentiation of B cells into plasma cells.

Non-immunological targets

Aquaporins

In order to treat the main symptoms of SS, dry mouth and dry eyes, gene therapy could be used to enhance fluid secretion by introducing water channels (aquaporins) into the ducts of the salivary glands. This re-engineering of the salivary gland has been successfully performed in rats, mini pigs and non-human primates for the treatment of radiation-induced xerostomia. In these stud-

ies, vectors encoding water channels were used weeks or months after radiation induced loss of salivary flow. The treatment restored salivary gland activity without any significant side effects [73]. Currently a Phase I trial is ongoing to evaluate the safety of this approach [74]. In this trial, patients with an history of damage of the salivary glands due to head and neck radiation and objective and subjective symptoms of dry mouth receive an adenoviral vector encoding aquaporin (AQP) 1 locally in the parotid gland. The primary outcome of this study is to determine safety of this novel treatment. The secondary outcome will be to measure the effectiveness of gene transfer of AQP1 to increase parotid gland salivary output and improve symptoms associated with irradiation-induced parotid hypofunction. If this proves to be safe, further studies will provide us with more information on the efficacy of the therapy and its applicability in SS.

Neuro-stimulatory pathway

Evidence is accumulating that the function of the muscarinic type 3 receptor (M3R) is impaired in SS [19, 75–78]. It is thought that antibodies blocking the M3R are responsible for the dysfunction of the receptor, leading to dryness of the secretory organs [79]. Enhancing the sensitivity of the receptor by using soluble receptors as decoy for possible auto-antibodies or introducing properly functioning receptors in excess to the gland may overcome the secretory dysfunction. However, at this moment the mechanism responsible for the malfunction of the receptor is not fully understood and further research will be necessary to identify its exact nature.

Future directions and limitations

The most difficult challenge facing the treatment of SS to date is the identification of the proper target(s). Since we understand little of the pathogenesis of SS and as we do not know the autoantigen that is recognized, choosing the correct target is largely empirical. However, by using local delivery of key immunomodulatory proteins or other potential therapeutic molecules directly to the gland in animal models we can begin to identify the critical pathways involved in the inflammatory process and the loss of secretory function.

Acknowledgements

The authors thank Kathrina Quinn for review and discussion of this chapter. NR and JAC are funded by an NIH NIDCR intramural research grant.

References

1 Fox RI (2005) Sjögren's syndrome. *Lancet* 366: 321–331

2 Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, Daniels TE, Fox PC, Fox RI, Kassan SS et al. (2002) Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 61: 554–558

3 Williams PH, Cobb BL, Namjou B, Scofield RH, Sawalha AH, Harley JB (2007) Horizons in Sjögren's syndrome genetics. *Clin Rev Allergy Immunol* 32: 201–209
4 Yamamoto K (2003) Pathogenesis of Sjögren's syndrome. *Autoimmun Rev* 2: 13–18
5 Roescher N, Tak PP, Illei GG (2009) Cytokines in Sjögren's syndrome. *Oral Dis* 15: 519–526
6 Daniels TE, Fox PC (1992) Salivary and oral components of Sjögren's syndrome. *Rheum Dis Clin North Am* 18: 571–589
7 Voulgarelis M, Dafni UG, Isenberg DA, Moutsopoulos HM (1999) Malignant lymphoma in primary Sjögren's syndrome: a multicenter, retrospective, clinical study by the European Concerted Action on Sjögren's Syndrome. *Arthritis Rheum* 42: 1765–1772
8 Tzioufas AG, Voulgarelis M (2007) Update on Sjögren's syndrome autoimmune epithelitis: from classification to increased neoplasias. *Best Pract Res Clin Rheumatol* 21: 989–1010
9 Mariette X (1999) Lymphomas in patients with Sjögren's syndrome: review of the literature and physiopathologic hypothesis. *Leuk Lymphoma* 33: 93–99
10 Melvin JE, Yule D, Shuttleworth T, Begenisich T (2005) Regulation of fluid and electrolyte secretion in salivary gland acinar cells. *Annu Rev Physiol* 67: 445–469
11 Tsunawaki S, Nakamura S, Ohyama Y, Sasaki M, Ikebe-Hiroki A, Hiraki A, Kadena T, Kawamura E, Kumamaru W, Shinohara M et al. (2002) Possible function of salivary gland epithelial cells as nonprofessional antigen-presenting cells in the development of Sjögren's syndrome. *J Rheumatol* 29: 1884–1896
12 Dimitriou ID, Kapsogeorgou EK, Moutsopoulos HM, Manoussakis MN (2002) CD40 on salivary gland epithelial cells: high constitutive expression by cultured cells from Sjögren's syndrome patients indicating their intrinsic activation. *Clin Exp Immunol* 127: 386–392
13 Matsumura R, Umemiya K, Goto T, Nakazawa T, Kagami M, Tomioka H, Tanabe E, Sugiyama T, Sueishi M (2001) Glandular and extraglandular expression of costimulatory molecules in patients with Sjögren's syndrome. *Ann Rheum Dis* 60: 473–482
14 Moutsopoulos HM (1994) Sjögren's syndrome: autoimmune epithelitis. *Clin Immunol Immunopathol* 72: 162–165
15 Humphreys-Beher MG, Brayer J, Yamachika S, Peck AB, Jonsson R (1999) An alternative perspective to the immune response in autoimmune exocrinopathy: induction of functional quiescence rather than destructive autoaggression. *Scand J Immunol* 49: 7–10
16 Fox PC, Speight PM (1996) Current concepts of autoimmune exocrinopathy: immunologic mechanisms in the salivary pathology of Sjögren's syndrome. *Crit Rev Oral Biol Med* 7: 144–158
17 Nakamura T, Matsui M, Uchida K, Futatsugi A, Kusakawa S, Matsumoto N, Nakamura K, Manabe T, Taketo MM, Mikoshiba K (2004) M(3) muscarinic acetylcholine receptor plays a critical role in parasympathetic control of salivation in mice. *J Physiol* 558: 561–575
18 Waterman SA, Gordon TP, Rischmueller M (2000) Inhibitory effects of muscarinic receptor autoantibodies on parasympathetic neurotransmission in Sjögren's syndrome. *Arthritis Rheum* 43: 1647–1654
19 Cha S, Singson E, Cornelius J, Yagna JP, Knot HJ, Peck AB (2006) Muscarinic acetylcholine type-3 receptor desensitization due to chronic exposure to Sjögren's syndrome-associated autoantibodies. *J Rheumatol* 33: 296–306
20 Kovacs L, Marczinovits I, Gyorgy A, Toth GK, Dorgai L, Pal J, Molnar J, Pokorny G (2005) Clinical associations of autoantibodies to human muscarinic acetylcholine receptor 3(213–228) in primary Sjögren's syndrome. *Rheumatology (Oxford)* 44: 1021–1025
21 Dawson LJ, Allison HE, Stanbury J, Fitzgerald D, Smith PM (2004) Putative anti-muscarinic antibodies cannot be detected in patients with primary Sjögren's syndrome using conventional immunological approaches. *Rheumatology (Oxford)* 43: 1488–1495
22 Vivino FB, Al-Hashimi I, Khan Z, LeVeque FG, Salisbury PL 3rd, Tran-Johnson TK, Muscoplat CC, Trivedi M, Goldlust B, Gallagher SC (1999) Pilocarpine tablets for the treatment of dry mouth and dry eye symptoms in patients with Sjögren syndrome: a randomized, placebo-controlled, fixed-dose, multicenter trial. P92-01 Study Group. *Arch Intern Med* 159: 174–181
23 Drosos AA, Skopouli FN, Costopoulos JS, Papadimitriou CS, Moutsopoulos HM (1986) Cyclosporin A (CyA) in primary Sjögren's syndrome: a double blind study. *Ann Rheum Dis* 45: 732–735
24 Skopouli FN, Jagiello P, Tsifetaki N, Moutsopoulos HM (1996) Methotrexate in primary Sjögren's syndrome. *Clin Exp Rheumatol* 14: 555–558
25 Zandbelt MM, van den Hoogen FH, de Wilde PC, van den Berg PJ, Schneider HG, van de Putte LB (2001) Reversibility of histological and immunohistological abnormalities in sublabial sali-

vary gland biopsy specimens following treatment with corticosteroids in Sjögren's syndrome. *Ann Rheum Dis* 60: 511–513
26 Fox PC, Datiles M, Atkinson JC, Macynski AA, Scott J, Fletcher D, Valdez IH, Kurrasch RH, Delapenha R, Jackson W (1993) Prednisone and piroxicam for treatment of primary Sjögren's syndrome. *Clin Exp Rheumatol* 11: 149–156
27 Tracey D, Klareskog L, Sasso EH, Salfeld JG, Tak PP (2008) Tumor necrosis factor antagonist mechanisms of action: a comprehensive review. *Pharmacol Ther* 117: 244–279
28 Mariette X, Ravaud P, Steinfeld S, Baron G, Goetz J, Hachulla E, Combe B, Puechal X, Pennec Y, Sauvezie B et al. (2004) Inefficacy of infliximab in primary Sjögren's syndrome: results of the randomized, controlled Trial of Remicade in Primary Sjögren's Syndrome (TRIPSS). *Arthritis Rheum* 50: 1270–1276
29 Niedermeyer J, Hoffmeyer F, Hertenstein B, Hoeper MM, Fabel H (2000) Treatment of lymphoproliferative disease with rituximab. *Lancet* 355: 499
30 Edwards JC, Szczepanski L, Szechinski J, Filipowicz-Sosnowska A, Emery P, Close DR, Stevens RM, Shaw T (2004) Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N Engl J Med* 350: 2572–2581
31 Arkfeld DG (2008) The potential utility of B cell-directed biologic therapy in autoimmune diseases. *Rheumatol Int* 28: 205–215
32 Pijpe J, van Imhoff GW, Spijkervet FK, Roodenburg JL, Wolbink GJ, Mansour K, Vissink A, Kallenberg CG, Bootsma H (2005) Rituximab treatment in patients with primary Sjögren's syndrome: an open-label phase II study. *Arthritis Rheum* 52: 2740–2750
33 Meijer J, Vissink A, Meiners P, Spijkervet F, Kallenberg C, Bootsma H (2008 ACR/ARHP Scientific meeting) Rituximab treatment in primary Sjögren's syndrome; a double-blind placebo controlled trial, San Francisco, CA, USA
34 Dass S, Bowman SJ, Vital EM, Ikeda K, Pease CT, Hamburger J, Richards A, Rauz S, Emery P (2008) Reduction of fatigue in Sjögren syndrome with rituximab: results of a randomised, double-blind, placebo-controlled pilot study. *Ann Rheum Dis* 67: 1541–1544
35 Isaksen K, Jonsson R, Omdal R (2008) Anti-CD20 treatment in primary Sjögren's syndrome. *Scand J Immunol* 68: 554–564
36 Moutsopoulos NM, Katsifis GE, Angelov N, Leakan RA, Sankar V, Pillemer S, Wahl SM (2008) Lack of efficacy of etanercept in Sjögren syndrome correlates with failed suppression of tumour necrosis factor alpha and systemic immune activation. *Ann Rheum Dis* 67: 1437–1443
37 Mavragani CP, Moutsopoulos HM (2007) Conventional therapy of Sjögren's syndrome. *Clin Rev Allergy Immunol* 32: 284–291
38 Giarratana N, Penna G, Adorini L (2007) Animal models of spontaneous autoimmune disease: type 1 diabetes in the nonobese diabetic mouse. *Methods Mol Biol* 380: 285–311
39 Robinson CP, Yamachika S, Bounous DI, Brayer J, Jonsson R, Holmdahl R, Peck AB, Humphreys-Beher MG (1998) A novel NOD-derived murine model of primary Sjögren's syndrome. *Arthritis Rheum* 41: 150–156
40 Lodde BM, Mineshiba F, Kok MR, Wang J, Zheng C, Schmidt M, Cotrim AP, Kriete M, Tak PP, Baum BJ (2006) NOD mouse model for Sjögren's syndrome: lack of longitudinal stability. *Oral Dis* 12: 566–572
41 Rott O, Fleischer B, Cash E (1994) Interleukin-10 prevents experimental allergic encephalomyelitis in rats. *Eur J Immunol* 24: 1434–1440
42 Bai XF, Zhu J, Zhang GX, Kaponides G, Hojeberg B, van der Meide PH, Link H (1997) IL-10 suppresses experimental autoimmune neuritis and down-regulates TH1-type immune responses. *Clin Immunol Immunopathol* 83: 117–126
43 Kok MR, Yamano S, Lodde BM, Wang J, Couwenhoven RI, Yakar S, Voutetakis A, Leroith D, Schmidt M, Afione S et al. (2003) Local adeno-associated virus-mediated interleukin 10 gene transfer has disease-modifying effects in a murine model of Sjögren's syndrome. *Hum Gene Ther* 14: 1605–1618
44 Gonzalez-Rey E, Delgado M (2007) Vasoactive intestinal peptide and regulatory T-cell induction: a new mechanism and therapeutic potential for immune homeostasis. *Trends Mol Med* 13: 241–251
45 Lodde BM, Mineshiba F, Wang J, Cotrim AP, Afione S, Tak PP, Baum BJ (2006) Effect of human vasoactive intestinal peptide gene transfer in a murine model of Sjögren's syndrome. *Ann Rheum Dis* 65: 195–200
46 Vosters JYH, Roescher N, Kok M, Tak P, Chiorini J (2009) Local expression of TNFR1:IgG can

induce salivary gland dysfunction in a murine model of Sjögren's Syndrome. *Arthritis Res Ther* 11: R189
47 Nedjai B, Hitman GA, Quillinan N, Coughlan RJ, Church L, McDermott MF, Turner MD (2009) Proinflammatory action of the antiinflammatory drug infliximab in tumor necrosis factor receptor-associated periodic syndrome. *Arthritis Rheum* 60: 619–625
48 Nguyen CQ, Cornelius JG, Cooper L, Neff J, Tao J, Lee BH, Peck AB (2008) Identification of possible candidate genes regulating Sjögren's syndrome-associated autoimmunity: a potential role for TNFSF4 in autoimmune exocrinopathy. *Arthritis Res Ther* 10: R137
49 Sakai A, Sugawara Y, Kuroishi T, Sasano T, Sugawara S (2008) Identification of IL-18 and Th17 cells in salivary glands of patients with Sjögren's syndrome, and amplification of IL-17-mediated secretion of inflammatory cytokines from salivary gland cells by IL-18. *J Immunol* 181: 2898–2906
50 Oxholm P, Daniels TE, Bendtzen K (1992) Cytokine expression in labial salivary glands from patients with primary Sjögren's syndrome. *Autoimmunity* 12: 185–191
51 van Woerkom JM, Kruize AA, Wenting-van Wijk MJ, Knol E, Bihari IC, Jacobs JW, Bijlsma JW, Lafeber FP, van Roon JA (2005) Salivary gland and peripheral blood T helper 1 and 2 cell activity in Sjögren's syndrome compared with non-Sjögren's sicca syndrome. *Ann Rheum Dis* 64: 1474–1479
52 Miceli-Richard C, Comets E, Loiseau P, Puechal X, Hachulla E, Mariette X (2007) Association of an IRF5 gene functional polymorphism with Sjögren's syndrome. *Arthritis Rheum* 56: 3989–3994
53 Bave U, Nordmark G, Lovgren T, Ronnelid J, Cajander S, Eloranta ML, Alm GV, Ronnblom L (2005) Activation of the type I interferon system in primary Sjögren's syndrome: a possible etiopathogenic mechanism. *Arthritis Rheum* 52: 1185–1195
54 Hjelmervik TO, Petersen K, Jonassen I, Jonsson R, Bolstad AI (2005) Gene expression profiling of minor salivary glands clearly distinguishes primary Sjögren's syndrome patients from healthy control subjects. *Arthritis Rheum* 52: 1534–1544
55 Gottenberg JE, Cagnard N, Lucchesi C, Letourneur F, Mistou S, Lazure T, Jacques S, Ba N, Ittah M, Lepajolec C et al. (2006) Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren's syndrome. *Proc Natl Acad Sci USA* 103: 2770–2775
56 Wildenberg ME, van Helden-Meeuwsen CG, van de Merwe JP, Drexhage HA, Versnel MA (2008) Systemic increase in type I interferon activity in Sjögren's syndrome: a putative role for plasmacytoid dendritic cells. *Eur J Immunol* 38: 2024–2033
57 Wu AJ, Chen ZJ, Baum BJ, Ambudkar IS (1996) Interferon-gamma induces persistent depletion of internal Ca^{2+} stores in a human salivary gland cell line. *Am J Physiol* 270: C514–521
58 Daniels PJ, Gustafson SA, French D, Wang Y, DePond W, McArthur CP (2000) Interferon-mediated block in cell cycle and altered integrin expression in a differentiated salivary gland cell line (HSG) cultured on Matrigel. *J Interferon Cytokine Res* 20: 1101–1109
59 Wu AJ, Kurrasch RH, Katz J, Fox PC, Baum BJ, Atkinson JC (1994) Effect of tumor necrosis factor-alpha and interferon-gamma on the growth of a human salivary gland cell line. *J Cell Physiol* 161: 217–226
60 Szekanecz Z, Strieter RM, Kunkel SL, Koch AE (1998) Chemokines in rheumatoid arthritis. *Springer Semin Immunopathol* 20: 115–132
61 Vergunst CE, van de Sande MG, Lebre MC, Tak PP (2005) The role of chemokines in rheumatoid arthritis and osteoarthritis. *Scand J Rheumatol* 34: 415–425
62 Kimura H, Caturegli P (2007) Chemokine orchestration of autoimmune thyroiditis. *Thyroid* 17: 1005–1011
63 Barone F, Bombardieri M, Rosado MM, Morgan PR, Challacombe SJ, De Vita S, Carsetti R, Spencer J, Valesini G, Pitzalis C (2008) CXCL13, CCL21, and CXCL12 Expression in Salivary Glands of Patients with Sjögren's Syndrome and MALT Lymphoma: Association with Reactive and Malignant Areas of Lymphoid Organization. *J Immunol* 180: 5130–5140
64 Saito M, Ota Y, Ohashi H, Dei Y, Shimoyama K, Suzuki D, Hayashi H, Ogawa N (2007) CD40–CD40 ligand signal induces the intercellular adhesion molecule-1 expression through nuclear factor-kappa B p50 in cultured salivary gland epithelial cells from patients with Sjögren's syndrome. *Mod Rheumatol* 17: 45–53
65 Vanbervliet B, Bendriss-Vermare N, Massacrier C, Homey B, de Bouteiller O, Briere F, Trinchieri G, Caux C (2003) The inducible CXCR3 ligands control plasmacytoid dendritic cell responsiveness to the constitutive chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12. *J Exp Med* 198: 823–830

66 Schiff M, Keiserman M, Codding C, Songcharoen S, Berman A, Nayiager S, Saldate C, Li T, Aranda R, Becker JC et al. (2008) Efficacy and safety of abatacept or infliximab vs placebo in ATTEST: a phase III, multi-centre, randomised, double-blind, placebo-controlled study in patients with rheumatoid arthritis and an inadequate response to methotrexate. *Ann Rheum Dis* 67: 1096–1103
67 Kremer JM, Genant HK, Moreland LW, Russell AS, Emery P, Abud-Mendoza C, Szechinski J, Li T, Teng J, Becker JC et al. (2008) Results of a two-year follow up study of patients with rheumatoid arthritis who received a combination of abatacept and methotrexate. *Arthritis Rheum* 58: 953–963
68 Leonardi C, Menter A, Hamilton T, Caro I, Xing B, Gottlieb AB (2008) Efalizumab: results of a 3-year continuous dosing study for the long-term control of psoriasis. *Br J Dermatol* 158: 1107–1116
69 Gupta AK, Cherman AM (2006) Efalizumab in the treatment of psoriasis. *J Cutan Med Surg* 10: 57–68
70 Sharpe A (2005) Costimulation and regulation of autoimmunity and tolerance. *J Pediatr Gastroenterol Nutr* 40 Suppl 1: S20–21
71 Turkcapar N, Sak SD, Saatci M, Duman M, Olmez U (2005) Vasculitis and expression of vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin in salivary glands of patients with Sjögren's syndrome. *J Rheumatol* 32: 1063–1070
72 Balasa B, Krahl T, Patstone G, Lee J, Tisch R, McDevitt HO, Sarvetnick N (1997) CD40 ligand-CD40 interactions are necessary for the initiation of insulitis and diabetes in nonobese diabetic mice. *J Immunol* 159: 4620–4627
73 Shan Z, Li J, Zheng C, Liu X, Fan Z, Zhang C, Goldsmith CM, Wellner RB, Baum BJ, Wang S (2005) Increased fluid secretion after adenoviral-mediated transfer of the human aquaporin-1 cDNA to irradiated miniature pig parotid glands. *Mol Ther* 11: 444–451
74 Effect of AdhAQP1 on salivary flow in patients treated with radiation for head and neck cancer (2009) Clinicaltrials.gov Identifier: NCT00372320
75 Cavill D, Waterman SA, Gordon TP (2004) Antibodies raised against the second extracellular loop of the human muscarinic M3 receptor mimic functional autoantibodies in Sjögren's syndrome. *Scand J Immunol* 59: 261–266
76 Koo NY, Li J, Hwang SM, Choi SY, Lee SJ, Oh SB, Kim JS, Lee EB, Song YW, Park K (2008) Functional epitope of muscarinic type 3 receptor which interacts with autoantibodies from Sjögren's syndrome patients. *Rheumatology (Oxford)* 47: 828–833
77 Schegg V, Vogel M, Didichenko S, Stadler MB, Beleznay Z, Gadola S, Sengupta C, Stadler BM, Miescher SM (2008) Evidence that anti-muscarinic antibodies in Sjögren's syndrome recognise both M3R and M1R. *Biologicals* 36: 213–222
78 Gao J, Cha S, Jonsson R, Opalko J, Peck AB (2004) Detection of anti-type 3 muscarinic acetylcholine receptor autoantibodies in the sera of Sjögren's syndrome patients by use of a transfected cell line assay. *Arthritis Rheum* 50: 2615–2621
79 Dawson L, Tobin A, Smith P, Gordon T (2005) Antimuscarinic antibodies in Sjögren's syndrome: where are we, and where are we going? *Arthritis Rheum* 52: 2984–2995

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Disease mechanisms, genetic susceptibility and therapeutic approaches in lupus disease

Rizgar A. Mageed[1], Taher E. Taher[1] and Ali S. Jawad[2]

[1] *Bone and Joint Research Unit, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, Queen Mary University of London, UK*
[2] *Department of Rheumatology, The Royal London Hospital, London, UK*

Abstract

Lupus is a prototype autoimmune disease with unknown etiology. The disease is complex; presented with diverse clinical manifestations caused by different disease mechanisms. The complex nature of lupus immunopathology has provided a fertile field to investigate disease mechanisms and explore new approaches for therapy. However, it is also this complexity that is hampering efforts to identify therapies that benefit most patients. In this chapter, we will briefly allude to some of the known immunological defects encountered in lupus and summarise recent advance in identifying new susceptibility genetic factors. We will also provide an overview of biological agents used for treatment of patient groups and new directions for gene therapy.

Introduction

Lupus is an autoimmune disease with diverse clinical manifestations and disease processes. The trigger for lupus autoimmunity remains unclear but there is evidence from murine models and human patients that susceptibility involves multiple factors; genetics, environmental, and hormonal. These factors cooperate to modify complex relations between the host, pathogens, and the environment. The disease mainly afflicts females during childbearing age with a prevalence of 0.4–2 in 1000 individuals in the population. The incidence of lupus is different in different ethnic groups, with Asian and Afro-Caribbean females in Western Europe and North America at higher risk than Caucasians.

Virtually all lupus patients manifest joint and/or skin involvement (reviewed in [1]). In addition, the kidneys, heart, lungs, and the central nervous system (CNS) are affected in about 30–70% of patients. About 90% of SLE patients experience generalised arthralgia, but with milder inflammation than patients with rheumatoid arthritis (RA). 60% of SLE patients complain of myalgia and muscle weakness and the kidneys are involved in 20–50% of patients. CNS involvement can affect up to 50% of patients, where involvement can range from migraine to grand mal seizures. Lymphocytopenia is common and although thrombocytopenia is uncommon, it is a well-recognised complication of lupus. Generally, when SLE involves many organs the prognosis is poor.

The cause of lupus and, indeed, the trigger of the characteristic immune defects remain unclear. However, the involvement of genetic factors is strong (reviewed in [2]). This is supported by early studies of twins with SLE that showed that the concordance rate is about 24–57% in monozygotic twins, but only about 3% in heterozygotic twins [3, 4]. Susceptibility genes include major histocompatibility complex (MHC) and non-MHC genes. Amongst the non-MHC loci are genes that regulate lymphocyte tolerance and responses and genes controlling the pattern of cytokine production [2]. Few possible exceptions to the involvement of multiple genes in lupus, perhaps, is seen in patients with homozygous deficiency in the early components of the complement system C1q, C2 and C4, FcγRIIIb deficiency or mutations in the DNA exonuclease TREX1 [5–7]. Thus, all individuals who have homozygous *C1q* deficiency develop lupus and half of those with homozygous deficiency of *C2* develop lupus-like diseases [5]. However, patients with complement deficiency comprise less than 5% of patients with lupus. In addition to complement genes, genes encoding co-receptors on B cells and macrophages, such as IgG Fc receptor IIA (FcγRIIA) and FcγRIIIA, and cytokines have been associated with susceptibility to lupus both in humans and murine models [5]. The importance of these findings exceeds the implication for understanding disease mechanisms into helping in designing and applying therapeutic strategies.

Immunological abnormalities in lupus

Studies from great many laboratories over many decades have noted that most compartments of the immune system have one or more abnormalities. It remains unclear, however, which immunological abnormalities have a causal relationship with lupus and which are secondarily to the disease process. For example, the production of anti-lymphocyte autoantibodies (auto-Abs) in human patients is thought to be secondary to the breakdown of tolerance. But these auto-Abs, in turn, have their effects in further undermining immune regulation by perturbing immune homeostasis.

Defective T lymphocyte responses

The most prominent immune abnormality in patients and in spontaneous animal models is lymphocyte hyper responsiveness and the production of auto-Abs. The IgG isotype of these auto-Abs, high avidity and accumulation of somatic mutations in the Ig variable (IgV) region genes are evidence for the involvement of T lymphocytes. However, there was no direct evidence until recently for autoreactive T lymphocytes driving the autoimmune response in lupus patients. Instead there has been evidence for defective immune regulation T lymphocyte regulation of immune responses. For example, lupus T lymphocytes have reduced suppressive properties such as reduced ability to produce

IL-2 and reduced ability to express CTLA-4 and defects in regulatory T cells [1]. There also has been evidence for reduced numbers and defective functions in T lymphocytes, particularly in $CD8^+$ T cells and in regulatory T cells (Tregs) in both human patients and murine models. Recently, however, T cells from mutant mice with a homozygous allele for a RING-type ubiquitin ligase (Roquin) were shown to directly induce auto-Abs production and cause lupus-like disease by promoting germinal centre formation and autoreactive B cells expansion [8].

There also is evidence for functional defects in T cells such as spontaneous activation with some evidence for intrinsic defects [9]. In addition, there is evidence for altered production of key cytokines by T cells in lupus, such as IL-2 and TGFβ. Reduced production of IL-2 has been attributed to the transcriptional inhibitor of IL-2 gene promoter, phosphorylated cAMP response element modifier (pCREM) [10] while reduced TGFβ levels have been attributed to reduced proliferative responses to CD2 engagement [11]. There is also evidence for defects in T lymphocyte signalling. For example, there is deficiency in the expression of the zeta chain of the CD3 complex and defects in calcium response to anti-CD3/CD28 and mitogen activation [12]. Activation of the transcription factor nuclear factor-kappa B (NF-κB) is also abnormal in patients with lupus [13]. Other abnormally expressed intracellular molecules include protein kinase C (PKC) and CD45. Further, a defect involving Cbl, an adaptor protein that negatively regulates transmembrane signalling, has been demonstrated. This abnormality may cause over expression of CD40 ligand (CD40L) and resistance to tolerance induction [14].

Defective B lymphocyte responses

Abnormalities within the B cell compartment have long been considered a characteristic feature of lupus. Initially, these considerations were based on the association of lupus with auto-Abs, but recent studies from congenic and gene knockout mice have indicated that many of the characteristic features of lupus immunopathology could be initiated by aberrant B cells regulation. However, it remains unclear whether B cell abnormalities in lupus patients are primary defects, or whether these are caused by defects in other compartments, such as T lymphocytes. Intriguingly, however, B cell responses to exogenous antigens have been shown to be either decreased or normal in lupus patients (reviewed in [1]). Studies in recent years have implicated defects in tolerance checkpoints during B cell development in patients with SLE [15]. These findings are, up to a point, consistent with evidence for primary defects in B cells in spontaneous murine models of lupus [16]. Furthermore, lupus-prone mice genetically modified to be deficient in $CD4^+$ cells still produce pathogenic autoantibodies. Genetic studies in congenic mice and gene knockout mice provide further evidence for a primary role for B lymphocytes as an independent contributor to the disease, apart from the production of auto-Abs. For exam-

ple, analyses of the congenic lupus-prone NZM2410 mouse strain, a sub-strain of the BWF1 mouse, have identified two major genetic susceptibility loci, *sle1* and *sle2* that relate to intrinsic B lymphocyte defects that result in the breakdown of tolerance to chromatin and IgM auto-Ab production [17].

In human patients with lupus, B lymphocytes are hyper-responsive and produce excess amounts of IL-10, which play an important role in lupus pathology [18]. The available evidence, however, indicates that whereas B cells hyperresponsiveness in lupus patients is disease-dependent, excess IL-10 production is disease-independent. These findings indicate that intrinsic defects underlie excess IL-10 production by B cells in lupus. In addition to altered cytokine production, B lymphocytes from both patients and spontaneous murine models manifest phenotypic changes such as aberrant expression of CD40L, CD80, and CD86 [19]. In addition, B lymphocytes from lupus patients spontaneously produce large amounts of immunoglobulins (Igs) when cultured *in vitro*. In contrast, lupus B lymphocytes are markedly deficient in their ability to proliferate further or increase Ig production in response to stimulation [1].

B lymphocytes from human patients with lupus also show evidence for intracellular signalling abnormalities. For example, stimulation through the B cell receptor (BCR) results in significantly higher Ca^{+2} mobilisation and protein tyrosine phosphorylation compared with patients with other systemic rheumatic diseases and healthy controls (reviewed in [1]). In addition, B cells in patients with lupus produce higher levels of inositol 1,4,5-triphosphate (IP3), but have reduced levels of negative regulators of BCR signaling Lyn, FcγRIIb and CD22 [20].

Defective removal of apoptotic bodies

In addition to defects in B and T lymphocytes, there are other immunological defects in lupus. For example, both patients and spontaneous murine models exhibit defective clearance of apoptotic bodies and immune complexes. The defects are complex in nature and causes. However, the role of the Fcγ receptors and CR1 in clearing immune complexes and apoptotic bodies has been identified, revealing that alleles of FcγRIIa that do not bind IgG well predispose to lupus [21]. In addition, particular CR1 alleles (discussed below) are susceptibility variants for lupus. Genetic polymorphisms in these receptors have generally been associated with defective removal of immune complexes. However, there is also evidence that the cross-linking of FcγR on B lymphocytes, which acts as a negative feedback mechanism, is abnormal in lupus [18].

Defects in the production and response to cytokines

One of the most noted defects in lupus, relevant both to understanding disease mechanisms and designing therapeutic strategies, has been dysregulated pro-

duction of and response to cytokines. For example, there is evidence for dysregulated production and response to IL-2, 4, 6, 10, IL-12, interferon alpha (IFNα), IFNγ, tumor necrosis factor alpha (TNFα) [1, 22]. Reduced IL-2 production by T cells was first identified in murine lupus [23] and, subsequently, in patients with lupus and attributed to impaired TCR signalling [24]. In addition, patients with lupus show a decreased response to IL-2 due to reduced expression of the β chain of the receptor [25].

One of the most consistent abnormalities in the cytokine network, both in patients and murine models is high expression levels of IFNγ [26] and IFNα [22]. IFNα has attracted much attention in recent years for its key role in promoting pro-inflammatory plasmacytoid dendritic cells [27]. The production of high levels of IFNα in lupus patients is mediated by immune complexes found in the blood of patients with lupus [22]. Excess IFNγ production has been associated with lupus and is thought that this cytokine exacerbates or even precipitates the disease through upregulation of MHC-II expression and promoting complement fixing auto-Abs [28]. The importance of IFNγ in lupus pathogenesis is supported by the demonstration that treatment of the spontaneous New Zealand black white F1 (BWF1) mouse model with anti- IFNγ antibody or soluble IFNγ receptor (IFNγR), during early life, delays disease progression [1].

There are contradictory results between mice and patients on the role of IL-4. Whereas levels of IL-4 are reduced in the lupus-prone MRL *lpr* and BWF1 mice, the level of IL-4 protein and mRNA is reported to be increased in patients [29]. Interestingly, nephritis was completely abrogated in lupus mice rendered transgenic for the IL-4 gene under the control of the Ig heavy chain (IgH) enhancer [30].

Increased levels of IL-6 also have been demonstrated in patients with active lupus [1]. However, the role of excess IL-6 in lupus has been somewhat unclear due to a lack of an association between high IL-6 levels and auto-Ab production or disease activity. Recently, however, evidence has emerged that elevated IL-6 levels can promote lupus immunopathology by promoting demethylation of genes encoding key regulatory proteins [31]. The cause of excess IL-6 production in lupus patients, however, is unknown, although there are suggestions that it could be the consequence of response to apoptosis [32].

The production of IL-10, important in lupus immunopathology, is increased in lupus patients and murine models. In addition to the role of IL-10 in B cell stimulation, IL-10 attenuates macrophage and antigen presenting cell activation, cytokine production and has both inhibitory and stimulatory effects on T-lymphocytes. The increase in IL-10 in lupus has been correlated with disease activity and with anti-DNA auto-Ab production [33].

Altered expression of IL-12 has also been seen in lupus although the data on this cytokine is inconsistent [1]. In BWF1 lupus mice, an intrinsic defect in the production of IL-12 has been reported [34]. However, peritoneal macrophages from MRL *lpr* mice produce high levels of IL-12 following stimulation with IFNγ and/or lipopolysaccharide (LPS) [35]. There are no consistent data

on the level of IL-12 in patients and the role of this cytokine in lupus remains unclear.

The production of TNFα, IL-1 and TGFβ also has been reported to be altered in lupus. TNFα production by macrophages from BWF1 lupus mice stimulated with LPS is reduced compared with non-autoimmune mice and repeated injections of recombinant TNFα delayed proteinuria and death in these mice [36]. In human patients, stimulated blood mononuclear cells from DQw1 and $DR2^+$ patients produce lower amounts of TNFα than DR3 and DR4 positive individuals. Interestingly, however, there has been a report to suggest that blockade of TNFα in some patients with lupus could be beneficial [37]. Increased production of IL-1 by monocytes from patients and murine models has also been reported [1]. The production of TGFβ, in both latent and active forms is decreased in patients with SLE and is associated with disease activity [38].

Genetic factors in lupus susceptibility

Susceptibility to develop lupus involves a strong genetic element. Early hypothesis-driven studies of candidate genes in patients with lupus were based on studies in murine models. These always involved genes with a strong genotype to phenotype relationship. Although the contribution of many of these genes in case control studies was confirmed, recent genome-wide linkage analyses (GWA) have identified important new genes, some with unclear functional implications for lupus. Two recent GWA identified nine lupus linkage regions [39, 40]. Interestingly, the analyses indicated that the effects arising from particular loci contribute to specific clinical or immunological phenotypes such as involvement of particular tissues, organs or the production of auto-Abs [2]. The model that has emerged is consistent with previous predictions in that susceptibility to lupus is genetic variants at multiple loci. This model also predicts that disease onset is triggered in susceptible individual by multiple environmental factors occurring either simultaneously, or sequentially. The nature of these environmental factors remains largely unknown. However, there is evidence that Epstein-Barr virus (EBV) could be one of the environmental factors in lupus [41].

MHC genes and variants on chromosome 6

The two GWA cited above involved DNA from over 2,000 patients with lupus and the genotyping of 300,000 and 500,000 single nucleotides, respectively [39, 40]. As predicted from previous case control studies, loci within the MHC region on chromosome 6 were strongly associated with lupus. Because of the strong linkage disequilibrium between genetic markers in this region suggesting that highly correlated variants involving multiple genes are inherited togeth-

er in single blocks it has been difficult to conclude whether lupus-associated variants on chromosome 6 are functional or simply reflect linkage disequilibrium with functional variants elsewhere. The analyses, nevertheless, provided evidence for the association of lupus with markers in both Class II and III regions [39]. Associated variants from the Class II region involved HLA-DRB1, HLA-DQA1 and HLA-DQB1 only. Markers in Class III region included the *SKIV2L* [superkiller viralicidic activity 2-like] gene and genes encoding the early complement components C2 and C4 [40]. In this respect, individuals carrying no or few copy numbers of the *C4A* gene that encode a C4 protein with better ability to bind immune complex than the *C4B* encoded protein are at a higher risk of developing lupus [42].

Variants for adhesion molecules and membrane co-receptors

There was a strong association between lupus susceptibility and variants of the *ITGAM* gene. This gene encodes the integrin α_M that forms a dimer with β2 integrin to form complement receptor 3 (CR3) or Mac-1, which is expressed on neutrophils, macrophages and dendritic cells. CR3 can bind a variety of ligands including intercellular adhesion molecule 1 (ICAM-1) and the C3 fragment C3bi. The CR3 variant associated with lupus was suggested to influence leucocyte trafficking mediated via ICAM-1 and/or uptake of apoptotic bodies, or immune complexes. Variants encoding the Fcγ receptor FcγRIIa, RIIb and IIIa were also associated with lupus. FcγRIIa is expressed on neutrophils, monocytes, macrophages, dendritic cells and platelets and the lupus variant encodes a protein with a lower binding affinity for IgG2 leading to impaired phagocytosis of IgG2 containing immune complexes [21]. A lupus-associated variant encoding FcγRIIb alters level of expression of the protein and regulation of B cell activation. In contrast, a mutation in the gene encoding FcγRIIIa results in reduced binding affinity of IgG to the receptor.

A variant of the gene encoding OX40 ligand (OX40L) has also been associated with lupus in a case control study [43]. OX40L is a member of the TNF super family of ligands and receptors (known as TNF super-family 4 or TNFSF4). It is a membrane-bound protein expressed on the surface of B lymphocytes and antigen presenting cells (APCs). It binds OX40 on helper T cells and provides an activating signal for both T cells and B cells. In T cells, OX-40-OX40L interaction leads to T cell activation and memory generation and promotes TH2-type cytokine production [44]. In addition, there is evidence that the interaction down regulates IL-10 production by regulatory T cells [45]. In B cells, OX40-OX40L interaction differentiation to plasma cells [46]. Both protective and risk *TNFSF4* variants have been identified and *in vitro* studies suggest that the risk variant is associated with increased OX40L transcript and membrane expression [43].

Variants of cytokine associated transcription factors

The involvement of interferon alpha (IFNα) in lupus immunopathology has attracted much interest in the last 5 years. Thus, IFNα has been shown to be induced by immune complex in the serum of patients with lupus, to be increased in the serum of patients and also to promote autoimmunity by inducing plasmacytoid dendritic cells [27]. In addition, there has been evidence for the upregulation of IFN-inducible genes in lupus patients [47]. The GWA revealed that IFN regulatory factor 5 gene (*IRF5*) is an important susceptibility gene in lupus. IRF5 is a transcription factor responsible for mediating expression of many genes. One model for the role of *IRF5* as a lupus risk factor proposes that mutations in the gene result in altered isoform expression which influences the effect of interferons in response to viral infections. These could include the induction of Th1-type responses and lowering the threshold for B cell activation through the BCR and promoting survival and differentiation [48]. Thus, genetic variants of IRF5 could help prolong proinflammatory responses and break self tolerance. Alternatively, altered IRF5 expression could influence cell cycle regulation and apoptosis which are key event in lupus pathogenesis [49].

Genes encoding signalling proteins

Lupus is associated with defects in intracellular signalling in immune cells. As cited earlier, there is evidence for abnormal intracellular signalling in both T and B lymphocytes. Case control and GWA have provided confirmation for such association. The B lymphoid tyrosine kinase (BLK), LYN and the B cell scaffold protein with ankyrin repeats (BANK1) were shown to be candidate genes for susceptibility to lupus. The existing functional data suggest that the risk alleles are associated with reduced expression. All three proteins play key roles in the regulation of BCR-mediated signalling. The best studied of these molecules is Lyn. In $Lyn^{-/-}$ mice B cells are hyperactive, produce IgM anti-DNA auto-Abs and the mice develop lupus nephritis [50]. There is also evidence that Lyn is reduced in B cells from patients with lupus [51]. Lyn plays a key role in mediating B cell signalling through the phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) on Igα/β. However, Lyn is also involved in negative regulation of BCR signalling through mediating the activation of inhibitory molecules such as CD22, SHP-1 and FcγRIIb [50].

There GWA also showed an association between signal transducer and activator of transcription 4 (*STAT4*) and lupus although no functional candidate polymorphism is yet identified. STAT4 is a transcription factor that mediates the expression of genes in response to signals by a number of important cytokines such as IFNα, IL-12 and 23 [52]. STAT4 activation by these cytokines leads to the induction of Th1 responses including IFNγ production which is strongly associated with lupus [53].

Genes for proteins with incompletely defined functions

In addition to the above gene variants that associate with lupus risk and whose function is relatively well-understood, there are a number of other genes whose functional association with the disease is less well characterised. For example, a gene that encodes a protein involved in DNA methylation, *MECP2* is a candidate risk factor. MECP2 protein is involved in turning off several other genes. This prevents the genes from making proteins when they are needed. Altered gene methylation has been reported in SLE and this could be a potentially important mechanism for over/under expression of cytokines and other proteins involved in lupus pathogenesis. Recently, we reported that expression of membrane CD5, which negatively regulates BCR and TCR signalling, is reduced in lupus B cells because of hypomethylation of genomic DNA [31]. In addition, *MECP2* is on the X-chromosome, which is of interest given that lupus is predominantly a disease of females.

The ubiquitin-carrier enzyme gene *UBE2L3* has also been identified as an SLE risk gene. There is little functional studies on *UBE2L3*, but the protein is widely expressed and has a role in the maturation of transcription factors such as the NF-κB precursor p105 [54]. Interestingly, although there is no direct evidence for the functional implication for this polymorphism, our studies have revealed increased ubiquitination and degradation of key regulatory signalling molecules in B and T lymphocytes in patients with SLE [51, 55]. Interestingly, in this respect, there is evidence that an autophagy gene, *ATG5* is also a candidate risk gene for lupus. Autophagy is a regulatory process by which intracellular proteins with long half-lives and organelles are degraded, but excessive autophagy activity can trigger apoptosis. This process is controlled by the autophagy genes that act in an ubiquitin-like conjugation system. ATG5 has been suggest to act as the switch that determines whether autophagy progresses to apoptosis, and whether overexpression may result in accelerated apoptosis as a potential disease process for lupus [56].

Biological agents for the treatment of lupus

The clinical management of lupus has improved significantly over the last decade. Thus, mortality rates have declined and organ damage is better controlled. This progress has been achieved as a result of the development and use of new generations of immunosuppressive agents. Despite this progress, however, organ damage, especially kidney damage and mortality remain unacceptably high. In addition, traditional immunosuppressive agents only provide symptomatic relief and have serious side effects. Therefore, there is a continuing need to develop therapies that target key immunological pathways in lupus and with fewer side effects. The development of biological agents, especially those that target B cells, are beginning to make an impact on lupus management. However, the heterogeneous nature of lupus and diverse disease

processes involved mean that we are a long way from identifying an ideal therapeutic strategy.

Biological agents targeting B lymphocytes

B lymphocytes are key players in lupus pathology and a number of new agents have been developed to target B cells. B cell ablation with mAbs has been in use for about 15 years mainly to treat patients with non-Hodgkin's lymphomas. The successful application and safe record of a mAb with specificity for CD20 (Rituximab) in the treatment of B cell lymphomas prompted its use in variety of autoimmune diseases including lupus (reviewed in [57]). Rituximab is a genetically engineered mAb with mouse variable regions and human IgG Fc. CD20 is a non-glycosylated membrane protein expressed exclusively on B lymphocyte throughout their development until the cells differentiate to plasma cell, whereupon CD20 expression is lost. CD20 is involved in B cell growth and differentiation although no know natural ligand has yet been identified. CD20 is neither cleaved nor endocytosed, thus, providing a stable target for therapy. B cells depletion with Rituximab is achieved through complement-mediated lysis, Ab-dependent cellular cytotoxicity and apoptosis. In open label studies of patients with lupus refractory to standard immunotherapy, the use of rituximab has proved to be highly successful when given in two separate administrations two weeks apart and in combination with cyclophosphamide and high doses of steroids [58]. The majority of patients improved for periods of 3–6 months or longer. There also were significant improvements to the kidneys in patients with nephritis, thus allowing the dose of steroids to be reduced. Interestingly, however, there was no immediate decrease in serum auto-Ab levels.

Targeting of B cell for treatment has also been performed using a mAb with specificity for CD22 (epratuzumab). CD22 is a type I transmembrane glycoprotein expressed on B cells from pre-lymphocyte to mature B cell stages but absent in plasma cells. The precise function of CD22 is not clear but there is some evidence that it negatively regulates intracellular signalling. Mechanisms of epratuzumab action include reducing B cell numbers though this does not lead to depletion and also modulation of B cell functions. In a Phase II clinical trial, 11 of 14 patients had significant clinical responses to epratuzumab [59].

In addition to the use of mAb to deplete, or modulate B cell responses, induction of tolerance in autoreactive B cells has been attempted. One such approach involves using a 'toleragen', LJP-394, or Abetimus sodium. LJP-394 is a synthetic biological agent made up of four double stranded oligonucleotides attached to polyethylene glycol [60]. It lacks epitopes for T cells and modulates B cell functions by cross-linking membrane-bound anti-dsDNA Abs. The theory is that cross-linking modulates BCR signalling and induces anergy. Treatment of lupus-prone BXSB mice with LJP-394 resulted in reduced auto-Ab levels, improved renal functions and extended survival [60].

In human patients, treatment with LJP-394 was shown to reduce anti-dsDNA Ab production, but no improvement in renal functions was seen [61].

Modulation of B cell survival and activation in lupus was also achieved by targeting the B lymphocyte stimulator (BLyS; also known as BAFF). BLyS is member of the TNF family with profound effects on B cell proliferation, differentiation and Ab production [62]. BLyS is produced by monocytes, macrophages, dendritic cells and neutrophils and its level is elevated in patients with lupus [63]. BLyS can bind to three different receptors BAFF-R, TACI (transmembrane activator and calcium modulator ligand interactor) and BMCA (B cell maturation Ag) with varying degrees of affinity. In BWF1 lupus mice, blockade of BLyS with soluble TACI-Ig temporarily reduced IgG anti-DNA auto-Ab levels and delayed the onset of proteinuria [64]. In human patients, open label trials of a human mAb with specificity to BLyS (LymphoStat B, or belimumab) was shown to be well-tolerated and to significantly reduce B cells numbers [65]. However, there have been disagreements on the therapeutic efficacy of belimumab, although the most recent studies show significant benefits [66]. Other studies using TACI-Ig fusion protein (Atacicept) led to reduction in the numbers of B cells, T cells and NK cells and a dose-dependent reduction in Ab levels [67].

Biological agents targeting cognate T–B lymphocyte interactions

The regulation of cognate interaction between B cells and T cells in lupus is defective. This is manifested by overexpression of CD40L on T cells and B cells and by dysregulated expression of CD80 and CD86 [68, 69]. Efforts to treat lupus by targeting cognate interactions with humanised antibodies have achieved mixed results. Treatment with anti-CD40L mAbs did not show significant therapeutic benefits in lupus patients compared with those treated with placebo [70]. In addition, treated patients had an increased risk of thromboembolism [71]. Better beneficial clinical effects were achieved by targeting CD28-B7 interactions using CTLA4-Ig. In BWF1 mice, soluble CTLA4-Ig decreased auto-Ab production, delayed proteinuria and extended survival [72]. The use of a fusion protein of human CTLA4-Ig (Abatacept) in RA has proved successful [73], but the outcome of trials in lupus patients is awaited.

Biological anti-cytokine agents

The range of abnormalities in expression and response to cytokines in lupus is extensive. Cytokines have, therefore, long been regarded as potential therapeutic targets in lupus. In this regard, IL-6, IL-10, IFNα, IFNγ and TGFβ have attracted interest as potential targets. In BWF1 mice, blockade of IL-6 with mAb was shown to reduce anti-DNA auto-Ab and improve kidney histology [74]. In human patients with RA, clinical trials with a mAb to the IL-6 recep-

tor (tocilizumab) have been successful and its application in the clinic in the UK was recently approved [75]. The application of tocilizumab in lupus is yet to start; however, its application is a matter of time because of the compelling evidence for its relevance to lupus immunopathology.

Although IL-10 is a key immunoregulatory cytokine, its role in promoting B lymphocyte activation and differentiation makes IL-10 a candidate target for therapy in lupus. Indeed, treatment of BWF1 mice with either recombinant TNFα, which suppresses IL-10 production, or with anti-IL-10 delays proteinuria and death [36, 76]. Treatment of lupus patients with a murine mAb to IL-10 resulted in decreased disease activity, improved cutaneous lesions and joint symptoms [77]. However, blockade of IL-10 for treatment in the clinic is complicated by the immunoregulatory role of IL-10 and its unpredictable effects on immune cells.

With the available evidence on IL-10 in lupus immunopathology it has been, perhaps, paradoxical that biological anti-TNFα agents should be considered for treatment of patients with lupus [78]. Although the logic that anti-TNFα agents target the inflammatory response in lupus is understandable, its long-term effects can be unpredictable. This is relevant in that a significant minority of RA patients treated with anti-TNFα agents develop auto-Abs to DNA [79]. Nevertheless, the data published by Aringer and colleagues show that treatment with anti-TNFα reduced proteinuria in nephritic patients after one week of treatment [78].

The importance of IFNα as a central player in lupus immunopathogenesis has been recognised. Thus, IFNα promotes autoimmunity through activating immature myeloid dendritic cells which, in turn, activate autoreactive T cells. Further, IFNα directly promotes B and T cell activation [27, 80]. Despite the potential of IFNα as a therapeutic target for lupus, however, treatment has been difficult because there are numerous genes that encode multiple IFNα proteins. In humans, for example, there are more than 20 genes encoding 13 different IFNα proteins. However, a recent study showed that immunisation of BWF1 mice with IFNα resulted in the generation of neutralising Abs that delayed, or prevented proteinuria, nephritis and death [81].

Biological agents targeting complement components

Lupus is associated both with deficiency in the early components of the complement system and with excess activation of C3 and C5 in the most patients. Thus, C5 is activated in lupus both through the classical and alternative pathways and this contributes to the inflammatory responses and tissue damage. A mAb to C5 that inhibited its cleavage to C5a and C5b and blocked the formation of the membrane attack complex (MAC) delayed the onset of proteinuria, improved renal histology and extended survival of BWF1 mice [82]. In addition, a small antagonist protein of C5a receptor delayed nephritis, migration of

inflammatory cells to the kidneys and suppressed the production of pro-inflammatory cytokines [83]. In patients with lupus, a humanised mAb (5G1.1) effective in interfering with C5 activation was shown in Phase I trials to be safe and well-tolerated but clinical benefits are yet to be reported [84].

Enzyme replacement

The production of DNase1, an enzyme produced by the pancreas and salivary glands that catalyses extracellular DNA hydrolysis, is reduced in BWF1 mice [85]. In addition, mice deficient in Dnase1 develop a lupus-like disease [86]. Some lupus patients have a nonsense mutation in exon 2 of the *DNASE1* gene, resulting in decreased DNase1 activity in and high IgG Ab levels against nuclear antigens [87]. In a double blind, placebo-controlled trial of 16 patients with lupus nephritis receiving recombinant human DNase there was reduction in proteinuria and serum creatinine and improvement in renal pathology. However, there was no improvement in disease activity [88].

Gene therapy

The wide-ranging immunological abnormalities in lupus have provided both opportunities and obstacles to developing new therapies for universal application. Thus, the diversity in disease pathways has provided opportunities to explore different therapeutic strategies. However, this has created its own problems in that predicted clinical benefits have not materialised in all patient groups. Disease in patients with apparently similar clinical phenotypes could be driven by different mechanisms. As a consequence, patient groups respond differently to similar therapies. Interestingly, this is not confined to the genetically diverse human patients, but is also seen in different murine models of lupus. With the advance in gene therapy and its successful application in many animal models of autoimmune diseases, gene therapy has become an obvious protocol to explore in lupus. Gene therapy provides obvious advantages over current therapies such as reduced costs, lack of a need for repeated injections of high levels of proteins and reduced side effects. Nevertheless, there are also safety concerns. In addition, there are issues with the optimal production levels of therapeutic molecules, site of production and regulation. Despite these drawbacks, however, gene therapy remains a forward-looking therapeutic approach for treating lupus. Indeed, the available evidence shows remarkable improvements in disease pathology in animal models with the application of gene therapy to deliver TGFβ1, IFNγ receptor or IL-4 (reviewed in [1]). Studies over the last 5 years have explored new means of gene delivery to target new immunological pathways in lupus. Results of gene therapy studies using new approaches carried out over the last 5 years are summarised below.

Modulation of T lymphocyte responses

T lymphocytes in patients and in murine models of lupus participate in the induction of pathogenic auto-Abs of the IgG isotypes. Many abnormalities in the T lymphocyte compartment have been reported. These include decreased level and activity of key signalling proteins such TCRζ, NF-κB p65, PKCθ and PKC dependent protein phosphorylation, cognate interactions and cytokine production. Although the primary abnormality(s) and causal relationships remain unclear, some of these abnormalities are connected. For example, reduced IL-2 production by T cells in lupus is associated with reduced TCRζ expression and its upregulation by gene therapy augments IL-2 production upon TCR/CD28 engagement and restore normal T cell functions [89]. Interestingly, this approach appears to be more beneficial for treating lupus mice as IL-2 gene delivery in MRL *lpr* mouse model of lupus was shown to result in exacerbated disease and accelerated death [90]. The approach of modifying T cells to treat lupus was also used to generate 'regulatory' type T cells. For example, a group of investigators used a retroviral vector to generate T cells with specificity for nucleosome, a target antigen in lupus, which also produced CTLA4-Ig as a suppressive molecule [91]. cDNAs for the V region genes of the α and β chains of the TCR from a T cell clone with specificity for the immunodominant I-A^d-restricted nucleosomal epitope were transfected together with cDNA for the CTLA4Ig to generate 'regulatory' T cells in recipient mice. Initial assessment showed that ~10% of the total CD4 cells expressed all three genes and manifested a Treg phenotype. When these T cells were transferred into 10 week old BWF1 mice there was evidence for suppression of T cells proliferation, auto-Ab production and nephritis [91].

Gene therapy to modulate cognate interaction

Cognate interactions between B and T lymphocytes have been targeted using recombinant proteins. Investigators have explored this same approach using gene therapy. A recombinant adenovirus containing the full-length mouse PD-L1 gene was used to engage the inhibitory PD-1 receptor on activated lymphocytes in BXSB lupus prone mice. The mice simultaneously received an anti-ICOS ligand mAb to block ICOS-mediated co-stimulation. The combination therapy delayed the onset of proteinuria, reduced IgG auto-Ab production, and suppressed nephritis [92].

Other investigators used adeno-associated virus (AAV) vectors to deliver CTLA4-Ig and/or CD40-Ig in BWF1 mice [93]. Treatment of neonatal BWF1 mice with the CTLA4-Ig vector delayed disease onset and suppressed auto-Ab production, proteinuria and nephritis and prolonged lifespan. Combination therapy with vectors for CTLA4-Ig and CD40-Ig achieved a synergistic effect. The therapeutic benefits were apparently achieved through suppression of T cell activation and memory generation [93].

Gene therapy to suppress B lymphocyte activation and responses

Excess BLyS (BAFF) production has been associated with lupus autoimmunity, and mice transgenic for the protein have increased numbers of B cells and effector T, develop auto-Abs and lupus nephritis [94]. Gene transfer with an adenoviral vector to deliver TACI-Ig to B6 *lpr* and MRL *lpr* mice reduced B cells numbers, blocked auto-Ab production and nephritis and improved survival [95]. However, these results could not be reproduced in BWF1 mice which developed neutralising antibodies to TACI [95]. Interestingly, these studies showed that the treatment did not have an effect on total IgG levels, but reduced the spontaneous production of IgG and IgM auto-Abs. This would be important for maintaining protection against exogenous pathogens. Thus, these data provide evidence for the potential of BLyS blockade in the treatment of human patients. The success of this approach will be consistent with the success of targeting B cells in the clinic.

Studies over the last 5 years have indicated that innate immune receptors and cells can participate in the induction of autoreactive B cells and the production of auto-Abs. Thus immune complexes that contain chromatin or ssRNA have been shown to activate Toll-like receptor-9 (TLR-9) or TLR-7 on B cells and specifically activate autoreactive B cells independent of T cell help [96]. To determine the potential of targeting TLR-dependent signalling pathways for the treatment of lupus, MRL *lpr* mice were given synthetic oligodeoxynucleotides with immunoregulatory sequences that specifically blocked signalling via TLR-7 or TLR-7 and TLR-9 [97]. Blockade of signalling through TLR-7, but not TLR7/9 significantly reduced serum levels of IL-12p40 and anti-DNA auto-Abs of the IgG2a, IgG2b subclasses. However, both treatments significantly reduced nephritis. These results highlight the potential of targeting signalling via TLR-7 as a therapeutic target in lupus patients.

Gene therapy to target cytokines and chemokines

Targeting cytokines associated with lupus immunopathology have been extensively used with the majority of earlier studies summarised previously [1]. This approach has continued with new molecules targeted and their therapeutic potentials assessed in animal models. For example, a truncated form of the recently discovered IK cytokine which inhibits IFNγ-induced MHC-II upregulation was used to treated MRL *lpr* mice. The study showed that implantation of a non-metastatic fibroblastoid cell transfected with cDNA for the truncated IK cytokine before the onset of nephritis reduced renal damage, decreased macrophage and T cell infiltration of the kidneys and reduced auto-Ab levels [98].

Chemokines have also been used as therapeutic targets in lupus mice. One such chemokine, the monocyte chemoattractant protein-1 (MCP-1), is upregulated in MRL *lpr* mice. This chemokine recruits and activates inflammatory

cells such as in nephritic kidneys. An N-terminal deletion mutant of the MCP-1 gene in pcDNA3 expression plasmid was injected into skeletal muscles of MRL *lpr* mice and effect on lupus pathology determined. The treatment protected the kidneys from injury due to reduced infiltration of leucocytes and prolonged survival [99].

References

1 Mageed RA, Prud'homme GJ (2003) Immunopathology and the gene therapy of lupus. *Gene Therapy* 10: 861–874
2 Rhodes B, Vyse TJ (2008) The genetics of SLE: an update in the light of genome-wide association studies. *Rheumatol* 47: 1603–1611
3 Deapen D, Escalante A, Weinrib L, Horwitz D, Bachman B, Roy-Burman P, Walker A, Mack TM (1992) A revised estimate of twin concordance in systemic lupus erythematosus. *Arthritis Rheum* 35: 311–318
4 Alarcon-Segovia D, Alarcon-Requelme ME, Cardiel MH, Caeiro F, Massardo L, Villa AR, Pons-Estel BA, Grupo Latinoamericano de Estudio del lupus Eritematoso (GLADEL) (2005) Familial aggregation of systemic lupus erythematosus, rheumatoid arthritis, and other autoimmune diseases in 1,177 lupus patients from the GLADEL cohort. *Arthritis Rheum* 52: 1138–1147
5 Manderson AP, Botto M, Walport MJ (2004) The role of complement in the development of systemic lupus erythematosus. *Annu Rev Immunol* 22: 431–456
6 Fanciulli M, Norsworthy PJ, Petretto E, Dong R, Harper L, Kamesh L, Heward JM, Gough SCL, de Smith A, Blakemore AIF et al. (2007) FCGR3B copy number variation is associated with susceptibility to systemic, but not organ-specific, autoimmunity. *Nat Genet* 39: 721–723
7 Lee-Kirsch MA, Gong M, Chowdhury D, Senenko L, Engel K, Lee YA, de Silva U, Bailey SL, Witte T, Vyse TJ et al. (2007) Mutations in the gene encoding the 30–50 DNA exonuclease TREX1 are associated with systemic lupus erythematosus. *Nat Genet* 39: 1065–1067
8 Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, Schwartzberg PL, Cook MC, Walters GD, Vinuesa CG (2009) Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* 206: 561–576
9 Mudd PA, Teague BN, Farris AD (2006) Regulatory T cells and systemic lupus erythematosus. *Scand J Immunol* 64: 211–218
10 Solomou EE, Juang YT, Gourley MF, Kammer GM, Tsokos GC (2001) Molecular basis of deficient IL-2 production in T cells from patients with systemic lupus erythematosus. *J Immunol* 166: 4216–4222
11 Horwitz DA, Tang FL, Stimmler MM, Oki A, Gray JD (1997) Decreased T cell response to anti-CD2 in systemic lupus erythematosus and reversal by anti-CD28: evidence for impaired T cell-accessory cell interaction. *Arthritis Rheum* 40: 822–833
12 Sierakowski S, Kucharz EJ, Lightfoot RW, Goodwin JS (1989) Impaired T-cell activation in patients with systemic lupus erythematosus. *J Clin Immunol* 9: 469–676
13 Wong HK, Kammer GM, Dennis G, Tsokos GC (1999) Abnormal NF-kappa B activity in T lymphocytes from patients with systemic lupus erythematosus is associated with decreased p65-RelA protein expression. *J Immunol* 163: 1682–1689
14 Yi Y, McNerney M, Datta SK (2000) Regulatory defects in Cbl and mitogen-activated protein kinase (extracellular signal-related kinase) pathways cause persistent hyperexpression of CD40 ligand in human lupus T cells. *J Immunol* 165: 6627–6634
15 Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, Nussenzweig MC (2005) Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J Exp Med* 201: 703–711
16 Reininger L, Winkler T, Kalberer CP, Jourdan M, Melchers F, Rolink AG (1996) Intrinsic B cell defects in NZB and NZW mice contribute to systemic lupus erythematosus in (NZB × NZW)F1 mice. *J Exp Med* 184: 853–861
17 Wakeland EK, Liu K, Graham RR, Behrens TW (2001) Delineating the genetic basis of systemic lupus erythematosus. *Immunity* 15: 397–408
18 Yanaba K, Bouaziz JD, Matsushita T, Magro CM, St Clair EW, Tedder TF (2008) B-lymphocyte contributions to human autoimmune disease. *Immunol Rev* 223: 284–299

19 Folzenlogen D, Hofer MF, Leung DY, Freed JH, Newell MK (1997) Analysis of CD80 and CD86 expression on peripheral blood B lymphocytes reveals increased expression of CD86 in lupus patients. *Clin Immunol Immunopathol* 83: 199–204
20 Zouali M, Sarmay G (2004) B lymphocyte signaling pathways in systemic autoimmunity: implications for pathogenesis and treatment. *Arthritis Rheum* 50: 2730–2741
21 Salmon JE, Millard S, Schachter LA, Arnett FC, Ginzler EM, Gourley MF, Ramsey-Goldman R, Peterson MG, Kimberly RP (1996) Fc gamma RIIA alleles are heritable risk factors for lupus nephritis in African Americans. *J Clin Invest* 97: 1348–1354
22 Rönnblom L, Eloranta ML, Alm GV (2006) The type I interferon system in systemic lupus erythematosus. *Arthritis Rheum* 54: 408–420
23 Dauphinee MJ, Kipper SB, Wofsy D, Talal N (1981) Interleukin 2 deficiency is a common feature of autoimmune mice. *J Immunol* 127: 2483–2487
24 Kammer GM (1999) High prevalence of T cell type I protein kinase A deficiency in systemic lupus erythematosus. *Arthritis Rheum* 42: 1458–1465
25 Tanaka T, Saiki O, Negoro S, Igarashi T, Kuritani T, Hara H, Suemura M, Kishimoto S (1989) Decreased expression of interleukin-2 binding molecules (p70/75) in T cells from patients with systemic lupus erythematosus. *Arthritis Rheum* 32: 552–559
26 Prud'homme GJ, Kono DH, Theofilopoulos AN (1995) Quantitative polymerase chain reaction analysis reveals marked overexpression of interleukin-1 beta, interleukin-1 and interferon-gamma mRNA in the lymph nodes of lupus-prone mice. *Mol Immunol* 32: 495–503
27 Rönnblom L, Pascual V (2008) The innate immune system in SLE: type I interferons and dendritic cells. *Lupus* 17: 394–399
28 Graninger WB, Hassfeld W, Pesau BB, Machold KP, Zielinski CC, Smolen JS (1991) Induction of systemic lupus erythematosus by interferon-gamma in a patient with rheumatoid arthritis. *J Rheumatol* 18: 1621–1622
29 Dueymes M, Barrier J, Besancenot JF, Cledes J, Conri C, Dien G, Drosos AA, Dueymes JM, Galanaud P, Grobois B et al. (1993) Relationship of interleukin-4 to isotypic distribution of anti-double-stranded DNA antibodies in systemic lupus erythematosus. *Int Arch Allergy Immunol* 101: 408–415
30 Santiago ML, Fossati L, Jacquet C, Muller W, Izui S, Reininger L (1997) Interleukin-4 protects against a genetically linked lupus-like autoimmune syndrome. *J Exp Med* 185: 65–70
31 Garaud S, Le Dantec C, Jousse-Joulin S, Hanrotel-Saliou C, Saraux A, Mageed RA, Youinou P, Renaudineau Y (2009) IL-6 modulates CD5 expression in B cells from patients with lupus by regulating DNA methylation. *J Immunol* 182: 5623–5632
32 Casciola-Rosen LA, Anhalt G, Rosen A (1994) Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J Exp Med* 179: 1317–1330
33 Hagiwara E, Gourley MF, Lee S, Klinman DK (1996) Disease severity in patients with systemic lupus erythematosus correlates with an increased ratio of interleukin-10:interferon-gamma-secreting cells in the peripheral blood. *Arthritis Rheum* 39: 379–385
34 Alleva DG, Kaser SB, Beller DI (1998) Intrinsic defects in macrophage IL-12 production associated with immune dysfunction in the MRL/++ and New Zealand Black/White F1 lupus-prone mice and the Leishmania major-susceptible BALB/c strain. *J Immunol* 161: 6878–6884
35 Huang FP, Feng GJ, Lindop G, Stott DI, Liew FY (1996) The role of interleukin 12 and nitric oxide in the development of spontaneous autoimmune disease in MRLMP-lprlpr mice. *J Exp Med* 183: 1447–1459
36 Jacob CO, McDevitt HO (1988) Tumour necrosis factor-α in murine autoimmune 'lupus' nephritis. *Nature* 331: 356–358
37 Aringer M, Steiner G, Graninger WB, Höfler E, Steiner CW, Smolen JS (2007) Effects of short-term infliximab therapy on autoantibodies in systemic lupus erythematosus. *Arthritis Rheum* 56: 274–279
38 Ohtsuka K, Gray JD, Stimmler MM, Toro B, Horwitz DA (1998) Decreased production of TGF-beta by lymphocytes from patients with systemic lupus erythematosus. *J Immunol* 160: 2539–2545
39 Harley JB, Alarcón-Riquelme ME, Criswell LA, Jacob CO, Kimberly RP, Moser KL, Tsao BP, Vyse TJ, Langefeld CD, Nath SK et al. (2008) Genome-wide association scan in women with systemic lupus erythematosus identifies susceptibility variants in ITGAM, PXK, KIAA1542 and other loci. *Nat Genet* 40: 204–210

40 Hom G, Graham RR, Modrek B, Taylor KE, Ortmann W, Garnier S, Lee AT, Chung SA, Ferreira RC, Pant PV et al. (2008) Association of systemic lupus erythematosus with C8orf13-BLK and ITGAM-ITGAX. *N Engl J Med* 358: 900–909
41 James JA, Harley JB, Scofield RH (2006) Epstein-Barr virus and systemic lupus erythematosus. *Curr Opin Rheumatol* 18: 462–467
42 Yang Y, Chung EK, Wu YL, Savelli SL, Nagaraja HN, Zhou B, Hebert M, Jones KN, Shu Y, Kitzmiller K et al. (2007) Gene copy-number variation and associated polymorphisms of complement component C4 in human systemic lupus erythematosus (SLE): low copy number is a risk factor for and high copy number is a protective factor against SLE susceptibility in European Americans. *Am J Hum Genet* 80: 1037–1054
43 Cunninghame Graham DS, Graham RR, Manku H, Wong AK, Whittaker JC, Gaffney PM, Moser KL, Rioux JD, Altshuler D, Behrens TW et al. (2008) Polymorphism at the TNF superfamily gene TNFSF4 confers susceptibility to systemic lupus erythematosus. *Nat Genet* 40: 83–89
44 Lane P (2000) Role of OX40 signals in coordinating CD4T cell selection, migration, and cytokine differentiation in T helper (Th)1 and Th2 cells. *J Exp Med* 191: 201–206
45 Ito T, Wang YH, Duramad O, Hanabuchi S, Perng OA, Gilliet M, Qin FX, Liu YJ (2006) OX40 ligand shuts down IL-10-producing regulatory T cells. *Proc Natl Acad Sci USA* 103: 13138–13143
46 Stuber E, Strober W (1996) The T cell-B cell interaction via OX40-OX40L is necessary for the T cell-dependent humoral immune response. *J Exp Med* 183: 979–989
47 Baechler EC, Batliwalla FM, Karypis G, Gaffney PM, Ortmann WA, Espe KJ, Shark KB, Grande WJ, Hughes KM, Kapur V et al. (2003) Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci USA*100: 2610–2615
48 Braun D, Caramalho I, Demengeot J (2002) IFN-alpha/beta enhances BCR-dependent B cell responses. *Int Immunol* 14: 411–419
49 Barnes BJ, Kellum MJ, Pinder KE, Frisancho JA, Pitha PM (2003) Interferon regulatory factor 5, a novel mediator of cell cycle arrest and cell death. *Cancer Res* 63: 6424–6431
50 Cornall R, Cyster J, Hibbs M, Dunn A, Otipoby K, Clark A, Goodnow CC (1998) Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection. *Immunity* 8: 497–508
51 Flores-Borja F, Kabouridis PS, Jury EC, Isenberg DA, Mageed RA (2005) Decreased Lyn expression and translocation to lipid raft signalling domains in B-lymphocytes from patients with systemic lupus erythematosus. *Arthritis Rheum* 52: 3955–3965
52 Darnell JE Jr, Kerr IM, Stark GR (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264: 1415–1421
53 Nguyen KB, Watford WT, Salomon R, Hofmann SR, Pien GC, Morinobu A, Gadina M, O'Shea JJ, Biron CA (2002) Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection. *Science* 297: 2063–2066
54 Moynihan TP, Cole CG, Dunham I, O'Neil L, Markham AF, Robinson PA (1998) Finemapping, genomic organization, and transcript analysis of the human ubiquitinconjugating enzyme gene UBE2L3. *Genomics* 51: 124–127
55 Jury EC, Kabouridis PS, Abba A, Mageed RA, Isenberg DA (2003) Increased ubiquitination and reduced expression of Lck in T lymphocytes from patients with systemic lupus erythematosus. *Arthritis Rheum* 48: 1343–1354
56 Yousefi S, Simon HU (2007) Apoptosis regulation by autophagy gene 5. *Crit Rev Oncol Hematol* 63: 241–244
57 Youinou P, Taher TE, Mankaï A, Berthou C, Mageed RA (2008) Anti-CD20 in targeting B-lymphocytes for the treatment of autoimmune diseases. Clinical benefits and insights into the role of B-lymphocytes in pathophysiology. *Immunol Endocrine Metabol Agents Med Chem* 8: 222–234
58 Lu T, Ng KP, Cambridge G, Leandro MJ, Edwards JCW, Ehrenstein M, Isenberg DA (2009) A retrospective seven-year analysis of the use of B cell depletion therapy in systemic lupus erythematosus at University College London Hospital: the first fifty patients. *Arthritis Rheum* 61: 482–487
59 Dörner T, Kaufmann J, Wegener WA, Teoh N, Goldenberg DM, Burmester GR (2006) Initial clinical trial of Epratuzumab (humanized anti-CD22 antibody) for immunotherapy of systemic lupus erythematosus. *Arthritis Res Ther* 8: R74
60 Coutts SM, Plunkett ML, Iverson GM, Barstad PA, Berner CM (1996) Pharmacological intervention in antibody mediated disease. *Lupus* 5: 158–159
61 Alarcón-Segovia D, Tumlin J, Furie RA, McKay JD, Cardiel MH, Strand V, Bagin RG, Linnik

MD, Hepburn B; LJP 394 Investigator Consortium (2003) LJP 394 for the prevention of renal flare in patients with systemic lupus erythematosus: results from a randomized, double-blind, placebo-controlled study. *Arthritis Rheum* 48: 442–454
62 Schiemann B, Gommerman JL, Vora K, Cachero TG, Shulga-Morskaya S, Dobles M, Frew E, Scott ML (2001) An essential role for BAFF in the normal development of B cells through a BCMA-independent pathway. *Science* 293: 2111–2114
63 Cheema GS, Roschke V, Hilbert DM, Stohl W (2001) Elevated serum B lymphocyte stimulator levels in patients with systemic immune-based rheumatic diseases. *Arthritis Rheum* 44: 1313–1319
64 Ramanujam M, Wang X, Huang W, Schiffer L, Grimaldi C, Akkerman A, Diamond B, Madaio MP, Davidson A (2004) Mechanism of action of transmembrane activator and calcium modulator ligand interactor-Ig in murine systemic lupus erythematosus. *J Immunol* 173: 3524–3534
65 Furie R, Stohl W, Ginzler E, Becker M, Mishra N, Chatham W, Merrill JT, Weinstein A, McCune WJ, Zhong J et al. (2008) Biologic activity and safety of belimumab, a neutralizing anti-B-lymphocyte stimulator (BLyS) monoclonal antibody: a phase I trial in patients with systemic lupus erythematosus. *Arthritis Res Ther* 10: R109
66 Ding C (2008) Belimumab, an anti-BLyS human monoclonal antibody for potential treatment of inflammatory autoimmune diseases. *Expert Opin Biol Ther* 8: 1805–1814
67 Dall'Era M, Chakravarty E, Wallace D Genovese M, Weisman M, Kavanaugh A, Kalunian K, Dhar P, Vincent E, Pena-Rossi C et al. (2007) Reduced B lymphocyte and immunoglobulin levels after atacicept treatment in patients with systemic lupus erythematosus: results of a multicenter, phase Ib, double-blind, placebo-controlled, dose-escalating trial. *Arthritis Rheum* 56: 4142–4150
68 Desai-Mehta A, Lu L, Ramsey-Goldman R, Datta SK (1996) Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. *J Clin Invest* 97: 2063–2073
69 García-Cózar FJ, Molina IJ, Cuadrado MJ, Marubayashi M, Peña J, Santamaría M (1996) Defective B7 expression on antigen-presenting cells underlying T cell activation abnormalities in systemic lupus erythematosus (SLE) patients. *Clin Exp Immunol* 104: 72–79
70 Kalunian KC, Davis Jr, JC, Merrill JT, Totoritis MC, Wofsy D, for the IDEC-131 Lupus Study Group (2002) Treatment of systemic lupus erythematosus by inhibition of T cell costimulation with anti-CD154. *Arthritis Rheum* 46: 3251–3258
71 Kawai T, Andrews D, Colvin R, Sachs D, Cosimi A (2000) Thromboembolic complications after treatment with monoclonal antibody against CD40 ligand. *Nat Med* 6: 114
72 Finck BK, Linsley PS, Wofsy D (1994) Treatment of murine lupus with CTLA4Ig. *Science* 265: 1225–1227
73 Genovese MC, Becker JC, Schiff M, Luggen M, Sherrer Y, Kremer J, Birbara C, Box J, Natarajan K, Nuamah I et al. (2005) Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. *N Engl J Med* 353: 1114–1123
74 Liang B, Gardner DB, Griswold DE, Bugelski PJ, Song XY (2006) Anti-interleukin-6 monoclonal antibody inhibits autoimmune responses in a murine model of systemic lupus erythematosus. *Immunol* 119: 296–305
75 Choy E (2009) RADIATE: more treatment options for patients with an inadequate response to tumor necrosis factor antagonists. *Nat Clin Pract Rheumatol* 5: 66–67
76 Ishida H, Muchamuel T, Sakaguchi S, Andrade S, Menon S, Howard M (1994) Continuous administration of anti-interleukin 10 antibodies delays onset of autoimmunity in NZB/W F1 mice. *J Exp Med* 179: 305–310
77 Llorente L, Richaud-Patin Y, García-Padilla C, Claret E, Jakez-Ocampo J, Cardiel MH, Alcocer-Varela J, Grangeot-Keros L, Alarcón-Segovia D, Wijdenes J et al. (2000) Clinical and biologic effects of anti-interleukin-10 monoclonal antibody administration in systemic lupus erythematosus. *Arthritis Rheum* 43: 1790–1800
78 Aringer M, Graninger WB, Steiner G, Smolen JS (2004) Safety and efficacy of TNFα blockage in systemic lupus erythematosus – an open label study. *Arthritis Rheum* 50: 3161–3169
79 Charles PJ, Smeenk RJ, De Jong J, Feldmann M, Maini RN (2000) Assessment of antibodies to double-stranded DNA induced in rheumatoid arthritis patients following treatment with infliximab, a monoclonal antibody to tumor necrosis factor alpha: findings in open-label and randomized placebo-controlled trials. *Arthritis Rheum* 43: 2383–2390
80 Pascual V, Farkas L, Banchereau J (2006) Systemic lupus erythematosus: all roads lead to type I interferons. *Curr Opin Immunol* 18: 676–682

81 Zagury D, Le Buanec H, Mathian A, Larcier P, Burnett R, Amoura Z, Emilie D, Peltre G, Bensussan A, Bizzini B et al. (2009) IFNalpha kinoid vaccine-induced neutralizing antibodies prevent clinical manifestations in a lupus flare murine model. *Proc Natl Acad Sci USA* 106: 5294–5299
82 Wang Y, Hu Q, Madri J, Rollins S, Chodera A, Matis L (1996) Amelioration of lupus-like autoimmune disease in NZB/W F1 mice after treatment with a blocking monoclonal antibody specific for complement component C5. *Proc Natl Acad Sci USA* 93: 8563–8568
83 Bao L, Osawe I, Puri T, Lambris JD, Haas M, Quigg RJ (2005) C5a promotes development of experimental lupus nephritis which can be blocked with a specific receptor antagonist. *Eur J Immunol* 35: 2496–2506
84 Robak E, Robak T (2009) Monoclonal antibodies in the treatment of systemic lupus erythematosus. *Curr Drug Targets* 10: 26–37
85 Macanovic M, Lachmann PJ (1997) Measurement of deoxyribonuclease I (DNase) in the serum and urine of systemic lupus erythematosus (SLE)-prone NZB/NZW mice by a new radial enzyme diffusion assay. *Clin Exp Immunol* 108: 220–226
86 Napirei M, Karsunky H, Zevnik B, Stephan H, Mannherz HG, Möröy T (2000) Features of systemic lupus erythematosus in Dnase1-deficient mice. *Nat Genet* 25: 177–181
87 Yasutomo K, Horiuchi T, Kagami S, Tsukamoto H, Hashimura C, Urushihara M, Kuroda Y (2001) Mutation of DNASE1 in people with systemic lupus erythematosus. *Nat Genet* 28: 313–314
88 Tinazzi E, Puccetti A, Gerli R, Rigo A, Migliorini P, Simeoni S, Beri R, Dolcino M, Martinelli N, Corrocher R et al. (2009) Serum DNase I, soluble Fas/FasL levels and cell surface Fas expression in patients with SLE: a possible explanation for the lack of efficacy of hrDNase I treatment. *Int Immunol* 21: 237–243
89 Nambiar MP, Fisher CU, Warke VG, Krishnan S, Mitchell JP, Delaney N, Tsokos GC (2003) Reconstitution of deficient T cell receptor zeta chain restores T cell signaling and augments T cell receptor/CD3-induced interleukin-2 production in patients with systemic lupus erythematosus. *Arthritis Rheum* 48: 1948–1955
90 Raz E, Dudler J, Lotz M, Baird SM, Berry CC, Eisenberg RA, Carson DA (1995) Modulation of disease activity in murine systemic lupus erythematosus by cytokine gene delivery. *Lupus* 4: 286–292
91 Fujio K, Okamura T, Okamoto A, Yamamotot K (2007) cell receptor gene therapy for autoimmune diseases. *Ann N Y Acad Sci* 1110: 222–232
92 Ding H, Wu X, Wu J, Yagita H, He Y, Zhang J, Ren J, Gao W (2006) Delivering PD-1 inhibitory signal concomitant with blocking ICOS co-stimulation suppresses lupus-like syndrome in autoimmune BXSB mice. *Clin Immunol* 118: 258–267
93 Ye X, Zhu T, Bastacky S, McHale T, Li J, Xiao X (2005) Prevention and reversal of lupus in NZB/NZW mice by costimulatory blockade with adeno-associated virus-mediated gene transfer. *Arthritis Rheum* 52: 3975–3986
94 Mackay F, Woodcock SA, Lawton P, Ambrose C, Baetscher M, Schneider P, Tschopp J, Browning JL (1999) Mice transgenic for BAFF develop lymphocytic disorders along with autoimmune manifestations. *J Exp Med* 190: 1697–1710
95 Liu W, Szalai A, Zhao L, Liu D, Martin F, Kimberly RP, Zhou T, Carter RH (1995) Control of spontaneous B lymphocyte autoimmunity with adenovirus-encoded soluble TACI. *Arthritis Rheum* 50: 1884–1896
96 Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A (2002) Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603–607
97 Pawar RD, Ramanjaneyulu A, Kulkarni OP, Lech M, Segerer S, Anders H-J (2007) Inhibition of toll-like receptor-7 (TLR-7) or TLR-7 plus TLR-9 attenuates glomerulonephritis and lung injury in experimental lupus. *J Am Soc Nephrol* 18: 1721–1731
98 Muraoka M, Hasegawa H, Kohno M, Inoue A, Miyazaki T, Terada M, Nose M, Yasukawa M (2006) IK cytokine ameliorates the progression of lupus nephritis in MRL/*lpr* Mice. *Arthritis Rheum* 54: 3591–3600
99 Shimizu S, Nakashima H, Masutani K, Inoue Y, Miyake K, Akahoshi M, Tanaka Y, Egashira K, Hirakata H, Otsuka T et al. (2004) Anti-monocyte chemoattractant protein-1 gene therapy attenuates nephritis in MRL/lpr mice. *Rheumatol* 43: 1121–1128

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Regulated promoters

Jeroen Geurts, Wim B. van den Berg and Fons A.J. van de Loo

Rheumatology Research and Advanced Therapeutics, Department of Rheumatology, Radboud University Nijmegen Medical Centre, Nijmegen Centre for Molecular Life Sciences, Nijmegen, The Netherlands

Abstract

Over the past decades gene therapy has emerged as a promising approach for treatment of a variety of diseases including monogenic diseases, cancer, neurodegenerative disorders, autoimmune and inflammatory diseases. However, efficacy and safety remain the major challenges for turning gene therapy into a clinical reality. Several advances in vectorology have provided opportunities to address these issues including transductional and transcriptional targeting of viral vectors. The prior involves the modification of the virus tropism in order to increase the efficiency and specificity of target cell transduction. The latter comprises the use of *cis*-regulatory elements, such as promoters and enhancers (Fig. 1), to restrict transgene expression to specific tissues or patho-physiological conditions. Here we focus on recent developments and applications of endogenously-regulated promoter systems in gene therapy for autoimmune and inflammatory diseases, in particular rheumatoid arthritis (RA).

Disadvantages of constitutively active promoters

The vast majority of preclinical gene therapy studies rely on high levels of therapeutic proteins using viral promoters derived from cytomegalovirus (CMV), Rous sarcoma virus (RSV) or cellular promoters such as the human phosphoglycerate kinase-1 (PGK), elongation factor 1α (EF1α) or chicken β-actin promoter. Despite their short-term effectiveness in animal models, the applicability of these promoters for long-term therapy is hampered by a number of issues. First, transgene expression *in vivo* is often transient due to promoter attenuation. A comprehensive study of Chen and co-workers demonstrated a dramatic drop in CMV-driven transgene expression in liver and spleen starting from 3 days after intravenous delivery of adenovirus [1]. Upon intramuscular injection, expression levels decreased between 100- and 1,000-fold within 3 weeks using both CMV, β-actin and EF1α promoters. A similar study, describing a rapid loss of CMV-driven transgene expression from muscle tissue, demonstrated rapid and extensive methylation of CpG sites in the promoter abolishing its transcriptional activity [2] (Fig. 1). In synovial tissue, which represents the target site of local gene therapy for RA, rapid silencing of the CMV promoter is frequently observed [3–7]. Second, strong constitutive promoters might transactivate endogenous (onco)genes upon integrating in the genome. Weber et al. established a cell-type specific correlation between

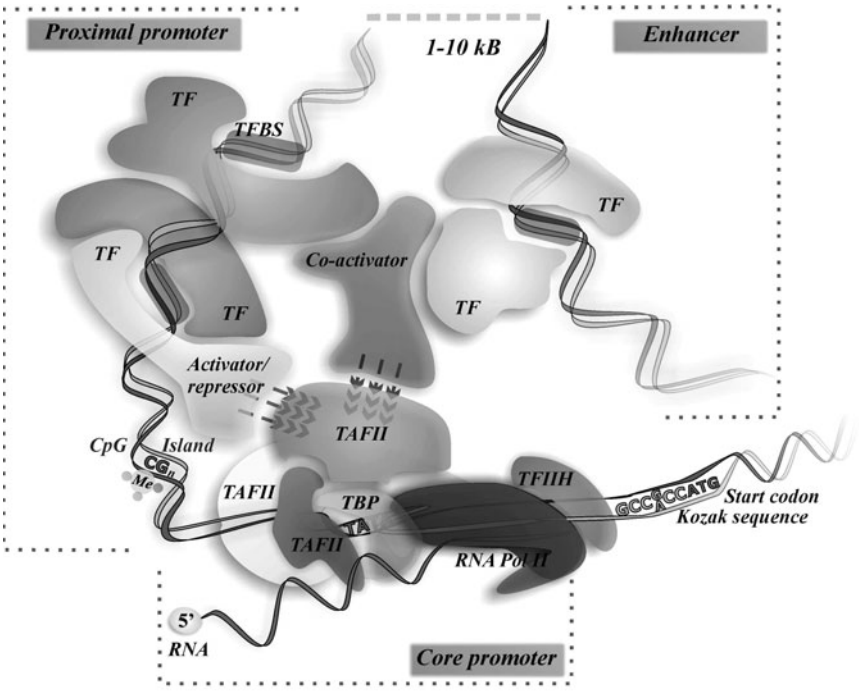


Figure 1. Schematic overview of a typical TATA-dependent promoter, its *cis*-regulatory sequences and interactions between regulatory proteins and the transcription initiation complex. The lower part displays the core promoter region which is the essential sequence for transcription initiation. In eukaryotes, the most common type core promoter is the TATA box that is the binding site for the transcription factor TATA binding protein (TBP). After TBP binds the TATA box, a number of TBP-associated factors (TAFII) and RNA polymerase (Pol II) combine around the TATA box to form the preinitiation complex (PIC). The TAFII, TFIIH has helicase activity and is involved in opening the double helical DNA strands. The PIC only drives a low rate of transcription. The transcriptional rate is further enhanced or inhibited by regulatory factors along with any associated co-activators or co-repressors. The *cis*-regulatory elements that drive tissue- or context-specific expression are predominantly located in the proximal-promoter region, depicted in the left part of the diagram. Regulatory factors consist of protein complexes, often hetero- or homodimers, that bind on their cognate binding sites (TFBS). Transcription factors (TF) can transactivate or repress the activity of the PIC and work cooperatively by stabilizing each other when forming DNA-protein complexes. Co-activators mediate the influence of regulatory factors through protein-protein interaction between a TF and the PIC. Additional fine-tuning of transcription is accomplished via more distal DNA elements as enhancers, displayed on the right part. These sequences exert their activating effects independently from position or orientation and are obtained by accomplishing a specific DNA formation. Extensive methylation of CG-rich regions in promoter sequences (CpG Islands), e.g., viral promoters, compromises the binding of regulatory factors and leads to inactivation of the promoter. The Kozak sequence (GCCRCC) is frequently placed directly upstream of the translation start codon (ATG) for enhanced translation of transgenes in gene therapy approaches.

transcriptional strength and transactivation potential of promoters, including PGK, EF1α and CMV [8]. A second study by Zychlinski and co-workers confirmed the transactivation potential of retroviral enhancer-promoters. Additionally, they demonstrated that the genotoxic risk of integrating vectors

is considerably reduced using constitutive cellular promoters (PGK, EF1α) in combination with a self-inactivating vector design [9]. Third, systemic or local overexpression of biologicals regardless of the physiological demand can elicit serious transgene-induced side effects. Our group has demonstrated several undesired side effects in cytokine-based gene therapy approaches, arising from the pleiotropic nature of these proteins. Intra-articular gene therapy for experimental arthritis and osteoarthritis using respectively interleukin-4 (IL-4) [5, 10] and transforming growth factor-β (TGFβ) [11], led to a protection against cartilage destruction. However, constitutive TGFβ expression also induced synovial fibrosis and osteophyte formation [12]. The chemotactic properties of IL-4 gave rise to a massive joint inflammation when this transgene was overexpressed in non-arthritic joints. Taken together, the development of long-term and safe gene therapeutic treatment requires a careful consideration of promoter selection. While strong ubiquitous promoters represent seemingly obvious candidates for gene therapy, tightly-regulated expression of transgene appears a key feature towards an effective and safe mode of gene therapy.

Transcriptional targeting

Transcriptional targeting strategies that are either based on cell-specificity or reactivity to physiological changes are explored extensively in cancer due to their unique expression of tumor specific (onco)genes and regional enriched expression of genes created by the hypoxic environment in tumors (reviewed by Robson and Hirst [13]). Although hypoxia and related processes such as neovascularisation also occur in the inflamed tissue, promoters of these genes or artificial promoters bearing consensus sequences of response elements for the hypoxia-inducible transcription factor (HIF-1α) have thus far not been explored in gene therapeutic approaches for chronic inflammatory of autoimmune diseases. Other strategies utilized in cancer gene therapy as radiation-, chemotherapy- and hyperthermia-inducible expression vectors make use of existing anti-cancer therapies [14] and are for this reason not used for transcriptional targeting of inflammation. Several chronic inflammatory diseases including RA, lupus and inflammatory bowel disease are characterized by a disease course which displays spontaneous periods of exacerbations and remission. Most ideally, expression of a therapeutic protein meets the variable demand during these diseases: high during a relapse and low during remission of the disease. For this, transcriptional targeting in these diseases is now based on the use inflammation-responsive rather than cell-specific promoters.

Acute phase gene promoters

The acute phase response (APR) is part of the innate immunity and comprises an early set of inflammatory reactions induced by infection or tissue injury

[15]. The APR is accompanied by a rapid increase in acute phase protein (APP) levels in plasma including serum amyloid A (*SAA*), complement factor 3 (*C3*) and C-reactive protein (*CRP*). The augmentation in serum SAA and CRP levels are sensitive biological laboratory markers in RA patients that correlate with disease activity [15]. The dramatic increase in APP levels are due to a strongly induced transcriptional rate arising from cytokine-mediated synergistic activation of APP promoters. Varley and co-workers pioneered the inflammation-inducible expression of recombinant proteins *in vivo* from APP promoters. Using intravenous administered adenoviral reporters harboring the firefly luciferase cDNA under transcriptional control of murine *Saa3* (–306/+33) or *C3* (–397/+45) promoters they demonstrated a strong response upon systemic injection of lipopolysaccharide (LPS). Burke and co-workers applied the human *CRP* (–122/+672) promoter to generate transgenic-mice showing inflammation-inducible expression of granulocyte macrophage colony-stimulating factor (*GM-CSF*) [16]. Upon systemic administration LPS, GM-CSF levels were increased approximately 150-fold within 6 h and protein levels were 100–500-fold higher compared to endogenous GM-CSF. Transgenic *Saa1-Luc* mice harboring a 7.7 kB promoter of murine *Saa1* demonstrated strong induction of luciferase activity in multiple tissues after systemic LPS and tumor necrosis factor-α (TNF-α) treatment [17]. The strongest induction of luciferase activity, approximately 5000-fold, was observed in the liver whereas brain and spleen showed a less than two-fold induction. During an acute arthritis induced by intra-articular injection of zymosan, luciferase levels were induced approximately 20-fold within 4 h and declined to seven- and six-fold induction at 1 and 5 days after arthritis induction, respectively.

Pro-inflammatory cytokine and enzyme gene promoters

Apart from APR genes, the promoter regions of genes that are differentially regulated in chronic inflammatory processes such as cytokines, chemokines and matrix degrading enzymes, represent attractive candidates for development of disease-specific gene therapeutic vectors. Experimental arthritis models showed that the pro-inflammatory cytokines TNFα, IL-1, IL-6, IL-18 belong to early-responsive genes, which are upregulated at the primary onset and secondary flare-ups of experimental arthritis [18]. Especially IL-6 is identified as a marker of disease activity and the principal cytokine responsible for the acute phase response in RA patients. Serum IL-6 levels correlated with serum CRP levels and erythrocyte sedimentation rate (ESR) [19, 20]. Also the synovial fluid levels of IL-6 correlate with serum CRP and ESR in patients with RA [21]. The synovial fluid concentration of IL-6 are higher than in serum with the synovial fibroblasts as the source of IL-6 with the highest production in the presence of lymphocytic follicles in the synovial tissue [19, 20, 22]. IL-6 is strictly regulated at the transcriptional level and several transcription factors,

including NF-κB, activator protein-1 (AP-1) and CCAAT/enhancer binding protein (C/EBP) contribute to the complex regulation of this gene. For NF-κB and AP-1 factors a pivotal role has been implied in human RA, murine collagen-induced arthritis (CIA) and immunity [23–27]. Enhanced expression and DNA binding activity of C/EBPβ in synovial tissue of RA patients has been implicated in the pathology [28] and chronicity [29] of disease. Therefore, the *IL-6* promoter appeared a promising candidate to achieve disease-regulated gene therapy. While the minimal promoter of human *IL-6* (–163/+12) showed only little responsiveness, the upstream addition of the human *IL-1β* enhancer (–3690/–2720) mounted a robust response towards pro-inflammatory stimuli *in vitro* and *in vivo* [7]. A comparison of *in vivo* performance between the hybrid (IL-1E/IL-6) and *C3*-Tat/HIV promoters revealed a comparable responsiveness and transcriptional strength. Geurts and co-workers exploited the properties of this hybrid promoter to enable the use of an otherwise disputable biological, IL-4 [5]. We demonstrated effective protection against cartilage erosion in CIA by injection of knee joints with adenoviral vector containing IL-1E/IL-6P-driven murine IL-4. Perhaps even more importantly, restriction of *IL-4* expression to inflammatory conditions minimized the deleterious effects of this transgene under non-diseased conditions, effectuating a safer mode of IL-4 gene therapy for RA. Adriaansen, Khoury and co-workers constructed adeno-associated viral vectors encoding human soluble p55 TNF receptor coupled to the Fc part of murine IgG1 under control of a minimal CMV promoter containing six upstream NF-κB binding motifs derived from the HIV-LTR promoter. Using a local intra-articular gene therapy approach, this construct delayed onset and decreased the incidence of CIA in mice [30]. The NF-κB-responsive promoter demonstrated a transient responsiveness to repetitive LPS injections at one and eight weeks post-transduction, respectively. In addition, the same vector was used to for local gene therapeutic treatment of adjuvant arthritis in rats [31]. Adriaansen and co-workers found a superior therapeutic effect for disease-regulated expression of transgenes and suggested his phenomenon to arise from a more favorable expression profile during the disease course.

Nitric oxide (NO) is produced by many cell types and has been implicated in host defense and immunity, including modulation of inflammatory processes. The compound is synthesized via nitric oxide synthases (NOS) and the inducible isoform (*iNOS*) is predominantly expressed after exposure to pro-inflammatory stimuli. The induction of *iNOS* is not a disease-specific event and has been demonstrated in several autoimmune and inflammatory diseases such as sepsis, arthritides, systemic lupus erythematosus and Type 1 diabetes [32]. Transgenic *iNOS*-luciferase mice showed a transient upregulation of luciferase activity in knee joints upon injection with zymosan. Luciferase expression peaked at 4 h after the challenge, showing six-fold upregulation, and returned to basal levels after 24 h [33]. This group also created a transgenic luciferase-reporter mouse for imaging of angiogenesis using the murine vascular endothelial growth factor receptor 2 (*Vegfr2*) [34]. Since angiogenesis is a hallmark of

a variety of inflammatory diseases, including RA, Vegf (receptor) promoters can serve as disease-regulated promoters for gene therapy. Considering the co-dependence of angiogenesis and chronic inflammation [35], angiogenesis-specific promoters may not be most suitable for warranting a rapid response to acute inflammatory processes. Prostaglandins (PGs), particularly PGE2 and prostacyclin (PGI2), are potent mediators of pain and inflammation. PGs are derived from arachidonic acid metabolism through constitutive and inducible cyclooxygenases: COX-1 and COX-2, respectively. An interesting development is the use of the *COX-2* promoter for the replication control of conditionally replicating adenoviruses (CRAds) [36]. CRAds are based on placement of essential early adenoviral E1 genes under specific promoters to mediate oncolytic potency on tumor cells. The COX-2-dependent CRAds might be applied for genetic synovectomy in a regulated fashion by killing the synovial cells when becoming active during inflammation. Rachakonda and co-workers used the canine *COX-2* (–1145/+93) promoter for cytokine-inducible expression of canine IL-4 in articular chondrocytes [37]. For the development of disease-specific gene therapy for canine osteoarthritis, Campbell and colleagues applied the matrix metalloproteinase-9 (*MMP9*) (–1984/-1) promoter [38]. In a human chondrosarcoma cell line this promoter responded strongly to TNFα but not IL-1β. Interestingly, the transcriptional strength of the promoter was significantly enhanced by addition of three or five tandem-arranged NFκB binding sites. However, the basal promoter activity increased accordingly indicating leakiness of the hybrid promoter *in vitro*.

Bioinformatics-driven promoter design

Undoubtedly, the availability of endogenous or artificial promoters that confer a range of transcriptional activities in an inflammation-regulated fashion would contribute substantially to tailor-made gene therapy. While the aforementioned studies have provided compelling evidence for the value of disease-regulated gene therapy, the number of experimentally-verified promoters is fairly low. In addition, the actual choice of promoter identity and region has been mostly an educated guess. The latter issues can be addressed by exploiting recent advances in bioinformatics that aid in inferring transcription regulatory networks [39–42] and understanding promoter architecture [43, 44]. Computational analyses of promoter regions from genes specifically expressed in human cartilage or nematode muscle successfully identified the relevant regulatory DNA elements [42, 45]. Using gene expression profiling of target tissues for gene therapy, computational analyses can provide useful information for promoter design. Our laboratory has pursued this approach and performed a gene expression profiling study of synovial tissue from murine CIA to elucidate disease-regulated genes. The proximal (–500/+200) promoter regions of these genes were analyzed with motif scanning algorithms to identify relevant DNA regulatory elements. Transcription factor binding sites for NF-κB, AP-1

and C/EBPβ were significantly enriched and evolutionary conserved in the promoters of arthritis-induced genes. The corresponding promoter regions that contained these motifs conferred inflammation-inducible expression *in vitro* and *in vivo* (unpublished observations). The strength of these approaches have additionally been demonstrated by the Pleiades promoter project, which is aimed at designing well-defined human promoters for brain region or cell type-specific gene therapy [46–48]. These studies focused on profiling region-enriched gene expression within 17 key areas of the adult mouse brain and used bioinformatics tools to elucidate the transcription factor combinations governing expression profiles. Li and co-workers managed to elucidate a regulatory network comprising 15 transcription factors and 153 target genes within the mouse brain, whose promoters or DNA regulatory elements will be tested for promoter design in brain-specific gene therapy [49].

Transcriptional amplification strategy

In the above computational approach promoter selection is based on their expression profile and promoter strength as determined by the level of their inducibility to obtain an efficacious gene therapy strategy. However, to obtain a side effect-free physiological response the basal expression of candidate promoters must be low and show no leakiness. To reach all these prerequisites a transcriptional amplification strategy might be necessary to integrate in the inflammation-responsive system. In order to couple inflammation-inducibility with sufficiently high expression levels required to achieve biological effects, Varley and Munford developed a two-component expression system in which the *C3* promoter regulates the production of the human immunodeficiency virus-1 (HIV) transactivator of transcription (Tat) protein, which in turn regulates the HIV-1 long terminal repeat (LTR) promoter to express the gene of interest [50]. This system was highly responsive towards various inflammatory stimuli as TNFα, IL-1β, IL-6, and LPS/turpentine-induced peritonitis. The HIV-LTR is also directly responsive to nuclear factor-kappaB (NF-κB) activating cytokines (IL-1/TNF) and other factors such as LPS. More importantly it was demonstrated that this two-component construct responded to an inflammatory reaction in a similar fashion in different organs as liver, spleen, kidney, lung and heart. We found that this system was also highly responsive in the joint towards a zymosan-induced joint inflammation in an IL-6-dependent fashion [51]. Miagkov and co-workers and our laboratory independently demonstrated the feasibility of this two-component system for auto-regulated expression of therapeutics in experimental arthritis models [51, 52]. Intra-articular injection of adenoviruses containing the *C3*-Tat/HIV-hIL-10 construct in arthritic paws of rats completely prevented reactivation of arthritis by an intravenous challenge with group A streptococcal peptidoglycan-polysaccharide (PG-APS) [52]. We used this system for adenoviral overexpression of human interleukin-1 receptor antagonist (IL-1Ra) in knee joints of murine col-

lagen-induced arthritis (CIA) and compared this with the effect of IL-1Ra expression under direct control of the conventional CMV promoter. In a prophylactic regimen we could demonstrate superior effectiveness of this two-component IL-1Ra expression system in CIA. While the *in vivo* studies using the Tat/HIV based-approach provided proof of principle of the efficacy of local disease-regulated gene therapy for arthritis, side effects of Tat expression in host cells including dysregulation of cytokine expression [53, 54] and promotion of chemotaxis [55] limit its applicability for a safe and long-term gene therapeutic treatment.

There are several new drug-inducible expression systems developed based on tetracycline, rapamycin and ecdysone that allows reversible and adjustable expression by an exogenous stimulus (extensively reviewed in [56]). These regulatable systems can be combined with cell-specific or physiologically responsive promoters for regulation of their respective transactivating proteins. Despite their robustness and specificity, long-term application of these systems might be hampered due to an immune-response against the artificial transactivators as has been described for tetracycline-regulated systems in larger animals [57, 58]. Regulating the transactivator expression using inducible promoters with low basal activities may delay or even prevent this immune-response and prolong the *in vivo* life time of these systems. Furthermore, these drug-controlled systems provide an additional safety switch to shut-down the system in case of malfunction or deleterious effects.

Conditional RNA interference-based gene therapy

Until now we reviewed the transcriptional targeting strategy for tunable expression of proteins but we envisage the application of inflammation-controlled promoters for development of a gene therapeutic treatment relying on conditional RNA interference-mediated gene silencing. Gene knockdown in experimental models of arthritis has been shown to be therapeutically efficacious [59, 60] but a systemic mode of treatment gives rise to the risk of impairing the normal physiological responses required to combat pathogens and injuries. Therefore, conditional knockdown of endogenous genes using inflammation-responsive promoters would be advantageous. However, viral systems for temporal and conditional knockdown are almost exclusively based on drug-controllable (tetracycline) expression of RNA polymerase (Pol) III promoter-driven short hairpin (sh) RNAs [61]. Due to tight restrictions for transcriptional start sites and the termination signal only a relative small number of RNA Pol II promoters are able to produce effective silencing (si) RNAs using the shRNA system. In contrast, microRNA-adapted shRNA systems, in which mature miRNA sequences are replaced with gene-specific duplexes, have no such limitations and therefore can accommodate Pol II-dependent cell- or conditions-specific promoters. Stegmeier and co-workers developed a sophisticated lentiviral platform for simultaneous production of a miR30-

adapted shRNA and transgene [62], which could be adapted for developing a dual-approach gene therapy. However, attempts for conditional expression of miRNAs using other than exogenously-controlled systems have been rarely undertaken. Yang and Paschen used the human heat shock protein 70 (*HSP70*) promoter for heat shock-induced silencing of genes [63]. Since the expression of *HSP70* is upregulated in almost all inflammatory diseases [64], this represents a strong candidate promoter for inflammation-induced gene silencing. Alternatively, Yoshizaki and co-workers used the human E-selectin (–160/+1) promoter for conditional knockdown of the *SELE* gene [65]. The promoter showed a two-fold increase in activity upon stimulation with TNFα, which proved sufficient for effectively reducing E-selectin expression.

Future perspectives

The efficacy of gene therapy for RA has been demonstrated extensively in animal models of disease using a variety of vectors and transgenes. These promising preclinical results have until now led up to the initiation of five human Phase I clinical trials in RA [66]. Without exception, these trials relied on strong constitutive viral promoters, e.g., the Moloney murine leukemia LTR [67, 68] and CMV [69] promoter. This indicates that the selection of an appropriate promoter remains an underestimated aspect in the development of gene therapeutic treatment for arthritis. In contrast, transcriptional targeting has been recognized as an essential prerequisite towards safe human gene therapy in other diseases, predominantly cancer. Gene therapy for inflammatory diseases is, however, not based on gene correction as in monogenic diseases but on restoring the balance by upregulating therapeutic – or downregulating disease-process implicated genes. In general, the latter genes often have important regulatory roles in normal physiology and immunity that needs to be maintained. For instance, current systemic anti-TNF-treatment in RA has been shown to cause side effects such as opportunistic infections, particularly tuberculosis, in a number of patients [70, 71]. For this, local and transcriptionally targeted gene therapy may circumvent the current problems seen with systemic delivery of protein biologicals. We have reviewed that hijacking the local inflammatory gene regulation is a feasible approach for transcriptional targeting, which has demonstrated efficacy in animal models. These studies are summarized in Table 1. Together with transductional targeting via vector modification or detargeting transgene expression from non-target tissues using miRNA-regulatory elements [72, 73], inflammation-responsive promoters will develop into a safer and efficient mode of gene therapy that is essential for treatment of non-lethal diseases.

Table 1. Inflammation-responsive promoters used in gene therapy approaches

Promoter	Vector	Inflammation model	Species	Ref.
Saa3	Adenovirus	LPS i.p. and turpentine s.c.	Mouse	[74]
C3	Adenovirus	LPS i.p. and turpentine s.c.	Mouse	[50–52,74]
CRP	Transgenic	LPS i.p.	Mouse	[16]
Saa1	Transgenic	LPS/TNFα i.p. and zymosan i.a.	Mouse	[17]
IL-1E/IL-6P	Adenovirus	SCW/zymosan i.a. and CIA	Mouse	[5,7]
6xNFkB/mCMV	Adeno-associated virus	Adjuvant arthritis	Rat	[31]
	Adeno-associated virus	CIA	Mice	[30]
iNOS	Transgenic	LPS/IFNγ i.p. and zymosan i.a.	Mice	[33]
Vegfr2	Transgenic	Oxazolone topical	Mice	[34]
cCox2	Plasmid	*In vitro*, TNFα/IL-1β	Dog	[37]
cMMP9	Plasmid	*In vitro*, TNFα/IL-1β	Human	[38]
HSP70	Plasmid	*In vitro*, heat shock	Mouse/Rat	[63]
SELE	Plasmid	*In vitro*, TNFα	Human	[65]

Abbreviations: LPS, lipopolysaccharide; SCW, streptococcal cell wall; CIA, collagen-induced arthritis; i.a., intra-articular; i.p., intraperitoneal; s.c., subcutaneous

References

1 Chen P, Tian J, Kovesdi I, Bruder JT (2008) Promoters influence the kinetics of transgene expression following adenovector gene delivery. *J Gene Med* 10: 123–131
2 Brooks AR, Harkins RN, Wang P, Qian HS, Liu P, Rubanyi GM (2004) Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med* 6: 395–404
3 Adriaansen J, Kuhlman RR, van HJ, Kaynor C, Vervoordeldonk MJ, Tak PP (2006) Intraarticular interferon-beta gene therapy ameliorates adjuvant arthritis in rats. *Hum Gene Ther* 17: 985–996
4 Adriaansen J, Fallaux FJ, de Cortie CJ, Vervoordeldonk MJ, Tak PP (2007) Local delivery of beta interferon using an adeno-associated virus type 5 effectively inhibits adjuvant arthritis in rats. *J Gen Virol* 88: 1717–1721
5 Geurts J, Arntz OJ, Bennink MB, Joosten LA, van den Berg WB, van de Loo FA (2007) Application of a disease-regulated promoter is a safer mode of local IL-4 gene therapy for arthritis. *Gene Ther* 14: 1632–1638
6 Pan RY, Xiao X, Chen SL, Li J, Lin LC, Wang HJ, Tsao YP (1999) Disease-inducible transgene expression from a recombinant adeno-associated virus vector in a rat arthritis model. *J Virol* 73: 3410–3417
7 van de Loo FA, de Hooge AS, Smeets RL, Bakker AC, Bennink MB, Arntz OJ, Joosten LA, van Beuningen HM, van der Kraan PK, Varley AW et al. (2004) An inflammation-inducible adenoviral expression system for local treatment of the arthritic joint. *Gene Ther* 11: 581–590
8 Weber EL, Cannon PM (2007) Promoter choice for retroviral vectors: transcriptional strength *versus* trans-activation potential. *Hum Gene Ther* 18: 849–860
9 Zychlinski D, Schambach A, Modlich U, Maetzig T, Meyer J, Grassman E, Mishra A, Baum C (2008) Physiological promoters reduce the genotoxic risk of integrating gene vectors. *Mol Ther* 16: 718–725
10 Lubberts E, Joosten LA, Chabaud M, van den Bersselaar L, Oppers B, Coenen-de Roo CJ, Richards CD, Miossec P, van den Berg WB (2000) IL-4 gene therapy for collagen arthritis suppresses synovial IL-17 and osteoprotegerin ligand and prevents bone erosion. *J Clin Invest* 105: 1697–1710

11 Blaney Davidson EN, Vitters EL, van den Berg WB, van der Kraan PM (2006) TGF beta-induced cartilage repair is maintained but fibrosis is blocked in the presence of Smad7. *Arthritis Res Ther* 8, R65
12 Bakker AC, van de Loo FA, van Beuningen HM, Sime P, van Lent PL, van der Kraan PM, Richards CD, van den Berg WB (2001) Overexpression of active TGF-beta-1 in the murine knee joint: evidence for synovial-layer-dependent chondro-osteophyte formation. *Osteoarthritis Cartilage* 9: 128–136
13 Robson T, Hirst DG (2003) Transcriptional targeting in cancer gene therapy. *J Biomed Biotechnol* 110–137
14 Guo ZS, Li Q, Bartlett DL, Yang JY, Fang B (2008) Gene transfer: the challenge of regulated gene expression. *Trends Mol Med* 14: 410–418
15 Suffredini AF, Fantuzzi G, Badolato R, Oppenheim JJ, O'Grady NP (1999) New insights into the biology of the acute phase response. *J Clin Immunol* 19: 203–214
16 Burke B, Pridmore A, Harraghy N, Collick A, Brown J, Mitchell T (2004) Transgenic mice showing inflammation-inducible overexpression of granulocyte macrophage colony-stimulating factor. *Clin Diagn Lab Immunol* 11: 588–598
17 Zhang N, Ahsan MH, Purchio AF, West DB (2005) Serum amyloid A-luciferase transgenic mice: response to sepsis, acute arthritis, and contact hypersensitivity and the effects of proteasome inhibition. *J Immunol* 174: 8125–8134
18 van de Loo FA, Joosten LA, van Lent PL, Arntz OJ, van den Berg WB (1995) Role of interleukin-1, tumor necrosis factor alpha, and interleukin-6 in cartilage proteoglycan metabolism and destruction. Effect of *in situ* blocking in murine antigen- and zymosan-induced arthritis. *Arthritis Rheum* 38: 164–172
19 Klimiuk PA, Sierakowski S, Latosiewicz R, Cylwik JP, Cylwik B, Skowronski J, Chwiecko J (2003) Interleukin-6, soluble interleukin-2 receptor and soluble interleukin-6 receptor in the sera of patients with different histological patterns of rheumatoid synovitis. *Clin Exp Rheumatol* 21: 63–69
20 Okamoto H, Yamamura M, Morita Y, Harada S, Makino H, Ota Z (1997) The synovial expression and serum levels of interleukin-6, interleukin-11, leukemia inhibitory factor, and oncostatin M in rheumatoid arthritis. *Arthritis Rheum* 40: 1096–1105
21 Uson J, Balsa A, Pascual-Salcedo D, Cabezas JA, Gonzalez-Tarrio JM, Martin-Mola E, Fontan G (1997) Soluble interleukin 6 (IL-6) receptor and IL-6 levels in serum and synovial fluid of patients with different arthropathies. *J Rheumatol* 24: 2069–2075
22 Madson KL, Moore TL, Lawrence JM III, Osborn TG (1994) Cytokine levels in serum and synovial fluid of patients with juvenile rheumatoid arthritis. *J Rheumatol* 21: 2359–2363
23 Aikawa Y, Morimoto K, Yamamoto T, Chaki H, Hashiramoto A, Narita H, Hirono S, Shiozawa S (2008) Treatment of arthritis with a selective inhibitor of c-Fos/activator protein-1. *Nat Biotechnol* 26: 817–823
24 Firestein GS, Manning AM (1999) Signal transduction and transcription factors in rheumatic disease. *Arthritis Rheum* 42: 609–621
25 Han Z, Boyle DL, Chang L, Bennett B, Karin M, Yang L, Manning AM, Firestein GS (2001) c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis. *J Clin Invest* 108: 73–81
26 Li Q, Verma IM (2002) NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2: 725–734
27 Tak PP, Gerlag DM, Aupperle KR, van de Geest DA, Overbeek M, Bennett BL, Boyle DL, Manning AM, Firestein GS (2001) Inhibitor of nuclear factor kappaB kinase beta is a key regulator of synovial inflammation. *Arthritis Rheum* 44: 1897–1907
28 Nishioka K, Ohshima S, Umeshita-Sasai M, Yamaguchi N, Mima T, Nomura S, Murata N, Shimizu M, Miyake T, Yoshizaki K et al. (2000) Enhanced expression and DNA binding activity of two CCAAT/enhancer-binding protein isoforms, C/EBPbeta and C/EBPdelta, in rheumatoid synovium. *Arthritis Rheum* 43: 1591–1596
29 Pope RM, Lovis R, Mungre S, Perlman H, Koch AE, Haines GK III (1999) C/EBP beta in rheumatoid arthritis: correlation with inflammation, not disease specificity. *Clin Immunol* 91: 271–282
30 Khoury M, Adriaansen J, Vervoordeldonk MJ, Gould D, Chernajovsky Y, Bigey P, Bloquel C, Scherman D, Tak PP, Jorgensen C et al. (2007) Inflammation-inducible anti-TNF gene expression mediated by intra-articular injection of serotype 5 adeno-associated virus reduces arthritis. *JGene Med* 9: 596–604

31 Adriaansen J, Khoury M, de Cortie CJ, Fallaux FJ, Bigey P, Scherman D, Gould DJ, Chernajovsky Y, Apparailly F, Jorgensen C et al. (2007) Reduction of arthritis following intra-articular administration of an adeno-associated virus serotype 5 expressing a disease-inducible TNF-blocking agent. *Ann Rheum Dis* 66: 1143–1150
32 Clancy RM, Amin AR, Abramson SB (1998) The role of nitric oxide in inflammation and immunity. *Arthritis Rheum* 41: 1141–1151
33 Zhang N, Weber A, Li B, Lyons R, Contag PR, Purchio AF, West DB (2003) An inducible nitric oxide synthase-luciferase reporter system for *in vivo* testing of anti-inflammatory compounds in transgenic mice. *JImmunol* 170: 6307–6319
34 Zhang N, Fang Z, Contag PR, Purchio AF, West DB (2004) Tracking angiogenesis induced by skin wounding and contact hypersensitivity using a Vegfr2-luciferase transgenic mouse. *Blood* 103: 617–626
35 Jackson JR, Seed MP, Kircher CH, Willoughby DA, Winkler JD (1997) The codependence of angiogenesis and chronic inflammation. *FASEB J* 11: 457–465
36 Kanerva A, Bauerschmitz GJ, Yamamoto M, Lam JT, Alvarez RD, Siegal GP, Curiel DT, Hemminki A (2004) A cyclooxygenase-2 promoter-based conditionally replicating adenovirus with enhanced infectivity for treatment of ovarian adenocarcinoma. *Gene Ther* 11: 552–559
37 Rachakonda PS, Rai MF, Schmidt MF (2008) Application of inflammation-responsive promoter for an *in vitro* arthritis model. *Arthritis Rheum* 58: 2088–2097
38 Campbell SE, Bennett D, Nasir L, Gault EA, Argyle DJ (2005) Disease- and cell-type-specific transcriptional targeting of vectors for osteoarthritis gene therapy: further development of a clinical canine model. *Rheumatology (Oxford)* 44: 735–743
39 Wang T, Stormo GD (2003) Combining phylogenetic data with co-regulated genes to identify regulatory motifs. *Bioinformatics* 19: 2369–2380
40 Wasserman WW, Sandelin A (2004) Applied bioinformatics for the identification of regulatory elements. *Nat Rev Genet* 5: 276–287
41 Zhang MQ (2007) Computational analyses of eukaryotic promoters. *BMCBioinformatics* 8 Suppl 6, S3
42 Zhao G, Schriefer LA, Stormo GD (2007) Identification of muscle-specific regulatory modules in *Caenorhabditis elegans*. *Genome Res* 17: 348–357
43 Carninci P, Sandelin A, Lenhard B, Katayama S, Shimokawa K, Ponjavic J, Semple CA, Taylor MS, Engstrom PG, Frith MC et al. (2006) Genome-wide analysis of mammalian promoter architecture and evolution. *Nat Genet* 38: 626–635
44 Ponjavic J, Lenhard B, Kai C, Kawai J, Carninci P, Hayashizaki Y, Sandelin A (2006) Transcriptional and structural impact of TATA-initiation site spacing in mammalian core promoters. *Genome Biol* 7, R78
45 Davies SR, Chang LW, Patra D, Xing X, Posey K, Hecht J, Stormo GD, Sandell LJ (2007) Computational identification and functional validation of regulatory motifs in cartilage-expressed genes. *Genome Res* 17: 1438–1447
46 D'Souza CA, Chopra V, Varhol R, Xie YY, Bohacec S, Zhao Y, Lee LL, Bilenky M, Portales-Casamar E, He A et al. (2008) Identification of a set of genes showing regionally enriched expression in the mouse brain. *BMCNeurosci* 9, 66
47 Portales-Casamar E, Kirov S, Lim J, Lithwick S, Swanson MI, Ticoll A, Snoddy J, Wasserman WW (2007) PAZAR: a framework for collection and dissemination of cis-regulatory sequence annotation. *Genome Biol* 8, R207
48 Yang GS, Banks KG, Bonaguro RJ, Wilson G, Dreolini L, de Leeuw CN, Liu L, Swanson DJ, Goldowitz D, Holt RA et al. (2009) Next generation tools for high-throughput promoter and expression analysis employing single-copy knock-ins at the Hprt1 locus. *Genomics* 93: 196–204
49 Li J, Liu ZJ, Pan YC, Liu Q, Fu X, Cooper NG, Li Y, Qiu M, Shi T (2007) Regulatory module network of basic/helix-loop-helix transcription factors in mouse brain. *Genome Biol* 8, R244
50 Varley AW, Geiszler SM, Gaynor RB, Munford RS (1997) A two-component expression system that responds to inflammatory stimuli *in vivo*. *Nat Biotechnol* 15: 1002–1006
51 Bakker AC, van de Loo FA, Joosten LA, Arntz OJ, Varley AW, Munford RS, van den Berg WB (2002) C3-Tat/HIV-regulated intraarticular human interleukin-1 receptor antagonist gene therapy results in efficient inhibition of collagen-induced arthritis superior to cytomegalovirus-regulated expression of the same transgene. *Arthritis Rheum* 46: 1661–1670
52 Miagkov AV, Varley AW, Munford RS, Makarov SS (2002) Endogenous regulation of a therapeutic transgene restores homeostasis in arthritic joints. *JClin Invest* 109: 1223–1229

53 Chen P, Mayne M, Power C, Nath A (1997) The Tat protein of HIV-1 induces tumor necrosis factor-alpha production. Implications for HIV-1-associated neurological diseases. *JBiol Chem* 272: 22385–22388
54 Nath A, Conant K, Chen P, Scott C, Major EO (1999) Transient exposure to HIV-1 Tat protein results in cytokine production in macrophages and astrocytes. A hit and run phenomenon. *JBiol Chem* 274: 17098–17102
55 Park IW, Wang JF, Groopman JE (2001) HIV-1 Tat promotes monocyte chemoattractant protein-1 secretion followed by transmigration of monocytes. *Blood* 97: 352–358
56 Toniatti C, Bujard H, Cortese R, Ciliberto G (2004) Gene therapy progress and prospects: transcription regulatory systems. *Gene Ther* 11: 649–657
57 le Guiner C, Stieger K, Snyder RO, Rolling F, Moullier P (2007) Immune responses to gene product of inducible promoters. *Curr Gene Ther* 7: 334–346
58 Stieger K, Belbellaa B, le Guiner C, Moullier P, Rolling F (2009) *In vivo* gene regulation using tetracycline-regulatable systems. *Adv Drug Deliv Rev* 61: 527–541
59 Courties G, Presumey J, Duroux-Richard I, Jorgensen C, Apparailly F (2009) RNA interference-based gene therapy for successful treatment of rheumatoid arthritis. *Expert Opin Biol Ther* 9: 535–538
60 Khoury M, Escriou V, Courties G, Galy A, Yao R, Largeau C, Scherman D, Jorgensen C, Apparailly F (2008) Efficient suppression of murine arthritis by combined anticytokine small interfering RNA lipoplexes. *Arthritis Rheum* 58: 2356–2367
61 Wiznerowicz M, Szulc J, Trono D (2006) Tuning silence: conditional systems for RNA interference. *Nat Methods* 3: 682–688
62 Stegmeier F, Hu G, Rickles RJ, Hannon GJ, Elledge SJ (2005) A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. *Proc Natl Acad Sci USA* 102: 13212–13217
63 Yang W, Paschen W (2008) Conditional gene silencing in mammalian cells mediated by a stress-inducible promoter. *Biochem Biophys Res Commun* 365: 521–527
64 van Eden W, van der Zee R, Prakken B (2005) Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol* 5: 318–330
65 Yoshizaki K, Wakita H, Takeda K, Takahashi K (2008) Conditional expression of microRNA against E-selectin inhibits leukocyte-endothelial adhesive interaction under inflammatory condition. *Biochem Biophys Res Commun* 371: 747–751
66 Evans CH, Ghivizzani SC, Robbins PD (2009) Orthopedic gene therapy in 2008. *Mol Ther* 17: 231–244
67 Evans CH, Robbins PD, Ghivizzani SC, Wasko MC, Tomaino MM, Kang R, Muzzonigro TA, Vogt M, Elder EM, Whiteside TL et al. (2005) Gene transfer to human joints: progress toward a gene therapy of arthritis. *Proc Natl Acad Sci USA* 102: 8698–8703
68 Wehling P, Reinecke J, Baltzer AA, Granrath M, Schulitz KP, Schultz C, Krauspe R, Whiteside T, Elder E, Ghivizzani SC et al. (2009) Clinical responses to gene therapy in joints of two subjects with rheumatoid arthritis. *Hum Gene Ther* 20: 97–101
69 Chan JM, Villarreal G, Jin WW, Stepan T, Burstein H, Wahl SM (2002) Intraarticular gene transfer of TNFR:Fc suppresses experimental arthritis with reduced systemic distribution of the gene product. *Mol Ther* 6: 727–736
70 Atzeni F, Bendtzen K, Bobbio-Pallavicini F, Conti F, Cutolo M, Montecucco C, Sulli A, Valesini G, Sarzi-Puttini P (2008) Infections and treatment of patients with rheumatic diseases. *Clin Exp Rheumatol* 26, S67–S73
71 Favalli EG, Desiati F, Atzeni F, Sarzi-Puttini P, Caporali R, Pallavicini FB, Gorla R, Filippini M, Marchesoni A (2009) Serious infections during anti-TNFalpha treatment in rheumatoid arthritis patients. *Autoimmun Rev* 8: 266–273
72 Brown BD, Venneri MA, Zingale A, Sergi SL, Naldini L (2006) Endogenous microRNA regulation suppresses transgene expression in hematopoietic lineages and enables stable gene transfer. *Nat Med* 12: 585–591
73 Brown BD, Gentner B, Cantore A, Colleoni S, Amendola M, Zingale A, Baccarini A, Lazzari G, Galli C, Naldini L (2007) Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. *Nat Biotechnol* 25: 1457–1467
74 Varley AW, Coulthard MG, Meidell RS, Gerard RD, Munford RS (1995) Inflammation-induced recombinant protein expression *in vivo* using promoters from acute-phase protein genes. *Proc Natl Acad Sci USA* 92: 5346–5350

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Development of AAV vectors for the therapy of autoimmune and inflammatory diseases

Chengwen Li[1], Matthew L. Hirsch[1] and R. Jude Samulski[1,2]

[1] *Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*
[2] *Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA*

Abstract

Adeno-associated virus (AAV) has been used as a gene delivery vehicle in over 60 human trials as a therapy for neurological, blood, lung, inflammatory, and muscle disorders. In these cases, the AAV vector has demonstrated both safety and efficacy; therapeutic transgene expression was observed in humans for greater than 5 years without associated side effects. Within the past decade, great progress has been achieved in AAV vector optimization for enhanced and targeted tissue transduction. Such advancements manifest at two levels, capsid and genome optimization, and include the characterization of new serotypes, rational and random capsid engineering, and the application of self-complementary, and split vector AAV genomes. These optimizations have allowed, not only enhanced and specific tissue transduction, but also the ability to evade the humoral response to the capsid and the delivery of payloads twice the packaging capacity. However, a recent clinical trial has put into question the immunogenicity of the AAV capsid in humans suggesting that transduced cells are often targeted by cytotoxic T cells, thus limiting the approaches efficacy *in vivo*. These advancements and limitations will be introduced and discussed with a focus on AAV's utility for treating auto-immune disorders including preclinical and clinical trials.

Introduction

Successful clinical trials using adeno-associated virus (AAV) vectors for gene delivery have been achieved due to several characteristics. First, AAV infects both dividing and non-dividing cells and has very broad tissue tropism. Secondly, AAV transduction initiates long-term transgene expression from episomal DNA with a very low tendency for host chromosome integration [1, 2]. Since the first AAV clone was generated in 1982, this platform has been extensively studied and refined as a gene therapy vehicle [3]. Although 12 serotypes of AAV have been isolated and characterized for gene delivery, the vector based on serotype 2 (AAV2) has been the most studied and is currently used in the majority of clinical trials for diseases such as hemophilia B, cystic fibrosis, alpha-1 antitrypsin, Canavan disease, Alzheimer's, Parkinson's, muscular dystrophy, rheumatoid arthritis, prostate and melanoma cancers, as well as a potential HIV vaccine [4] (Tab. 1). The clinical trials have shown that the application of AAV vectors is safe and therapeutic effects have been achieved in patients with hemophilia and blind resulted from Leber's congenital amaurosis (LCA) [5, 6]. The first successful clinical trial used AAV2 to deliver the coagulation

factor IX (F9) gene into the liver of patients with hemophilia. In this case therapeutic levels of circulating Factor IX were detected [5]. The second successful trial employed AAV2 vectors to deliver the retinal pigment epithelium-specific 65-kDa protein gene (RPE65) into the subretina for treatment of Leber's congenital amaurosis. All three patients treated with AAV2/RPE65 had modest improvements in retinal function [6]. Recently, a great deal of progress in AAV vector development has been achieved and has allowed the application of AAV vectors for the treatment of autoimmune disorders and inflammatory diseases.

AAV biology

Adeno-associated virus (AAV) is a small (20 nm) non-enveloped virus of the family *Parvoviridae* genus *Dependovirus*. The AAV capsid is packaged with a 4.7 kb single-strand linear DNA genome that is flanked at both ends by 145 nucleotide (nt) inverted terminal repeats (ITRs). The viral genome encodes two viral genes, Rep and Cap, which contain multiple reading frames and splice variants. The Rep proteins are involved in replication and packaging as well as host chromosome integration. The capsid proteins Vp1, Vp2, Vp3 assemble in a ratio of 1:1:10, respectively, to form the 60 subunit icosohedral virion. AAV, by itself, is capable of infection and the genomes persist in the nucleus as double-stranded circular monomers, concatamers and in some cases will integrate at a specific location on human chromosome 19. To initiate viral replication, co-infection by a helper virus, such as adenovirus or herpes virus, is required which ultimately results in packaged AAV capsids. At this point, the AAV particles cannot leave the host cell and rely on other viruses or cellular death for release.

The exact mechanism of AAV transduction is largely unknown, however, the multi-step pathway is initiated by AAV binding to the cell surface via primary receptors and co-receptors [1, 2]. This event results in endocytosis, and once inside the endosome, intact AAV particles accumulate perinuclear. It remains unknown where AAV particle uncoating occurs, although intact particles can be observed in the cell nucleus. The infection pathway results in the presence of single-stranded AAV genomes within the nucleus which must synthesize the other strand by extension of the 3' ITR or through opposite polarity genome annealing. At this point the virus is capable of transcription and host enzymes process the viral ITRs resulting in intra- and inter- AAV genome linkages.

The most detailed information about the AAV transduction pathway was obtained from AAV2 characterization. Heparin sulphate proteoglycan (HSPG) is a primary attachment receptor for AAV2 binding [7], other co-receptors are also involved to mediate particle entry including integrins, fibroblast growth factor receptor 1, hepatocyte growth factor receptor, and the laminin receptor [8–12]. After binding, AAV2 is endocytosed via the clathrin-dependent pathway. The endosomal trafficking pathway is poorly characterized however, the activation of Rac1 protein and the phosphatidylinositol 3 kinase pathway is required [13]. It is generally thought that a structural change of the virus parti-

Table 1. Clinical trials with adeno-associated virus vector

Disease	Transgene	Serotype	Phase	Delivery route
AAT deficiency	AAT	AAV1 AAV2	I	IM
ALS	EAAT2	AAV2	I	Intrathecal
Alzheimer's disease	Beta nerve growth factor	AAV2	I/II	Intracranial
AMD	Unknown	AAV2	I/II	Unknown
Arthritis	TNFR:Fc	AAV2	I	Intraarticular
Canavan's disease	Aspartoacylase	AAV2	I	Intracranial
CF	CFTR	AAV2	I/II	Intranasal, Intrabronchinal
Early onset retinal degeneration	RPE65	AAV2	I	Subretinal
Epilepsy	Neuropeptide Y	AAV2	I	Intracranial
Heart failure	SERCA-2a	AAV1	I	Intracoronary
	Intramyocardial			
Hemophilia B	Factor IX	AAV2	I	IM, Intrahepatic
		scAAV8	I	Intravenous
HIV	HIV gag	AAV2	I	IM
Late infantile neuronal lipofuscinosis	CLN2	AAV2	I	Intraparenchymal
Leber congenital amaurosis	RPE65	AAV2	I/II	Subretinal
Lipoprotein lipase deficiency	LP6	AAV1	I/II	IM
Macular degeneration	sFlt01	AAV2	I	Intravitreal
Melanoma	GM-CSF B7.2	AAV2	I	Intratumoural
Muscular dystrophy	Minidystrophin	AAV2.5	I	IM
	Sarcoglycan	AAV2	I	IM
Nasopharyngeal carcinoma	LMP1, LMP2	AAV2	I	CTL *in vitro*
Parkinson's disease	GAD65	AAV2	I/II	Intracranial
	Neurturin	AAV2	II	Intracranial
	GDNF	AAV2	I	Intracranial
Pompe Disease	GAA	AAV1	I/II	IM
Prostate cancer	GM-CSF	AAV2	I/II/III	Intratumoural
	HSV-TK	AAV2	I/II	Intratumoural
	IL-2	AAV2	I	Unknown

Abbreviations: AAV, adeno-associated virus; AAT, alpha-1 antitrypsin; IM, intramuscular; AMD, age-related macular degeneration; TNFR:Fc, tumor necrosis factor receptor:immunoglobulin Fc fusion gene; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; RPE65-retinal pigment epithelium; SERCA, sacroplasmic reticulum calcium ATPase; sc, self-complementary; HIV, human immunodeficiency virus; CLN, neuronal ceroid lipofuscinosis; GM-CSF, granulocyte monocyte colony stimulating factor; LMP, latent membrane protein derived from Epstein-Barr virus; GAD, glutamic acid decarboxylase; GDNF, glial cell line derived neurotrophic factor; GAA, acid-alpha glycosidase; HSV-TK, herpes simplex virus thymidine kinase; IL-2, interleukin-2

cle occurs within the endosome to expose a phospho-lipase (PLA2) domain of the Vp1 protein [14]. This PLA2 domain may play a role for AAV escape from the late endosome or even perhaps for nuclear entry. In addition, endosomal

cystine proteases, cathepsins B and L, may also be involved in AAV trafficking [15]. After the AAV virion is released from the endosome, it will be ubiquitinated, marking it for degradation [16]. There is little information about remaining steps in the infection pathway until the DNA genomes appear in the nucleus.

Improvement of AAV vector transduction

The AAV vector, or recombinant AAV (rAAV), was created over 20 years ago by replacing the viral genes, *rep* and *cap* gene, with a DNA sequence of interest. In such a construct the only remaining genetic viral elements are the ITRs which allow replication and packaging in conjunction with the Rep and Cap proteins provided *in trans*. As such, the capsid can package at most, the size of the wild type genome (4.7 kb). Since the initial description/discovery of AAV vectors, several achievements have been made to improve transduction at the genome and capsid levels.

Development of double-stranded AAV cassettes

AAV transduction results in the presence of single-stranded genomes, of both polarities, within the host nucleus. At this point the genomes can either anneal or undergo second-strand synthesis to generate a double-stranded template capable of gene transcription. The process of second-strand synthesis was deemed a rate limiting step in AAV transduction in a manner that is perhaps cell type dependent. The generation of self-complementary (or duplex) AAV genomes solved this obstacle [17, 18]. Mutation of the Rep protein nicking site in either one of the ITRs in the AAV plasmid context generates a replication intermediate during Rep-mediated AAV genome excision from the plasmid [18]. This replication intermediate, or self-complementary AAV (scAAV), is essentially an inverted repeat capable of 'snapping back' to form a duplex region capable of initiating transcription immediately upon capsid uncoating (thus bypassing second-strand synthesis). The utilization of a scAAV vector induces faster onset of gene expression and results in 10–100-fold higher transgene synthesis compared to the conventional single-strand AAV vectors *in vitro* and *in vivo* [19]. The substantial increase in the transduction efficiency of scAAV allows a decrease in the dose needed for effective gene transfer. As such, a scAAV vector has recently been approved for a clinical trial to deliver the coagulation factor 9 (F9) to patients with hemophilia B (Tab. 1). A drawback of scAAV is the decreased packaging size of the transgene, which is about half of the size of the wild type capacity (around 2.2 kb). However, a recent study has demonstrated that the scAAV packaging capacity can be larger than 2.2 kb and that a 3.3 kb scAAV2 cassette is successfully packaged [20].

AAV packaging capacity/AAV split vectors

Recently, several investigators have tried to package large genomes into the AAV virion despite that the wild type, or optimal, size is about 4.7 kb. We have demonstrated that a 5.9 kb genome can be packaged into AAV1 to 5 capsids, however, increasing the packaging size is often accompanied by a decrease in the production perhaps due to vector genome partial package [21]. Allocca et al. have reported that an 8.9 kb genome was packaged into a single AAV5 virion [22]. Although these studies have reported the packaging of large genomes, in general, these reports are isolated and the packaging limitation of 4.7 kb remains a major hurdle in AAV therapy applications requiring large transgenes.

To address the AAV capsid packaging limitation, a novel approach has been developed by exploiting the natural ability of the AAV genomes to form concatamers [23–34]. In this approach, termed AAV split vectors, a promoter and a partial transgene coding sequence are packaged separately from the remaining transgene fragment and poly-adenylation sequence. The co-transduction of the mentioned particles results in the nuclear appearance of the gene fragments that can then concatemerize, or undergo homologous recombination, to reconstruct the intact, large (up to 10 kb) transgene intracellular. There are two types of AAV split vectors: i) overlapping vectors that each contain partial gene fragments with a region of overlapping sequence and ii) trans-splicing vectors that contain intron elements to 'buffer' the functional orientation. Despite the success of these approaches *in vivo*, there is no bias for the different genomes to reconstruct the large transgene in the functional orientation. This strategy has been applied to the cDNA sequence of coagulation factor VIII (7 kb), dystrophin (>10 kb) and CFTR (4.5 kb). Optimization of AAV split vectors has been demonstrated by utilization of synthetic introns, rational selection of the gene splitting site and employing two different ITRs in one vector [30, 31, 35]. Also, insertion of a 'recombinogenic' alkaline phosphatase (AP) sequence into AAV cassettes, at the functional junction, has been reported to enhance homologous recombination and thus the efficiency of the approach [28].

AAV serotypes

AAV2 was the first serotype isolated as a contaminant in a preparation of adenovirus. Since then, 12 AAV serotypes and over 100 AAV variants have been isolated from adenovirus stocks or directly from human/primates tissues, some of which have been investigated for gene therapy applications. These novel serotypes and variants demonstrate altered cellular tropism and often can be used to achieve increased transduction [1, 2]. Increased transduction potency results in lower viral doses needed for efficient transduction increasing the clinical safety. In addition, the isolated capsid variants demonstrate differing immune profiles such as the evasion of pre-existing neutralizing antibodies generated from natural infection or prior treatment with AAV-based vectors.

Additionally, they can serve as templates to develop tissue-specific capsids and expand the current range of AAV vectors.

The different AAV serotypes have different tissue tropism and use different machinery for transduction including different receptor and co-receptors for binding the cell surface, intracellular trafficking and capsid uncoating (Tab. 2) [2]. For instance, sialic acid is used by AAV1, 4, 5 and 6 for cell surface binding [36, 37]. The platelet derived growth factor receptor-α and the laminin receptor are used as co-receptors for AAV5, AAV8 and 9, respectively [12, 38]. Also, the kinetics of transgene expression varies from one serotype to another *in vivo* [39]. For example, AAV6 and AAV8 initiate faster transgene expression than AAV2 in mouse liver [39]. In addition, higher transgene expression with one serotype can be achieved compared to other serotypes depending on the specific tissue transduced due to different AAV trafficking after AAV vector binding to cell receptors [2]. For example, AAV1 and AAV7 can induce strong transgene expression in muscle, while AAV8 and AAV9 result in efficient transduction in the heart (Tab. 2) [2].

Table 2. Tissue tropism of AAV vector

Tissue	Serotype (s)
Liver	AAV6, AAV8, AAV9
Skeletal muscle	AAV1, AAV6, AAV7
CNS	AAV1, AAV4, AAV5, AAV9
Eye	AAV2, AAV4, AAV5
Lung	AAV1, AAV5, AAV6, AAV9
Heart	AAV8, AAV9
Pancreas	AAV8, AAV9
Kidney	AAV2

AAV mosaic and chimeric capsids

Mosaic AAV vectors are produced by the assembly of capsid subunits from different serotypes, or from the same serotype with different capsid mutations, to form a single particle. Such a vector combines the advantages of different serotypes (e.g., tropism, cellular trafficking pathway) and, depending on the combination and the target cell, exhibits novel tissue tropism and/or enhanced transgene expression compared to parent serotypes. In addition, these mosaic AAV capsids may be easily purified using a heparin or sialic acid column if the mosaic virions contain capsid subunits from AAV2 or AAV5, respectively. The outstanding example is the AAV1/2 mosaic vector which is generated from capsid subunits of AAV1 and AAV2. This vector binds to heparin and induces higher transduction in the liver and muscles than either AAV1 or AAV2 vector alone [40]. In our lab, Rabinowitz et al. has extended the mosaic vector generation to the combination of serotype 1 to 5 subunits and classified different

groups based on compatibility to form mosaic virions at different subunit ratios [41]. These types of experiments may allow the generation of mosaic AAV virions with novel tropisms and/or high transduction efficiencies using subunits from the newly identified AAV serotypes and variants.

Although mosaic vectors can improve the transduction efficiency and may allow easier purification, there remain limitations to use the approach. First, it is currently not possible to standardize the ratios of subunits assembled in a particle (the current method is based on differing the amount of helper plasmids). Second, mosaic capsids can sometimes offer the worst of both worlds such as the mosaic virus can be neutralized by antibodies against all parent subunits.

Another approach using rational capsid design is the generation of chimeric capsids. In these cases, a single capsid gene contains genetic information from different serotypes, including point mutations and large domain swaps. For example, a chimeric virus was generated by swapping AAV1 capsid amino acids 350 to 430, into the corresponding region of the AAV2 capsid. This AAV1/2 chimeric capsid was able to be purified by a heparin column (like AAV2) and demonstrated high transduction in muscle (similar to AAV1) [42]. By alignment of the capsid sequence of AAV1 and AAV2, we have rationally designed novel mutants of AAV2 virion with muscle tropism by substitution or insertion of AAV1 sequences into the AAV2 capsid. In fact, the first clinical trial using this chimeric virus is underway for the treatment of Duchenne muscular dystrophy (Tab. 1). It is interesting to note that this chimeric virus has a distinct immune profile compared to either parent and does not interact with the neutralizing antibodies from AAV1 or AAV2 immunization. The further characterization of chimeric capsids can also be used to define the domains that are responsible for AAV transduction in specific cells.

Development of targeted AAV vectors

In general, most serotypes of AAV have a broad tissue tropism regardless of their transduction efficiency. For example, AAV8 transduces the liver, muscle, heart and pancreas. Thus, for specific cell transduction, there are two important aspects that need to be reconciled, de-targeting of off-target tissue while targeting the desired tissue. Many approaches have been explored to generate such capsids including cellular receptor targeting, direct evolution coupled to a specific cell selection, insertion of a peptide derived from a phage library into the capsid, and insertion of a random peptide coupled to a specific cell selection.

Receptor targeting via chemical linker

AAV transduction initially relies on the particle binding to the cell surface receptors. However, the usage of different AAV receptors by different sero -

types is, in general, unknown. Therefore to target known receptors in a specific manner several approaches have been explored including chemical cross-linked bi-functional antibodies and genetic manipulation of the capsid gene. The utilization of bi-functional antibodies has been used to bridge AAV2 virions to target a non-permissive cell line. Bartlett et al. used a bispecific $f(ab'\gamma)_2$ antibody which had specific affinity for the AAV2 capsid and the surface receptor $\alpha_{IIb}\beta_3$ integrin to enhance AAV2 transduction on human megakaryoblast cells (DAMI and MO7e) [43]. Ponnazhagan et al. applied a conjugate-based targeting method to increase AAV2 transduction in specific target cells [44]. The strategy was designed to cross-link purified targeting ligands with the high-affinity biotin–avidin interaction as a molecular bridge. The core-streptavidin was fused to a recombinant bi-specific protein which contained the sequence of the human epidermal growth factor (EGF) or the human fibroblast growth factor 1α (FGF 1α) as a target cell ligand. The incubation of biotinylated AAV2 particles with conjugated protein significantly improved AAV2 transduction in SKOV3.ip1 cells and M07e cells, which are EGF receptor-positive or FGF receptor 1 α-positive, respectively [44]. However there are limitations of this approach including the stability and efficiency of the intermediate molecule to interact with the virus and the binding affinity of the intermediate to cell-specific receptors [45].

Receptor targeting via genetic modification

Another approach for receptor targeting is to genetically introduce a targeting peptide into the AAV capsid via insertion or substitution at tolerated locations. Before the elucidation of the AAV three-dimensional structure, capsid domains in which alterations could be accepted were identified by several approaches including: i) random mutagenesis by insertion of a linker throughout the entire AAV2 capsid gene [46, 47], ii) definition of immunogenic regions on the virion surface via incubation of AAV2 neutralizing antibodies with AAV2 virions in the presence of capsid peptides [48], and iii) the alignment of the AAV2 capsid sequence to other parvoviruses with known crystal structures [49, 50].

In a different targeting approach that also relies on sequence insertion into the capsid gene, a single-chain antibody against CD34 was engineered into the N-terminal of Vp2 and particles were produced by mixing the above mutant and wild-type capsid subunits (similar to mosaic capsids) [51]. This modification resulted in increased infectivity on the $CD34^+$ human leukemic cell line KG-1. A similar approach was used to insert the serpin receptor ligand at the N-terminus of Vp2 [46]. This virus demonstrated increased transduction for the lung epithelial cell line IB3 compared to the wild type AAV2 parent [46]. These data indicate that the N-terminus of Vp2 is exposed on the surface of the virion similar to canine parvovirus (CPV) [52, 53] and can tolerate insertions of various sizes.

Based on the alignment of the capsid sequences between AAV2 and CPV, Girod et al. inserted a 14 amino-acid peptide (L14) into six different locations of the AAV capsid (amino acid 261, 381, 447, 534, 573 and 587) [49]. Increased transduction was observed with one insertion mutant (at residue 587) on tumor cell lines exhibiting the L14 specific RGD receptor on the cell surface [49]. Grifman's work further supported Girod's finding by the capsid alignment of AAV2 to AAV serotype 1, 3, 4, and 5 [50]. Later studies revealed that the 587 residue is located on the surface of the AAV2 virion and responsible for heparin sulfate binding [54]. The recent elucidation of the crystal structures for AAV2, and other AAV serotypes, have provided valuable information in regards to genetic modification of the AAV virion surface in order to target specific cells or purify the vectors. In addition to the N-terminus of Vp2 and the heparin binding residue 587 for insertion, Shi et al. inserted the RGD-4C peptide at the 520 residue to eliminate heparin sulphate binding and increase integrin binding, which resulted in altered tropism [55]. Another amenable position of the AAV capsid is the HI loop. Diprimio et al. substituted the HI loop of the AAV2 virion with histidine residues and demonstrated that the insertion did not change virus production or the transduction profile [56].

Receptor targeting via the combination of a chemical linker and genetic modification

Recently, the combination of a chemical linker and genetic manipulation has been performed to target specific cells for transduction or for virus purification. Arnold et al. inserted a small peptide into the AAV capsid and the peptide was biotinylated intracellularly during AAV vector production [57]. Since the biotinylated peptide was exposed on the surface of AAV virion, the generated virus could be used to target cells expressing an artificial avidin–biotin receptor. Gigout et al. inserted a Z34C immunoglobulin (IgG) binding domain at residue 587 of the capsid gene and made AAV vectors in a mosaic approach with the wild type AAV2 capsid [58]. These vectors had the capacity to bind to different antibodies via their Fc regions and selectively transduced MO7e and Jurkat cells at high efficiencies in the presence of antibodies against CD117 or CD29, respectively.

Directed evolution

DNA shuffling and related techniques have been investigated to improve protein stability, yields or novel features, vaccines with strong immunogenicity or the generation of viruses with new properties. This approach was first applied to AAV by Schaffer et al. to develop AAV variants, using an error-prone capsid mutant library and a staggered extension process (analogous to DNA shuf-

fling), to escape AAV2 neutralizing antibodies [59]. We have extended this technique by performing error-prone PCR to amplify capsid genes from nine different AAV serotypes and shuffling them to generate a chimeric/mutant capsid library [60]. Then, the DNA library was used to make an AAV vector library. After cycling transduction of the AAV library on the CS-1 cell line, which is poorly transduced by AAV2 due to lack of the co-receptor $\alpha_v\beta_5$, a unique single infectious clone was isolated and sequenced. The isolated capsid was a chimeric of capsid regions from AAV1, 8, 2, and 9 (dubbed chimeric 1829). *In vitro* experiments demonstrated higher transduction efficiency with chimeric 1829 than any of its parents in CS-1 cells. Consistent with this *in vitro* result, chimeric 1829 induced strong transduction in a xenografted CS-1 tumor, but low transduction in muscles and the liver compared to its parents. Most importantly, chimeric 1829 showed a different immune profile from all parents and neutralizing antibodies induced from chimeric 1829 infection did not react with the parent serotypes or *vice versa* [60]. This AAV shuffling library has been extensively exploited to develop tissue specific variants for heart, airway epithelial cells and liver cells [61, 62].

Insertion of a peptide derived from a phage library into the AAV capsid

Bacteriophages (phages) have been engineered to incorporate specific ligands for mammalian cell transduction. However, these phage-based vectors are, in general, poor gene delivery vehicles. To improve phage for gene delivery and transgene expression in mammalian cells, Arap's group inserted AAV2 ITRs into the phage genome to create an AAV/phage hybrid (termed AAVP) [63]. An AAVP vector displaying an RGD-4C peptide transduced tumor cells very efficiently. The superior transduction efficiency by targeted AAVP over conventional phage-based vectors on mammalian cells is associated with an improved fate of the delivered transgene by formation of transgene cassette concatamers and maintenance of the transgene stability [63]. The application of such vectors can be expanded by the successful isolation of targeting peptides to the murine brain and kidney vasculature from a phage display library [64].

Insertion of random peptides into the AAV capsid

Another approach to re-target the capsid is the use of random peptides inserted at a tolerated and surface exposed capsid location. To this end, Perabo et al. established a library of mutant AAV capsids with random peptide insertions at amino acid 587 of the AAV2 capsid [65]. This mutant AAV capsid library was used to select a receptor targeting specific cells. After multiple rounds of selection on different AAV2 non-permissive cell lines, infectious mutant capsids were obtained [65]. These resulting mutants demonstrated about 100-fold higher transduction efficiency as compared to the wild type AAV2 parent.

Cellular immune response to AAV vectors

The AAV capsid mounts a very strong humoral immune response in humans and mice. In fact, 35–70% of the human population has neutralizing antibodies against AAV2 [66]. As described above, technologies, such as direct-evolution and rational design of the AAV vector or the use of recently described serotypes can be used for neutralizing antibody evasion [59]. In regards to innate immunity, rAAV induces less of a response than adenoviral vectors following liver transduction [67]. However, a recent study demonstrated the activation of inflammatory cytokine gene expression after macrophage uptake of AAV due to AAV interactions with the complement component C3 [68].

In AAV vectors, all viral genes are replaced with a therapeutic cassette, such that the only remaining *cis* elements are the ITRs. Such genomes are capable of long-term transgene expression (at this point 5 yrs in human, 7 yrs in canine and 9 yrs in primate subjects). As such, it has been thought that the AAV transduction does not elicit a cytotoxic T lymphocyte (CTL) response against the capsid. However, that assumption has been recently challenged by the results of a single patient given rAAV therapy for severe hemophilia B [5]. rAAV2 vectors, expressing clotting factor IX cDNA from a liver specific promoter, were injected into the hepatic artery. Increased circulating factor IX was detected within the first week and peaked to therapeutic values 14 days after administration. This peak level of circulating factor IX was largely unchanged up to week 4, however levels slowly returned to baseline values over the following 8 weeks. Concomitant with the factor IX decrease were increased liver transaminases, which indicate liver damage. Further examination of this result detected CTLs specific for the AAV2 capsid but not for factor IX, the transgene product. From these results it was suggested that a CTL response specific for the AAV capsid mediated elimination of AAV2/factor IX vector transduced hepatocytes and, in turn, resulted to the therapeutic failure [5]. Subsequent work confirmed a CTL response to the AAV2 capsid using an adenovirus vector to deliver the AAV capsid gene or direct injection of AAV2 vectors into animal models [69, 70]. However, inconsistent to what was observed in the clinical trial, the capsid specific CTLs, although detectable, could not eliminate AAV transduced target cells in mouse models [71, 72]. The discrepancies between the results from different animal models with the observation from the patient in the AAV/factor IX clinical trial may be reconciled by alternative explanations, such as the contamination of the human vector preparation with wild type replication-competent AAV2. Another possible explanation is that the immunogenicity of the AAV vector differs between the tested animal model and in human subjects [73]. A better understanding of the mechanisms of AAV2 capsid cross-presentation following transduction, has allowed the exploration of effective approaches to prevent the elimination of AAV2 vector transduced target cells by capsid specific CTLs.

Therapy of autoimmune diseases by AAV-mediated gene delivery

Autoimmune diseases arise from over-reactive immune responses to self-antigens. Traditionally, such diseases are treated with drugs that inhibit the host's global immune response, termed immunosuppressants. However, a better understanding of autoimmune disease development regarding the balance of the expression of pro-inflammatory cytokines has led to the promise of AAV gene therapy applications for autoimmune diseases. In such cases, the delivery of a therapeutic gene locally, or systemically, decreases the overexpression of inflammatory proteins or alternatively, induce tolerance to the self-antigen. AAV vectors can also be used to express therapeutic molecules that block the interaction of CTL effector cells with target cells.

Arthritis

Rheumatoid arthritis (RA) is a systemic autoimmune disorder associated with the over-production of inflammatory cytokines that principally manifests in the joints of those afflicted. The development of RA is associated with increased production of cytokines including tumor necrosis factor (TNF)-α and interleukin (IL)-1, which induce potent pro-inflammatory effects and contribute to the pathogenesis of the disease. Although TNF-α seems to be the major cytokine in the inflammatory process, IL-1 is the key mediator with regards to cartilage and bone destruction. To block TNF-α function, Zhang et al. administrated intra-articularly AAV2 vectors encoding the human soluble TNFR1 gene (soluble TNFR1 is the extracellular domain of TNFR1 and sequesters TNF-α) into a transgenic mouse expressing human TNF-α [74]. A significant reduction in the synovial cell hyperplasia and cartilage and bone destruction was observed. More recently it was demonstrated in arthritic rats that intra-muscular injection of an AAV1 vector encoding a rat TNFR-immunoglobulin Fc (TNFR:Fc) fusion gene resulted in sustained circulation of TNFR:Fc for at least a year. In this study, the application of AAV1/TNFR:Fc resulted in complete and long-term suppression of chronic inflammatory disease for at least 6 months following vector administration [75]. In two different animal models, these collective results demonstrate that AAV vectors used to deliver a soluble TNFR dramatically reduce the severity of RA. As such, a Phase I clinical trial using TNFR:Fc for the treatment of arthritis has been carried out (Tab. 1). In a similar approach, the use of AAV vectors to deliver an IL-1 receptor antagonist (IL-1Ra) was also recently investigated in a model of arthritis. After injection of scAAV2/IL-1Ra vectors into the joints of rabbits, substantial production of IL-1Ra was detected. The levels of IL-1Ra were adequate to inhibit inflammation in the disease model and offer promise for the translation of this approach for treatment of RA in humans [76].

Beside direct blockage/sequestration of IL-1/TNF, expression of IL-4 and IL-10 has also been shown effective in the treatment of RA by inhibition of

TNF-α and IL-1 expression. As IL-4 has a short half-life *in vivo*, IL-4 expression was investigated following AAV delivery [77]. Intra-muscular injection of AAV/IL-4 vectors in a mouse model of RA demonstrated therapeutic effects including a reduction in paw swelling, attenuated histological synovitis and a delayed onset of arthritis [77]. Anti-angiogenic gene therapy using AAV vectors provide an alternative and new approach for the effective treatment of RA. A direct joint injection of AAV expressing the angiostatin gene efficiently inhibited the development of collagen-induced arthritis in a mouse model [78].

Diabetes

Diabetes mellitus Type 1 (Type 1 diabetes, T1D) results from the destruction of insulin-producing beta cells in the pancreas by an autoimmune response. The lower insulin in circulation leads to high levels of glucose in the blood and urine. The traditional therapy for TID is injection of insulin and utilization of AAV vectors for insulin gene delivery has been investigated. An early study proved efficacy for this approach in which an AAV/insulin vector directly injected into the liver parenchyma of diabetic mice resulted in decreased blood glucose levels [79]. Jindal et al. administered an AAV vector encoding the full-length rat preproinsulin gene in non-obese diabetic (NOD) mice via muscular injection. Insulin mRNA was detected in the injected muscles, and 70% of treated mice had normal blood glucose levels [80]. Recently, Li et al. used AAV to deliver the pancreatic and duodenal homeobox gene 1 (pdx-1), a crucial transcription factor in pancreatic islet development and differentiation, into the liver of STZ-induced diabetic rats via portal vein injection. The expression of insulin was increased in the treated liver and an overall improved disease phenotype was demonstrated by several additional clinical manifestations [81].

T cells are involved in the development of T1D. Specifically, Th1 cells mediate autoimmunity in NOD mice, whereas Th2 and regulatory T (T-reg) cells prevent the development of diabetes. The anti-inflammatory cytokines secreted from Th2 cells, such as IL-4 and IL-10, have been used to prevent the onset of T1D. Goudy et al. injected AAV2/IL-10 into mouse muscle and demonstrated that insulin auto-antibodies and pancreatic insulitis were decreased and islet insulin content was maintained [82]. Rehman et al. used a scAAV8 vector to deliver the murine IL-4 (mIL-4) gene driven by the murine insulin promoter (mIP) to endogenous beta cells in NOD mice [83]. This group used the AAV8 capsid, as it transduces the islet very efficiently, and the scAAV genomes to maximize transduction at different levels. After IP delivery of scAAV8-mIP-IL4, the onset of hyperglycemia in NOD mice was delayed and the severity of insulitis was reduced. It was also shown that normal levels of $CD4^+CD25^+FoxP3^+$ T-reg cells were maintained in the treated NOD mice, in contrast to lower level of these cells in the non-treated NOD mice. Additionally, diabetes induced by splenocytes from NOD mice was blocked by the adoptive transfer of splenocytes from non-diabetic mice treated with scAAV8-

mIP-IL-4 vectors. These results demonstrate that local expression of mIL-4 prevents beta cell destruction and blocks autoimmunity, partly through the regulation of T cell function [83].

Alpha-1 antitrypsin (AAT) deficiency, is often associated with liver and lung complications. AAT has been considered to modulate the immune response in NOD mice including the attenuation of cell-mediated autoimmunity and alteration of the T cell receptor repertoire. After AAV delivery of the AAT gene into the muscle, the intensity of insulitis and the levels of insulin autoantibodies were dramatically reduced [84]. Consequently, a low frequency of diabetes development was observed [84]. AAV vectors were also employed to deliver the gene encoding haeme-oxygenase-1 (HO-1), a potent immunoregulatory enzyme into NOD mice [85]. After intravenous injection of AAV/HO-1, the insulitis and the development of diabetes were decreased, which was related to the prevention of a Th1-mediated response [85].

Glutamic acid decarboxylase (GAD) has been found to be a dominant autoantigen that triggers the development of T1D. Thus, researchers have used AAV vectors to deliver the GAD immunodominant epitope (GAD 500–585) to induce immune tolerance. After injection of AAV/GAD500–585 vectors into muscle, the onset of T1D was delayed and severe insulitis was decreased [86]. The therapeutic effect was associated with increased levels of IL-4, IL-10 and TGF-β. Most importantly, T-reg cells were induced in the GAD immunized mice. This indicates that utilization of rAAV to deliver the auto-antigen $GAD_{500–585}$ induces potent immunological tolerance through active suppression of effector T cells and results in prevention of T1D [86].

In addition to correcting the imbalance of the immune response or providing insulin using AAV vectors, AAV has also been explored to treat diabetes related complications. Chu et al. used AAV vectors to deliver the insulin-like growth factor 1 (IGF-1) into the liver and observed improved mobility in mice with diabetic peripheral neuropathy [87]. To prevent diabetic retinopathy, the soluble VEGF receptor, sflt-1, was delivered into the subretinal space using AAV in the spontaneously diabetic non-obese Torii (SDT) rat. In this study, less changes in avascular area, hyperfluorescein and arterial narrowing was observed [88].

Islet transplantation is an effective treatment for T1D. However, the destruction of the grafted islet is still observed due to persisting alloimmune and autoimmune responses. AAV-mediated gene delivery after islet transplantation has been used to inhibit the host immune response mediated destruction of the grafted islet. After AAV vector delivery of IL-10, significant prolongation of graft survival was observed. IL-10-mediated protection was associated with suppression of T cell activation [89].

Inflammatory bowel diseases

Inflammatory bowel diseases (IBDs), like the name suggests, are a group of inflammatory conditions affecting the colon and small intestine. In general,

AAV2 demonstrates poor transduction in most colon cell lines. Therefore, to improve the AAV2 infection efficiency, AAV2 particles were attached to microbeads and conjugated to concanavalin A (Con A). This platform not only enhanced the transduction efficiency in the colon cell lines, but also demonstrated efficient transduction of an inflamed colon in mice following intracolon administration [90]. The utilization of the more recently described AAV serotypes also improved AAV transduction in intestinal epithelial cells [91]. An additional approach to enhance AAV transduction for IBDs is the conjugation of AAV2 vectors to a heparinized small intestinal submucosa (H-SIS) matrix. Such a composition resulted in AAV2 transduction in culture cells and provides a unique, modified tissue substrate for rAAV2 which can perhaps be used for targeted gene transfer to the intestine [92]. Overall these preliminary results have laid the foundation for the development of AAV vectors for the treatment of IBDs.

Other autoimmune diseases

Sjögren's syndrome is an autoimmune disorder in which there is a destructive immune response against the exocrine glands, which produce both tears and saliva. AAV vector delivery of IL-10 to the salivary glands and muscles markedly improved saliva production in NOD mice [93]. Salivary flow was also improved and pro-inflammatory cytokines were reduced after AAV delivery of a vasoactive intestinal peptide (VIP) into salivary gland as well [94]. These data demonstrate that the AAV vector can be used to deliver therapeutic genes into the salivary glands to treat Sjögren's syndrome.

Systemic lupus erythematosus is a chronic disease in which the immune system attacks primarily connective tissue resulting in inflammation and tissue damage. Ye et al. delivered AAV vectors encoding CTLA-4Ig or CD40Ig into NZB/NZW mice to disrupt the interaction between effector and target cells by blocking co-stimulatory recognition [95]. After injection of AAV8-CTLA-4Ig vectors into neonatal NZB/NZW mice, the onset of lupus was delayed and autoantibody production was suppressed. The combined administration of AAV8-CTLA-4Ig and AAV8-CD40Ig vectors exerted a synergistic therapeutic effect. This result was contributed by inhibiting $CD4^+$ T cell activation. This study demonstrated that delivery of co-stimulatory inhibitor transgenes by AAV vectors can prevent and reverse lupus complications in a murine model [95].

Conclusion

AAV is a safe and efficient vector for delivery of therapeutic genes to treat a wide variety of human diseases (Tab. 1). The development of targeted vectors has facilitated transduction of previously non-permissive tissue while also restricting widespread/off-target transduction which further increases the safe-

ty of rAAV in clinical trials. However, these promising studies have focused on specific receptor targeting, while overlooking the intracellular, and perhaps rate-limiting, aspects of transduction which have been shown to be somewhat variable in different cell types. Thus, an understanding of the AAV intracellular trafficking mechanism will facilitate the development of more efficient AAV vectors. In addition, the utilization of chemical compounds, such as proteasome inhibitors, may improve AAV transduction even further [21]. The immune response to the AAV capsid is another obstacle for the successful advancement of rAAV in clinical trials at two levels; i) pre-existing neutralizing antibodies and partial serotype cross-reactivity and ii) the epitopes thought important for a capsid-mediated T cell response are shared among most serotypes. However, as described herein, recent technologies are addressing these limitations and the elucidation of the AAV transduction mechanism, especially AAV uncoating, will aid in the development of additional strategies. Furthermore, genetically engineered AAV capsids are currently being designed to create a novel AAV vector with enhanced transduction in specific tissue and the capacity to escape the immune response for future clinical applications. In conclusion, the understanding of AAV transduction machinery and development of AAV vectors will expedite their application for human therapy of autoimmune diseases and inflammatory disorders.

References

1 Li C, Bowles DE, van Dyke T, Samulski RJ (2005) Adeno-associated virus vectors: potential applications for cancer gene therapy. *Cancer Gene Ther* 12: 913–925
2 Wu Z, Asokan A, Samulski RJ (2006) Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther* 14: 316–327
3 Samulski RJ, Berns KI, Tan M, Muzyczka N (1982) Cloning of adeno-associated virus into pBR322: rescue of intact virus from the recombinant plasmid in human cells. *Proc Natl Acad Sci USA* 79: 2077–2081
4 http://www.wiley.co.uk/genetherapy/clinical/
5 Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JJ, Ozelo MC, Hoots K, Blatt P, Konkle B et al. (2006) Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med* 12: 342–347
6 Maguire AM, Simonelli F, Pierce EA, Pugh EN Jr, Mingozzi F, Bennicelli J, Banfi S, Marshall KA, Testa F, Surace EM et al. (2008) Safety and efficacy of gene transfer for Leber's congenital amaurosis. *N Engl J Med* 358: 2240–2248
7 Summerford C, Samulski RJ (1998) Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 72: 1438–1445
8 Summerford C, Bartlett JS, Samulski RJ (1999) AlphaVbeta5 integrin: a co-receptor for adeno-associated virus type 2 infection. *Nat Med* 5: 78–82
9 Qing K, Mah C, Hansen J, Zhou S, Dwarki V, Srivastava A (1999) Human fibroblast growth factor receptor 1 is a co-receptor for infection by adeno-associated virus 2. *Nat Med* 5: 71–77
10 Kashiwakura Y, Tamayose K, Iwabuchi K, Hirai Y, Shimada T, Matsumoto K, Nakamura T, Watanabe M, Oshimi K, Daida H (2005) Hepatocyte growth factor receptor is a coreceptor for adeno-associated virus type 2 infection. *J Virol* 79: 609–614
11 Asokan A, Hamra JB, Govindasamy L, Agbandje-McKenna M, Samulski RJ (2006) Adeno-associated virus type 2 contains an integrin alpha5beta1 binding domain essential for viral cell entry. *J Virol* 80: 8961–8969
12 Akache B, Grimm D, Pandey K, Yant SR, Xu H, Kay MA (2006) The 37/67-kilodalton laminin

receptor is a receptor for adeno-associated virus serotypes 8, 2, 3, and 9. *J Virol* 80: 9831–9836
13 Sanlioglu S, Benson PK, Yang J, Atkinson EM, Reynolds T, Engelhardt JF (2000) Endocytosis and nuclear trafficking of adeno-associated virus type 2 are controlled by rac1 and phosphatidylinositol-3 kinase activation. *J Virol* 74: 9184–9196
14 Girod A, Wobus CE, Zadori Z, Ried M, Leike K, Tijssen P, Kleinschmidt JA, Hallek M (2002) The VP1 capsid protein of adeno-associated virus type 2 is carrying a phospholipase A2 domain required for virus infectivity. *J Gen Virol* 83: 973–978
15 Akache B, Grimm D, Shen X, Fuess S, Yant SR, Glazer DS, Park J, Kay MA (2007) A two-hybrid screen identifies cathepsins B and L as uncoating factors for adeno-associated virus 2 and 8. *Mol Ther* 15: 330–339
16 Yan Z, Zak R, Luxton GW, Ritchie TC, Bantel-Schaal U, Engelhardt JF (2002) Ubiquitination of both adeno-associated virus type 2 and 5 capsid proteins affects the transduction efficiency of recombinant vectors. *J Virol* 76: 2043–2053
17 McCarty DM, Monahan PE, Samulski RJ (2001) Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. *Gene Ther* 8: 1248–1254
18 McCarty DM, Fu H, Monahan PE, Toulson CE, Naik P, Samulski RJ (2003) Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction *in vivo*. *Gene Ther* 10: 2112–2118
19 McCarty DM (2008) Self-complementary AAV vectors; advances and applications. *Mol Ther* 16: 1648–1656
20 Wu J, Zhao W, Zhong L, Han Z, Li B, Ma W, Weigel-Kelley KA, Warrington KH, Srivastava A (2007) Self-complementary recombinant adeno-associated viral vectors: packaging capacity and the role of rep proteins in vector purity. *Hum Gene Ther* 18: 171–182
21 Grieger JC, Samulski RJ (2005) Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. *J Virol* 79: 9933–9944
22 Allocca M, Doria M, Petrillo M, Colella P, Garcia-Hoyos M, Gibbs D, Kim SR, Maguire A, Rex TS, Di Vicino U et al. (2008) Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. *J Clin Invest* 118: 1955–1964
23 Duan D, Yue Y, Engelhardt JF (2001) Expanding AAV packaging capacity with trans-splicing or overlapping vectors: a quantitative comparison. *Mol Ther* 4: 383–391
24 Duan D, Yue Y, Engelhardt JF (2003) Dual vector expansion of the recombinant AAV packaging capacity. *Methods Mol Biol* 219: 29–51
25 Duan D, Yue Y, Yan Z, Engelhardt JF (2000) A new dual-vector approach to enhance recombinant adeno-associated virus-mediated gene expression through intermolecular cis activation. *Nat Med* 6: 595–598
26 Duan D, Yue Y, Yan Z, Engelhardt JF (2003) Trans-splicing vectors expand the packaging limits of adeno-associated virus for gene therapy applications. *Methods Mol Med* 76: 287–307
27 Ghosh A, Duan D (2007) Expanding adeno-associated viral vector capacity: a tale of two vectors. *Biotechnol Genet Eng Rev* 24: 165–177
28 Ghosh A, Yue Y, Lai Y, Duan D (2008) A hybrid vector system expands adeno-associated viral vector packaging capacity in a transgene-independent manner. *Mol Ther* 16: 124–130
29 Lai Y, Li D, Yue Y, Duan D (2008) Design of trans-splicing adeno-associated viral vectors for Duchenne muscular dystrophy gene therapy. *Methods Mol Biol* 433: 259–275
30 Lai Y, Yue Y, Liu M, Duan D (2006) Synthetic intron improves transduction efficiency of trans-splicing adeno-associated viral vectors. *Hum Gene Ther* 17: 1036–1042
31 Lai Y, Yue Y, Liu M, Ghosh A, Engelhardt JF, Chamberlain JS, Duan D (2005) Efficient *in vivo* gene expression by trans-splicing adeno-associated viral vectors. *Nat Biotechnol* 23: 1435–1439
32 Xu Z, Yue Y, Lai Y, Ye C, Qiu J, Pintel DJ, Duan D (2004) Trans-splicing adeno-associated viral vector-mediated gene therapy is limited by the accumulation of spliced mRNA but not by dual vector coinfection efficiency. *Hum Gene Ther* 15: 896–905
33 Yan Z, Zhang Y, Duan D, Engelhardt JF (2000) Trans-splicing vectors expand the utility of adeno-associated virus for gene therapy. *Proc Natl Acad Sci USA* 97: 6716–6721
34 Yue Y, Duan D (2003) Double strand interaction is the predominant pathway for intermolecular recombination of adeno-associated viral genomes. *Virology* 313: 1–7
35 Yan Z, Zak R, Zhang Y, Engelhardt JF (2005) Inverted terminal repeat sequences are important for intermolecular recombination and circularization of adeno-associated virus genomes. *J Virol* 79: 364–379

36 Wu Z, Miller E, Agbandje-McKenna M, Samulski RJ (2006) Alpha2,3 and alpha2,6 N-linked sialic acids facilitate efficient binding and transduction by adeno-associated virus types 1 and 6. *J Virol* 80: 9093–9103
37 Kaludov N, Brown KE, Walters RW, Zabner J, Chiorini JA (2001) Adeno-associated virus serotype 4 (AAV4) and AAV5 both require sialic acid binding for hemagglutination and efficient transduction but differ in sialic acid linkage specificity. *J Virol* 75: 6884–6893
38 Di Pasquale G, Davidson BL, Stein CS, Martins I, Scudiero D, Monks A, Chiorini JA (2003) Identification of PDGFR as a receptor for AAV-5 transduction. *Nat Med* 9: 1306–1312
39 Wu Z, Sun J, Zhang T, Yin C, Yin F, Van Dyke T, Samulski RJ, Monahan PE (2008) Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. *Mol Ther* 16: 280–289
40 Hauck B, Chen L, Xiao W (2003) Generation and characterization of chimeric recombinant AAV vectors. *Mol Ther* 7: 419–425
41 Rabinowitz JE, Bowles DE, Faust SM, Ledford JG, Cunningham SE, Samulski RJ (2004) Cross-dressing the virion: the transcapsidation of adeno-associated virus serotypes functionally defines subgroups. *J Virol* 78: 4421–4432
42 Hauck B, Xiao W (2003) Characterization of tissue tropism determinants of adeno-associated virus type 1. *J Virol* 77: 2768–2774
43 Bartlett JS, Kleinschmidt J, Boucher RC, Samulski RJ (1999) Targeted adeno-associated virus vector transduction of nonpermissive cells mediated by a bispecific F(ab'gamma)2 antibody. *Nat Biotechnol* 17: 181–186
44 Ponnazhagan S, Mahendra G, Kumar S, Thompson JA, Castillas M Jr, (2002) Conjugate-based targeting of recombinant adeno-associated virus type 2 vectors by using avidin-linked ligands. *J Virol* 76: 12900–12907
45 Huttner NA, Girod A, Perabo L, Edbauer D, Kleinschmidt JA, Buning H, Hallek M (2003) Genetic modifications of the adeno-associated virus type 2 capsid reduce the affinity and the neutralizing effects of human serum antibodies. *Gene Ther* 10: 2139–2147
46 Wu P, Xiao W, Conlon T, Hughes J, Agbandje-McKenna M, Ferkol T, Flotte T, Muzyczka N (2000) Mutational analysis of the adeno-associated virus type 2 (AAV2) capsid gene and construction of AAV2 vectors with altered tropism. *J Virol* 74: 8635–8647
47 Rabinowitz JE, Xiao W, Samulski RJ (1999) Insertional mutagenesis of AAV2 capsid and the production of recombinant virus. *Virology* 265: 274–285
48 Moskalenko M, Chen L, van Roey M, Donahue BA, Snyder RO, McArthur JG, Patel SD (2000) Epitope mapping of human anti-adeno-associated virus type 2 neutralizing antibodies: implications for gene therapy and virus structure. *J Virol* 74: 1761–1766
49 Girod A, Ried M, Wobus C, Lahm H, Leike K, Kleinschmidt J, Deleage G, Hallek M (1999) Genetic capsid modifications allow efficient re-targeting of adeno-associated virus type 2. *Nat Med* 5: 1438
50 Grifman M, Trepel M, Speece P, Gilbert LB, Arap W, Pasqualini R, Weitzman MD (2001) Incorporation of tumor-targeting peptides into recombinant adeno-associated virus capsids. *Mol Ther* 3: 964–975
51 Yang Q, Mamounas M, Yu G, Kennedy S, Leaker B, Merson J, Wong-Staal F, Yu M, Barber JR (1998) Development of novel cell surface CD34-targeted recombinant adenoassociated virus vectors for gene therapy. *Hum Gene Ther* 9: 1929–1937
52 Weichert WS, Parker JS, Wahid AT, Chang SF, Meier E, Parrish CR (1998) Assaying for structural variation in the parvovirus capsid and its role in infection. *Virology* 250: 106–117
53 Warrington KH Jr, Gorbatyuk OS, Harrison JK, Opie SR, Zolotukhin S, Muzyczka N (2004) Adeno-associated virus type 2 VP2 capsid protein is nonessential and can tolerate large peptide insertions at its N terminus. *J Virol* 78: 6595–6609
54 Opie SR, Warrington KH Jr, Agbandje-McKenna M, Zolotukhin S, Muzyczka N (2003) Identification of amino acid residues in the capsid proteins of adeno-associated virus type 2 that contribute to heparan sulfate proteoglycan binding. *J Virol* 77: 6995–7006
55 Shi X, Fang G, Shi W, Bartlett JS (2006) Insertional mutagenesis at positions 520 and 584 of adeno-associated virus type 2 (AAV2) capsid gene and generation of AAV2 vectors with eliminated heparin-binding ability and introduced novel tropism. *Hum Gene Ther* 17: 353–361
56 DiPrimio N, Asokan A, Govindasamy L, Agbandje-McKenna M, Samulski RJ (2008) Surface loop dynamics in adeno-associated virus capsid assembly. *J Virol* 82: 5178–5189
57 Arnold GS, Sasser AK, Stachler MD, Bartlett JS (2006) Metabolic biotinylation provides a unique

platform for the purification and targeting of multiple AAV vector serotypes. *Mol Ther* 14: 97–106
58 Gigout L, Rebollo P, Clement N, Warrington KH Jr, Muzyczka N, Linden RM, Weber T (2005) Altering AAV tropism with mosaic viral capsids. *Mol Ther* 11: 856–865
59 Maheshri N, Koerber JT, Kaspar BK, Schaffer DV (2006) Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. *Nat Biotechnol* 24: 198–204
60 Li W, Asokan A, Wu Z, Van Dyke T, DiPrimio N, Johnson JS, Govindaswamy L, Agbandje-McKenna M, Leichtle S, Redmond DE Jr, et al. (2008) Engineering and selection of shuffled AAV genomes: a new strategy for producing targeted biological nanoparticles. *Mol Ther* 16: 1252–1260
61 Excoffon KJ, Koerber JT, Dickey DD, Murtha M, Keshavjee S, Kaspar BK, Zabner J, Schaffer DV (2009) Directed evolution of adeno-associated virus to an infectious respiratory virus. *Proc Natl Acad Sci USA* 106: 3865–3870
62 Yang L, Jiang J, Drouin LM, Agbandje-McKenna M, Chen C, Qiao C, Pu D, Hu X, Wang DZ, Li J et al. (2009) A myocardium tropic adeno-associated virus (AAV) evolved by DNA shuffling and *in vivo* selection. *Proc Natl Acad Sci USA* 106: 3946–3951
63 Hajitou A, Trepel M, Lilley CE, Soghomonyan S, Alauddin MM, Marini FC 3rd, Restel BH, Ozawa MG, Moya CA, Rangel R et al. (2006) A hybrid vector for ligand-directed tumor targeting and molecular imaging. *Cell* 125: 385–398
64 Pasqualini R, Ruoslahti E (1996) Organ targeting *in vivo* using phage display peptide libraries. *Nature* 380: 364–366
65 Perabo L, Buning H, Kofler DM, Ried MU, Girod A, Wendtner CM, Enssle J, Hallek M (2003) *In vitro* selection of viral vectors with modified tropism: the adeno-associated virus display. *Mol Ther* 8: 151–157
66 Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM (2009) Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. *J Infect Dis* 199: 381–390
67 Zaiss AK, Liu Q, Bowen GP, Wong NC, Bartlett JS, Muruve DA (2002) Differential activation of innate immune responses by adenovirus and adeno-associated virus vectors. *J Virol* 76: 4580–4590
68 Zaiss AK, Cotter MJ, White LR, Clark SA, Wong NC, Holers VM, Bartlett JS, Muruve DA (2008) Complement is an essential component of the immune response to adeno-associated virus vectors. *J Virol* 82: 2727–2740
69 Chen J, Wu Q, Yang P, Hsu HC, Mountz JD (2006) Determination of specific CD4 and CD8 T cell epitopes after AAV2- and AAV8-hF.IX gene therapy. *Mol Ther* 13: 260–269
70 Sabatino DE, Mingozzi F, Hui DJ, Chen H, Colosi P, Ertl HC, High KA (2005) Identification of mouse AAV capsid-specific $CD8^+$ T cell epitopes. *Mol Ther* 12: 1023–1033
71 Li C, Hirsch M, Asokan A, Zeithaml B, Ma H, Kafri T, Samulski RJ (2007) Adeno-associated virus type 2 (AAV2) capsid-specific cytotoxic T lymphocytes eliminate only vector-transduced cells coexpressing the AAV2 capsid *in vivo*. *J Virol* 81: 7540–7547
72 Wang L, Figueredo J, Calcedo R, Lin J, Wilson JM (2007) Cross-presentation of adeno-associated virus serotype 2 capsids activates cytotoxic T cells but does not render hepatocytes effective cytolytic targets. *Hum Gene Ther* 18: 185–194
73 Li C, Hirsch M, DiPrimio N, Asokan A, Goudy K, Tisch R, Samulski RJ (2009) Cytotoxic-T-lymphocyte-mediated elimination of target cells transduced with engineered adeno-associated virus type 2 vector *in vivo*. *J Virol* 83: 6817–6824
74 Zhang HG, Xie J, Yang P, Wang Y, Xu L, Liu D, Hsu HC, Zhou T, Edwards CK 3rd, Mountz JD (2000) Adeno-associated virus production of soluble tumor necrosis factor receptor neutralizes tumor necrosis factor alpha and reduces arthritis. *Hum Gene Ther* 11: 2431–2442
75 Sandalon Z, Bruckheimer EM, Lustig KH, Burstein H (2007) Long-term suppression of experimental arthritis following intramuscular administration of a pseudotyped AAV2/1-TNFR:Fc Vector. *Mol Ther* 15: 264–269
76 Kay JD, Gouze E, Oligino TJ, Gouze JN, Watson RS, Levings PP, Bush ML, Dacanay A, Nickerson DM, Robbins PD et al. (2009) Intra-articular gene delivery and expression of interleukin-1Ra mediated by self-complementary adeno-associated virus. *J Gene Med* 11: 605–614
77 Cottard V, Mulleman D, Bouille P, Mezzina M, Boissier MC, Bessis N (2000) Adeno-associated virus-mediated delivery of IL-4 prevents collagen-induced arthritis. *Gene Ther* 7: 1930–1939
78 Takahashi H, Kato K, Miyake K, Hirai Y, Yoshino S, Shimada T (2005) Adeno-associated virus vector-mediated anti-angiogenic gene therapy for collagen-induced arthritis in mice. *Clin Exp Rheumatol* 23: 455–461
79 Sugiyama A, Hattori S, Tanaka S, Isoda F, Kleopoulos S, Rosenfeld M, Kaplitt M, Sekihara H,

Mobbs C (1997) Defective adenoassociated viral-mediated transfection of insulin gene by direct injection into liver parenchyma decreases blood glucose of diabetic mice. *Horm Metab Res* 29: 599–603

80 Jindal RM, Karanam M, Shah R (2001) Prevention of diabetes in the NOD mouse by intra-muscular injection of recombinant adeno-associated virus containing the preproinsulin II gene. *Int J Exp Diabetes Res* 2: 129–138

81 Li H, Li X, Lam KS, Tam S, Xiao W, Xu R (2008) Adeno-associated virus-mediated pancreatic and duodenal homeobox gene-1 expression enhanced differentiation of hepatic oval stem cells to insulin-producing cells in diabetic rats. *J Biomed Sci* 15: 487–497

82 Goudy K, Song S, Wasserfall C, Zhang YC, Kapturczak M, Muir A, Powers M, Scott-Jorgensen M, Campbell-Thompson M, Crawford JM et al. (2001) Adeno-associated virus vector-mediated IL-10 gene delivery prevents type 1 diabetes in NOD mice. *Proc Natl Acad Sci USA* 98: 13913–13918

83 Rehman KK, Trucco M, Wang Z, Xiao X, Robbins PD (2008) AAV8-mediated gene transfer of interleukin-4 to endogenous beta-cells prevents the onset of diabetes in NOD mice. *Mol Ther* 16: 1409–1416

84 Song S, Goudy K, Campbell-Thompson M, Wasserfall C, Scott-Jorgensen M, Wang J, Tang Q, Crawford JM, Ellis TM, Atkinson MA et al. (2004) Recombinant adeno-associated virus-mediated alpha-1 antitrypsin gene therapy prevents type I diabetes in NOD mice. *Gene Ther* 11: 181–186

85 Hu CM, Lin HH, Chiang MT, Chang PF, Chau LY (2007) Systemic expression of heme oxygenase-1 ameliorates type 1 diabetes in NOD mice. *Diabetes* 56: 1240–1247

86 Han G, Li Y, Wang J, Wang R, Chen G, Song L, Xu R, Yu M, Wu X, Qian J et al. (2005) Active tolerance induction and prevention of autoimmune diabetes by immunogene therapy using recombinant adenoassociated virus expressing glutamic acid decarboxylase 65 peptide GAD(500–585). *J Immunol* 174: 4516–4524

87 Chu Q, Moreland R, Yew NS, Foley J, Ziegler R, Scheule RK (2008) Systemic Insulin-like growth factor-1 reverses hypoalgesia and improves mobility in a mouse model of diabetic peripheral neuropathy. *Mol Ther* 16: 1400–1408

88 Ideno J, Mizukami H, Kakehashi A, Saito Y, Okada T, Urabe M, Kume A, Kuroki M, Kawakami M, Ishibashi S et al. (2007) Prevention of diabetic retinopathy by intraocular soluble flt-1 gene transfer in a spontaneously diabetic rat model. *Int J Mol Med* 19: 75–79

89 Carter JD, Ellett JD, Chen M, Smith KM, Fialkow LB, McDuffie MJ, Tung KS, Nadler JL, Yang Z (2005) Viral IL-10-mediated immune regulation in pancreatic islet transplantation. *Mol Ther* 12: 360–368

90 Farlow SJ, Jerusalmi A, Sano T (2007) Enhanced transduction of colonic cell lines *in vitro* and the inflamed colon in mice by viral vectors, derived from adeno-associated virus serotype 2, using virus-microbead conjugates bearing lectin. *BMC Biotechnol* 7: 83

91 Polyak S, Mah C, Porvasnik S, Herlihy JD, Campbell-Thompson M, Byrne BJ, Valentine JF (2008) Gene delivery to intestinal epithelial cells *in vitro* and *in vivo* with recombinant adeno-associated virus types 1, 2 and 5. *Dig Dis Sci* 53: 1261–1270

92 Yue TW, Chien WC, Tseng SJ, Tang SC (2007) EDC/NHS-mediated heparinization of small intestinal submucosa for recombinant adeno-associated virus serotype 2 binding and transduction. *Biomaterials* 28: 2350–2357

93 Kok MR, Yamano S, Lodde BM, Wang J, Couwenhoven RI, Yakar S, Voutetakis A, Leroith D, Schmidt M, Afione S et al. (2003) Local adeno-associated virus-mediated interleukin 10 gene transfer has disease-modifying effects in a murine model of Sjögren's syndrome. *Hum Gene Ther* 14: 1605–1618

94 Lodde BM, Mineshiba F, Wang J, Cotrim AP, Afione S, Tak PP, Baum BJ (2006) Effect of human vasoactive intestinal peptide gene transfer in a murine model of Sjögren's syndrome. *Ann Rheum Dis* 65: 195–200

95 Ye X, Zhu T, Bastacky S, McHale T, Li J, Xiao X (2005) Prevention and reversal of lupus in NZB/NZW mice by costimulatory blockade with adeno-associated virus-mediated gene transfer. *Arthritis Rheum* 52: 3975–3986

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Delivery and application of plasmid DNA in arthritis gene therapy

Maria C. Subang and David J. Gould

Queen Mary University of London, Barts and The London School of Medicine and Dentistry, Bone and Joint Research Unit, William Harvey Research Institute, London, UK

Abstract

Plasmids are simple circular double-stranded DNA that can be generated in large quantities in bacteria and can be highly purified at relatively low cost. The simplicity of its structure make it a safe vehicle for gene delivery, but this same attribute also means that delivery methods must be employed for plasmids to enter cells and gain access to the cell nucleus. These hurdles are overcome by a variety of chemical and physical delivery methods many of which have been utilised in gene therapy studies in arthritis models. In this chapter we outline the different gene therapy strategies that have employed plasmid DNA as a vector in arthritis models. We comprehensively review the array of studies in which plasmid DNA was utilised to demonstrate therapeutic effect. The article also introduces innovative concepts in plasmid delivery that have yet to be applied in gene therapy studies but could feasibly unlock the full potential of plasmid DNA in future gene therapy investigations.

Introduction

Plasmid DNA was first described by Nobel Prize winner Joshua Lederberg in 1952 [1]. Plasmids are extrachromosomal double stranded circular DNA from bacteria that replicate independently of genomic DNA so that multiple copies are produced in a single bacterial cell. They naturally encode antibiotic resistance genes or fertility factors and can be transferred horizontally between bacteria through the process of conjugation.

Since their discovery numerous plasmids have been constructed to facilitate recombinant DNA applications. As vectors for gene therapy, plasmid DNA has several advantages over viral vectors. Firstly, plasmids are simple DNA molecules that can be produced in large amounts and high quality through straightforward, inexpensive techniques. The nucleotide sequence of plasmids is identical to that of human DNA with the exception of methylation patterns. Plasmid DNA remains ‘naked’ and does not need to be packaged into small particles like viral genomes. This means that there are no size constraints on transgenes that can be incorporated in plasmids. Importantly, this also means that when delivered *in vivo* there are no protein components that activate the adaptive immune system. The lack of immune response has the advantage that it permits re-delivery of plasmids *in vivo*. Within cells plasmids need to access

the nucleus where they remain episomal and harness the cells own transcriptional system to express transgenes. The lack of genomic integration is another important safety feature of plasmid DNA. However, in dividing cells plasmids are rapidly shed during cell division so like other episomal vectors, such as adeno-associated viruses (AAV), it is important that plasmids are delivered to terminally differentiated cells in order to achieve long-term transgene expression.

In terms of safety profile plasmids clearly top the table of existing gene therapy vectors, but their simplicity is detrimental when it comes to cell delivery. Where viruses have evolved a variety of mechanisms such as cell binding, internalisation, endosomal escape, nuclear uptake, reverse transcription, second strand DNA synthesis, genomic integration and replication, plasmid DNA is essentially an inert molecule when combined with cells. In order to use plasmid DNA as a gene therapy vector it must be delivered to the cell nucleus by chemical or physical techniques as is the case for *in vitro* cell transfection studies.

Chemical delivery of plasmids

Chemical methods have traditionally utilised cationic lipids that condense DNA into small complexes to facilitate uptake into cells by endocytosis, within endosomes DNA dissociates from the complex and the endosomal membrane is destabilised enabling plasmid escape and enhancing nuclear uptake. In the collagen-induced arthritis (CIA) model lipid complexes have been used to deliver plasmid encoding human IL-10 (hIL-10). In this study, plasmid DNA was complexed in cationic liposomes with the reagent cytofectin ACHx, liposomes containing 300 μg plasmid DNA were then delivered by intraperitoneal (i.p.) injection after onset of disease which achieved a prolonged inhibition of disease progression. Distribution studies revealed the presence of plasmid in inflamed paws and other organs including liver, spleen and kidneys, with the main target cell being macrophages, nonetheless IL-10 protein was not detected in paws or serum [2].

DNA can also be delivered using small synthetic and natural peptides rich in arginine and/or lysine [3, 4] that can efficiently condense DNA and facilitate DNA delivery to cells. These peptides often have membrane translocating properties which also facilitate cell entry. Some sequences target their cargo to the nucleus and other domains enable endosomal escape [5]. Currently the best peptides display similar efficiency to that of lipids [6], but with further development these multidomain small peptides could potentially endow plasmids with the delivery efficiency of virus without triggering adaptive immunity due to their small size. The efficiency of their function will need to be determined in more rigorous systems because most testing is still limited to cultured cells with only a few examples of testing *in vivo*.

Nanoparticles of complex polyelectrolyte structures are another recent innovation that have been applied to plasmid delivery. These complexes are

assembled by the process of layer-by-layer (LbL) adsorption, which utilises electrostatic forces to fabricate defined structures from charged materials which include proteins, nucleic acids, saccharides, and organic polymers. Importantly, these structures can be generated relatively simply and at low cost as either films or particles (50 nm to 50 μm in diameter) [7]. Through control of particle assembly they can be designed to undergo structural changes and release contents when certain conditions are encountered. This biosensing property can be in response to mechanical stimuli such as pressure, optical activation or ultrasound or it could be a cellular environment such as alkali pH or intracellular degradation. Clearly, they have the potential to be important tools in DNA delivery. A recent study assembled an LbL film with plasmid DNA and a reducible polycation to achieve efficient transfection of invading fibroblasts with significant expression of secreted alkaline phosphatase (SEAP) reporter in the rat circulation [8]. The delivery in this study proved very efficient with 1.2 μg plasmid DNA incorporated in the film and peak levels of SEAP measured after 5 days at 160 ng/ml although the duration of expression was short. An interesting development in targeting particles to sites of inflammation was recently reported. DNA condensed with poly(L-lysine) was then coated with polymer and surface modified with recombinant P-selectin glycoprotein ligand-1 immunoglobulin chimera. When delivered *in vivo* intravital microscopy showed enhanced accumulation within inflamed cremastic venules [9] this study indicates a feasible approach to target multiple inflamed sites with plasmid DNA following systemic administration.

Physical delivery of plasmids

These methods crudely force DNA into cells by propulsion, pressure or permeability. The first efficient method demonstrated to deliver plasmid DNA *in vivo* was simple intramuscular injection of a DNA solution. In this study the DNA was injected in large volumes and it appears that the pressure that is created is sufficient to force the DNA into the cells. Because DNA was transfected into terminally differentiated myotubes long-term gene expression approaching a year was demonstrated by this approach [10]. This ground breaking observation led to plasmid delivery being utilised in two contrasting gene therapy approaches – genetic immunisation and therapeutic gene expression.

Genetic immunisation

Short-term muscle expression from plasmid DNA has been developed as an innovative vaccination strategy. Following plasmid delivery to muscle transgene expression occurs in among other cells the myotubes and resident dendritic cells. Transgenes are then presented to the immune system by MHC

Class I and II on myotubes or through migration of transfected dendritic cells to local lymph nodes. When plasmid delivery is repeated twice in 2 weeks the immune response can be sufficient to break tolerance to endogenous molecules. Immunisation strategies in arthritis models have either aimed to produce an immune response (break tolerance) to pro-inflammatory mediators with the aim of inhibiting their function and having an anti-inflammatory effect, whilst other studies have aimed at protecting against the Ag immunisation for disease development. Based on rheumatoid arthritis (RA) clinical treatment, TNFα is a good target, vaccination with a plasmid encoding hTNFα was shown to inhibit CIA with reduced inflammation, lower levels of TNFα in sera and reduced lymphocyte proliferation observed. Despite these observations cross-reacting antibodies were not demonstrated in this study [11].

In the rat adjuvant-induced arthritis (AA) model the myobacterial 65-kDa heat shock protein (HSP65) contains at least one epitope associated with disease in the model. Interestingly, an epitope from amino acids 180–188 of HSP65 recognised by a T cell clone also reacts with self cartilage and can transfer disease. Quintana et al. (2003) demonstrated that human HSP60 or a regulatory peptide Hu3 which could be expressed from plasmid to vaccinate and protect rats from AA [12]. In a subsequent study a similar vaccination effect was demonstrated with human HSP70 and HSP90 [13]. Similarly, in the rat CIA model, a plasmid (200 μg/kg) encoding chicken type II collagen (CII) delivered intravenous (i.v.) after onset of disease (day 21) caused an inhibition in disease progression and reduction in inflammation. In terms of immune response there was a shift Th1 to Th2 response with associated cytokine changes and also an increase in $CD4^+CD25^+$ Treg cells [14].

Vaccination studies have also been performed to investigate the role of mediators in arthritis models. Vaccination with a plasmid encoding the p28 subunit of IL-27 in the AA model in rats led to production of IL-27 neutralising Ab and suppression of disease. Further studies revealed a role of IL-27 in directing polarisation of naïve T cells, but also had effects on proliferation and cytokine production from Ag-specific effector/memory Th1 cells [15].

Vaccination studies have provided an interesting approach in experimental models, but this strategy is unlikely to progress to clinical treatment of RA patients where ongoing autoimmune responses are already out of control and pathogenic immunogens have not been identified.

Therapeutic gene expression from skeletal muscle

In the second approach direct injection of plasmid DNA has been utilised in gene therapy studies to express therapeutic molecules. Several experimental studies demonstrated therapeutic effect with plasmid DNA injected into muscle using rodent models. The first application of plasmid DNA in the treatment of an arthritis model was demonstrated with a plasmid encoding human TGF-β1 in the rat streptococcal cell wall-induced model. In this study plasmid

(300 μg) was injected intramuscular (i.m.) at the peak of the acute phase or as the chronic phase commenced and in both cases treatment greatly inhibited the arthritis response. Increased circulating levels of active hTGF-β1 peaking at 1 ng/ml after 3 days was observed with inhibition of inflammation, cartilage and bone destruction [16]. The same plasmid delivery method has been used in subsequent studies to express IL-1ra in the CIA model. A total of 400 μg of plasmid injected in four sites and inhibited paw swelling, joint erosion and expression of IL-1β was reduced in joints [17]. Plasmid injection into muscle of CIA mice was also utilised to express soluble complement receptor 1 at onset of disease with significant inhibition of disease progression observed [18]. In an innovative approach latent IFNβ was expressed from plasmid injected i.m. at the time of disease onset in the CIA model, and achieved better therapeutic effect than the normal IFNβ [19].

Despite the relative efficiency of this plasmid delivery route in rodents the same efficiency did not translate when attempted in man. In a clinical trial for treatment of critical limb ischemia, expression of VEGF following i.m. injection of encoding plasmid (4 μg) gave a transient increase 1–2 weeks after plasmid delivery [20].

In order to improve the efficiency of this transfection route combination with other physical treatments was examined. Electroporation (EP) of the muscle soon after DNA injection significantly enhanced transfection by a factor of 100-fold [21]. EP opens ion channels in the cell membrane which permits entry of plasmid DNA, this entry may also be facilitated by the movement of negatively charged DNA molecules in the electrical field generated. Again, long-term transgene expression was observed in skeletal muscle and the combination with DNA injection proved effective in many other tissues and organs [22]. EP has been applied widely in rodents with great success, more recently the method has be employed in a Phase I clinical trial for the local delivery of hIL-12 encoding plasmid into metastatic melanoma lesions with encouraging observations [23]. A side effect of EP is co-lateral damage at the EP site which initiates an inflammatory response and cell necrosis followed by tissue remodelling and regeneration. Due to the recruitment of inflammatory cells and more efficient transfection there is evidence that EP may also enhance the process of DNA vaccination. Indeed there are several companies that have devised EP devices for clinical application and clinical trials are about to be initiated [24].

Expression of therapeutic genes by electroporation

The combination of i.m. injection and EP has been widely exploited in experimental arthritis studies. Inhibition of TNFα has been effective in several studies. Kim et al. (2003) showed that expression of hTNFRII-hIgG Fc molecule from treated gastrocnemius muscle achieved peak levels of 2.3 ng/ml after 5 days and reduced swelling, synovitis and cartilage erosion in the mouse CIA model. In addition, expression of inflammatory cytokines in paws was also

reduced [25]. Similarly, long-term expression of variants of hTNFRI either as soluble receptor, dimeric or on the Fc-IgG1 backbone of up to 6 months was demonstrated in mice. In the CIA model, 50 μg of the Fc-IgG1 version delivered at onset of disease led to a decrease in clinical and histological signs of disease [26]. Similarly, expression of a small dimeric version of the hTNFRII expressed from plasmid DNA either constitutively or in a pharmacologically regulated manner achieved therapeutic effects in the CIA model [27, 28]. Expression of hIL-1Ra in the CIA model from plasmid (15 μg) delivered with EP into the gastrocnemius muscle achieved peak expression in the blood at about 1.6 ng/ml with decline to basal levels by day 20. Therapeutic effects included reduction in paw swelling and severity and inflammatory cytokine levels in paws [29]. When murine IL-4 was delivered using a plasmid, serum levels peaked at 340 pg/ml on day 1, but again returned to baseline by day 12. Disease was inhibited when plasmid was delivered prior to onset with reduction in disease incidence, paw swelling and pro-inflammatory mediators released from ankle joint explants [30]. Expression of vIL-10 was achieved by injection of plasmid (total 400 μg) at four sites in the anterior tibialis muscles and delivered with needle electrodes with expression levels of viral IL-10 (vIL-10) peaking in the blood after 15 days at 1.5 ng/ml then rapidly declining. In the CII Ab mouse arthritis model, disease was dramatically inhibited by this vIL-10 treatment with reduction in histological score, and levels of mRNA for pro-inflammatory cytokines in joints [31].

Hydrodynamic delivery

Wolff's group also developed the idea of forcing DNA into muscle by rapid i.v. injection of a large volume of DNA solution into limbs with occluded blood flow. This approach has the advantage that the whole muscle bed can be accessed through the extensive vasculature that supplies skeletal muscle. This method has proven effective through both the venous and arterial route [32, 33], most importantly delivery through the venous route has also proven effective in larger species and in primates, which is a good indication that efficient transfection could be achieved in man. The method requires the injection of DNA in a volume equivalent to a third of the volume of the limb to be injected so clinical studies are necessary to determine how well the procedure is tolerated in patients. There are no reports utilising this delivery method in arthritis models to date.

Hydrodynamic delivery to liver

The ability to force DNA through cells membranes has been most efficiently demonstrated following rapid injection of plasmid DNA i.v. in rodents. By this method, volumes of DNA solution equivalent to the entire blood volume of the

mouse are injected at rates up to 0.5 ml/sec by tail vein injection. Following rapid injection there are transient irregularities in heart rate and a sharp increase in venous pressure along with liver fenestrae enlargement and generation of membrane pores in the hepatocytes [34]. Whilst the nature of the procedure would initially suggest that this procedure could not be scaled up for gene delivery in the clinic it may be feasible for a more localised infusion to be performed in the liver following occlusion of the hepatic vein [35]. As an *in vivo* gene delivery approach in experimental models the method has been utilised widely [36]. Hydrodynamic plasmid delivery was utilised in one CIA study where plasmid (25 μg) encoding the amino terminal 15 amino acids of fibronectin was rapidly injected i.v. in a volume of 3 ml PBS in 5 seconds. Plasmid was delivered after disease onset and was able to inhibit progression of disease. The idea behind this study was that cell–cell adhesion would be disrupted by the short fibronectin peptide containing the heparin-binding domain, and indeed inhibition of cell recruitment was observed in the study. Interestingly, levels of the peptide were only detectable in the blood up to 5 days post injection suggesting only short-term expression in this model [37].

Massage delivery

An interesting twist on hydrodynamic delivery to the liver has been the application of external pressure by massage in the hepatic area following intravenous injection of DNA. This simple non-invasive manipulation achieved efficient transfection of hepatocytes and indicates that simple procedures could be utilised for effective gene delivery *in vivo* [38].

Ballistic delivery

At the other extreme, plasmid DNA can also be directly delivered to cells by bombardment with coated gold microparticles. Utilising a gene gun, tissues for targeting are skin of exposed internal organs. Gold particles from 0.6 μm in diameter are propelled with helium pulses up to 600 psi with gene expression resulting from particles that are delivered to the cell nucleus. Unlike other mechanical methods, the preparation of particles is a relatively time consuming process where other physical procedures require direct injection of a DNA solution.

Intradermal (i.d.) delivery of plasmid has been shown to be efficacious in the CIA model with of plasmid encoding IL-10 injected (i.d.) a week before CII immunisation. Treated mice showed reduced footpad thickness, less histopathological changes and suppression of the Th1 response determined by IgG2a/IgG1 ratios [39]. In another study plasmid (50 μg) encoding mouse IL-4 was injected i.d. on days 0 and 21 the time points for primary and booster immunisation with CII. For comparison the same amount of plasmid was

delivered at the same time points by gene gun delivery of coated gold particles (1.6 μm diameter) to the abdomen. Both treatments reduced disease incidence, severity and anti-CII levels, but with greater immunosuppression observed with gene gun delivery [40].

Sonoporation

Ultrasound is an imaging technique that enables visualisation of tissue and organs by measurement of the reflection signature of an ultrasound wave. In some instances, ultrasound resolution is improved by using microbubbles which are small (1–8 μm) gas filled microspheres that are used as contrast agents. These microbubbles can be constructed with different materials such as lipid, protein or polymer and indeed the structure of the bubbles can also be controlled such that DNA can be incorporated in the shell or core of the bubbles. Due to there size microbubbles can be administered to the blood supply and can freely circulate in the body. Release from the bubbles can be triggered by application of high intensity ultrasound which causes their collapse and consequent release of contents. Ultrasound alone increases cell membrane permeability, but in combination with microbubbles there is a further increase in permeability. This effect is termed ‘sonoporation’. These characteristics make it possible to deliver micobubbles systemically, but trigger plasmid release to a target site though the local application of ultrasound. The application of sonoporation for plasmid delivery to skeletal muscle was first described in by Lu et al. (2003) [41], and has received increasing support since. The method has been used to deliver DNA *in vitro* and *in vivo*, but the relative efficiency of gene delivery is low compared to adenoviral and lipofection methods [42].

Plasmid delivery to joints

Local delivery to joints is obviously of interest in order to achieve local expression of therapeutic proteins at the disease site. Injection of plasmid DNA into knee joints of rats in combination with EP was shown to achieve short-term (up to 9 days) expression of a GFP reporter in the superficial, middle and deep zones of the patellar cartilage, while expression only persisted (1–2 months) in the deep zone [43]. In another study, it was proposed that plasmid DNA associates with LPS and the complex interacts with LPS-binding protein (LBP) in order to transfect cells, this theory was supported by the observation of lower gene expression in knee joints of mice deficient in the LBPs MD-2 and CD14 [44]. Gene therapy studies have also utilised this delivery route. However, expression of hTNFRI variants or hIL-10 expressed from plasmid DNA (20–25 μg) delivered by i.a. injection with EP was sufficient to achieve short-term transgene expression, but even repeat administrations did not have significant therapeutic effect in the mouse CIA model [45, 46]. These studies

are complicated by the fact that 10 µl intraarticular (i.a.) injection will cause leakage from the joint even with the most careful technique (unpublished data) and in one of these published studies a bioluminescent image appears to show transgene expression is actually in the muscle next to the joint [46]. Studies with AAV vectors clearly show there are cells in joints that can be transduced to achieve long-term transgene expression. With further refinement of methods it maybe possible to transfect the same cells with plasmid DNA and achieve long-term local gene expression.

How efficient are plasmid delivery methods?

When delivered to the appropriate cells, plasmids can achieve long-term gene expression. There are few studies that directly compare the efficiency of gene delivery methods. One recent report revealed that expression of the transgene Apo E3 from plasmid DNA (20 µg) delivered by i.m. injection with EP was several-fold less efficient than i.m. delivery with AAV2/7 (10^{10} viral genomes) (<15 ng/ml *versus* 1.4 µg/ml) [47]. However, the efficiency of plasmid DNA delivery to liver following i.v. injection is a 1,000-fold greater than i.m. injection with EP (Tab. 1). This liver delivery achieves systemic transgene expression with an efficiency approaching that observed with viruses. Less efficient plasmid delivery may suffice with local delivery to inflamed joints where a more local expression of therapeutic molecules could have great effect.

Table 1. Comparison of SEAP levels in the blood following plasmid DNA delivery by different methods.

Delivery method	SEAP version	Vector amount	Approximate copy number	Expression level in blood	Duration	Ref
Hydrodynamic i.v. liver	Human SEAP	Plasmid 10 µg	1.85×10^{12}	1,000 µg/ml	Approx 200 µg/ml at 38 weeks	[48]
Skeletal muscle EP	Human SEAP	Plasmid 10 µg	1.85×10^{12}	375 ng/ml	Approx 100 ng/ml at 24 weeks	[49]
Polyelectrolyte film implanted s.c.	Human SEAP	Plasmid 1.2 µg	2.2×10^{11}	160 ng/ml	Short-term Peak at day 3	[8]

Calculations of copy number are based on the assumption of a vector size of 5000 bp

Conclusion

Plasmid DNA has great potential as a safe gene therapy vector in the treatment of non-fatal chronic diseases such as RA. In terms of RA treatment, systemic expression from plasmids has huge potential to provide a viable alternative to deliver existing biological molecules in cheaper genetic form. Key to the suc-

cessful application of plasmid DNA in clinical use will be the demonstration of effective plasmid delivery in patients.

References

1 Lederberg J (1952) Cell genetics and hereditary symbiosis. *Physiol Rev* 32: 403–430
2 Fellowes R, Etheridge CJ, Coade S, Cooper RG, Stewart L, Miller AD, Woo P (2000) Amelioration of established collagen induced arthritis by systemic IL- 10 gene delivery. *Gene Ther* 7: 967–977
3 Parker AL, Collins L, Zhang X, Fabre JW (2005) Exploration of peptide motifs for potent non-viral gene delivery highly selective for dividing cells. *J Gene Med* 7: 1545–1554
4 Futaki S (2005) Membrane-permeable arginine-rich peptides and the translocation mechanisms. *Adv Drug Deliv Rev* 57: 547–558
5 Kichler A, Leborgne C, Danos O, Bechinger B (2007) Characterization of the gene transfer process mediated by histidine-rich peptides. *J Mol Med* 85: 191–201
6 Lo SL, Wang S (2008) An endosomolytic Tat peptide produced by incorporation of histidine and cysteine residues as a nonviral vector for DNA transfection. *Biomaterials* 29: 2408–2414
7 Ariga K, Hill JP, Ji Q (2008) Biomaterials and biofunctionality in layered macromolecular assemblies. *Macromol Biosci* 8: 981–990
8 Blacklock J, You YZ, Zhou QH, Mao G, Oupicky D (2009) Gene delivery *in vitro* and *in vivo* from bioreducible multilayered polyelectrolyte films of plasmid DNA. *Biomaterials* 30: 939–950
9 Newman CM, Crosdale DJ, Fisher KD, Briggs SS, Norman KE, Seymour LW, Hellewell PG (2009) P-selectin dependent targeting to inflamed endothelium of recombinant P-selectin glycoprotein ligand-1 immunoglobulin chimera-coated poly[N-(2-hydroxypropyl) methacrylamide]-DNA polyplexes *in vivo* visualised by intravital microscopy. *J Gene Med* 11: 326–334
10 Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL (1990) Direct gene transfer into mouse muscle *in vivo*. *Science* 247: 1465–1468
11 Shen Y, Chen J, Zhang X, Wu X, Xu Q (2007) Human TNF-alpha gene vaccination prevents collagen-induced arthritis in mice. *Int Immunopharmacol* 7: 1140–1149
12 Quintana FJ, Carmi P, Mor F, Cohen IR (2003) DNA fragments of the human 60-kDa heat shock protein (HSP60) vaccinate against adjuvant arthritis: identification of a regulatory HSP60 peptide. *J Immunol* 171: 3533–3541
13 Quintana FJ, Carmi P, Mor F, Cohen IR (2004) Inhibition of adjuvant-induced arthritis by DNA vaccination with the 70-kd or the 90-kd human heat-shock protein: immune cross-regulation with the 60-kd heat-shock protein. *Arthritis Rheum* 50: 3712–3720
14 Song X, Liang F, Liu N, Luo Y, Xue H, Yuan F, Tan L, Sun Y, Xi C, Xi Y (2009) Construction and characterization of a novel DNA vaccine that is potent antigen-specific tolerizing therapy for experimental arthritis by increasing $CD4^{+}CD25^{+}$Treg cells and inducing Th1 to Th2 shift in both cells and cytokines. *Vaccine* 27: 690–700
15 Goldberg R, Wildbaum G, Zohar Y, Maor G, Karin N (2004) Suppression of ongoing adjuvant-induced arthritis by neutralizing the function of the p28 subunit of IL-27. *J Immunol* 173: 1171–1178
16 Song XY, Gu M, Jin WW, Klinman DM, Wahl SM (1998) Plasmid DNA encoding transforming growth factor-beta1 suppresses chronic disease in a streptococcal cell wall-induced arthritis model. *J Clin Invest* 101: 2615–2621
17 Kim JM, Jeong JG, Ho SH, Hahn W, Park EJ, Kim S, Yu SS, Lee YW (2003) Protection against collagen-induced arthritis by intramuscular gene therapy with an expression plasmid for the interleukin-1 receptor antagonist. *Gene Ther* 10: 1543–1550
18 Dreja H, Annenkov A, Chernajovsky Y (2000) Soluble complement receptor 1 (CD35) delivered by retrovirally infected syngeneic cells or by naked DNA injection prevents the progression of collagen-induced arthritis. *Arthritis Rheum* 43: 1698–1709
19 Adams G, Vessillier S, Dreja H, Chernajovsky Y (2003) Targeting cytokines to inflammation sites. *Nat Biotechnol* 21: 1314–1320
20 Baumgartner I, Pieczek A, Manor O, Blair R, Kearney M, Walsh K, Isner JM (1998) Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia. *Circulation* 97: 1114–1123

21 Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud JM, Delaere P, Branellec D, Schwartz B, Scherman D (1999) High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc Natl Acad Sci USA* 96: 4262–4267
22 Trollet C, Scherman D, Bigey P (2008) Delivery of DNA into muscle for treating systemic diseases: advantages and challenges. *Methods Mol Biol* 423: 199–214
23 Daud AI, DeConti RC, Andrews S, Urbas P, Riker AI, Sondak VK, Munster PN, Sullivan DM, Ugen KE, Messina JL et al. (2008) Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. *J Clin Oncol* 26: 5896–5903
24 Luxembourg A, Evans CF, Hannaman D (2007) Electroporation-based DNA immunisation: translation to the clinic. *Expert Opin Biol Ther* 7: 1647–1664
25 Kim JM, Ho SH, Hahn W, Jeong JG, Park EJ, Lee HJ, Yu SS, Lee CS, Lee YW, Kim S (2003) Electro-gene therapy of collagen-induced arthritis by using an expression plasmid for the soluble p75 tumor necrosis factor receptor-Fc fusion protein. *Gene Ther* 10: 1216–1224
26 Bloquel C, Bessis N, Boissier MC, Scherman D, Bigey P (2004) Gene therapy of collagen-induced arthritis by electrotransfer of human tumor necrosis factor-alpha soluble receptor I variants. *Hum Gene Ther* 15: 189–201
27 Gould DJ, Bright C, Chernajovsky Y (2004) Inhibition of established collagen-induced arthritis with a tumour necrosis factor-alpha inhibitor expressed from a self-contained doxycycline regulated plasmid. *Arthritis Res Ther* 6: R103–113
28 Gould DJ, Yousaf N, Fatah R, Subang MC, Chernajovsky Y (2007) Gene therapy with an improved doxycycline regulated plasmid encoding a TNFalpha inhibitor in experimental arthritis. *Arthritis Res Ther* 9: R7
29 Jeong JG, Kim JM, Ho SH, Hahn W, Yu SS, Kim S (2004) Electrotransfer of human IL-1Ra into skeletal muscles reduces the incidence of murine collagen-induced arthritis. *J Gene Med* 6: 1125–1133
30 Ho SH, Hahn W, Lee HJ, Kim DS, Jeong JG, Kim S, Yu SS, Jeon ES, Kim JM (2004) Protection against collagen-induced arthritis by electrotransfer of an expression plasmid for the interleukin-4. *Biochem Biophys Res Commun* 321: 759–766
31 Kuroda T, Maruyama H, Shimotori M, Higuchi N, Kameda S, Tahara H, Miyazaki J, Gejyo F (2006) Effects of viral interleukin 10 introduced by *in vivo* electroporation on arthrogen-induced arthritis in mice. *J Rheumatol* 33: 455–462
32 Zhang G, Budker V, Williams P, Subbotin V, Wolff JA (2001) Efficient expression of naked dna delivered intraarterially to limb muscles of nonhuman primates. *Hum Gene Ther* 12: 427–438
33 Hagstrom JE, Hegge J, Zhang G, Noble M, Budker V, Lewis DL, Herweijer H, Wolff JA (2004) A facile nonviral method for delivering genes and siRNAs to skeletal muscle of mammalian limbs. *Mol Ther* 10: 386–398
34 Zhang G, Gao X, Song YK, Vollmer R, Stolz DB, Gasiorowski JZ, Dean DA, Liu D (2004) Hydroporation as the mechanism of hydrodynamic delivery. *Gene Ther* 11: 675–682
35 Eastman SJ, Baskin KM, Hodges BL, Chu Q, Gates A, Dreusicke R, Anderson S, Scheule RK (2002) Development of catheter-based procedures for transducing the isolated rabbit liver with plasmid DNA. *Hum Gene Ther* 13: 2065–2077
36 Herweijer H, Wolff JA (2007) Gene therapy progress and prospects: hydrodynamic gene delivery. *Gene Ther* 14: 99–107
37 Imagawa T, Watanabe S, Katakura S, Boivin GP, Hirsch R (2002) Gene transfer of a fibronectin peptide inhibits leukocyte recruitment and suppresses inflammation in mouse collagen-induced arthritis. *Arthritis Rheum* 46: 1102–1108
38 Liu F, Huang L (2002) Noninvasive gene delivery to the liver by mechanical massage. *Hepatology* 35: 1314–1319
39 Miyata M, Sasajima T, Sato H, Saito A, Iriswa A, Sato Y, Kasukawa R (2000) Suppression of collagen induced arthritis in mice utilizing plasmid DNA encoding interleukin 10. *J Rheumatol* 27: 1601–1605
40 Kageyama Y, Koide Y, Uchijima M, Nagata T, Yoshida A, Taiki A, Miura T, Nagafusa T, Nagano A (2004) Plasmid encoding interleukin-4 in the amelioration of murine collagen-induced arthritis. *Arthritis Rheum* 50: 968–975
41 Lu QL, Liang HD, Partridge T, Blomley MJ (2003) Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle *in vivo* with reduced tissue damage. *Gene Ther* 10: 396–405
42 Hernot S, Klibanov AL (2008) Microbubbles in ultrasound-triggered drug and gene delivery. *Adv*

Drug Deliv Rev 60: 1153–1166
43 Grossin L, Cournil-Henrionnet C, Mir LM, Liagre B, Dumas D, Etienne S, Guingamp C, Netter P, Gillet P (2003) Direct gene transfer into rat articular cartilage by *in vivo* electroporation. *Faseb J* 17: 829–835
44 Kolka JA, Vreede AP, Roessler BJ (2005) Lipopolysaccharide recognition protein, MD-2, facilitates cellular uptake of E. coli-derived plasmid DNA in synovium. *J Gene Med* 7: 956–964
45 Khoury M, Bigey P, Louis-Plence P, Noel D, Rhinn H, Scherman D, Jorgensen C, Apparailly F (2006) A comparative study on intra-articular *versus* systemic gene electrotransfer in experimental arthritis. *J Gene Med* 8: 1027–1036
46 Bloquel C, Denys A, Boissier MC, Apparailly F, Bigey P, Scherman D, Bessis N (2007) Intra-articular electrotransfer of plasmid encoding soluble TNF receptor variants in normal and arthritic mice. *J Gene Med* 9: 986–993
47 Evans V, Foster H, Graham IR, Foster K, Athanasopoulos T, Simons JP, Dickson G, Owen JS (2008) Human apolipoprotein E expression from mouse skeletal muscle by electrotransfer of non-viral DNA (plasmid) and pseudotyped recombinant adeno-associated virus (AAV2/7). *Hum Gene Ther* 19: 569–578
48 Wooddell CI, Reppen T, Wolff JA, Herweijer H (2008) Sustained liver-specific transgene expression from the albumin promoter in mice following hydrodynamic plasmid DNA delivery. *J Gene Med* 10: 551–563
49 Fabre EE, Bigey P, Orsini C, Scherman D (2006) Comparison of promoter region constructs for *in vivo* intramuscular expression. *J Gene Med* 8: 636–645

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Helper-dependent adenoviral vectors

Nicola Brunetti-Pierri and Philip Ng

Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA

Abstract

Helper-dependent adenoviral (HDAd) vectors have several characteristics making them attractive for human gene therapy. These vectors are completely devoid of viral coding sequences and are able to mediate high efficiency transduction *in vivo* to direct high level transgene expression with negligible chronic toxicity. However, clinical translation is complicated by the dose-dependent acute toxic response following systemic vector injection. With a better understanding of vector-mediated toxicity and improved delivery methods, HDAds may emerge as an important vector for gene therapy of human diseases.

Introduction

Gene therapy vectors derived from the adenovirus (Ad) are the most often used in clinical trials [1]. The majority of these applications are for cancer treatment and very few are for non-cancer diseases [1]. First generation adenoviral (FGAd) vectors were rendered replication-deficient by the deletion of the viral early region 1 (E1). FGAd can efficiently transduce a wide variety of cell types from many different species independent of the cell cycle to direct high levels of transgene expression. However, low levels of viral gene expression from the vector backbone result in loss of transgene expression due to immune-mediated clearance of transduced cells. In contrast, helper-dependent adenoviral (HDAd) vectors, which are devoid of all viral sequences, are safer and more effective for disease applications requiring long-term expression of the therapeutic gene [2]. A multitude of small and large animal models of genetic disorders can be corrected effectively and long-term by HDAd vectors without signs of chronic toxicity [3]. HDAd can mediate high efficiency transduction, do not integrate in the host genome, and have a large cloning capacity of up to ~37 kb which allows for the delivery of whole genomic loci, multiple transgenes, and large *cis*-acting elements to enhance, prolong, and regulate transgene expression. This chapter will present the general features of the HDAd and will focus on recently developed applications for liver, lung, and brain gene therapy.

HDAd

HDAd are derived from the Ad, a non-enveloped icosahedral capsid containing a linear double-stranded DNA genome of ~30–40 kb. The Ad genome is flanked by inverted terminal repeats (ITRs) which are the only sequences required in *cis* for viral DNA replication. A *cis*-acting packaging (ψ) signal, required for encapsidation of the genome, is located near the left ITR (relative to the conventional map of Ad). The Ad genome can be divided into two sets of genes (Fig. 1): the early region genes (E1A, E1B, E2, E3, and E4) expressed before DNA replication and the late region genes (L1 to L5) expressed after initiation of DNA replication. The early region genes are expressed during viral infection and are involved in transcriptional regulation of the viral genome. The late region genes mostly encode virion structural proteins.

The first and most efficient method for generating HDAd is the Cre/loxP system [4] (Fig. 2). In this system the HDAd genome, constructed in a bacterial plasmid, contains: i) the ITRs and ψ signal, ii) the expression cassette of interest, and iii) stuffer DNA up to ~36 Kb required for efficient packaging [5, 6]. To convert the plasmid form of the HDAd genome into the viral form 293

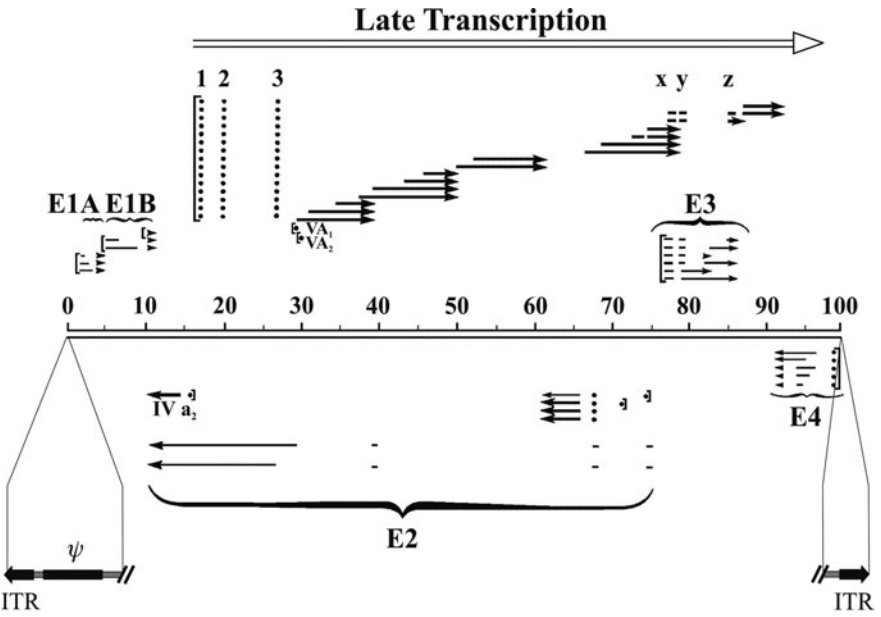


Figure 1. Transcription map of human adenovirus serotype 5. The 100 map unit (~36 kb) genome is divided into four early region transcription units, E1–E4, and five families of late mRNA, L1–L5, which are alternative splice products of a common late transcript expressed from the major late promoter located at 16 map units. Four smaller transcripts, pIX, IVa, and VA RNA's I and II, are also produced. The 103 bp inverted terminal repeats (ITRs) are located at the termini of the genome and are involved in viral DNA replication, and the packaging signal (ψ) located from nucleotides 190 to 380 at the left end is involved in packaging of the genome into virion capsids.

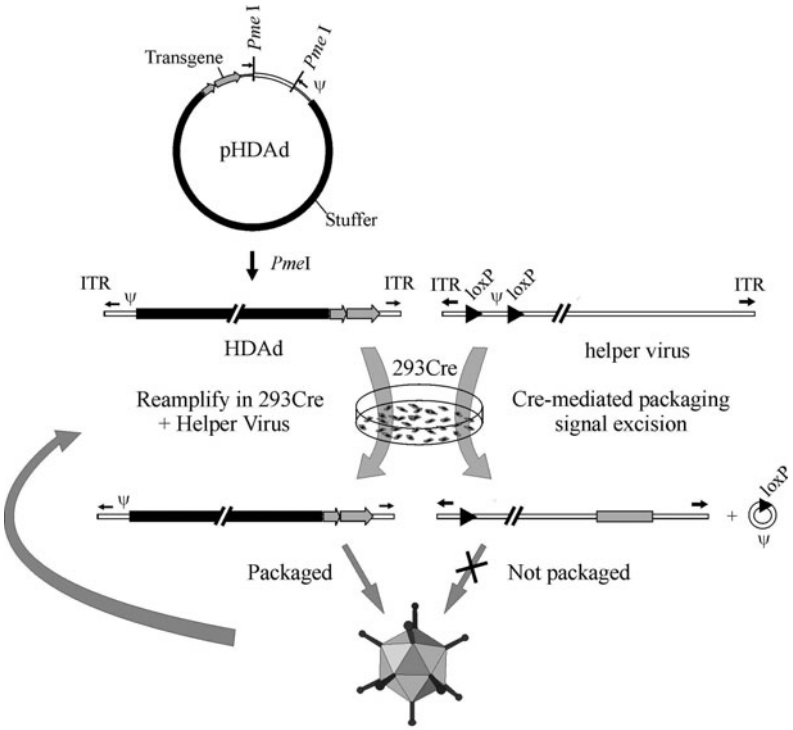


Figure 2. The Cre/loxP system for generating HDAds. The HDAd contains only ~500 bp of *cis*-acting Ad sequences required for DNA replication (ITRs) and packaging (ψ), the remainder of the genome consists of the desired transgene and non-Ad *stuffer* sequences. The HDAd genome is constructed as a bacterial plasmid (pHDAd) and is liberated by restriction enzyme digestion (e.g., *Pme*I). To rescue the HDAd, the liberated genome is transfected into 293 cells expressing Cre and infected with a helper virus bearing a packaging signal (ψ) flanked by loxP sites. Cre-mediated excision of ψ renders the helper virus genome unpackageable, but still able to provide all of the necessary *trans*-acting factors for propagation of the HDAd. The titer of the HDAd is increased by serial coinfections of 293Cre cells with the HDAd and the helper virus.

cells expressing Cre are transfected with the linearized HDAd genome and subsequently infected with the helper virus. The helper virus is a FGAd bearing a packaging signal flanked by loxP sites and following infection of 293Cre cells, the packaging signal is excised from the helper viral genome by Cre-mediated site-specific recombination between the loxP sites. This renders the helper viral genome unpackageable but still able to undergo DNA replication and thus *trans*-complement the replication and encapsidation of the HDAd genome. The production of large quantities of HDAd vectors with extremely low levels of helper virus contamination can be obtained through a rapid and efficient production method which makes the preparation of large vector stocks possible for large animal experiments and potentially for human applications [7].

In vivo studies with HDAd

As of this writing, numerous examples of *in vivo* HDAd-mediated gene transfer through different routes of administration (intravenous, intramuscular, brain and intratumoral injection, airway administration) in various small and large animal disease models have been reported. The purpose of this chapter is not to provide a comprehensive review of all of these studies. Instead, recent examples of particular significance or interest are described.

Liver directed gene therapy

The liver is a very attractive target for gene therapy because it is the affected organ in many genetic and acquired diseases and it can be used as a factory organ for systemic delivery through vascular circulation of vector-encoded therapeutic proteins. To date numerous examples of *in vivo* liver-directed gene therapy using HDAd in several monogenic disease models have been reported. In general, all these studies have demonstrated long-term phenotypic correction in the absence of chronic toxicity thus supporting the potential of HDAd for clinical applications [8, 9]. Importantly, these results have also been recapitulated in clinically relevant large animal models [10–14]. HDAd-mediated hepatocyte transduction can be exploited for numerous diseases beyond monogenic disorders. An interesting application has been reported for the treatment of Type 1 diabetes mellitus. In this study, two HDAds, one expressing *Neurod1* (a transcription factor expressed in developing and adult β-cells of the pancreas), and the other expressing *betacellulin* (a β-cell growth factor), co-injected systemically into diabetic mice, resulted in the formation, within the liver, of cell clusters exhibiting immunohistochemical and ultrastructural properties of the pancreatic islets [15]. Remarkably, the diabetic mice also showed a normalization of glucose levels.

In liver-directed approaches, HDAds expressing short hairpin RNA (shRNA) to silence specific target genes have also been used. For example, HDAd-driven expression of shRNA to the specific mouse genes resulted in approximately 75–90% silencing [16, 17] and in a mouse model of obesity and Type 2 diabetes (*db/db* mice) silencing of the transcription factor sterol regulatory element-binding protein-1c (SREBP1), which is upregulated in obese mice, resulted in a reduction in the body weight [16]. These initial studies could pave the way to a multitude of applications directed at silencing of specific genes for the treatment of a variety of genetic and acquired disorders. Interestingly, in contrast with previous reports showing severe toxicity and lethality following administration of AAV encoding shRNA [18], the HDAd expressing shRNA was clinically well tolerated in mice with only mild pathological and biochemical signs of hepatotoxicity [16, 17]. Moreover, saturation of the exportin-5 pathway, which shuttles cellular micro-RNA (miRNA) from the nucleus to the cytoplasm, was found in the case of AAV [18] and was

thought to be involved in the observed toxicity. In contrast, saturation of the exportin-5 pathway was not seen with HDAd expressing shRNA [17].

Recent studies have uncovered the opportunity to treat autoimmune disorders by expressing functional therapeutic protein into hepatocytes to induce tolerance to a specific protein [19–21]. Hepatic expression of a brain protein, for example, was found to be protective against the neuroinflammatory disease in a mouse model of multiple sclerosis [22]. The suppression of autoimmunity from transgene expression in the liver suggests that the introduction of antigens to the liver may have potential as a preventative or therapeutic intervention against autoimmune disease. Given their high efficiency of hepatic transduction and the ability to drive long-term expression, HDAd would be well suited for this type of application.

A major problem with HDAd-mediated liver directed gene therapy which is preventing various therapeutic strategies to be translated into the clinic is the acute toxicity. High vector doses are required to achieve efficient hepatic transduction following systemic intravascular delivery because of a nonlinear dose response. Kupffer cells of the liver [23, 24], and antibodies both specific and nonspecific for Ad [23, 24] are involved in this nonlinear response. Unfortunately, systemic injection of high vector doses results in a potentially lethal inflammatory response secondary to the activation of the innate immunity and to the interactions with multiple cell types and bloodborne factors. The interaction with red blood cells appears to be particularly important as the majority of the Ad particles of serotype 5 are sequestered by human erythrocytes preventing liver infection [25–27]. Intravenous administration of Ad vectors also results in rapid recruitment of neutrophils in blood and peripheral tissues leading to acute liver inflammation and injury [28, 29], thrombocytopenia [14, 29–31], and widespread transduction of a large number of various other cell types (e.g., endothelium, spleen, lung, etc.). All these interactions play an important role in the activation of the toxic response. Furthermore, several blood factors interact with the Ad particles including proteins of the classical and alternative complement pathways [32–34] and several vitamin K-dependent serine proteases such as factors VII, IX, X, and protein C [35–37]. The interactions with these factors also play an important role in the transduction of target tissues because Ad5 hexon has high-affinity for human coagulation Factor X which facilitate virus entry into hepatocytes [37, 38].

Several groups have investigated various strategies to overcome the threshold to hepatocyte transduction and the obstacle of the acute toxicity. Because the severity of the acute response is dose-dependent, some of these approaches are aimed at preferential targeting of the vector to the liver thereby allowing the use of lower vector doses. For example, injection of HDAd directly into the surgically isolated liver of nonhuman primates was shown to achieve higher efficiency hepatic transduction with reduced systemic vector dissemination, and stable, multi-year transgene expression without chronic toxicity [11]. An alternative, minimally invasive, and clinically more attractive method to deliv-

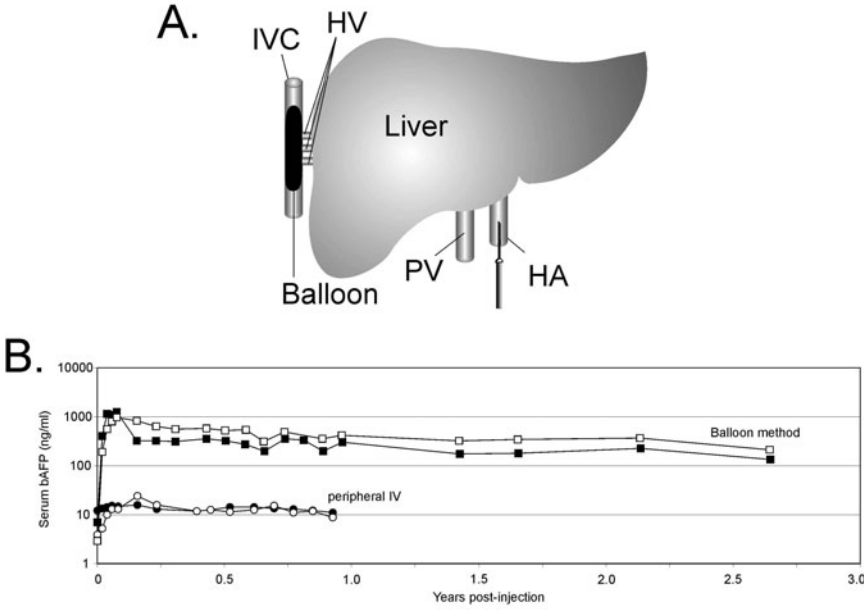


Figure 3. Minimally invasive method to achieve preferential liver transduction in nonhuman primates. (A) A sausage-shaped balloon catheter is positioned in the inferior vena cava (IVC) under fluoroscopic guidance. Inflation of the balloon results in hepatic venous outflow occlusion from the hepatic veins (HV). The HDAd is administered by injection through a percutaneously positioned hepatic artery (HA) catheter. (B). Serum levels of the reporter baboon α-fetoprotein (bAFP) following administration of 3×10^{10} vp/kg of a HDAd expressing bAFP into baboons using the balloon method described above (squares) or by simple peripheral intravenous injection (circles). The balloon method of vector delivery yielded up to 80-fold higher level of transgene expression compared to peripheral intravenous injection of vector, and transgene expression persisted at high levels for at least 2.5 years. Adapted from [13].

er HDAd preferentially to the liver has been developed using balloon occlusion catheters (Fig. 3) [10, 13].

Other strategies such as *masking* the viral capsid through liposome encapsidation [39] or PEGylation [40, 41] seem to attenuate the acute inflammatory response. Given the multiple factors involved, it appears more difficult to manipulate the innate immune response to systemic Ad injection. Nevertheless, a simple approach using pre-treatment with anti-inflammatory glucocorticoids (dexamethasone) before Ad administration, has been shown to significantly reduce Ad-induced acute responses, at least in mice [42].

Gene therapy for cystic fibrosis

The lung is an attractive target for gene transfer with the goal of treating cystic fibrosis (CF), one of the most common genetic disorders due to recessive

mutations in the cystic fibrosis transmembrane conductance regulatory (CFTR) gene. Several CF gene therapy clinical trials have been conducted [1] but no single class of gene therapy vector or vector delivery strategy has yet emerged as obviously superior and the results to date have been disappointing.

FGAd, extensively studied for CF gene therapy, have a number of serious shortcomings. First, pulmonary delivery of FGAd is inefficient because the cellular receptor for Ad (and other viral vectors) resides on the basolateral surface of the airway epithelial cells and the tight junctions prevent vector-receptor interactions required for transduction [43]. Second, pulmonary delivery of FGAd results in dose-dependent inflammation and pneumonia [44–48] beginning about 3 to 4 days post-administration and becoming progressively more severe before eventually resolving. This latter problem has been attributed to the expression of the viral genes of the FGAd vector backbone which are cytotoxic and cause an adaptive cellular immune response against the transduced cells resulting in loss of transgene expression and chronic toxicity [49, 50]. The first obstacle was addressed using molecules that disrupt the tight junctions which resulted in extensive Ad-mediated transduction of the proximal and distal airways (Figs 4A and B) and submucosal glands (Fig. 4C). The second obstacle was solved with the use of HDAd: while administration of FGAd results in pulmonary inflammation with focal peribronchial lymphocytic infiltrates and focal alveolar macrophages, the lungs of mice given HDAd are free of inflammation and indistinguishable from saline treated animals presumably because of the absence of viral gene expression from HDAd [51]. Moreover, the duration of HDAd-mediated pulmonary transgene expression persisted for at least 15 weeks [51]. The studies with HDAd have also indicated that the human cytokeratin 18 (K18) promoter is expressed, similarly to the mouse Cftr, in the epithelium of large airways and bronchioles and in submucosal glands with little expression in the alveoli [52]. In contrast to commonly used viral promoters, the K18 promoter is less likely to suffer host shut-off and could reduce immune stimulation resulting from inappropriate expression in

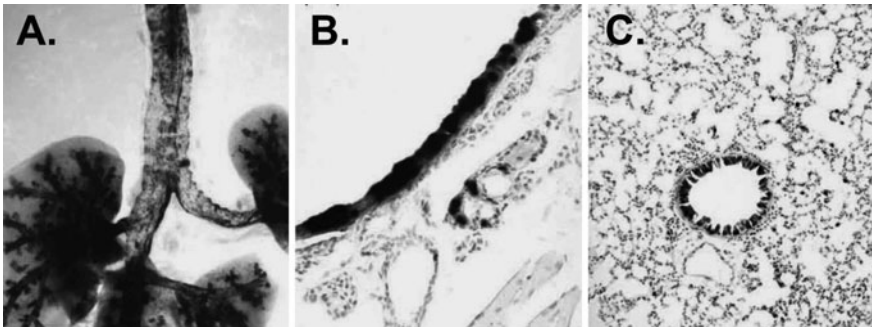


Figure 4. Airway transduction by HDAd. Epithelia transduction of the proximal and distal airway (A), trachea (B), and bronchiole (C) of mice 3 days post-intranasal administration of HDAd-K18LacZ. Blue areas represent HDAd transduced cells. From [51].

antigen presenting cells. The large cloning capacity of HDAd makes this vector ideal to accommodate the relatively large K18 control elements (4.1 kb) and the reporter or therapeutic cDNAs.

An HDAd vector bearing the human CFTR cDNA under the control of the K18 was also found to express properly localized CFTR in cultured cells and in the apical airway epithelia of mice following intranasal administration [53]. Importantly, this vector was also found to improve resistance to acute lung infection in CFTR knockout mice [53]. High efficiency transduction of the airway epithelium has also been demonstrated in a large animal model (rabbit) using an HDAd, formulated in 0.1% L-α-lysophosphatidylcholine (LPC) to open the tight junctions, and delivered by an intracorporeal nebulizing catheter called the AeroProbe (Trudell Medical International) to aerosolize material directly into the trachea and lungs [54]. Although high interlobular variation was present, the delivery of HDAd revealed exceedingly high and unprecedented transduction from the trachea to terminal bronchioles (Figs 5A–E). All rabbits, including those given only LPC as controls showed a transient decrease in dynamic lung compliance immediately following aerosol delivery. Fever and mild-to-moderate patchy pneumonia without edema were also observed. It is possible that LPC may have contributed to these effects which may be eliminated or minimized by optimizing the LPC and/or vector doses. Nevertheless, this study significantly demonstrated for the first time high efficiency transduction of the airway epithelium in a large animal which had previously been a major obstacle to CF gene therapy. This strategy has been applied to nonhuman primates and has yielded similar encouraging results [55]. A uniform vector distribution to all lung lobes was also achieved in the nonhuman primate model by targeting HDAd aerosolization individually into each lung lobe. This strategy resulted in an exceedingly high transduction efficiency to all lung lobes with negligible toxicity [56]. It should be pointed out that the aforementioned studies were performed in animal models with generally intact airways and that transduction will likely be reduced in the lungs affected by multiple bacterial colonizations and thick mucus such as the human CF lungs. Up to now efficacy of gene therapy has been only addressed in animal models with unaffected airways such as the CFTR knockout mice and the nonhuman primates. The recently developed pig model for CF could potentially provide a better model for assessing the efficacy of experimental treatments in the CF lung disease [57]. However, several strategies can be envisioned to address this obstacle in the clinical setting. For example, severely affected CF patients may undergo commonly employed regimens to clear their lungs before gene transfer. This could include inhaled antibiotics (such as tobramycin) and systemic intravenous anti-pseudomonal antibotics (such as aminoglycosides, beta lactams, fluoroquinolones), pulmonary treatment with mucolytic agents (such as pulmozyme), along with mechanical airway clearance to reduce the amount of mucus. Conducting gene transfer in CF patients with less affected lungs may be an alternative option, including the enrollment of younger CF patients with little or no lung disease. While somewhat contro-

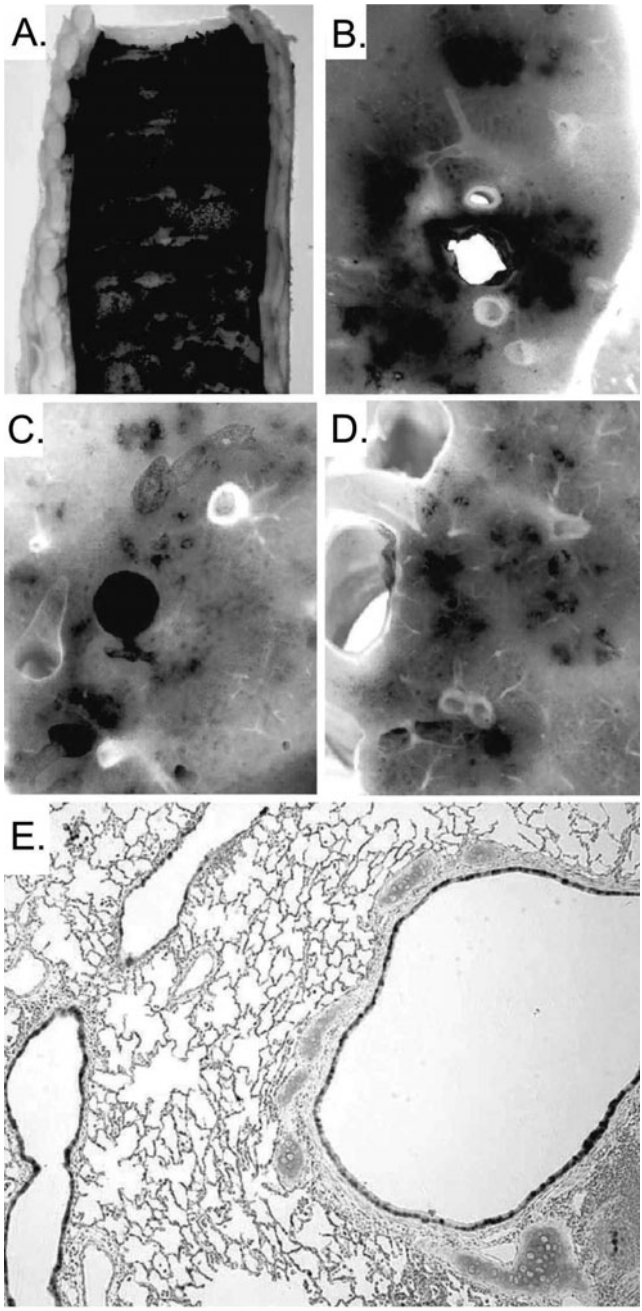


Figure 5. Pulmonary transduction in rabbits following AeroProbe-mediated intratracheal aerosolization of HDAd-K18LacZ formulated in 0.1% LPC. X-gal stained trachea (A), right upper lobe (B), left lower lobe (C), right lower lobe (C) and bronchus and bronchioles (D). Blue areas represent HDAd transduced cells. From [54].

versial, this is not without precedence. Indeed, in a recent clinical trial using AAV, CF patients as young as 12 years of age were enrolled [58]. In summary, while the thickened mucus remains a barrier for all gene transfer vectors (viral or nonviral) as well as for small molecule therapeutics, we do not believe it to be insurmountable, especially considering the low levels of gene transfer that may be required for CF phenotypic correct.

Brain gene therapy

The use of Ad vectors to deliver genes to the central nervous system (CNS) holds great promise for therapeutic applications. Because of their ability to infect post-mitotic cells, including cells of the CNS [59], and to mediate long-term transgene expression, Ad-based vectors are particularly attractive for these applications. Moreover, the delivery to CNS cells of anti-inflammatory genes is attractive for the treatment of inflammatory disorders such as multiple sclerosis. Unlike the rapid decline observed in transgene expression in peripheral organs following intravenous administration, FGAd-mediated transduction of adult brain cells leads to stable transgene expression [60, 61]. It is thought that FGAd-mediated long-term transgene expression occurs because the brain is relatively protected from the effects of the immune response, and in fact, Ad injection into the brain results in an ineffective T cell response against brain-transduced cells [62]. However, the immune system can respond to antigenic stimuli in the brain [63] and if a peripheral immune response against Ad is elicited after natural infection or vector readministration, loss of transgene expression and chronic inflammation are observed [64]. Interestingly, these problems are not seen when HDAd is used [64, 65]. For example, in naïve animals, the expression of β-galactosidase in the brain from FGAd or HDAd is sustained. However, in animals immunized prior to vector delivery, transgene expression is abolished in FGAd injected mice but not in the mice injected with HDAd. These results indicate that long-term (up to 1 year) HDAd mediated transgene expression in the brain occurs even in animals that had been immunized systemically against Ad before the delivery of HDAd into the brain. Therefore, HDAd vectors could turn out to be effective for gene therapy of chronic neurological disorders, even in patients who had been pre-exposed to Ad prior to gene therapy [66].

There are few examples of applications of HDAd for brain-directed gene therapy. Among these, encouraging results have been reported in a Huntington's disease mouse model showing a significant inhibition of Huntington protein aggregation following stereotactic injection into the striatum of a HDAd vector expressing a short hairpin RNA to silence the Huntington disease gene [67].

Besides the potential applications for neurodegenerative disorders, HDAd have also potential for the treatment of inflammatory diseases of the brain. Intrathecal administration of an HDAd expressing interleukin 4, for example,

has a protective role in mice against chronic or relapsing–remitting experimental autoimmune encephalomyelitis, modeling the most common clinical subtypes of multiple sclerosis [68]. Intrathecal HDAd administration is an attractive delivery method because the injection of viral vectors into the cisterna magna (in rodents) or trough lumbar puncture (in nonhuman primates) [69] allows viral vector transduction of neuroepithelial cells and delivery of transgene products to the whole CNS through the ventricular circulation.

Concluding remarks

HDAd possess many characteristics that make them attractive vectors for gene therapy of a wide variety of genetic and acquired diseases. For systemic delivery, the acute toxicity due to a multi-factorial reaction is the most significant obstacle currently hindering the clinical application of this otherwise promising technology. However, the potential of using HDAd for liver-directed gene therapy should not be dismissed but should instead proceed with caution considering the encouraging and compelling studies generated so far. Regardless of the multiple mechanisms involved, strategies to improve the efficiency of gene transfer using lower vector doses are clinically attractive because the acute toxic response is dose-dependent. Improvements in current technologies and development of novel strategies must be pursued to make HDAd gene therapy a clinical reality.

Acknowledgements
PN is supported by grants from the National Institutes of Health (R01 DK067324 and R01 HL083047). NB-P is supported by grants from the National Institutes of Health (R00 HL088692), the Texas Affiliate of the American Heart Association (0765032Y), and the Public Health Service Grant DK56338, which funds the Texas Medical Center Digestive Diseases Center.

References

1 Gene Therapy Clinical Trials Worldwide. *Journal of Gene Medicine*. Available at www.wiley.co.uk/genmed/clinical (accessed April 2009)
2 Segura MM, Alba R, Bosch A, Chillon M (2008) Advances in helper-dependent adenoviral vector research. *Curr Gene Ther* 8: 222–235
3 Brunetti-Pierri N, Ng P (2008) Progress and prospects: gene therapy for genetic diseases with helper-dependent adenoviral vectors. *Gene Ther* 15: 553–560
4 Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL (1996) A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci USA* 93: 13565–13570
5 Bett AJ, Prevec L, Graham FL (1993) Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol* 67: 5911–5921
6 Parks RJ, Graham FL (1997) A helper-dependent system for adenovirus vector production helps define a lower limit for efficient DNA packaging. *J Virol* 71: 3293–3298
7 Palmer D, Ng P (2007) Methods for the production and characterization of helper-dependent adenoviral vectors. Cold Spring Harbor Press
8 Kim IH, Jozkowicz A, Piedra PA, Oka K, Chan L (2001) Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci USA*

98: 13282–13287
9 Toietta G, Mane VP, Norona WS, Finegold MJ, Ng P, McDonagh AF, Beaudet AL, Lee B (2005) Lifelong elimination of hyperbilirubinemia in the Gunn rat with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci USA* 102: 3930–3935
10 Brunetti-Pierri N, Stapleton GE, Palmer DJ, Zuo Y, Mane VP, Finegold MJ, Beaudet AL, Leland MM, Mullins CE, Ng P (2007) Pseudo-hydrodynamic delivery of helper-dependent adenoviral vectors into non-human primates for liver-directed gene therapy. *Mol Ther* 15: 732–740
11 Brunetti-Pierri N, Ng T, Iannitti DA, Palmer DJ, Beaudet AL, Finegold MJ, Carey KD, Cioffi WG, Ng P (2006) Improved hepatic transduction, reduced systemic vector dissemination, and long-term transgene expression by delivering helper-dependent adenoviral vectors into the surgically isolated liver of nonhuman primates. *Hum Gene Ther* 17: 391–404
12 Morral N, O'Neal W, Rice K, Leland M, Kaplan J, Piedra PA, Zhou H, Parks RJ, Velji R, Aguilar-Cordova E et al. (1999) Administration of helper-dependent adenoviral vectors and sequential delivery of different vector serotype for long-term liver-directed gene transfer in baboons. *Proc Natl Acad Sci USA* 96: 12816–12821
13 Brunetti-Pierri N, Stapleton GE, Law M, Breinholt J, Palmer DJ, Zuo Y, Grove NC, Finegold MJ, Rice K, Beaudet AL et al. (2009) Efficient, long-term hepatic gene transfer using clinically relevant HDAd doses by balloon occlusion catheter delivery in nonhuman primates. *Mol Ther* 17: 327–333
14 Brunetti-Pierri N, Nichols TC, McCorquodale S, Merricks E, Palmer DJ, Beaudet AL, Ng P (2005) Sustained phenotypic correction of canine hemophilia B after systemic administration of helper-dependent adenoviral vector. *Hum Gene Ther* 16: 811–820
15 Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, Chan L (2003) NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. *Nat Med* 9: 596–603
16 Ruiz R, Witting SR, Saxena R, Morral N (2009) Robust hepatic gene silencing for functional studies using helper-dependent adenovirus vectors. *Hum Gene Ther* 20: 87–94
17 Witting SR, Brown M, Saxena R, Nabinger S, Morral N (2008) Helper-dependent adenovirus-mediated short hairpin RNA expression in the liver activates the interferon response. *J Biol Chem* 283: 2120–2128
18 Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, Davis CR, Marion P, Salazar F, Kay MA (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* 441: 537–541
19 Brown BD, Cantore A, Annoni A, Sergi LS, Lombardo A, Della Valle P, D'Angelo A, Naldini L (2007) A microRNA-regulated lentiviral vector mediates stable correction of hemophilia B mice. *Blood* 110: 4144–4152
20 Cerullo V, McCormack W, Seiler M, Mane V, Cela R, Clarke C, Rodgers JR, Lee B (2007) Antigen-specific tolerance of human alpha1-antitrypsin induced by helper-dependent adenovirus. *Hum Gene Ther* 18: 1215–1224
21 Mingozzi F, Liu YL, Dobrzynski E, Kaufhold A, Liu JH, Wang Y, Arruda VR, High KA, Herzog RW (2003) Induction of immune tolerance to coagulation factor IX antigen by *in vivo* hepatic gene transfer. *J Clin Invest* 111: 1347–1356
22 Luth S, Huber S, Schramm C, Buch T, Zander S, Stadelmann C, Bruck W, Wraith DC, Herkel J, Lohse AW (2008) Ectopic expression of neural autoantigen in mouse liver suppresses experimental autoimmune neuroinflammation by inducing antigen-specific Tregs. *J Clin Invest* 118: 3403–3410
23 Tao N, Gao GP, Parr M, Johnston J, Baradet T, Wilson JM, Barsoum J, Fawell SE (2001) Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol Ther* 3: 28–35
24 Schiedner G, Hertel S, Johnston M, Dries V, van Rooijen N, Kochanek S (2003) Selective depletion or blockade of Kupffer cells leads to enhanced and prolonged hepatic transgene expression using high-capacity adenoviral vectors. *Mol Ther* 7: 35–43
25 Lyons M, Onion D, Green NK, Aslan K, Rajaratnam R, Bazan-Peregrino M, Phipps S, Hale S, Mautner V, Seymour LW et al. (2006) Adenovirus type 5 interactions with human blood cells may compromise systemic delivery. *Mol Ther* 14: 118–128
26 Seiradake E, Henaff D, Wodrich H, Billet O, Perreau M, Hippert C, Mennechet F, Schoehn G, Lortat-Jacob H, Dreja H et al. (2009) The cell adhesion molecule "CAR" and sialic acid on human erythrocytes influence adenovirus *in vivo* biodistribution. *PLoS Pathog* 5: e1000277

27 Carlisle RC, Di Y, Cerny AM, Sonnen AF, Sim RB, Green NK, Subr V, Ulbrich K, Gilbert RJ, Fisher KD et al. (2009) Human erythrocytes bind and inactivate type 5 adenovirus by presenting coxsackievirus-adenovirus receptor and complement receptor 1. *Blood* 113: 1909–1918
28 Muruve DA, Barnes MJ, Stillman IE, Libermann TA (1999) Adenoviral gene therapy leads to rapid induction of multiple chemokines and acute neutrophil-dependent hepatic injury *in vivo*. *Hum Gene Ther* 10: 965–976
29 Brunetti-Pierri N, Palmer DJ, Beaudet AL, Carey KD, Finegold M, Ng P (2004) Acute toxicity after high-dose systemic injection of helper-dependent adenoviral vectors into nonhuman primates. *Hum Gene Ther* 15: 35–46
30 O'Neal WK, Zhou H, Morral N, Aguilar-Cordova E, Pestaner J, Langston C, Mull B, Wang Y, Beaudet AL, Lee B (1998) Toxicological comparison of E2a-deleted and first-generation adenoviral vectors expressing alpha1-antitrypsin after systemic delivery. *Hum Gene Ther* 9: 1587–1598
31 Wolins N, Lozier J, Eggerman TL, Jones E, Aguilar-Cordova E, Vostal JG (2003) Intravenous administration of replication-incompetent adenovirus to rhesus monkeys induces thrombocytopenia by increasing *in vivo* platelet clearance. *Br J Haematol* 123: 903–905
32 Cichon G, Boeckh-Herwig S, Schmidt HH, Wehnes E, Muller T, Pring-Akerblom P, Burger R (2001) Complement activation by recombinant adenoviruses. *Gene Ther* 8: 1794–1800
33 Jiang H, Wang Z, Serra D, Frank MM, Amalfitano A (2004) Recombinant adenovirus vectors activate the alternative complement pathway, leading to the binding of human complement protein C3 independent of anti-ad antibodies. *Mol Ther* 10: 1140–1142
34 Kiang A, Hartman ZC, Everett RS, Serra D, Jiang H, Frank MM, Amalfitano A (2006) Multiple innate inflammatory responses induced after systemic adenovirus vector delivery depend on a functional complement system. *Mol Ther* 14: 588–598
35 Shayakhmetov DM, Gaggar A, Ni S, Li ZY, Lieber A (2005) Adenovirus binding to blood factors results in liver cell infection and hepatotoxicity. *J Virol* 79: 7478–7491
36 Parker AL, Waddington SN, Nicol CG, Shayakhmetov DM, Buckley SM, Denby L, Kemball-Cook G, Ni S, Lieber A, McVey JH et al. (2006) Multiple vitamin K-dependent coagulation zymogens promote adenovirus-mediated gene delivery to hepatocytes. *Blood* 108: 2554–2561
37 Kalyuzhniy O, Di Paolo NC, Silvestry M, Hofherr SE, Barry MA, Stewart PL, Shayakhmetov DM (2008) Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes *in vivo*. *Proc Natl Acad Sci USA* 105: 5483–5488
38 Waddington SN, McVey JH, Bhella D, Parker AL, Barker K, Atoda H, Pink R, Buckley SM, Greig JA, Denby L et al. (2008) Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell* 132: 397–409
39 Yotnda P, Chen DH, Chiu W, Piedra PA, Davis A, Templeton NS, Brenner MK (2002) Bilamellar cationic liposomes protect adenovectors from preexisting humoral immune responses. *Mol Ther* 5: 233–241
40 Mok H, Palmer DJ, Ng P, Barry MA (2005) Evaluation of polyethylene glycol modification of first-generation and helper-dependent adenoviral vectors to reduce innate immune responses. *Mol Ther* 11: 66–79
41 Croyle MA, Le HT, Linse KD, Cerullo V, Toietta G, Beaudet A, Pastore L (2005) PEGylated helper-dependent adenoviral vectors: highly efficient vectors with an enhanced safety profile. *Gene Ther* 12: 579–587
42 Seregin SS, Appledorn DM, McBride AJ, Schuldt NJ, Aldhamen YA, Voss T, Wei J, Bujold M, Nance W, Godbehere S et al. (2009) Transient pretreatment with glucocorticoid ablates innate toxicity of systemically delivered adenoviral vectors without reducing efficacy. *Mol Ther* 17: 685–696
43 Walters RW, Grunst T, Bergelson JM, Finberg RW, Welsh MJ, Zabner J (1999) Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia. *J Biol Chem* 274: 10219–10226
44 Yei S, Mittereder N, Wert S, Whitsett JA, Wilmott RW, Trapnell BC (1994) *In vivo* evaluation of the safety of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA to the lung. *Hum Gene Ther* 5: 731–744
45 Wilmott RW, Amin RS, Perez CR, Wert SE, Keller G, Boivin GP, Hirsch R, De Inocencio J, Lu P, Reising SF et al. (1996) Safety of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA to the lungs of nonhuman primates. *Hum Gene Ther* 7: 301–318

46 Simon RH, Engelhardt JF, Yang Y, Zepeda M, Weber-Pendleton S, Grossman M, Wilson JM (1993) Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: toxicity study. *Hum Gene Ther* 4: 771–780
47 Joseph PM, O'Sullivan BP, Lapey A, Dorkin H, Oren J, Balfour R, Perricone MA, Rosenberg M, Wadsworth SC, Smith AE et al. (2001) Aerosol and lobar administration of a recombinant adenovirus to individuals with cystic fibrosis. I. Methods, safety, and clinical implications. *Hum Gene Ther* 12: 1369–1382
48 Harvey BG, Maroni J, O'Donoghue KA, Chu KW, Muscat JC, Pippo AL, Wright CE, Hollmann C, Wisnivesky JP, Kessler PD et al. (2002) Safety of local delivery of low- and intermediate-dose adenovirus gene transfer vectors to individuals with a spectrum of morbid conditions. *Hum Gene Ther* 13: 15–63
49 Yang Y, Nunes FA, Berencsi K, Gonczol E, Engelhardt JF, Wilson JM (1994) Inactivation of E2a in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. *Nat Genet* 7: 362–369
50 Yang Y, Li Q, Ertl HC, Wilson JM (1995) Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses. *J Virol* 69: 2004–2015
51 Toietta G, Koehler DR, Finegold MJ, Lee B, Hu J, Beaudet AL (2003) Reduced inflammation and improved airway expression using helper-dependent adenoviral vectors with a K18 promoter. *Mol Ther* 7: 649–658
52 Koehler DR, Hannam V, Belcastro R, Steer B, Wen Y, Post M, Downey G, Tanswell AK, Hu J (2001) Targeting transgene expression for cystic fibrosis gene therapy. *Mol Ther* 4: 58–65
53 Koehler DR, Sajjan U, Chow YH, Martin B, Kent G, Tanswell AK, McKerlie C, Forstner JF, Hu J (2003) Protection of Cftr knockout mice from acute lung infection by a helper-dependent adenoviral vector expressing Cftr in airway epithelia. *Proc Natl Acad Sci USA* 100: 15364–15369
54 Koehler DR, Frndova H, Leung K, Louca E, Palmer D, Ng P, McKerlie C, Cox P, Coates AL, Hu J (2005) Aerosol delivery of an enhanced helper-dependent adenovirus formulation to rabbit lung using an intratracheal catheter. *J Gene Med* 7: 1409–1420
55 Hiatt P, Brunetti-Pierri N, Koehler D, McConnell R, Katkin J, Palmer DJ, Dimmock D, Hu J, Fine-Gold M, Beaudet AL et al. (2005) Aerosol delivery of helper-dependent adenoviral vector into nonhuman primate lungs results in high efficiency pulmonary transduction with minimal toxicity. *Mol Ther* 11: S317
56 Hiatt P, Brunetti-Pierri N, McConnell R, Palmer D, Zuo Y, Finegold M, Beaudet A, Ng P (2007) Bronchoscope-guided, targeted lobar aerosolization of HDAd into nonhuman primate lungs results in uniform, high level pulmonary transduction, long term transgene expression and negligible toxicity. *Mol Ther* 15: S161
57 Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA et al. (2008) Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. *Science* 321: 1837–1841
58 Moss RB, Rodman D, Spencer LT, Aitken ML, Zeitlin PL, Waltz D, Milla C, Brody AS, Clancy JP, Ramsey B et al. (2004) Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest* 125: 509–521
59 Persson A, Fan X, Widegren B, Englund E (2006) Cell type- and region-dependent coxsackie adenovirus receptor expression in the central nervous system. *J Neurooncol* 78: 1–6
60 Le Gal La Salle G, Robert JJ, Berrard S, Ridoux V, Stratford-Perricaudet LD, Perricaudet M, Mallet J (1993) An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* 259: 988–990
61 Davidson BL, Allen ED, Kozarsky KF, Wilson JM, Roessler BJ (1993) A model system for *in vivo* gene transfer into the central nervous system using an adenoviral vector. *Nat Genet* 3: 219–223
62 Byrnes AP, Wood MJ, Charlton HM (1996) Role of T cells in inflammation caused by adenovirus vectors in the brain. *Gene Ther* 3: 644–651
63 Perry VH, Andersson PB, Gordon S (1993) Macrophages and inflammation in the central nervous system. *Trends Neurosci* 16: 268–273
64 Thomas CE, Schiedner G, Kochanek S, Castro MG, Lowenstein PR (2000) Peripheral infection with adenovirus causes unexpected long-term brain inflammation in animals injected intracranially with first-generation, but not with high-capacity, adenovirus vectors: toward realistic long-term neurological gene therapy for chronic diseases. *Proc Natl Acad Sci USA* 97: 7482–7487

65 Xiong W, Goverdhana S, Sciascia SA, Candolfi M, Zirger JM, Barcia C, Curtin JF, King GD, Jaita G, Liu C et al. (2006) Regulatable gutless adenovirus vectors sustain inducible transgene expression in the brain in the presence of an immune response against adenoviruses. *J Virol* 80: 27–37
66 Barcia C, Jimenez-Dalmaroni M, Kroeger KM, Puntel M, Rapaport AJ, Larocque D, King GD, Johnson SA, Liu C, Xiong W et al. (2007) One-year expression from high-capacity adenoviral vectors in the brains of animals with pre-existing anti-adenoviral immunity: clinical implications. *Mol Ther* 15: 2154–2163
67 Huang B, Schiefer J, Sass C, Landwehrmeyer GB, Kosinski CM, Kochanek S (2007) High-capacity adenoviral vector-mediated reduction of huntingtin aggregate load *in vitro* and *in vivo*. *Hum Gene Ther* 18: 303–311
68 Butti E, Bergami A, Recchia A, Brambilla E, Del Carro U, Amadio S, Cattalini A, Esposito M, Stornaiuolo A, Comi G et al. (2008) IL4 gene delivery to the CNS recruits regulatory T cells and induces clinical recovery in mouse models of multiple sclerosis. *Gene Ther* 15: 504–515
69 Butti E, Bergami A, Recchia A, Brambilla E, Franciotta D, Cattalini A, Stornaiuolo A, Lachapelle F, Comi G, Mavilio F et al. (2008) Absence of an intrathecal immune reaction to a helper-dependent adenoviral vector delivered into the cerebrospinal fluid of non-human primates. *Gene Ther* 15: 233–238

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Cells as carriers of gene therapy

Ingo H. Tarner

Department of Internal Medicine and Rheumatology, Justus-Liebig-University Giessen, Division of Rheumatology, Clinical Immunology, Osteology and Physical Medicine, Kerckhoff-Klinik, Bad Nauheim, Germany

Abstract

In order to improve safety and target specificity of gene therapy in autoimmune and inflammatory diseases, various cell types have been used as carriers after *ex vivo* modification. Tissue cells such as fibroblasts, immune cells including lymphocytes, macrophages and dendritic cells (DCs) as well as stem cells, primarily mesenchymal stem cells (MSCs), all have been used for cellular gene therapy. The use of immune cells has been evaluated extensively since these cells have the intrinsic ability to migrate into inflamed tissues and lymphoid organs, the key sites for therapeutic intervention using anti-inflammatory (e.g., cytokine inhibitors) and tissue-protective (e.g., enzyme inhibitors) gene products. Among the immune cells, DCs are powerful tools not only as gene carriers but also because of their own immunomodulatory capacity. Mesenchymal stem cells are attractive because of their potential for tissue regeneration in addition to gene product delivery. Further research is required to optimise the treatment strategies based on these cells and to utilise and control the special features of DCs and MSCs in order to advance towards human application.

Concept and rationale of cells as carriers of gene therapy

The autoimmune and inflammatory diseases covered in the first section of this volume all represent truly systemic diseases. 'Systemic' means that the entire body as a biological system is affected. This systemic involvement manifests in two ways, directly and indirectly. A direct involvement can occur because most autoimmune diseases can affect virtually all tissues and organ systems and frequently do so over time. Systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) are probably the best examples of that. An indirect systemic involvement is virtually inevitable in all of these diseases since affection of the kidneys in lupus will eventually have effects on the cardiovascular system, long-standing diabetes will cause damage to the kidneys, eyes or nervous system, and joint diseases such as RA and osteoarthritis (OA) cause disability. On the other hand, each individual autoimmune and inflammatory disease entity is characterised by its individual avidity for a specific organ or organ system, such as the joints in RA, the pancreatic islet cells in Type 1 diabetes (T1D) or the gut in inflammatory bowel disease (IBD).

From the therapeutic point of view, this situation poses a dilemma. On the one hand, a broad, systemic affection necessitates a similarly broad, systemic treatment. Conventional systemic immunosuppression using agents such as

methotrexate, azathioprine or cyclophosphamide is frequently very effective in improving all aspects of disease. On the other hand, such non-selective treatments come at the price of a high risk of systemic side-effects and toxicity. Particularly at an early stage, a locally targeted and functionally specific treatment of the primarily affected organ system would be an ideal treatment for the majority of the autoimmune and inflammatory diseases.

Local therapy would thus be advantageous over systemic therapy in most diseases because it would avoid affection of other, non-diseased organs, and afford reduction of the required doses of the therapeutic agents of choice, thereby reducing toxicity. Gene therapy has been investigated extensively in the context of autoimmune and inflammatory diseases because it allows long-term, and by now even on-demand expression of therapeutic gene products that avoids the necessity of repeated dosing in these chronic and relapsing-remitting diseases.

For the purpose of locally targeted gene therapy, several strategies have been developed and tested [1–3], one of which is the use of cells as carriers of gene therapy. The use of cells in gene therapy has several advantages. Firstly, cells provide a packaging for the therapeutic gene of interest serving as 'vehicles' for site-specific gene delivery after systemic administration. They can shield their cargo from premature degradation *en route* and, conversely, protect the recipient from unwanted effects of the transported gene outside the target organ. Secondly, many cells, in particular immune cells such as lymphocytes and dendritic cells, have the inborn capacity to home to target organs. In addition, cells can be modified *ex vivo* to either gain or improve this capacity. Thirdly, cells not only can be used as mere vehicles that shuttle genes or gene products to specific organs but they may also serve as therapeutic effectors at the target site, such as stem cells that can be genetically modified to mediate tissue repair.

This chapter provides an overview of different cell-based strategies in gene therapy, using tissue cells, lymphocytes, macrophages, dendritic cells, cell-derived particles, and stem cells (Tab. 1). Due to vast development in the field, it is beyond the scope of this chapter to cover all studies in all disease entities. Instead, a variety of examples are presented that illustrate important developments and strategies in cellular gene therapy. Most examples are taken from RA research since RA represents an archetypical autoimmune disease characterised by systemic inflammation and local tissue damage and thus illustrates several therapeutic challenges in cellular gene therapy.

Tissue cells

The most simple concept for cellular gene therapy is the use of genetically modified tissue cells that are either injected systemically or locally, e.g., intra-articularly in arthritis, into recipients.

Examples from the field of RA research demonstrate that systemic application by intraperitoneal [4, 5] or subcutaneous [6, 7] injection of murine fibroblasts engineered to express interferon (IFN-) β [4], galectin 1 [5], interleukin

Table 1. Overview of the different approaches to using cells as carriers of gene therapy as reviewed in this chapter

Cell type	Vector	Transgene	Animal model	Ref.
Tissue cells				
Syngeneic fibroblasts	Retrovirus	IFNβ	CIA	[4]
Syngeneic fibroblasts	Plasmid transfection	Galectin-1	CIA	[5]
Syngeneic fibroblasts	Plasmid transfection	IL-4	CIA	[6, 7]
Autologous human RASF	Retrovirus	huIL-1Ra	Application to human RA patients	[8]
Human RASF	Adenovirus	IL-1Ra, IL-10	SCID mouse model	[10]
Lympocytes				
DBA/1 splenocytes	Retrovirus	sTNFR	CIA transfer into SCID mice	[12, 14]
Splenocytes, isolated T cells	Retrovirus	TGFβ1	CIA	[13]
Islet-specific T cells	Retrovirus	IL-10	NOD mice	[15]
Ag-specific T cells	Retrovirus	IL-4	EAE	[16]
Ag-specific T cells	Retrovirus	latentTGFβ1	EAE	[17]
Splenocytes	Retrovirus	IL-4	NOD mice	[18]
Ag-specific T cells	Retrovirus	IL-12p40	EAE	[19]
Ag-specific T cells	Retrovirus	IL-12p40	CIA	[20]
Ag-specific T cells	Retrovirus	IL-4	CIA	[21]
Ag-specific T cells	Retrovirus	anti-TNF scFv	CIA	[22]
T cells	Retrovirus	Anti-CII scFv/TCRζ	*In vitro* assays	[24]
Ag-specific T cells	Retrovirus	IL-1Ra, sTNFR-Ig, IL-4, IL-10,	Proteoglycan-induced arthritis	[25]
Antigen-presenting cells				
B cells, macrophages +/– Ag pulsing	Plasmid transfection	IL-4	CIA	[26]
BmDC	Retrovirus	IL-4	CIA	[28]
BmDC	Adenovirus	IL-4	CIA	[29]
BmDC	Lentivirus	IL-4	NOD mice	[30]
BmDC	Lentivirus	IL-12p40, Galectin-1	CIA	[31]
BmDC	Adenovirus	FasL	CIA	[32]
BmDC + Ag pulsing	Adenovirus	TRAIL	CIA	[33]
BmDC	Adenovirus	Galectin-1	NOD mice	[34]
Cell-derived particles				
Exosomes from BmDC	Adenovirus	IL-10	CIA	[37]
Exosomes from BmDC	Adenovirus	IL-FasL	DTH	[38]
Exosomes from BmDC	Adenovirus	IL-4	CIA, DTH	[39]

(continued on next page)

Table 1. (continued)

Cell type	Vector	Transgene	Animal model	Ref.
Stem cells				
MSC	Retrovirus	IL-10	CIA	[42]
MSC	Plasmid transfection	bFGF	*In vitro* assays, rabbits with cartilage lesions	[44]
MSC	Plasmid transfection	TGFβ1	*In vitro* assays, rabbits with cartilage lesions	[45]
Mesenchymal cells	Adenovirus	Bmp-2, IGF-1	Rats with cartilage lesions	[46]

Abbreviations: Ag, antigen; bFGF, basic fibroblast growth factor; BmDC, bone marrow-derived dendritic cells; Bmp-2, bone morphogenetic protein; CIA, collagen-induced arthritis; CII, collagen type II; DBA/1, dilute brown non-agouti/1 mouse strain; DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalomyelitis; FasL, Fas ligand; huIL-1Ra, human interleukin-1 receptor antagonist; IFNβ, interferon-β; Ig, immunoglobulin; IGF-1, insulin-like growth factor-1; IL-12p40, 40kD subunit of interleukin-12; MSC, mesenchymal stem cells; NOD, non-obese diabetic mouse strain; RASF, rheumatoid arthritis synovial fibroblasts; scFv, single-chain immunoglobulin variable fragment; sTNFR, soluble tumour necrosis factor receptor; TCR, T cell receptor; TGFβ1, transforming growth factor-β1; TRAIL, TNF-related apoptosis-inducing ligand

(IL-) 4 [6] or IL-4 and osteoprotegerin [7] is effective in preventing or attenuating collagen-induced arthritis (CIA) in mice.

Though effective, this systemic approach cannot be expected to avoid systemic side-effects. For that purpose, intra-articular transfer of genetically modified rheumatoid arthritis synovial fibroblasts (RASF) has been tested as an alternative. This approach has led to a milestone trial of human application by the Pittsburgh group who injected *ex vivo* modified IL-1 receptor antagonist (IL-1Ra) expressing autologous RASF into the metacarpophalangeal (MCP) joints of patients with RA who were scheduled for routine MCP replacement surgery shortly after [8]. This treatment study proved the feasibility and safety of this therapeutic approach to localised cellular gene therapy.

The disadvantage of this approach is the relatively high degree of invasiveness for the patients if all arthritic joints are to be treated this way in polyarticular RA. Genetic modification of RASF is, however, a promising strategy since we and others have been able to demonstrate that RASF are key mediators of articular destruction in RA [9] and adenoviral transduction of human RASF with genes encoding anti-inflammatory and anti-destructive mediators such as IL-1Ra and IL-10 significantly reduces their invasiveness into human articular cartilage in the severe combined immunodeficiency (SCID) mouse model of RA [10]. Of particular interest, our group have been able to demonstrate that in the SCID mouse model, RASF have the capability to migrate long distance from one subcutaneous implant of RASF and cartilage to another implant of cartilage without RASF via the blood-stream [11]. In addition,

adoptive transfer of RASF by either intravenous, subcutaneous or intraperitoneal injection, respectively, results in trafficking of these cells to subcutaneous cartilage implants. These findings indicate that RASF may be suited for targeted cellular gene therapy by their ability to migrate to inflamed joints after *ex vivo* genetic modification and subsequent systemic application. Further studies are required to verify this strategy.

A group of cells that are characterised by an intrinsic capability of migration into different tissues is the cellular arm of the immune system, in particular lymphocytes and antigen-presenting cells, because they naturally patrol the body and accumulate at sites of inflammation.

Lymphocytes

Lymphocytes were probably the earliest used cell type for local delivery of gene therapy. Their application was based on the hypothesis that their pathogenic specificity for autoantigens could be utilised to guide them specifically to the sites of antigen expression and thus autoimmune inflammation. Previous genetic modification *ex vivo* was intended to prevent their pathogenic behaviour at the target site and to convey an anti-inflammatory activity instead.

Early examples date back to the 1990s and comprise studies in animal models of RA [12–14], diabetes [15] and multiple sclerosis (MS) [16, 17]. Lymphocytes, particularly T cells, are known to play an important role in the pathogenesis of these diseases in humans and even more so in their animal models CIA, non-obese diabetic (NOD) mice and experimental autoimmune encephalomyelitis (EAE), respectively. Adoptive transfer of autoantigen-specific splenocytes can induce or aggravate disease in the respective animal models. In arthritis, transfer of splenocytes from CIA mice can even induce joint inflammation in SCID mice, which are deficient in T and B cells.

The basic principle of all studies using lymphocytes was to modify their behaviour and biological effect by introducing genes encoding cytokines or cytokine antagonists that exert primarily anti-inflammatory effects. Chernajovsky and co-workers demonstrated quite elegantly that the concept of modifying pathogenic splenocytes is effective in the models of CIA and lymphocyte transfer into SCID mice. In their studies, retroviral transduction of splenocytes mediating the expression of the soluble p75 tumour necrosis factor receptor (TNF-R) [12, 14] or of transforming growth factor (TGF-) β1 [13] prevented exacerbation of CIA as well as arthritis induction in SCID mice as compared with non-modified lymphocytes or lymphocytes transduced to express TNF or murine interleukin 10 (mIL-10). Joint swelling, anti-collagen type II (CII) antibody levels, histologic joint damage and articular gelatinase levels were reduced. Similarly, Moritani et al. [15] demonstrated that the induction of insulitis and diabetes in non-diabetic NOD mice by adoptive transfer of islet specific T cells could be clearly reduced if the cells were modified to express mIL-10. Yamamoto and co-workers [18] proved the homing of

genetically modified lymphocytes to the pancreatic islets with local expression of the transgene. Furthermore, they found that in the NOD mouse model among the pool of splenocytes one has to differentiate between cluster of differentiation (CD) 62L non-expressing diabetogenic and CD62L expressing immunoregulatory T cells. Retroviral transfer of the gene encoding IL-4 had no beneficial effect when $CD62L^-$ cells were transduced and transferred into recipient NOD mice but only abrogated diabetes development when $CD62L^+$ cells were transduced and co-transferred with the $CD62L^-$ cells [18].

A whole series of studies on the use of genetically modified T-lymphocytes in EAE and CIA was conducted by the Fathman laboratory at Stanford University [16, 19–22]. This experimental series explored the concept of adoptive cellular gene therapy based on the hypothesis that genetically modified T cells with specificity for the disease-associated autoantigen would specifically home to the site of inflammation and thus deliver the immune-modulating molecules they were transduced to express in a localised fashion for the prevention of systemic side-effects.

Expression of IL-4 [16, 21], the IL-12 receptor inhibitor IL-12p40 [19, 20] and an anti-TNF antibody-derived single chain variable fragment (scFv) by myelin basic protein (MBP)- or CII-specific T-cell receptor (TCR) transgenic (tg) T cell lines or T cell hybridomas proved to be very effective in preventing and attenuating EAE and CIA, respectively. T-lymphocyte transduction was facilitated by a novel bicistronic retroviral construct developed by Costa et al. [23] expressing the gene of interest and a marker protein, green fluorescent protein (GFP), in equivalent amounts. It was shown that antigen specificity was required for achieving the therapeutic effect since T cells with specificity for an irrelevant antigen did not confer disease amelioration in either model [16, 19, 20]. The same was found by Chen et al. [17] who used latent TGF-β1 expressing T cell clones that were only effective if specific for MBP and also previously stimulated with the antigen.

In addition, cellular homing to the sites of inflammation was demonstrated by PCR detection of the marker gene in inflamed paws in arthritis [22] and also in the central nervous system (CNS) or the joints in EAE and CIA, respectively, by *in vivo* bioluminescence imaging using antigen specific T-lymphocytes transduced to express firefly luciferase [19–21]. Interestingly, this imaging technology showed that T-lymphoctytes with specificity for an irrelevant antigen also homed to inflamed joints in arthritis but, in contrast to the CII-specific cells, were not retained there [20]. The adoptively transferred cells were shown to express several chemokine receptors and to respond to chemokines in transwell assays [21] indicating that the cells may follow chemokine gradients into inflamed tissues and remain there if they recognise their cognate antigen. *In vivo* bioluminescence imaging demonstrated that the antigen-specific cells persisted in the target tissue long-term, reaching 50 days in EAE [19].

Lack of effect of the adoptively transferred cells on systemic cytokine and anti-CII antibody levels [16, 20–22] as well as on antigen-specific lymphocyte

proliferation in the recipient CIA animals [20, 21] supported the notion that this treatment strategy had less systemic effects than conventional treatment. By contrast, the local cytokine profile in the inflamed paws was altered [22].

Taken together, these studies provided great hopes for the concept of local cytokine modulation by lymphocyte delivery of anti-inflammatory agents. Important advantages of lymphocytes as gene carriers thus comprise target specificity, probably facilitated by their capability to follow chemokine gradients and to cross endothelial barriers, together with long-term persistence at the target site, and their proliferation upon encounter with their specific antigen, which may enhance transgene expression and thus therapeutic efficacy.

The disadvantage of using antigen-specific T cells is that, in contrast to the well-defined animal models, in human disease the relevant autoantigen(s) are not (yet) identified and the phenomenon of epitope spreading adds further complexity to this situation. Engineering of T cells to create specificity for antigens that are known to be expressed at the target site irrespective of their pathogenic relevance may provide a solution for this problem. Annenkov and Chernajovsky [24] provide evidence for this concept by their anti-CII scFv/TCR ζ chimeric receptor construct that, by retroviral transduction, renders T cells responsive to CII, an antigen that can be expected to be exposed in arthritic joints.

Apart from the issue of antigen specificity, the majority of the studies quoted above also share another weakness. They all applied genetically engineered T cells in a preventative fashion, but none demonstrated efficacy in established disease including the most recent study on the use of cartilage proteoglycan-specific T cells [25]. In preliminary experiments, our group was unable to effectively treat advanced CIA using either IL-12p40 or IL-4 expressing T cell hybridomas (unpublished observations). Since disease prevention does not reflect the situation in clinical medicine, further studies will be necessary before lymphocyte-based gene transfer becomes an option for human application.

In addition, sufficient numbers of autologous T cells for human application may be difficult to obtain. Therefore, other cell types can be envisioned as alternatives. Antigen-presenting cells, such as macrophages and dendritic cells (DCs), appear to be quite promising for human application since they can be derived from monocytes that can be isolated in meaningful numbers from leukapheresis.

Antigen-presenting cells

Macrophages and dendritic cells are appealing for cellular gene therapy because they have two useful characteristics. As immune cells they have the ability to cross the vascular endothelium and to migrate into target tissues, similar to lymphocytes. In addition, as antigen-presenting cells (APC) they have the potential to present (auto)antigens in an immunogenic or tolerogenic fashion.

Based on that, Guéry and co-workers [26] engineered murine bone marrow-derived macrophages to express IL-4 and to present CII on their major histocompatibility complex Class II (MHC II). Adoptive transfer of these cells into CIA mice around the time of immunisation with CII was intended to present the antigen in a T-helper type 2 (Th2) cytokine context thus skewing the CII-specific immune response, and proved to be effective. Of note, the modified macrophages were found to migrate to the inflamed joints although only to a certain extent since it is known that significant numbers of adoptively transferred cells are also retained in the lungs, liver and spleen [27]. Application of IL-4 expressing, CII-pulsed macrophages after clinical disease onset, however, did not have a therapeutic effect. This could mean that at later stages of disease, the antigen-specific interaction between the transferred macrophages and the T cells of the recipient animals is of less importance, at least locally in the inflamed tissues, whereas local delivery of sufficient amounts of the anti-inflammatory IL-4 becomes dominant.

The extent to which an interaction between lymphocytes and the gene carrier cells is important also depends on the cell type that is used for gene delivery. Morita et al. [28] used DCs instead of macrophages for IL-4 expression. Application of these cells also prevented CIA development. However, this effect was achieved without prior pulsing of the DCs with CII. It was shown in this study that the clinical effect correlated with migration of the modified DCs to the lymphoid organs, in particular to the spleen, suggesting that modulation of the immune system outside the inflamed joints is of great importance, at least in this disease model. These findings were supported by Kim and co-workers [29] who also applied IL-4 expressing DCs in CIA and who were able, in contrast to all previous studies, to significantly ameliorate established disease. Further analyses showed that the DCs accumulated in the liver, spleen and lymph nodes and caused a reduction in lymphocyte production of IFN-γ and anti-CII antibodies. Of note, transferred DCs were only found in small numbers in other organs such as lungs, heart, kidneys and muscles, and serum levels of IL-4 were not significantly increased, which indicates that cellular gene therapy using DCs can be expected to have limited systemic side effects.

Similar results were obtained by Creusot et al. [30] in the NOD mice model of diabetes. Here, IL-4 transduced DCs were also found to migrate to the lymphoid system, specifically to the pancreatic lymph nodes and the spleen, where they exerted a regulatory effect on a whole variety of genes that are over- and under-expressed in the NOD model, though no effect on IFN-γ was seen. Interestingly, these authors clearly demonstrated that expression of MHC by the DCs is required for their therapeutic effect. Similarly, Morita et al. [28] showed that, in contrast to IL-4 expressing DCs, the use of IL-4 expressing 3 T3 cells had no therapeutic effect in CIA.

Thus, DCs do not merely serve as vehicles for gene transfer but contribute actively to the therapeutic effect by interacting with lymphocytes. Nevertheless, local migration to inflamed joints can be observed as well [31] and DCs transduced to express IL-12p40 and galectin-1 were found to increase

the expression of IL-10 while reducing the expression of IFN-γ in the inflamed paws (unpublished observations).

The choice of the therapeutic transgene should, therefore, be based on a concept that considers the combined effect of the transgene and the DCs. Successful examples include the expression of apoptosis-inducing molecules such as FasL [32], TNF-related apoptosis-inducing ligand (TRAIL) [33] or galectin-1 [34] by DCs in CIA and NOD mice, all of which resulted in suppression of IFN-γ and antigen-specific T cell proliferation.

Taking these data together, DCs appear to hold great promise for cellular gene therapy with regard to future human application. Their disadvantage lies in their limited proliferative capacity *in vitro* and the high technical complexity of *ex vivo* culture and gene transfer under appropriate good medical practice (GMP) conditions. Furthermore, it remains to be established to which degree the state of DC maturity is important, whether antigen pulsing is required and which is the optimal number of transferred cells.

Also, the route of administration appears to be of importance. Intravenous (i.v.) injection, a commonly used route in inflammatory disease facilitates accumulation of DC in the spleen [28, 35] but also access to local sites of inflammation such as arthritic joints [31], whereas subcutaneous (s.c.) injection favours accumulation in the draining lymph nodes [35]. While in cancer therapy with the goal of eliciting potent anti-tumour immune-responses, s.c. application of DCs has been shown to be more effective than i.v. application [35, 36], the reverse has been reported in the inflammatory setting by Morita et al. [28] for DC-based gene therapy of arthritis. Interestingly, the authors found the highest effectiveness for intraperitoneal (i.p.) transfer of DC and concluded that this was due to effective migration to the spleen by this route. In addition, simultaneous access to the inguinal lymph nodes from the intraperitoneal compartment may have contributed to the superior effectiveness of i.p. DC transfer.

Thus, several parameters and conditions in DC-based gene transfer need to be taken into account and to be clarified in order to achieve optimal therapeutic success in individual autoimmune diseases.

Cell-derived particles

Of interest, Kim and co-workers [37–39] described that therapeutic effects can be achieved not only by whole DCs but also by DC-derived particles, so-called exosomes. Exosomes are small membrane-derived vesicles that are released by a variety of cells including DCs. They contain cytosolic proteins from their parental cells as well as membrane-bound molecules including MHC II and costimulatory molecules and thus are able to present antigen and to stimulate or regulate T cell function either directly or indirectly by fusion with other DCs.

Injection of exosomes that were derived from DCs modified *in vitro* to express IL-4, IL-10 or FasL resulted in a marked improvement of delayed-type

hypersensitivity as well as CIA in respective animal models [37–39] that was remarkably similar to the therapeutic effect obtained by the use of whole genetically modified DCs. Further analysis showed that adoptively transferred exosomes are taken up by hepatic and splenic DCs and macrophages of the recipient when applied intravenously. Upon intradermal injection, they were also internalised by DCs and macrophages of the local draining lymph nodes.

While their efficacy appeared to be somewhat lower than that of whole DCs, the use of exosomes may have some advantages over the use of DCs for human applications. One aspect is safety. Since exosomes are only small membrane-derived particles they do not contain genetic material. Thus, their use would avoid transfer of a whole genetically modified organism including the transgene material into the recipient. This can be significant, for instance, when retroviral constructs are used for gene transfer that carry a certain risk for insertional mutagenesis. In addition, the *in vivo* behaviour can be assumed to be somewhat more predictable because exosomes appear to retain the phenotype of the parental DCs at the time of release whereas DCs might change their phenotype after adoptive transfer into the recipient. Further studies are warranted on the application of exosomes in autoimmune and inflammatory diseases.

Stem cells

Another approach of cellular gene therapy that utilises a certain cell type not only as mere carriers but also takes advantages of specific cellular properties is the use of genetically modified stem cells. Stem cells are particularly appealing for use in diseases that cause irreversible tissue damage such as cartilage and bone destruction in OA and RA. Mesenchymal stem cells (MSCs) isolated from bone marrow or other mesenchymal tissues are thus far most commonly used. Though present only in very low numbers *in vivo*, these cells can be readily amplified to meaningful numbers *in vitro* and have the ability to differentiate into bone, cartilage, muscle or adipose cells.

In addition, MSC have immunoregulatory potential [40] and can ameliorate CIA even without genetic modification by decreasing the levels of proinflammatory cytokines and inducing regulatory T cells [41]. Improved efficacy in CIA was achieved when MSC were transduced to express IL-10 [42].

These studies did, however, not make use of the tissue regenerating potential of MSCs. An important step in exploiting this potential for therapeutic use is the application of signals that drive the differentiation of MSCs towards the desired cell type such as cartilage or bone. Important examples include TGF-β and insulin-like growth factor (IGF-) I for cartilage differentiation and basic fibroblast growth factor (bFGF) and bone morphogenetic protein (BMP-) 2 and 7 for bone formation [43]. Introduction of the respective encoding genes into MSCs by genetic engineering and subsequent implantation into cartilage/bone defects can be utilised to provide the modified MSCs with an autocrine signal and the local cells with a paracrine signal.

Guo et al. [44, 45] investigated TGF-β and bFGF transfected MSC, respectively, *in vivo*. When seeded onto an appropriate biodegradable, porous matrix such as a certain type of ceramics or polylactide, and implanted into cartilage and bone defects, the engineered cells were able to proliferate, to form matrix proteins, to stimulate vascularisation and ultimately to fill cartilage and bone defects with osteoblasts, chondrocytes and hyaline cartilage matrix. At the same time, inflammatory reactions towards the implants were suppressed, a phenomenon attributed to the immunoregulatory potential of the MSC.

Despite this success, a more detailed understanding of the mechanisms that drive MSC differentiation and possible ways to manipulate these mechanisms for therapeutic purposes will be required to make full use of the potential of stem cells and to avoid unwanted effects such as the formation of osteophytes as has been observed with BMP-2 expressing MSCs in an OA model [46].

For the purpose of utilising MSCs to promote formation of a three-dimensional repair tissue in cartilage and bone defects, provision of a scaffold structure as described above is required. Application of MSC without a scaffold can only be expected to improve small lesions [47]. Therefore, in complex lesions such as polyarticular arthritic erosions, implantation of such scaffold-seeded MSC implants represents a much more complex and invasive undertaking than an adoptive transfer of modified MSCs by intravenous or intra-articular injection. In addition, the ideal scaffold material is still a matter of ongoing research.

Conclusions

Gene therapy is a very complex modality. However, if successful, it holds promise for the treatment of equally complex diseases. Due to this complexity, the field has suffered numerous setbacks including human fatalities that seem to have retarded progress towards human application, at least in autoimmune and inflammatory diseases that are usually regarded as being non-fatal. This general view leads to an underestimation of the severity and significance of these diseases as well as to a disproportionate retardation of development when compared to cancer research where gene therapy appears to be more vigorously investigated.

Nevertheless, there have been groundbreaking developments in the field of gene therapy of autoimmune diseases which are reported and summarised in this volume. Among these, cellular gene therapy stands out for two reasons, safety and selectivity. *Ex vivo* gene transfer into vehicle cells avoids direct and systemic exposure of the recipient to the genetic material and to the transfer vector and allows for extensive testing and selection of carrier cells prior to adoptive transfer. In addition, the choice of an adequate cell type such as migrating RASF or DCs can improve directed delivery to desired target sites. Furthermore, certain cell types such as DCs and MSCs feature distinct intrinsic properties that, if used appropriately, can be utilised to broaden their therapeutic value beyond gene delivery.

The advantages of cellular gene therapy do, of course, come at the price of high cost and technical complexity. On the other hand, given the physical and psychological strain and the economic burden caused by chronic autoimmune and inflammatory diseases and comparing cellular gene therapy with current therapy in malignant diseases such as bone marrow transplantation, further development of cellular gene therapy may well be worth the effort and expenses. The necessary basis has definitely been provided by previous research as presented in this chapter.

Acknowledgements
The author is most grateful to his scientific mentors Drs. Ulf Müller-Ladner and C. Garrison Fathman for their continuous and invaluable support.

References

1 Vervoordeldonk MJ, Tak PP (2001) Gene therapy in rheumatic diseases. *Best Pract Res Clin Rheumatol* 15: 771–788
2 Tarner IH, Müller-Ladner U, Fathman CG (2004) Targeted gene therapy: frontiers in the development of 'smart drugs'. *Trends Biotechnol* 22: 304–310
3 Tarner IH, Müller-Ladner U (2008) Drug delivery systems for the treatment of rheumatoid arthritis. *Expert Opin Drug Deliv* 5: 1027–1037
4 Triantaphyllopoulos KA, Williams RO, Tailor H, Chernajovsky Y (1999) Amelioration of collagen-induced arthritis and suppression of interferon-gamma, interleukin-12, and tumor necrosis factor alpha production by interferon-beta gene therapy. *Arthritis Rheum* 42: 90–99
5 Rabinovich GA, Daly G, Dreja H, Tailor H, Riera CM, Hirabayashi J, Chernajovsky Y (1999) Recombinant galectin-1 and its genetic delivery suppress collagen-induced arthritis via T cell apoptosis. *J Exp Med* 190: 385–398
6 Bessis N, Cottard V, Saidenberg-Kermanach N, Lemeiter D, Fournier C, Boissier MC (2002) Syngeneic fibroblasts transfected with a plasmid encoding interleukin-4 as non-viral vectors for anti-inflammatory gene therapy in collagen-induced arthritis. *J Gene Med* 4: 300–307
7 Saidenberg-Kermanac'h N, Bessis N, Lemeiter D, de Vernejoul MC, Boissier MC, Cohen-Solal M (2004) Interleukin-4 cellular gene therapy and osteoprotegerin decrease inflammation-associated bone resorption in collagen-induced arthritis. *J Clin Immunol* 24: 370–378
8 Evans CH, Robbins PD, Ghivizzani SC, Wasko MC, Tomaino MM, Kang R, Muzzonigro TA, Vogt M, Elder EM, Whiteside TL et al. (2005) Gene transfer to human joints: progress toward a gene therapy of arthritis. *Proc Natl Acad Sci USA* 102: 8698–8703
9 Huber LC, Distler O, Tarner I, Gay RE, Gay S, Pap T (2006) Synovial fibroblasts: key players in rheumatoid arthritis. *Rheumatology (Oxford)* 45: 669–675
10 Neumann E, Judex M, Kullmann F, Grifka J, Robbins PD, Pap T, Gay RE, Evans CH, Gay S, Scholmerich J et al. (2002) Inhibition of cartilage destruction by double gene transfer of IL-1Ra and IL-10 involves the activin pathway. *Gene Ther* 9: 1508–1519
11 Lefèvre S, Knedla A, Tennie C, Tarner IH, Stürz H, Steinmeyer J, Gay S, Müller-Ladner U, Neumann E (2007) Long distance migration of RASF: Role of extracellular matrix. *Arthritis Rheum* 56: S436
12 Chernajovsky Y, Adams G, Podhajcer OL, Mueller GM, Robbins PD, Feldmann M (1995) Inhibition of transfer of collagen-induced arthritis into SCID mice by *ex vivo* infection of spleen cells with retroviruses expressing soluble tumor necrosis factor receptor. *Gene Ther* 2: 731–735
13 Chernajovsky Y, Adams G, Triantaphyllopoulos K, Ledda MF, Podhajcer OL (1997) Pathogenic lymphoid cells engineered to express TGF beta 1 ameliorate disease in a collagen-induced arthritis model. *Gene Ther* 4: 553–559
14 Mageed RA, Adams G, Woodrow D, Podhajcer OL, Chernajovsky Y (1998) Prevention of collagen-induced arthritis by gene delivery of soluble p75 tumour necrosis factor receptor. *Gene Ther* 5: 1584–1592

15 Moritani M, Yoshimoto K, Ii S, Kondo M, Iwahana H, Yamaoka T, Sano T, Nakano N, Kikutani H, Itakura M (1996) Prevention of adoptively transferred diabetes in nonobese diabetic mice with IL-10-transduced islet-specific Th1 lymphocytes. A gene therapy model for autoimmune diabetes. *J Clin Invest* 98: 1851–1859
16 Shaw MK, Lorens JB, Dhawan A, DalCanto R, Tse HY, Tran AB, Bonpane C, Eswaran SL, Brocke S, Sarvetnick N et al. (1997) Local delivery of interleukin 4 by retrovirus-transduced T lymphocytes ameliorates experimental autoimmune encephalomyelitis. *J Exp Med* 185: 1711–1714
17 Chen LZ, Hochwald GM, Huang C, Dakin G, Tao H, Cheng C, Simmons WJ, Dranoff G, Thorbecke GJ (1998) Gene therapy in allergic encephalomyelitis using myelin basic protein-specific T cells engineered to express latent transforming growth factor-beta1. *Proc Natl Acad Sci USA* 95: 12516–12521
18 Yamamoto AM, Chernajovsky Y, Lepault F, Podhajcer O, Feldmann M, Bach JF, Chatenoud L (2001) The activity of immunoregulatory T cells mediating active tolerance is potentiated in nonobese diabetic mice by an IL-4-based retroviral gene therapy. *J Immunol* 166: 4973–4980
19 Costa GL, Sandora MR, Nakajima A, Nguyen EV, Taylor-Edwards C, Slavin AJ, Contag CH, Fathman CG, Benson JM (2001) Adoptive immunotherapy of experimental autoimmune encephalomyelitis via T cell delivery of the IL-12 p40 subunit. *J Immunol* 167: 2379–2387
20 Nakajima A, Seroogy CM, Sandora MR, Tarner IH, Costa GL, Taylor-Edwards C, Bachmann MH, Contag CH, Fathman CG (2001) Antigen-specific T cell-mediated gene therapy in collagen-induced arthritis. *J Clin Invest* 107: 1293–1301
21 Tarner IH, Nakajima A, Seroogy CM, Ermann J, Levicnik A, Contag CH, Fathman CG (2002) Retroviral gene therapy of collagen-induced arthritis by local delivery of IL-4. *Clin Immunol* 105: 304–314
22 Smith R, Tarner IH, Hollenhorst M, Lin C, Levicnik AU, Fathman CG, Nolan GP (2003) Localized expression of an anti-TNF single-chain antibody prevents development of collagen-induced arthritis. *Gene Ther* 10: 1248–1257
23 Costa GL, Benson JM, Seroogy CM, Achacoso P, Fathman CG, Nolan GP (2000) Targeting rare populations of murine antigen-specific T lymphocytes by retroviral transduction for potential application in gene therapy for autoimmune disease. *J Immunol* 164: 3581–3590
24 Annenkov A, Chernajovsky Y (2000) Engineering mouse T lymphocytes specific to type II collagen by transduction with a chimeric receptor consisting of a single chain Fv and TCR zeta. *Gene Ther* 7: 714–722
25 Guichelaar T, ten Brink CB, van Kooten PJ, Berlo SE, Lafeber FP, Broeren CP, van Eden W, Broere F (2008) Cartilage proteoglycan-specific T cells as vectors of immunomodulatory biologicals in chronic proteoglycan-induced arthritis. *Mol Immunol* 45: 3526–3535
26 Guéry L, Chiocchia G, Batteux F, Boissier MC, Fournier C (2001) Collagen II-pulsed antigen-presenting cells genetically modified to secrete IL-4 down-regulate collagen-induced arthritis. *Gene Ther* 8: 1855–1862
27 Burke B, Sumner S, Maitland N, Lewis CE (2002) Macrophages in gene therapy: cellular delivery vehicles and *in vivo* targets. *J Leukoc Biol* 72: 417–428
28 Morita Y, Yang J, Gupta R, Shimizu K, Shelden EA, Endres J, Mule JJ, McDonagh KT, Fox DA (2001) Dendritic cells genetically engineered to express IL-4 inhibit murine collagen-induced arthritis. *J Clin Invest* 107: 1275–1284
29 Kim SH, Kim S, Evans CH, Ghivizzani SC, Oligino T, Robbins PD (2001) Effective treatment of established murine collagen-induced arthritis by systemic administration of dendritic cells genetically modified to express IL-4. *J Immunol* 166: 3499–3505
30 Creusot RJ, Yaghoubi SS, Kodama K, Dang DN, Dang VH, Breckpot K, Thielemans K, Gambhir SS, Fathman CG (2008) Tissue-targeted therapy of autoimmune diabetes using dendritic cells transduced to express IL-4 in NOD mice. *Clin Immunol* 127: 176–187
31 Purath U, Neumann E, Creusot R, Yaghoubi S, Härle P, Straub R, Fathman CG, Muller-Ladner U, Tarner I (2006) Immature dendritic cells as vehicles for application in adoptive cellular gene therapy. *Clin Immunol* 119: S34
32 Kim SH, Kim S, Oligino TJ, Robbins PD (2002) Effective treatment of established mouse collagen-induced arthritis by systemic administration of dendritic cells genetically modified to express FasL. *Mol Ther* 6: 584–590
33 Liu Z, Xu X, Hsu HC, Tousson A, Yang PA, Wu Q, Liu C, Yu S, Zhang HG, Mountz JD (2003) CII-DC-AdTRAIL cell gene therapy inhibits infiltration of CII-reactive T cells and CII-induced

arthritis. *J Clin Invest* 112: 1332–1341
34 Perone MJ, Bertera S, Tawadrous ZS, Shufesky WJ, Piganelli JD, Baum LG, Trucco M, Morelli AE (2006) Dendritic cells expressing transgenic galectin-1 delay onset of autoimmune diabetes in mice. *J Immunol* 177: 5278–5289
35 Eggert AA, Schreurs MW, Boerman OC, Oyen WJ, de Boer AJ, Punt CJ, Figdor CG, Adema GJ (1999) Biodistribution and vaccine efficiency of murine dendritic cells are dependent on the route of administration. *Cancer Res* 59: 3340–3345
36 Wang TL, Ling M, Shih IM, Pham T, Pai SI, Lu Z, Kurman RJ, Pardoll DM, Wu TC (2000) Intramuscular administration of E7-transfected dendritic cells generates the most potent E7-specific anti-tumor immunity. *Gene Ther* 7: 726–733
37 Kim SH, Lechman ER, Bianco N, Menon R, Keravala A, Nash J, Mi Z, Watkins SC, Gambotto A, Robbins PD (2005) Exosomes derived from IL-10-treated dendritic cells can suppress inflammation and collagen-induced arthritis. *J Immunol* 174: 6440–6448
38 Kim SH, Bianco N, Menon R, Lechman ER, Shufesky WJ, Morelli AE, Robbins PD (2006) Exosomes derived from genetically modified DC expressing FasL are anti-inflammatory and immunosuppressive. *Mol Ther* 13: 289–300
39 Kim SH, Bianco NR, Shufesky WJ, Morelli AE, Robbins PD (2007) Effective treatment of inflammatory disease models with exosomes derived from dendritic cells genetically modified to express IL-4. *J Immunol* 179: 2242–2249
40 Le Blanc K (2006) Mesenchymal stromal cells: Tissue repair and immune modulation. *Cytotherapy* 8: 559–561
41 Gonzalez MA, Gonzalez-Rey E, Rico L, Buscher D, Delgado M (2009) Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. *Arthritis Rheum* 60: 1006–1019
42 Choi JJ, Yoo SA, Park SJ, Kang YJ, Kim WU, Oh IH, Cho CS (2008) Mesenchymal stem cells overexpressing interleukin-10 attenuate collagen-induced arthritis in mice. *Clin Exp Immunol* 153: 269–276
43 Nixon AJ, Goodrich LR, Scimeca MS, Witte TH, Schnabel LV, Watts AE, Robbins PD (2007) Gene therapy in musculoskeletal repair. *Ann N Y Acad Sci* 1117: 310–327
44 Guo X, Zheng Q, Kulbatski I, Yuan Q, Yang S, Shao Z, Wang H, Xiao B, Pan Z, Tang S (2006) Bone regeneration with active angiogenesis by basic fibroblast growth factor gene transfected mesenchymal stem cells seeded on porous beta-TCP ceramic scaffolds. *Biomed Mater* 1: 93–99
45 Guo X, Zheng Q, Yang S, Shao Z, Yuan Q, Pan Z, Tang S, Liu K, Quan D (2006) Repair of full-thickness articular cartilage defects by cultured mesenchymal stem cells transfected with the transforming growth factor beta1 gene. *Biomed Mater* 1: 206–215
46 Gelse K, von der Mark K, Aigner T, Park J, Schneider H (2003) Articular cartilage repair by gene therapy using growth factor-producing mesenchymal cells. *Arthritis Rheum* 48: 430–441
47 Nöth U, Steinert AF, Tuan RS (2008) Technology insight: adult mesenchymal stem cells for osteoarthritis therapy. *Nat Clin Pract Rheumatol* 4: 371–380

Gene Therapy for Autoimmune and Inflammatory Diseases
Edited by Y. Chernajovsky and P.D. Robbins

Perspectives for the future developments of gene therapy for autoimmune and inflammatory therapy

Yuti Chernajovsky[1] and Paul D. Robbins[2]

[1] *Bone and Joint Research Unit, Barts and The London School of Medicine, Queen Mary University of London, London, UK*
[2] *Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA, USA*

Abstract

Important scientific advances in the understanding of the molecular and cellular events that lead to the development of autoimmune inflammatory conditions have identified a number of targets that could be used for the development of therapeutic interventions that exploit gene and cell-mediated delivery. Relevant to these advances are the results of clinical trials using biological agents, genetic and cellular interventions and more recently the discovery of new genetic predisposition genes in multinational genome-wide association studies, epigenetics and its role in disease progression and finally, therapeutic targeting and exploitation of microRNAs. Application of this broad knowledge is being facilitated by the improvement of gene delivery vectors whose characteristics are being constantly improved and transformed to achieve tissue targeting, long-term expression, transcriptional control and low immunogenicity.

New findings from genome-wide association studies

Most autoimmune inflammatory diseases are of unknown aetiology and their susceptibility has both genetic and environmental factors. Clarifying the genetic factors is very difficult due to the heterogeneity of the human population and the majority of the current large studies only include Caucasian populations. A recent analysis of several published genome-wide association studies and genome wide non- synonymous SNP scans (single nucleotide polymorphisms of non-silent mutations) has brought together important conclusions regarding the similarities in genes involved and shared immunological pathways [1].

23 genes encoding proteins were identified that are shared between two or more autoimmune diseases (including rheumatoid arthritis, Type 1 diabetes, ankylosing spondylitis, Crohn's disease, ulcerative colitis, SLE, coeliac disease, multiple sclerosis, autoimmune thyroid disease and asthma). These 23 genes fall into three groups: genes involved in T cell differentiation, immune cell signalling, and innate immunity and TNF signalling [1].

These genetic studies however, have to be brought into biological context. Firstly, the penetrance or effect of these identified genes will vary among different human populations of different genetic background. Secondly, these

interactions will be affected differentially in different pathological situations, timing and locality as expression of different genes is being modulated. For example the R602W allele of the PTPN22 gene is considered extremely important in the susceptibility to autoimmune diseases [2]. Yet, recent data suggests that the same allele may have a protective effect in Crohn's disease [3].

Interestingly, most of the genetic variants identified in complex genetic disorders are located in non-coding regions of the genome and hence their biological effects are unknown. This indicates that the current genetic knowledge is just the tip of the iceberg on what is really contributing as genetic predisposing factor(s). The need for deep DNA sequencing technologies that can also assess changes in copy number of genes and changes in copies of sequence repeats is still needed. This fact points to the most interesting discovery in molecular biology in the 21st century, the existence of microRNAs and their important role in regulation of gene expression and cell differentiation.

MicroRNAS

microRNAs (miRNAs or miRs) are small RNAs that regulate gene expression by translational repression and/or mRNA degradation. miRs are mainly transcribed by RNA polymerase II, after processing by drosha-DGCR8 into 70 bp mRNA, exported to the cytoplasm and further processed by the nuclease Dicer and then loaded into the RNA-induced silencing complex (with Argonaute and other proteins). Then, a specific single strand of the miR duplex is selected as a guide to direct targeting of mRNA 3' untranslated (UTR) region. A single miR can target many mRNAs containing binding sites (for a review see [4]). miR expression have major effects on cell function and have been reported to serve as tumour suppressors, oncogenes, regulate haematopoietic cell development, T cell sensitivity to antigen stimulation and overall cell metabolism [5–10]. Data from gene knockout mice of the miR nuclease Dicer has clearly demonstrated the importance of this regulatory pathway in the immune system [11–13] and in the control of autoimmunity [14].

miRs have been conserved through evolution and about 700 miRs control about 30% of the genome [15]. Interestingly, but not surprisingly, the miR profile changes between naïve, effector and memory T cells [9]. The transcriptional control of miRs is not fully understood and is an important topic of investigation. Bioinformatic analysis of miR targets of 613 immune-related genes has identified 285 genes that are miR targets. Hence there is preferential targeting of immune genes compared to the genome. Major targets include transcription factors, cofactors involved in mRNA stability and chromatin modifiers (histone deacetylase genes) whereas upstream factors, such as ligands and receptors (cytokines, chemokines and TLRs), are in general, non-targets. About 10% of the immune genes are 'hubs' with eight or more different miRNAs predicted to target their 3' UTRs [15]. All this bioinformatic data has

yet to be validated experimentally. A database of validated miR targets is being updated regularly [16].

In humans, most of the research has focused on differences of miR expression in cancer. However, understanding the role of miRs has become an important step for elucidating additional mechanisms of gene regulation in autoimmune disease [17–19]. miR expression and changes have been recently noted in autoimmune conditions [20–24].

Importantly, mRNAs with miR target sequences can be delivered by gene therapy (for review see [25]) to prevent transgene expression in particular cell types such as antigen presenting cells and avoid immunogenicity, to express in neurons *versus* glia, immature *versus* mature T cells. Normally, miR targets expression is also combined with transcription driven by tissue-specific promoters. Overexpression of miR target sequences in a transgene can serve as a 'decoy' and release the endogenous genes from miR regulation [26]. In addition, expression can be made inducible [27, 28].

The majority of the applications of gene therapy, to date, involve the use of secreted transgenes that act in a paracrine fashion on the affected tissue [29]. In order to properly target miRs *in vivo*, vectors capable of very efficient transduction of all cells affected by the pathology will need to be developed. For targeting miR expression *ex vivo,* current vectors are sufficient.

The understanding of regulation of miR expression will probably have major impact in the treatment of inflammatory conditions as miRs target many genes concomitantly and through miR regulation many target genes could be modulated at the same time. miR function could possibly explain the genetic penetrance effects of certain genes.

Epigenetics

The role of chromatin remodelling in controlling gene expression during differentiation of the immune system is well documented. Cytokine genes (IL-10, IL-4, IFN-γ) [30], accessory genes such as CD4, CD8 are also regulated by DNA methylation and histone modifications via histone acetylases (HATs), deacetylases (HDACs) and histone methylases [31]. In addition, epigenetic changes are affected by ageing [32, 33] and environmental factors that exert epigenetic effects can cause inflammation [34], autoimmunity [35] and cancer [36]. Interestingly, a DNA methylase inhibitor, i.e., 5-azacytidine, can inhibit experimental arthritis [37] as can Trichostatin A, which is a HDAC inhibitor, [38–40]. The mechanisms of action of these drugs *in vivo* are not fully understood and the exact target(s) in these experiments are unknown.

The manipulation of the 'methylome' for therapeutic purposes using gene therapy approaches is a goal that has yet to be achieved, and poses a big challenge, because we do not understand fully the biochemical intricacies of gene-specific silencing [41].

Mechanisms of resistance to therapies

Pro-inflammatory cytokines can affect DNA methylation [42, 43] and regulate HDAC expression [44] as does LPS treatment of macrophages [45]. Changes in DNA methylation, gene silencing and changes in gene expression are common mechanisms of drug resistance with steroids and other chemotherapeutic agents [46–48]. Biologicals such as IFNs [46] and TGFβ [49] affect DNA methylation as well.

Resistance to monoclonal antibody therapies have been reported in cancer treatments [50–55] and mechanisms of resistance vary from downmodulation of the target antigen to mutations in seemingly disparate signalling pathways. Importantly, a genetic study of Rituximab-resistant leukaemia concluded that seven of ten pairs of recurrent CD20-negative cases showed identical Ig heavy chain and Igkappa gene rearrangements by PCR assay, strongly suggesting that the pre- and post-Rituximab treated B cell neoplasms are clonally-related [56], indicating clonal selection of resistant cells. However, there are no reports looking directly into epigenetic changes of gene expression. Intriguingly, the most commonly used anti-inflammatory drug, corticosteroid, recruits HDAC to suppress inflammatory genes [57].

Recently it has been shown that drug resistance is also accompanied by changes in miR expression [58] which affects the expression of the multidrug resistance gene (MDR) 1. The accumulating evidence of drug resistance to biologicals in cancer and autoimmune conditions [59–63], points towards the need of developing gene therapies that are tightly regulated and targeted to the pathological site/tissue both because autoimmune chronic conditions have relapse-remitting cycles [64, 65] and also for safety reasons to prevent long-term side effects [29]. For example, whereas anti-TNF therapies have fundamentally changed the way RA patients are managed, 25–40% of patients fail to respond and there is no clear understanding of the mechanisms responsible for this outcome [59, 62, 63]. Recent results indicate that there is a reduced therapeutic response to Infliximab after the first 6 months of treatment, suggesting acquired drug resistance [60]. This is a very active area or research within the rheumatology community.

Improving on delivery vectors for autoimmune inflammatory disease

In the chapters by Li, Hirsch and Samulsky and by Brunetti-Pierri and Ng above the use of non-immunogenic, gutted adenovirus and AAV vectors has been covered and the current state of their development and use discussed. Retroviral vectors, derived from Moloney murine leukemia virus (MMLV) also have been used extensively for gene therapy applications including an *ex vivo* approach to treat arthritis with genetically modified autologous synovial cells. The primary benefit of retroviral vectors is the integration of their genetic material into the chromosomal DNA of infected cells, thus allowing long-

term expression of transgenes. However, MMLV-based retroviral vectors only infect dividing cells, limiting their utilisation to costly *ex vivo* protocols in which patient cells are isolated, grown and transduced in culture, then returned to the patient. Lentiviral vectors, including human, simian and feline immunodeficiency virus-based vectors (HIV, SIV and FIV) as well as equine infectious anaemia virus (EIAV), overcome this issue due to their ability to infect quiescent cells. The utility of lentiviral vector for efficient intra-articular gene delivery already has been demonstrated. Direct injection of a HIV based lentiviral vector pseudotyped with VSV-G protein into knees of rats resulted in efficient transduction of quiescent synovial lining cells, but not other articular tissues such as cartilage [66]. The level of synovial transduction was similar to that observed with adenoviral vectors with expression of a therapeutic transgene, IL-1Ra, persisted for over a year.

The efficiency of stable transduction with lentiviral vectors previously has been dependent on provirus integration in the cell genome where there is a risk of mutagenesis, either activation of proto-oncogene expression or inactivation of tumour suppressor. To circumvent the risk associated with integration, integration-deficient lentiviral vectors (IDLVs) have been generated recently through the use of specific integrase mutations that prevent proviral integration. Following transduction of target cells, the lentiviral proviral DNA is maintained in circular, vector episomes [67, 68]. Although these lentiviral episomes are unable to replicate and thus are eventually lost in dividing cells, they are maintained stably in quiescent cells. It is also possible that in the future, IDLVs can be modified to allow for replication during the cell cycle, allowing for stable transduction of proliferating tissues.

Conclusion

Long-term gene therapy of autoimmune inflammatory conditions require the development of non-immunogenic vectors (chapters by Subang and Gould; Li, Hirsch and Samulsky and by Brunetti-Pierri and Ng) and the awareness that any long-term therapy could result on unwanted side effects unless the delivery is well controlled and can be terminated if necessary. Advances in vector development including AAV vectors, in particular, the identification of novel serotypes and modified capsids, non-integrating lentiviral vectors and non-viral plasmid formulations should lead to effective *in vivo* gene therapy approaches. Delivery of therapeutic small RNAs such as miRs and siRNA also can be used as novel therapies for inflammatory and autoimmune diseases. Preclinical and clinical trials already have demonstrated the feasibility of gene therapy using both *in vivo* and *ex vivo* approaches [69] (see chapters by Subang and Gould; Li, Hirsch and Samulsky and by Brunetti-Pierri and Ng). Indeed, the Phase I *ex vivo* trials for RA and OA have shown hints of efficacy whereas the Phase II trial for RA using a suboptimal AAV2 based also showed evidence of efficacy. Thus, it is likely that clinically applications of gene therapy

for inflammatory and autoimmune disease will continue to develop, becoming part of standard medical care.

References

1 Zhernakova A, van Diemen CC, Wijmenga C (2009) Detecting shared pathogenesis from the shared genetics of immune-related diseases. *Nat Rev Genet* 10(1): 43–55
2 Criswell LA, Pfeiffer KA, Lum RF, Gonzales B, Novitzke J, Kern M, Moser KL, Begovich AB, Carlton VE, Li W et al. (2005) Analysis of families in the multiple autoimmune disease genetics consortium (MADGC) collection: the PTPN22 620W allele associates with multiple autoimmune phenotypes. *Am J Hum Genet* 76(4): 561–571
3 Barrett JC, Hansoul S, Nicolae DL, Cho JH, Duerr RH, Rioux JD, Brant SR, Silverberg MS, Taylor KD, Barmada MM et al. (2008) Genome-wide association defines more than 30 distinct susceptibility loci for Crohn's disease. *Nat Genet* 40(8): 955–962
4 Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD (2008) MicroRNAs: new regulators of immune cell development and function. *Nat Immunol* 9(8): 839–845
5 Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL, Massague J (2008) Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* 451(7175): 147–152
6 Zheng Y, Josefowicz SZ, Kas A, Chu TT, Gavin MA, Rudensky AY (2007) Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. *Nature* 445(7130): 936–940
7 Chen CZ (2005) MicroRNAs as oncogenes and tumor suppressors. *N Engl J Med* 353(17): 1768–1771
8 Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT et al. (2009) c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature* 458(7239): 762–765
9 Wu H, Neilson JR, Kumar P, Manocha M, Shankar P, Sharp PA, Manjunath N (2007) miRNA profiling of naive, effector and memory CD8 T cells. *PLoS ONE* 2(10): e1020
10 Li QJ, Chau J, Ebert PJ, Sylvester G, Min H, Liu G, Braich R, Manoharan M, Soutschek J, Skare P et al. (2007) miR-181a is an intrinsic modulator of T cell sensitivity and selection. *Cell* 129(1): 147–161
11 Cobb BS, Nesterova TB, Thompson E, Hertweck A, O'Connor E, Godwin J, Wilson CB, Brockdorff N, Fisher AG, Smale ST et al. (2005) T cell lineage choice and differentiation in the absence of the RNase III enzyme Dicer. *J Exp Med* 201(9): 1367–1373
12 Koralov SB, Muljo SA, Galler GR, Krek A, Chakraborty T, Kanellopoulou C, Jensen K, Cobb BS, Merkenschlager M, Rajewsky N et al. (2008) Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. *Cell* 132(5): 860–874
13 Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, Rajewsky K (2005) Aberrant T cell differentiation in the absence of Dicer. *J Exp Med* 202(2): 261–269
14 Zhou X, Jeker LT, Fife BT, Zhu S, Anderson MS, McManus MT, Bluestone JA (2008) Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity. *J Exp Med* 205(9): 1983–1991
15 Asirvatham AJ, Gregorie CJ, Hu Z, Magner WJ, Tomasi TB (2008) MicroRNA targets in immune genes and the Dicer/Argonaute and ARE machinery components. *Mol Immunol* 45(7): 1995–2006
16 Papadopoulos GL, Reczko M, Simossis VA, Sethupathy P, Hatzigeorgiou AG (2009) The database of experimentally supported targets: a functional update of TarBase. *Nucleic Acids Res* 37 (Database issue): D155–158
17 Sheedy FJ, O'Neill LA (2008) Adding fuel to fire: microRNAs as a new class of mediators of inflammation. *Ann Rheum Dis* 67 Suppl 3: iii50–55
18 Rigby RJ, Vinuesa CG (2008) SiLEncing SLE: the power and promise of small noncoding RNAs. *Curr Opin Rheumatol* 20(5): 526–531
19 Yu D, Tan AH, Hu X, Athanasopoulos V, Simpson N, Silva DG, Hutloff A, Giles KM, Leedman PJ, Lam KP et al. (2007) Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. *Nature* 450(7167): 299–303
20 Karouzakis E, Gay RE, Gay S, Neidhart M (2009) Epigenetic control in rheumatoid arthritis synovial fibroblasts. *Nat Rev Rheumatol* 5(5): 266–272

21 Nakamachi Y, Kawano S, Takenokuchi M, Nishimura K, Sakai Y, Chin T, Saura R, Kurosaka M, Kumagai S (2009) MicroRNA-124a is a key regulator of proliferation and monocyte chemoattractant protein 1 secretion in fibroblast-like synoviocytes from patients with rheumatoid arthritis. *Arthritis Rheum* 60(5): 1294–1304
22 Alsaleh G, Suffert G, Semaan N, Juncker T, Frenzel L, Gottenberg JE, Sibilia J, Pfeffer S, Wachsmann D (2009) Bruton's tyrosine kinase is involved in miR-346-related regulation of IL-18 release by lipopolysaccharide-activated rheumatoid fibroblast-like synoviocytes. *J Immunol* 182(8): 5088–5097
23 Chan EK, Satoh M, Pauley KM (2009) Contrast in aberrant microRNA expression in systemic lupus erythematosus and rheumatoid arthritis: is microRNA-146 all we need? *Arthritis Rheum* 60(4): 912–915
24 Pauley KM, Cha S, Chan EK (2009) MicroRNA in autoimmunity and autoimmune diseases. *J Autoimmun* 32(3–4): 189–194
25 Brown BD, Naldini L (2009) Exploiting and antagonizing microRNA regulation for therapeutic and experimental applications. *Nat Rev Genet* 10(8): 578–585
26 Scherr M, Venturini L, Battmer K, Schaller-Schoenitz M, Schaefer D, Dallmann I, Ganser A, Eder M (2007) Lentivirus-mediated antagomir expression for specific inhibition of miRNA function. *Nucleic Acids Res* 35(22): e149
27 Gray DC, Hoeflich KP, Peng L, Gu Z, Gogineni A, Murray LJ, Eby M, Kljavin N, Seshagiri S, Cole MJ et al. (2007) pHUSH: a single vector system for conditional gene expression. *BMC Biotechnol* 7: 61
28 Aagaard L, Amarzguioui M, Sun G, Santos LC, Ehsani A, Prydz H, Rossi JJ (2007) A facile lentiviral vector system for expression of doxycycline-inducible shRNAs: knockdown of the pre-miRNA processing enzyme Drosha. *Mol Ther* 15(5): 938–945
29 Chernajovsky Y, Gould DJ, Podhajcer OL (2004) Gene therapy for autoimmune diseases: quo vadis? *Nat Rev Immunol* 4(10): 800–811
30 Villagra A, Cheng F, Wang HW, Suarez I, Glozak M, Maurin M, Nguyen D, Wright KL, Atadja PW, Bhalla K et al. (2009) The histone deacetylase HDAC11 regulates the expression of interleukin 10 and immune tolerance. *Nat Immunol* 10(1): 92–100
31 Kioussis D, Ellmeier W (2002) Chromatin and CD4, CD8A and CD8B gene expression during thymic differentiation. *Nat Rev Immunol* 2(12): 909–919
32 Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suner D, Cigudosa JC, Urioste M, Benitez J et al. (2005) Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci USA* 102(30): 10604–10609
33 Chen WY, Wang DH, Yen RC, Luo J, Gu W, Baylin SB (2005) Tumor suppressor HIC1 directly regulates SIRT1 to modulate p53-dependent DNA-damage responses. *Cell* 123(3): 437–448
34 Veldhoen M, Hirota K, Westendorf AM, Buer J, Dumoutier L, Renauld JC, Stockinger B (2008) The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. *Nature* 453(7191): 106–109
35 Ballestar E, Esteller M, Richardson BC (2006) The epigenetic face of systemic lupus erythematosus. *J Immunol* 176(12): 7143–7147
36 Grolleau-Julius A, Ray D, Yung RL (2009) The role of epigenetics in aging and autoimmunity. *Clin Rev Allergy Immunol*
37 Kroger H, Dietrich A, Gratz R, Wild A, Ehrlich W (1999) The effect of tryptophan plus methionine, 5-azacytidine, and methotrexate on adjuvant arthritis of rat. *Gen Pharmacol* 33(2): 195–201
38 Nasu Y, Nishida K, Miyazawa S, Komiyama T, Kadota Y, Abe N, Yoshida A, Hirohata S, Ohtsuka A, Ozaki T (2008) Trichostatin A, a histone deacetylase inhibitor, suppresses synovial inflammation and subsequent cartilage destruction in a collagen antibody-induced arthritis mouse model. *Osteoarthritis Cartilage* 16(6): 723–732
39 Halili MA, Andrews MR, Sweet MJ, Fairlie DP (2009) Histone deacetylase inhibitors in inflammatory disease. *Curr Top Med Chem* 9(3): 309–319
40 Chung YL, Lee MY, Wang AJ, Yao LF (2003) A therapeutic strategy uses histone deacetylase inhibitors to modulate the expression of genes involved in the pathogenesis of rheumatoid arthritis. *Mol Ther* 8(5): 707–717
41 Haberland M, Montgomery RL, Olson EN (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. *Nat Rev Genet* 10(1): 32–42
42 Hmadcha A, Bedoya FJ, Sobrino F, Pintado E (1999) Methylation-dependent gene silencing induced by interleukin 1beta via nitric oxide production. *J Exp Med* 190(11): 1595–1604

43 Qian X, Huang C, Cho CH, Hui WM, Rashid A, Chan AO (2008) E-cadherin promoter hypermethylation induced by interleukin-1beta treatment or H. pylori infection in human gastric cancer cell lines. *Cancer Lett* 263(1): 107–113
44 Gopal YN, Van Dyke MW (2006) Depletion of histone deacetylase protein: a common consequence of inflammatory cytokine signaling? *Cell Cycle* 5(23): 2738–2743
45 Aung HT, Schroder K, Himes SR, Brion K, van Zuylen W, Trieu A, Suzuki H, Hayashizaki Y, Hume DA, Sweet MJ et al. (2006) LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. *Faseb J* 20(9): 1315–1327
46 Reu FJ, Leaman DW, Maitra RR, Bae SI, Cherkassky L, Fox MW, Rempinski DR, Beaulieu N, MacLeod AR, Borden EC (2006) Expression of RASSF1A, an epigenetically silenced tumor suppressor, overcomes resistance to apoptosis induction by interferons. *Cancer Res* 66(5): 2785–2793
47 Worm J, Kirkin AF, Dzhandzhugazyan KN, Guldberg P (2001) Methylation-dependent silencing of the reduced folate carrier gene in inherently methotrexate-resistant human breast cancer cells. *J Biol Chem* 276(43): 39990–40000
48 Teitz T, Lahti JM, Kidd VJ (2001) Aggressive childhood neuroblastomas do not express caspase-8: an important component of programmed cell death. *J Mol Med* 79(8): 428–436
49 Chen G, Ghosh P, Osawa H, Sasaki CY, Rezanka L, Yang J, O'Farrell TJ, Longo DL (2007) Resistance to TGF-beta 1 correlates with aberrant expression of TGF-beta receptor II in human B-cell lymphoma cell lines. *Blood* 109(12): 5301–5307
50 Linardou H, Dahabreh IJ, Kanaloupiti D, Siannis F, Bafaloukos D, Kosmidis P, Papadimitriou CA, Murray S (2008) Assessment of somatic k-RAS mutations as a mechanism associated with resistance to EGFR-targeted agents: a systematic review and meta-analysis of studies in advanced non-small-cell lung cancer and metastatic colorectal cancer. *Lancet Oncol* 9(10): 962–972
51 Lu Y, Li X, Liang K, Luwor R, Siddik ZH, Mills GB, Mendelsohn J, Fan Z (2007) Epidermal growth factor receptor (EGFR) ubiquitination as a mechanism of acquired resistance escaping treatment by the anti-EGFR monoclonal antibody cetuximab. *Cancer Res* 67(17): 8240–8247
52 Meyer zum Buschenfelde C, Feuerstacke Y, Gotze KS, Scholze K, Peschel C (2008) GM1 expression of non-Hodgkin's lymphoma determines susceptibility to rituximab treatment. *Cancer Res* 68(13): 5414–5422
53 Ritter CA, Perez-Torres M, Rinehart C, Guix M, Dugger T, Engelman JA, Arteaga CL (2007) Human breast cancer cells selected for resistance to trastuzumab *in vivo* overexpress epidermal growth factor receptor and ErbB ligands and remain dependent on the ErbB receptor network. *Clin Cancer Res* 13(16): 4909–4919
54 Vazquez-Martin A, Oliveras-Ferraros C, Menendez JA (2009) Autophagy facilitates the development of breast cancer resistance to the anti-HER2 monoclonal antibody trastuzumab. *PLoS One* 4(7): e6251
55 Villamor N, Montserrat E, Colomer D (2003) Mechanism of action and resistance to monoclonal antibody therapy. *Semin Oncol* 30(4): 424–433
56 Chu PG, Chen YY, Molina A, Arber DA, Weiss LM (2002) Recurrent B-cell neoplasms after Rituximab therapy: an immunophenotypic and genotypic study. *Leuk Lymphoma* 43(12): 2335–2341
57 Ito K, Lim S, Caramori G, Cosio B, Chung KF, Adcock IM, Barnes PJ (2002) A molecular mechanism of action of theophylline: Induction of histone deacetylase activity to decrease inflammatory gene expression. *Proc Natl Acad Sci USA* 99(13): 8921–8926
58 Kovalchuk O, Filkowski J, Meservy J, Ilnytskyy Y, Tryndyak VP, Chekhun VF, Pogribny IP (2008) Involvement of microRNA-451 in resistance of the MCF-7 breast cancer cells to chemotherapeutic drug doxorubicin. *Mol Cancer Ther* 7(7): 2152–2159
59 Di Poi E, Perin A, Morassi MP, Del Frate M, Ferraccioli GF, De Vita S (2007) Switching to etanercept in patients with rheumatoid arthritis with no response to infliximab. *Clin Exp Rheumatol* 25(1): 85–87
60 Finckh A, Simard JF, Gabay C, Guerne PA (2006) Evidence for differential acquired drug resistance to anti-tumour necrosis factor agents in rheumatoid arthritis. *Ann Rheum Dis* 65(6): 746–752
61 Gartlehner G, Hansen RA, Jonas BL, Thieda P, Lohr KN (2006) The comparative efficacy and safety of biologics for the treatment of rheumatoid arthritis: a systematic review and metaanalysis. *J Rheumatol* 33(12): 2398–2408
62 Hyrich KL, Lunt M, Watson KD, Symmons DP, Silman AJ (2007) Outcomes after switching from one anti-tumor necrosis factor alpha agent to a second anti-tumor necrosis factor alpha agent in patients with rheumatoid arthritis: results from a large UK national cohort study. *Arthritis Rheum*

56(1): 13–20
63 Taylor PC (2006) Is abatacept an effective treatment for patients with RA who do not respond to other anti-TNF treatments? *Nat Clin Pract Rheumatol* 2(3): 128–129
64 Gould D, Yousaf N, Fatah R, Subang MC, Chernajovsky Y (2007) Gene therapy with an improved doxycycline-regulated plasmid encoding a tumour necrosis factor-alpha inhibitor in experimental arthritis. *Arthritis Res Ther* 9(1): R7
65 Gould DJ, Bright C, Chernajovsky Y (2004) Inhibition of established collagen-induced arthritis with a tumour necrosis factor-alpha inhibitor expressed from a self-contained doxycycline regulated plasmid. *Arthritis Res Ther* 6(2): R103–113
66 Gouze E, Pawliuk R, Pilapil C, Gouze JN, Fleet C, Palmer GD, Evans CH, Leboulch P, Ghivizzani SC (2002) *In vivo* gene delivery to synovium by lentiviral vectors. *Mol Ther* 5(4): 397–404
67 Wanisch K, Yanez-Munoz RJ (2009) Integration-deficient lentiviral vectors: a slow coming of age. *Mol Ther* 17(8): 1316–1332
68 Yanez-Munoz RJ, Balaggan KS, MacNeil A, Howe SJ, Schmidt M, Smith AJ, Buch P, MacLaren RE, Anderson PN, Barker SE et al. (2006) Effective gene therapy with nonintegrating lentiviral vectors. *Nat Med* 12(3): 348–353
69 Evans CH, Ghivizzani SC, Robbins PD (2009) Progress and Prospects: genetic treatments for disorders of bones and joints. *Gene Ther* 16(8): 944–952

Index

The MDT-Series
Milestones in Drug Therapy

The discovery of drugs is still an unpredictable process. Breakthroughs are often the result of a combination of factors, including serendipidity, rational strategies and a few individuals with novel ideas. *Milestones in Drug Therapy* highlights new therapeutic developments that have provided significant steps forward in the fight against disease. Each book deals with an individual drug or drug class that has altered the approach to therapy. Emphasis is placed on the scientific background to the discoveries and the development of the therapy, with an overview of the current state of knowledge provided by experts in the field, revealing also the personal stories behind these milestone developments. The series is aimed at a broad readership, covering biotechnology, biochemistry, pharmacology and clinical therapy.

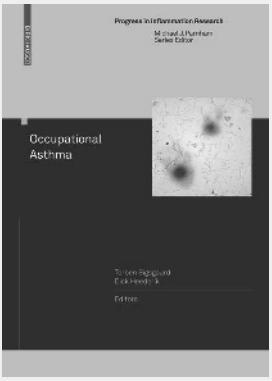
Forthcoming titles:

Drugs for HER2-positive Breast Cancer, C.C. Zielinski, M. Sibilia, T. Grunt, R. Bartsch (Editors), 2010
Influenza Virus Sialidase – A Drug Discovery Target, M. von Itzstein (Editor), 2010
Bortezomib in the Treatment of Multiple Myeloma, K.C. Anderson, P.G. Richardson, I. Ghobrial (Editors), 2010
Glutamate-based Therapies for Psychiatric Disorders, P. Skolnick (Editor), 2010
Treatment and Prevention of Malaria, H.M. Staines, S. Krishna (Editors), 2011

Published volumes:

PEGylated Protein Drugs: Basic Science and Clinical Applications, F.M. Veronese (Editor), 2009
Erythropoietins, Erythropoietic Factors, and Erythropoiesis, 2nd Revised and Extended Edition, S. Elliott, M. Foote, G. Molineux (Editors), 2009
Bipolar Depression: Molecular Neurobiology, Clinical Diagnosis and Pharmacotherapy, C.A. Zarate, H.K. Manji (Editors), 2009
Treatment of Psoriasis, J.M. Weinberg (Editor), 2008
Aromatase Inhibitors, 2nd revised edition, B.J.A. Furr (Editor), 2008
Pharmacotherapy of Obesity, J.P.H. Wilding (Editor), 2008
Entry Inhibitors in HIV Therapy, J.D. Reeves, C.A. Derdeyn (Editors), 2007
Drugs affecting Growth of Tumours, H.M. Pinedo, C. Smorenburg (Editors), 2006
TNF-alpha Inhibitors, J.M. Weinberg, R. Buchholz (Editors), 2006

Aromatase Inhibitors, B.J.A. Furr (Editor), 2006
Cannabinoids as Therapeutics, R. Mechoulam (Editor), 2005
St. John's Wort and its Active Principles in Anxiety and Depression, W.E. Müller (Editor), 2005
Drugs for Relapse Prevention of Alcoholism, R. Spanagel, K. Mann (Editors), 2005
COX-2 Inhibitors, M. Pairet, J. Van Ryn (Editors), 2004
Calcium Channel Blockers, T. Godfraind (Author), 2004
Sildenafil, U. Dunzendorfer (Editor), 2004
Hepatitis Prevention and Treatment, J. Colacino, B.A. Heinz (Editors), 2004
Combination Therapy of AIDS, E. De Clercq, A.M. Vandamme (Editors), 2004
Cognitive Enhancing Drugs, J. Buccafusco (Editor), 2004
Fluoroquinolone Antibiotics, A.R. Ronald, D. Low (Editors), 2003
Erythropoietins and Erythropoiesis, G. Molineux, M. Foote, S. Elliott (Editors), 2003
Macrolide Antibiotics, W. Schönfeld, H. Kirst (Editors), 2002
HMG CoA Reduktase Inhibitors, G. Schmitz, M. Torzewski (Editors), 2002
Antidepressants, B.E. Leonard (Editor), 2001
Recombinant Protein Drugs, P. Buckel (Editor), 2001
Glucocorticoids, N. Goulding, R.J. Flower (Editors), 2001
Modern Immunosuppressives, H.-J. Schuurman (Editor), 2001
ACE Inhibitors, P. D'Orleans-Juste, G. Plante (Editors), 2001
Atypical Antipsychotics, A.R. Cools, B.A. Ellenbroek (Editors), 2000
Methotrexate, B.N. Cronstein, J.R. Bertino (Editors), 2000
Anxiolytics, M. Briley, D. Nutt (Editors), 2000
Proton Pump Inhibitors, L. Olbe (Editor), 1999
Valproate, W. Löscher (Editor), 1999



BIRKHÄUSER

PROGRESS IN INFLAMMATION RESEARCH

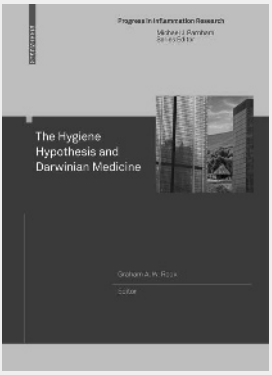
Occupational Asthma

Sigsgaard, T., University of Aarhus, Denmark /
Heederik, D., University of Utrecht,
The Netherlands (Eds)

Sigsgaard, T./
Heederik, D. (Eds)
Occupational Asthma
2010. 376 p. 31 illus. Hardcover
ISBN 978-3-7643-8555-2
PIR — Progress in Inflammation Research

Recent developments in occupational asthma research and clinical practice are the focus of this publication. It discusses the contribution of occupational exposures to chemical and biological agents to the burden of asthma in adults, powerful approaches like the use of apprentices studies, which include young individuals, naïve with regard to occupational exposures, and diagnostic criteria for work related and work aggravated asthma. Specific emphasis is given to exposure response relationships for allergen exposure, large gene-environment studies and whole genome screening approaches. Management, prevention and surveillance are covered as well. Diagnostic rules are introduced to rationalize the diagnostic workup and improve the cost benefit of surveillance schemes. Issues in the design of population studies are discussed to assist the readers in designing and conducting their own study. This comprehensive volume presents new developments changing the occupational asthma field in the coming years.

From the Contents:
The history of research on asthma in the workplace.– Epidemiology and risk factors of occupational respiratory asthma and occupational sensitization.– Epidemiology of laboratory animal allergy.– Population-attributable fraction for occupation and asthma.– Definition and diagnosis of occupational asthma.– Work-exacerbated asthma.– Natural history and prognosis.– Mechanisms of allergic occupational asthma.– Mechanisms of occupational asthma caused by low molecular weight (LMW) chemicals.– Asthma-like diseases in agriculture.– Allergen and irritant exposure and exposure-response relationships.– Gene-environment interactions in occupational asthma.– Asthma in apprentice workers.– Management of an individual worker with occupational asthma.– Social consequences and quality of life in work-related asthma.– Prevention of work-related asthma seen from the workplace and the public health perspective.– Prevention and regulatory aspects of exposure to asthmagens in the workplace.– Design, conduct and analysis of surveys on work-related asthma.



BIRKHÄUSER

PROGRESS IN INFLAMMATION RESEARCH

The Hygiene Hypothesis and Darwinian Medicine

Graham A.W. Rook,
University College London, UK (Ed)

Graham A.W. Rook (Ed)
The Hygiene Hypothesis and Darwinian Medicine
2009. 320 p. 24 illus. Hardcover
ISBN 978-3-7643-8902-4
PIR — Progress in Inflammation Research

Man has moved rapidly from the hunter-gatherer environment to the living conditions of industrialised countries. The hygiene hypothesis suggests that the resulting reduced exposure to micro-organisms has led to disordered regulation of the immune system, and hence to increases in certain chronic inflammatory disorders, like allergic disorders, autoimmunity, inflammatory bowel disease, atherosclerosis, depression, some cancers and perhaps Alzheimer and Parkinson. This book discusses the evidence for and against in the context of Darwinian medicine, which uses knowledge of evolution to cast light on human diseases. The approach is interdisciplinary, looking at man's microbiological history, at the biology of the effects of microorganisms on the immune system, and at the implications for chronic inflammatory disorders in multiple organ systems. Finally, the authors describe progress in the exploitation of microorganisms or their components as novel prophylactics and treatments.

From the Contents: