ADVANCES IN IMMUNOLOGY

VOLUME 103

Advances in
IMMUNOLOGY

VOLUME 103

Advances in
IMMUNOLOGY

VOLUME **103**

Edited by

FREDERICK W. ALT
Howard Hughes Medical Institute, Boston, Massachusetts, USA

Associate Editors

K. FRANK AUSTEN
Harvard Medical School, Boston, Massachusetts, USA

TASUKU HONJO
Kyoto University, Kyoto, Japan

FRITZ MELCHERS
University of Basel, Basel, Switzerland

JONATHAN W. UHR
University of Texas, Dallas, Texas, USA

EMIL R. UNANUE
Washington University, St. Louis, Missouri, USA

ELSEVIER

AMSTERDAM • BOSTON • HEIDELBERG • LONDON
NEW YORK • OXFORD • PARIS • SAN DIEGO
SAN FRANCISCO • SINGAPORE • SYDNEY • TOKYO
Academic Press is an imprint of Elsevier

Academic Press is an imprint of Elsevier

525 B Street, Suite 1900, San Diego, CA 92101-4495, USA
30 Corporate Drive, Suite 400, Burlington, MA 01803, USA
32 Jamestown Road, London, NW1 7BY, UK
Radarweg 29, PO Box 211, 1000 AE Amsterdam, The Netherlands

First edition 2009

ISBN: 978-0-12-374832-4
ISSN: 0065-2776 (series)

Printed and bound in USA
09 10 11 12 10 9 8 7 6 5 4 3 2 1

CONTENTS

CONTRIBUTORS

Numbers in parentheses indicate the pages on which the authors' contributions begin.

Suzanne L. Cassel
Department of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA (*49*)

Gillian Kay
Department of Biochemistry, The Institute for Medical Research Israel-Canada, Hebrew University Hadassah Medical School, Jerusalem, Israel (*1*)

Sunghoon Kim
Center for Medicinal Protein Network and Systems Biology, College of Pharmacy, Seoul National University, Seoul, Korea (*1*)

Mingfang Lu
Departments of Internal Medicine and Microbiology, UT-Southwestern Medical Center, Dallas, Texas, USA; *and* Laboratory of Clinical Infectious Diseases, NIAID/NIH, Bethesda, Maryland, USA (*29*)

Robert Munford
Departments of Internal Medicine and Microbiology, UT-Southwestern Medical Center, Dallas, Texas, USA; *and* Laboratory of Clinical Infectious Diseases, NIAID/NIH, Bethesda, Maryland, USA (*29*)

Hovav Nechushtan
Oncology Department, Hadassah Hebrew University Medical Center, Jerusalem, Israel (*1*)

Raimund J. Ober
Department of Immunology, University of Texas Southwestern Medical Center, Dallas, Texas, USA; *and* University of Texas at Dallas, Richardson, Texas, USA (*77*)

Ehud Razin
Department of Biochemistry, The Institute for Medical Research Israel-Canada, Hebrew University Hadassah Medical School, Jerusalem, Israel (*1*)

Paul B. Rothman
Department of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA (*49*)

Alan Varley
Departments of Internal Medicine and Microbiology, UT-Southwestern Medical Center, Dallas, Texas, USA (*29*)

E. Sally Ward
Department of Immunology, University of Texas Southwestern Medical Center, Dallas, Texas, USA (*77*)

CHAPTER 1

The Physiological Role of Lysyl tRNA Synthetase in the Immune System

Hovav Nechushtan,* **Sunghoon Kim,†** **Gillian Kay,‡** and **Ehud Razin‡**

Contents

Abstract

Lysyl tRNA synthetase (LysRS) is an aminoacyl-tRNA synthetase (AaRS). This group of ancient proteins, known for their critical role in translation, was found in recent years to function in a variety of other roles.

* Oncology Department, Hadassah Hebrew University Medical Center, Jerusalem, Israel
† Center for Medicinal Protein Network and Systems Biology, College of Pharmacy, Seoul National University, Seoul, Korea
‡ Department of Biochemistry, The Institute for Medical Research Israel-Canada, Hebrew University Hadassah Medical School, Jerusalem, Israel

Advances in Immunology, Volume 103
ISSN 0065-2776, DOI: 10.1016/S0065-2776(09)03001-6

Besides its enzymatic activity in aminoacylation of tRNA, LysRS can produce dinucleotide diadenosine tetraphosphate (Ap_4A). Intracellularly, it is found mainly in the cytoplasm as a part of a multisynthetase complex where it interacts with several proteins, most notably AIMP2.

Besides its role in translation it has been demonstrated that LysRS can act as a cytokine-like molecule, secreted by cells and having distinct effects on macrophages. Moreover, LysRS can bind to the transcription factors USF2 and MITF and can influence their transcriptional activities following immunological stimulation of mast cells.

In this review, we focus on the nontranslational functions of LysRS related to the immune system. We begin with a short discussion of "gene sharing," proceed to a description of its structural and enzymatic function and then describe some of the *in vivo* functions of this enzyme.

Lysyl tRNA synthetase (LysRS) is an aminoacyl-tRNA synthetase (AaRS). This ancient family of enzymes has diverged to take on alternate and very diverse functions in the cell that extend well beyond their primary role in protein synthesis. Modules of synthetase ancestors or paralogs have also been adapted for other cellular purposes. One of these alternate roles for an AaRS was discovered when it was shown that AlaRS could bind to an upstream region of its own gene and autoregulate transcription (Putney and Schimmel, 1981). Another example is that human TyrRS is specifically cleaved to produce two distinct cytokines (Wakasugi and Schimmel, 1999). The cleavage itself unmasks the cytokine activities that are imbedded in the native protein, but not elucidated until cleavage occurs. A further example has been described whereby a secreted alternative splice fragment of TrpRS is a potent regulator of angiogenesis (Wakasugi *et al.*, 2002). Such observations have powerful implications on a link between protein synthesis, the immune system, and apoptosis.

In this review, we focus on the nontranslational functions of LysRS that are important to the immune system. We will first briefly discuss ''gene sharing,'' then describe the structural and enzymatic functions of LysRS and finally examine the *in vivo* functions of this enzyme.

1. GENE SHARING AND NONCANONICAL PROTEIN ROLES

Although the initial paradigm relating genes and proteins was that one gene encodes for one protein with one specific function, it has become clear that through alternative splicing one gene can be the source of

numerous, sometimes radically different, proteins. Piatigorsky was a pioneer in the study of alternative functions of similar proteins. For many years, he studied the crystallins, proteins with a critical role in the lens and cornea. His group (Wistow *et al.*, 1987) discovered that duck ε-crystallin was essentially identical to lactate dehydrogenase B4 (LDH64), an ancient, highly conserved glycolytic enzyme. Since ε-crystallin seemed to be confined to birds and crocodiles, it was possible that this represented a recent, atypical event in evolution. These researchers later revealed, however, this was not the case and sequence relationships have been found between all crystallins and specific enzymes (Piatigorsky and Wistow, 1989). The basic reason for the evolution of these enzymes into an alternative role is still not obvious since no common catalytic function is evident among these enzymes/crystallins. It seems that properties such as the ability to accumulate to high intracellular concentrations without precipitation, and thermodynamic stability, a characteristic of many enzymes, are probably the basis for their selection as crystallins. Thus this secondary protein function, which is not ''stable'' during evolution, might seem unrelated to what we perceive as the main attributes of the double function protein but nevertheless may play a critical role in certain biological systems.

This phenomenon was termed ''gene sharing,'' which is defined as a situation whereby a gene may acquire and maintain a second function without duplication or loss of the primary function (Piatigorsky, 2007). While the term gene sharing has been used to define the use of a single protein for two different functions, other names for a similar phenomena are also used such as ''moonlighting'' or noncanonical roles. Most people describing the alternative roles of AaRSs tend to use these terms perhaps due to the critical role of these proteins in translation.

It is important to note that the use of ''off the shelf'' proteins as components of new complexes with novel functions has possible important evolutionary advantages. It allows relatively rapid adaptation for new roles without the need for the creation of totally new proteins. Thus, it is not surprising that gene sharing or noncanonical roles of proteins are widespread.

2. AMINOACYL-tRNA SYNTHETASES (AaRSs) AND THE MULTISYNTHETASE COMPLEX

2.1. General description

The first function described for AaRSs was aminoacylation of their specific tRNAs. AaRSs are essential for decoding the genetic code during protein translation. Researchers trying to obtain insights as to the

beginning of life have studied in great detail these ancient proteins in a range of different organisms as probes for understanding basic aspects relating to the evolution of the genetic code (Ataide and Ibba, 2004; Pezo *et al.*, 2004; Ribas de Pouplana and Schimmel, 2001; Schimmel and Ribas de Pouplana, 2001; Stathopoulos *et al.*, 2001). There are two classes of tRNA synthetases, I and II, that are distinguished by the architecture of their active-site catalytic cores.

The AaRSs are essential housekeeping genes and thus critical for cellular survival. As mentioned earlier, many of these proteins have additional role/roles. These are related either to their catalytic activity or to an additional functional activity.

Decades ago, it was reported that during evolution many of the AaRSs incorporated additional domains (Schimmel and Ribas de Pouplana, 1995). The initially recognized roles of those domains were tRNA recognition and modification functions. However, later it was revealed that these appended domains can help in the regulation of important biological processes, including cell cycle control, tissue differentiation, cellular cemotaxis, and inflammation (Park *et al.*, 2005b, 2008; Ribas de Pouplana and Geslain, 2008).

As described earlier for crystallins, the reason for the evolution of a specific additional role of an ancient protein is not always obvious to us. In many cases, it is not linked to the known enzymatic activity of that specific protein. One suggestion has been that the critical role that AaRSs play in translational regulation evolved to provide a link between translation and a regulatory process such as cell cycle progression. This might be the case in some modes of regulation, but as will be described in the following section, the relationship between LysRS's noncanonical roles and translation is not straightforward. The alternative functions of this protein are probably related also to some other attributes of this protein and not to its role in translation *per se*.

Already in the 1970s, it was reported that most AaRSs reside in large protein complexes in the cytoplasm (Bandyopadhyay and Deutscher, 1971). A stable cytosolic complex of nine AaRSs (leucyl-, lysyl-, prolyl-, isoleucyl-, methionyl-, glutamyl-, glutaminyl-, arginyl-, and aspartyl-tRNA synthetases) was found in mammals (Mirande *et al.*, 1985). Moreover, it was suggested that the AaRSs responsible for coupling to those tRNA molecules that are charged hydrophobic and nonaromatic amino acids, are all present within the complex, while those aminoacylating the smallest and largest amino acids are absent (Wolfson and Knight, 2005). In addition to the nine AaRSs, the complex contains three nonsynthetase proteins: AIMP1, AIMP2, and AIMP3 (until recently, these proteins were known as P43, P38, and P18, respectively).

A variety of tRNA synthetase complexes have been identified in organisms varying from prokaryotes to archea and to eukaryotes, yet it

seems that the larger multisynthetase complex appeared only later in evolution and thus is found only in mammalians (Hausmann and Ibba, 2008a). It was postulated that the existence of the multisynthetase complex is important for efficient translation. Since depletion of any of the synthetases can cause a significant decrease in translation and cellular viability assessing the physiological importance of the multisynthetase complex for translation is difficult.

Only in 2008, there was more direct evidence for the importance of the multisynthetase complex in translational regulation finally provided (Kyriacou and Deutscher, 2008). Already in the 1980s, it was shown that in addition to its role in translation, ArgRS has a role in posttranslational modification, as it arginylates the NH_2 terminus of some proteins, a modification that can serve as a signal for ubiquitin-dependent protein degradation (Ferber and Ciechanover, 1987). Interestingly, there are two forms of ArgRS: one of around 72 Kd, which is found inside the multisynthetase complex, and the other of around 60 Kd found in the cytoplasm (Deutscher and Ni, 1982; Vellekamp *et al.*, 1985). Both forms have similar *in vitro* in catalytic activities (Vellekamp *et al.*, 1985). Kyriacou and Deutscher hypothesized that the shorter cytoplasmatic form is probably important for protein arginylation, while the longer form found in the multisynthetase complex is responsible for translation and the fact that it is part of this complex is critical for this role. A Chinese hamster ovary (Cho) cell line encoding temperature sensitive full-length arginine tRNA synthetase was used in this study. ArgRS is nearly deleted at higher temperatures in these cells. The cells were complemented with recombinant DNA encoding either the short 60 Kd or the longer 72 Kd form of ArgRS. They found that the shorter ArgRS, though just as active *in vitro* as the longer form, was significantly less efficient in complementing the translational activities of ArgRs *in vivo*. Although these results were obtained only for one type of AaRS, they certainly support the notion that the existence of the multisynthetase complex allows much more efficient translational efficacy.

It is interesting to note here the two aspects of the study described earlier. Firstly, in order to obtain insights about the specific function of ArgRS, it was essential to use an approach of reconstitution of the endogenously depleted AaRS with transfected mutated forms of the same plasmid. In recent experiments, we have used a similar approach to try to delineate noncanonical roles of LysRS. Secondly, the protein modifier role of ArgRS needs mention. In the same way that this enzyme can arginylate certain proteins at their NH_2 terminus, it has been postulated that LysRS adenylates NH_2 residues (Chou *et al.*, 2007).

Whatever be the initial important evolutionary advantage of allowing the formation of this multimeric complex, it is clear now that such a complex has advantages for the cells besides its importance in translation.

While the main role of the multisynthetase complex may have been initially to enable efficient protein translation, perhaps through the ''tRNA channeling,'' there are other attributes to proteins found in a complex such as this, for example, their stability might be totally different (Han *et al.*, 2008). The multisynthetase complex not only allows a more efficient functioning of its enzyme components in translation, but can also be viewed as a depot of 11 proteins.

2.2. The multisynthetase complex as a protein "depot"

The depot hypothesis postulates that there are some common features of macromolecular depots and their released daughter proteins (Lee *et al.*, 2004a; Ray *et al.*, 2007). While the macromolecular depots may allow efficient processes that demand close proximity of related proteins to take place, the same proteins released from the multimeric complexes (daughter proteins) commonly acquire a totally different role outside the complex either as monomers or as parts of newly formed complexes. The best studied example of a new complex which includes ''daughter proteins'' released from a multimeric complex is that of the large protein assembly called ''interferon-γ-activated inhibitor of translation'' (the GAIT complex) (Ray *et al.*, 2007). This is an assembly of four proteins that can bind specific structures at the 3′ end of at least several scores of messenger RNAs. The complex was initially identified through the study of proteins bound to the 30-nucleotide untranslated region of the ceruloplasmin mRNA in human monocytic cells. This sequence was demonstrated to be involved in the inactivation of translation following IFN-γ stimulation of those cells. The components of the GAIT complex assemble in two steps: initially, GluProRS is released from the multisynthetase complex and together with a known RNA binding protein: NS1-associated protein-1 (NSAP1) form a pre-GAIT complex after around 2 h of IFN-γ treatment. The complex is inactive until approximately 14 h later in human monocytes, when ribosomal protein L13a, which is released from the large ribosome units, together with another protein which is known mainly for its enzymatic roles, glyceraldehydes 3-phosphate dehydrogenase (GAPDH), join to form the active GAIT complex. Therefore, the GAIT complex contains two daughter proteins each released from a different depot of large multicomplex proteins that are involved in protein translation to ultimately regulate translational activity of several mRNAs. Interestingly, the trigger for release of both GluProRS and ribosomal protein 13a from the large complexes seems to be specific phosphorylation of these proteins (Mazumder *et al.*, 2003; Sampath *et al.*, 2004).

Mammalian GluProRS is unusual because it is a large protein containing two different AaRSs. These AaRSs are linked by a linker containing three homologous protein domains, called WHEP domains (Ribas de Pouplana

and Geslain, 2008). The complex mechanism involved in the binding of GluProRS to the GAIT element in mRNA and the translational inactivation of the related mRNA was recently described in detail (Jia *et al.*, 2008). It seems that all the RNA binding of the GAIT complex to GAIT element RNA is directly mediated by the WHEP domains. The first two WHEP domains are expressed in isolation and can compete with the GAIT complex for binding to the GAIT element, however, these two domains are insufficient for silencing mRNA translation since for that the whole complex is needed. The second and third WHEP domains were found to be used for GluProRS–NSAP1 binding, an association that later undergoes a conformational switch with the addition of phospho-L13a and GAPDH. Thus, a complex picture emerges in which WHEP domains change their interactions and conformation over the period of time following IFN-γ stimulation.

It seems that the important role in the GAIT complex is carried out only by the AaRS appended domains and not by the AaRSs themselves. In this regard, it is interesting to note that, so far, the WHEP domains have only been found to AaRS and not to other proteins. Thus, the large GluProRS released from the multisynthetase complex is used for cellular regulation due to its appended domains and not for any enzymatic activity. A further study identified the single site of L13a phosphorylation responsible for its release from ribosomes and for activation of the GAIT system in IFN-γ-treated monocytic cells (Mukhopadhyay *et al.*, 2008). This site is phosphorylated by the death-associated protein kinase-1 (DAPK) and zipper-interacting protein kinase (ZIPK), both of which contain a functional 30UTR GAIT element. This study revealed the existence of an RNA-based negative-feedback module.

It is interesting to note that the GAIT complex seems to have evolved only later in evolution as it is not found in rodents. While the enzymatic domains of tRNA synthetase are highly conserved through evolution, it would seem that the acquisition of additional, more specific function occurred later in evolution (Ribas de Pouplana and Geslain, 2008).

Several of the multisynthetase complex proteins have been demonstrated to have additional roles besides those related to translation in the multisynthetase complex. Thus, GlutRS and MetRS were shown to be involved in antiapoptotic regulation and rRNA biogenesis (Kim *et al.*, 2000; Ko *et al.*, 2001). Various roles have been described for the AaRS-interacting factors AIMP1–3. AIMP1 acts have several roles as an extracellular cytokine/hormone-like molecule (Kim *et al.*, 2008a; Lee *et al.*, 2008; Park *et al.*, 2006b). AIMP2 has important intracellular roles as a downregulator of c-Myc during lung cell differentiation (Kim *et al.*, 2003) and also as a positive regulator of p53 (Han *et al.*, 2008), while AIMP3 is a tumor suppressor that activates ATM/ATR, which is required for repair of DNA damage (Kim *et al.*, 2008b; Park *et al.*, 2005a, 2006a). Although it would seem that the multisynthetase complex may be the

source of these functional proteins, this has not yet been verified experimentally for proteins other than GluProRS and LysRS. Of all the multisynthetase complex proteins, the enzyme with the most diverse activities besides translational regulation is LysRS on which the rest of this review will focus.

3. LysRS

3.1. Structural and molecular characteristics

As mentioned earlier, the two classes of AaRSs are distinguished by the architectures of their active-site catalytic cores. LysRS in mammals is a class II synthetase, though there are some organisms whose LysRS is a class I synthetase. Like all AaRSs, LysRS is also organized as a modular arrangement of functional domains. The class II LysRS (and the closely related AspRS and AsnRS) have an arrangement of functional domains in which the order of the functional domains is the opposite of that found in most AaRSs; that is, the catalytic domain is at the C-terminal end of the polypeptide, whereas the anticodon-binding site is encoded by the N-terminal part of the protein (Cusack, 1995; Guo *et al.*, 2008). Class II LysRS is one of the most conserved AaRSs (Guo *et al.*, 2008). When human and *Escherichia coli* enzymes are compared, the highest similarity exists for the C-terminal aminoacylation domain (50%), while there is still 26% similarity for the N-terminal anticodon-binding domain (Guo *et al.*, 2008).

In eukaryotes, LysRS contains an N-terminal appendage which is lysine-rich and has been shown to enable nonspecific tRNA binding and thus allow increased catalytic efficiency of the enzyme, especially at the low concentration of deacylated tRNA prevailing *in vivo* (Francin and Mirande, 2003; Francin *et al.*, 2002). Recently, the crystal structure of a tetrameric form of LysRS has been described (Guo *et al.*, 2008). The crystals revealed an unusual tetramer, a structure not seen with any of the other class II AaRS. This crystal structure has already revealed important insights regarding possible interaction mechanisms between LysLysRS and proteins such as AIMP2—one of the three nonenzymatic components of the MSC complex (Guo *et al.*, 2008). There are two eukaryote-specific insertions, one of which is embedded in the tetramer interface from the side of the catalytic domain. This seems to be a hotspot for variations during evolution, and is different even from structures in the related AspRS. This region could thus be involved in the building of new protein interfaces during evolution (Guo *et al.*, 2008).

Unlike several other AaRSs, both mitochondrial and cytoplasmatic LysRS are encoded by the same gene, with the difference being the inclusion of exon 2 between exons 1 and 3 for the mitochondrial isoform encoding. In humans, it has been shown that the mature LysLysRS mRNA

consists of 70% of the cytoplasmic isoform and 30% of the mitochondrial isoform (Tolkunova *et al.*, 2000).

In summary, LysRS in mammalians is a class II AaRS, which has an N-terminal appendage (lacking in LysRS of lower eukaryotes) allowing higher catalytic efficiency due to nonspecific binding of tRNA, and several other nonconserved regions, which allow new protein–protein interactions.

3.2. Interacting proteins

LysRS is found in mammalian cells mainly as a part of the multisynthetase complex. Numerous studies have been performed to map this complex's localization and binding partners (see, e.g., Han *et al.*, 2006; Quevillon *et al.*, 1999) and it may also be found in a tetrameric form (Guo *et al.*, 2008) (Fig. 1.1).

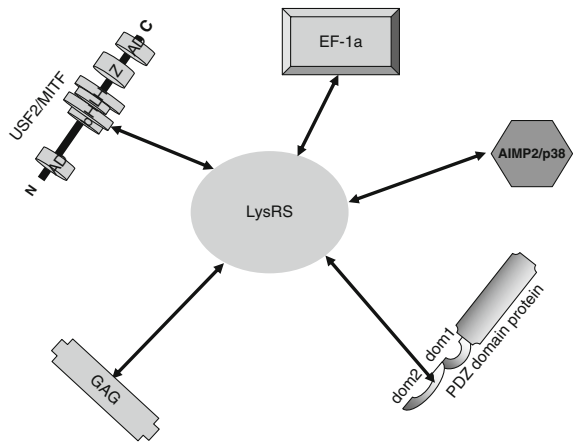

FIGURE 1.1 LysRS interactions. LysRS has been demonstrated to have direct interactions with several proteins. In the multisynthetase complex it has direct interaction with AIMP2/p38, and has a critical role in the maintenance of stability of the complex. A role for EF-1a in channeling tRNA–aminoacids complexes to the ribosome has been suggested by several researchers, direct interactions with mammalian truncated LysRS has only been recently demonstrated *in vitro*. We have demonstrated that LysRS can bind to two bHLH-Leucine zipper transcription factors-MITF and USF2. The HIV virions (and some other retroviruses) contain LysRS. Critical for this incorporation is the interaction with the GAG protein. LysRS is the only AaRS for which interactions with PDZ domain containing proteins has been described. Two independent groups have described interactions with PDZ domain proteins—one with PSD-15 and TIP-15 (two proteins derived by alternative splicing of the same gene), and the other one with syntegenin/mda-9. It seems that the binding to the PDZ domains is mediated at least in part by a PDZ domain binding element found in the C-terminus of LysRS.

Besides these interactions, which are probably critical to the canonical roles of LysRS, several other protein–protein interactions of LysLysRS have been described over the years by different research groups. The protein interactions of LysRS whose functional role has not yet been studied in detail will be described now. Later in this review, we will detail our own work regarding the function of the interactions of LysRS with the transcription factor MITF (Carmi-Levy *et al.*, 2008; Lee *et al.*, 2004b) and with components of the HIV virus in which it is packaged.

3.2.1. PDZ domain interactions

Proteins harboring the postsynaptic density-95/discs large/zonula occludens-1 (PDZ) domain function as scaffolds in organizing multiprotein complexes (Nourry *et al.*, 2003). The PDZ domain-containing proteins have a critical role in the connections of various membrane proteins such as cell adhesion molecules, receptors, and ion channels with downstream signaling molecules (Nourry *et al.*, 2003; Zimmermann, 2006). Interestingly, PDZ-containing proteins usually contain more than just PDZ domains and include other protein interaction domains, such as SH3, PTB, and WW (Nourry *et al.*, 2003). In most cases, the PDZ domains recognize the C-terminus of their protein binding partners, though by now it is known that some PDZ domains can recognize internal peptide sequences and sometimes even lipids (Nourry *et al.*, 2003). Specific roles for PDZ domain-containing proteins have been described in various biological systems. Of note is the particular role of some PDZ domain proteins in cancer.

Two independent groups, who have tried to isolate proteins interacting with the PDZ domain proteins, reported the specific binding of LysRS to those proteins. We will now describe their findings in some detail as they reveal a possible important connection between LysRS and signal-transduction pathways.

The first group performed a yeast two-hybrid screen with HTLV-1 TAX as a bait and isolated a PDZ domain protein called TIP-15, which harbors the first two PDZ domains of a larger protein known as PSD-95 (Rousset *et al.*, 1998). TIP-15 was then used as bait itself in another two-hybrid screen, resulting in the isolation of 12 proteins with high affinity to this protein, including LysRS (Fabre *et al.*, 2000). Coimmunoprecipitation experiments in mammalian cells confirmed the interactions of four of these proteins, one of them being LysRS. TIP-1, a truncated protein containing only one PDZ domain, did not bind to LysRS. A canonical PDZ domain binding motif at the C-terminus of LysRS was identified in this study for the first time (Fabre *et al.*, 2000).

Recently, another group (Meerschaert *et al.*, 2008) independently described an interaction of LysRS with a PDZ domain protein known as both syntenin-1 (isolated as a protein that binds syndecan

(Grootjans *et al.*, 1997)) and as mda-9 (isolated as a melanoma differentiation associated gene (Lin *et al.*, 1998). This specific PDZ domain protein has been shown to be overexpressed in breast and gastric cancer cells and in melanoma, where it promotes the migration and metastasis of cancer cells (Boukerche *et al.*, 2005; Koo *et al.*, 2002). Numerous proteins have been found to be able to bind syntenin-1/mda-9 including various glutamate receptors, the serine/threonine kinase Unc51.1 and rab5,19 (Meerschaert *et al.*, 2008). Meerschaert's group used mass spectrometry to discover genes interacting *in vivo* with syntenin-1/mda-9 in mammalian cells (Meerschaert *et al.*, 2008). They identified several AaRSs derived from the multisynthetase complex as binding partners of this protein. Subsequent experiments identified LysRS as the direct syntenin-1/mda-9 binding protein on which the binding of other multisynthetase complex proteins is dependent. They also identified the PDZ domain binding motif in the C-terminus of LysRS (Meerschaert *et al.*, 2008).

As an initial functional assay, this group studied the effect of syntenin-1/mda-9 binding on LysRS aminoacylation activity. They found that recombinant syntenin-1/mda-9, when used at an equimolar ratio to LysRS, inhibited LysRS activity by approximately 40%. This assay, however, was performed *in vitro* without any further *in vivo* studies (Meerschaert *et al.*, 2008).

The findings that two different PDZ domain proteins bind specifically to LysRS and not to other AaRSs, strongly suggest that LysRS–PDZ interactions have a role in signal transduction. Syntenin-1/mda-9 binding to LysRS seems to result in decreased translation rates (Meerschaert *et al.*, 2008), yet there might be other roles for this interaction, such as facilitating the interaction of LysRS with other signal-transduction molecules. In this regard, it is interesting to note that syntenin-1/mda-9 was originally isolated as a melanoma differentiation gene and that LysRS binds to the transcription factor MITF and is involved in its regulation. This transcription factor has been shown to have an important role both as a melanocyte differentiation regulation and as a melanoma oncogene. All this suggests that the any connection between LysRS, MITF, and syntenin-1/mda-9 in melanoma should be investigated.

3.2.2. Elongation factor 1A (EF-1A) and LysRS

A role for elongation factor 1A (EF-1A) in channeling the tRNA–aminoacyl complex from the tRNA to the ribosome has been proposed (Hausmann and Ibba, 2008b). The archeal multisynthetase complex, which is much smaller than its mammalian counterpart and contains only three synthetases, LeuRS, LysRS, and ProRS, was recently investigated (Hausmann and Ibba, 2008b). EF-1A was found to bind to LeuRS in a stable complex which increased Leu-tRNA synthesis to several folds. The interaction of EF-1A with the archaeal multisynthetase complex

contributes to the translational process by enhancing the aminoacylation rates of the AaRSs in the complex and by subsequent channeling of the cognate tRNA–aminoacid complexes to the ribosome (Hausmann *et al.*, 2007). Interactions between EF-1A and various AaRSs have been described in eukaryotes. Already in 1994, the importance of the N-terminal appendage of human AspRS EF-1A was described (Reed and Yang, 1994). It is important to mention that both LysRS and AspRS are similar class II AaRSs with a terminal appendage to the original structure which has a role in the binding of tRNA (Francin *et al.*, 2002). Yang and his colleagues in 2008 published their research regarding the effect of EF-1A and AspRS on stimulated lysylation *in vitro*. They noted that only upon the removal of the amino-terminal appendage from human LysRS could it bind to EF-1A and its lysylation activity was stimulated by this binding (Guzzo and Yang, 2008). The observed stimulation of lysylation activity was unlikely due to stabilization of synthetase by EF-1A since the half-life of LysRS under the assay conditions was several hours while the aminoacylation assays were completed in minutes. Since it was the truncated LysRS which was influenced by EF-1A binding, the physiological relevance of EF-1A binding to LysRS are still not clear. Furthermore, this group did not study any other activity of LysRS such as its ability to synthesize Ap_4A.

It is interesting to note that similar to LysRS, EF-1A has been implicated in several critical biological processes. For example, a truncated form of this enzyme has been found to be an important oncogene in prostate cancer (Mansilla *et al.*, 2005), and it has been claimed that the effect of G-rich GT oligonucleotides on cellular cytotoxicity correlated with their binding to nuclear EF-1A. We found strong binding of EF-1A to MITF in a yeast two-hybrid screen (Razin, unpublished data). We have not yet studied this *in vitro* interaction further, but it could be postulated that under certain conditions not only LysRS but also an associated elongation factor might have a role in transcriptional regulation.

3.2.3. Mutated superoxide dismutase

Amyotrophic lateral sclerosis or as it is popularly known, Lou Gehrig's disease, is a progressive motor neuron degenerative disease which invariably results in death. The majority of the familial cases of this disease were found to be related to mutations in the gene encoding Cu, Zn superoxide dismutase. In 1997, mouse models of ALS were used to try and locate the downstream effectors of mutated superoxide dismutase. It was demonstrated that mutated superoxide dismutase can specifically bind to mitochondrial LysRS (Kunst *et al.*, 1997). More than 10 years later, it was found that LysRS that is bound to mutated superoxide dismutase displays a high propensity to misfold and aggregate prior to its import into mitochondria (Kawamata *et al.*, 2008). This misfolded LysRS can then

undergo proteasomal degradation. Ultimately, the interactions between mutated superoxide dismutase and mitochondrial LysRS result in mitochondrial morphological abnormalities and cellular toxicity. Thus, it seems that mitochondrial LysRS is the first protein whose abnormal binding to mutated superoxide dismutase leads to mitochondrial toxicity and might play a significant role in the development of mutated superoxide dismutase-related ALS.

3.3. LysRS and the autoimmune response

Autoantibodies can cause a variety of diseases. A surprising finding regarding autoantibodies is that their repertoire is rather limited and has been assessed as less than 2% of total human proteins (Plotz, 2003). Interestingly, mouse models of autoimmunity have displayed similar autoantibodies to those found in humans, again emphasizing the nonrandomness of this repertoire. However, it seems that for most autoantigens, the reason that specific proteins turn into antigens is far from obvious since there is no clear cut functional relationship between the different proteins. Several AaRSs have been found to be autoantigens. The most studied are antibodies to HistRS (known as anti-Jo-1 antibodies), which are characteristic of autoimmune inflammatory myopathy (myositis) in humans (Nagaraju *et al.*, 2000), and anti-IleRS (known as anti-OJ antibodies). Patients with anti-OJ antibodies have been shown to have antibodies to other constituents of the multisynthetase complex (Gelpi *et al.*, 1996; Targoff *et al.*, 1993). The reason for the common occurrence of AaRSs as autoantigens was postulated by Plotz and colleagues to be linked to their ability to act as chemoattractants to immune cells (Plotz, 2003). Unknown to Plotz at the time of writing of his review was the possible activity of LysRS as a chemokine-like molecule. Autoantibodies were isolated from the sera of patients who had developed transplant-associated coronary artery disease (TxCAD) following cardiac transplantation (Linke *et al.*, 2001). They isolated a total of six positive clones out of 40,000 clones from a HUVEC cDNA library, one of which was LysRS, and two were ribosomal proteins. So, LysRS would also seem to be an autoantigens, though it is not as prominent as several other AaRSs.

3.4. Functional roles

A variety of roles for LysRS have been described. We will initially describe some alternate biochemical roles and later its physiological functions.

3.4.1. Lysine residue adenylation

Wagner and colleagues recently described the ability of LysRS to adenylate lysine residues (Chou and Wagner, 2007). They used both human and *E. coli* LysRS to study adenylation of Hint. Hint-1 is a member of the histidine triad (HiT) superfamily of proteins, all of which can bind and hydrolyze nucleotides or their derivatives. It has great structural similarity to the tumor suppressor FHIT, which can bind both Ap_4A and Ap_3A, though it is much more efficient in the hydrolysis of Ap_3A. All the reactions were carried out *in vitro* with purified recombinant proteins. The adenylated proteins are found for relatively short times and that complicates any efforts to identify them *in vivo*. Radiolabled nucleotides were added to the purified proteins to prove that the Hint is labeled by the addition of AMP, in a manner that is dependent upon the formation of lysyl-AMP. They found that Hint labeling was dependent on Mg^{2+}, known to be required for aminoacyl–adenylate formation, whereas addition of Zn^{2+}, which favors Ap_4A formation, was inhibitory. Ap_4A inhibited the formation of lysyl-AMP with an IC_{50} value in the low micromolar range. This *in vitro* study also revealed that the lysyl-AMP intermediate formed by LysRS could be a natural substrate for the Hint proteins. Therefore, the same protein that can be adenylated by LysRS can also hydrolyze adenylated lysine residues.

The physiological importance of the adenylation of proteins at lysine residues is still not clear. As mentioned, this adenylation is short lived and hence hard to detect *in vivo.* Further studies may reveal a very important role for LysRS in this regard once *in vivo* studies can provide the hard evidence.

3.4.2. Synthesis of dinucleotides

Already in the 1960s, Zamecnik demonstrated that under certain conditions LysRS in purified *E. coli* could produce dinucleotides *in vitro*, and characterized the important product of this reaction as adenosine tetraphosphate, a compound which had been synthesized chemically just a short time before (Randerath *et al.*, 1966; Zamecnik *et al.*, 1966). Much later in the 1980s, other groups studied the role of LysRS from mammalian sources as a producer of Ap_4A. At this time, they were limited to *in vitro* studies due to their inability to efficiently knockdown LysRS. Zinc was shown to be critical for the activation of the production of Ap_4A via LysRS and PhenylRS in sheep's liver (Brevet *et al.*, 1982). Zinc greatly stimulates the initial rate of *in vitro* synthesis of Ap_4A. Later, however, it was shown that AMP can be omitted from the reaction and that zinc levels can be markedly reduced provided catalytic amounts of LysRS are added to the reaction mixture (Hilderman and Ortwerth, 1987). Therefore, the *in vivo*

role of zinc in the production of Ap_4A might be much less critical than initially thought. As mentioned earlier, other AaRSs apart from LysRS were able to produce Ap_4A *in vitro.* Hilderman and colleagues demonstrated that one type of tRNAlys (tRNALys) was much more efficient in inducing Ap_4A production by the ArgRS/LysRS complex than the more common tNAlys and tRNALys (Hilderman and Ortwerth, 1987). These experiments suggested a role for LysRS in efficient Ap_4A synthesis. Furthermore, two studies demonstrated the differential efficacy of LysRS in the production of Ap_4A within the complex and external to it (Wahab and Yang, 1985a,b). According to their calculations, based on *in vitro* experiments with purified multisynthetase complexes, LysRS was six times more efficient in the production of Ap_4A once unbound from the complex. One must, of course, note that these are the results of *in vitro* studies where different concentrations of reaction reagents, such as zinc, might significantly alter the final results; but still one might expect an increase in cellular Ap_4A levels if free LysRS levels are increased intracellularly.

3.4.3. LysRS as a cytokine-like molecule

Several AaRSs have been implicated as having a possible role as secreted extracellular modulators of cellular function (Kleeman *et al.*, 1997; Tolstrup *et al.*, 1995; Wakasugi and Schimmel, 1999; Wakasugi *et al.*, 2002). Media was obtained from HEK293 cells overexpressing one of 11 different synthetases, and screened to determine which synthetases were secreted (Park *et al.*, 2005b). Only AlaRS and LysRS were detected in the culture media. Various other cell lines were used to investigate whether endogenous LysRS was secreted in a signal-dependent manner. HCT116 colon cancer cells, DU145, SKN-SH, and MCF-7 cells had substantially increased LysRS secretion rates in response to treatment with TNF-α but not TGF-β. Recombinant LysRS was shown to bind to the membranes of two macrophage-like cell lines (RAW264.7 and THP-1). Following LysRS stimulation, increased secretion of TNF by these two cell lines was detected. Thus, it seems that LysRS and TNF-α form a positive feedback loop in these cells. This study further demonstrated that RAW264.7 macrophage-like cells had increased migrational capacity following LysRS stimulation. The activity of MMP-9, but not MMP-2, was significantly induced by LysRS in these cells. LysRS also induced the migration of peripheral blood mononuclear cells. Selective inhibitors were used to determine that the MAPK pathway has a pivotal role in this LysRS-cytokine signal-transduction pathway. Altogether, the results of this study suggest that LysRS can act as a cytokine-like molecule under certain circumstances. This activity might be related to LysRS's role as an autoantigen (as mentioned earlier).

3.4.4. Physiological roles of LysRS

LysRS is probably the AaRS to which the largest number of physiological roles has been attributed in addition to aminoacylation (Freist and Gauss, 1995). One example is the unique heat shock inducible LysRS gene (lysU) in bacteria (Charlier and Sanchez, 1987) and another is the existence of a specific iron inducible LysRS in tomato roots with a role in plant adaptation (Giritch *et al.*, 1997). Here, we will focus on LysRS's functions in higher eukaryotes and then discuss in some detail the proposed role of LysRS in transcriptional regulation.

3.4.4.1. LysRS and transcription factors We have been studying transcription factor networks in mast cells for more than a decade (Nechushtan and Razin, 1998). We began our studies by assessing the fate of c-Fos and c-Jun proteins following IgE-Ag stimulation of mast cells. These proteins are well known as both oncogenes and early response transcription factors. Surprisingly, at the time we found out that IgE-Ag stimulation leads to the binding of c-Fos not to Jun but to what was then a newly discovered transcription factor known at that time as Fos interacting protein (FIP) and now known by the name of USF2 (Lewin *et al.*, 1993). This transcription factor is a bHLH-leucine zipper transcription factor found in most cells that has an important role in many biological processes. The transcription factor MITF was cloned around this time (Hodgkinson *et al.*, 1993; Tachibana *et al.*, 1994). This is also a bHLH-leucine zipper transcription factor, which is structurally most similar to the TFE transcription factors. TFE transcription factors and MITF are now considered part of a transcription factor family known as MiT (Hemesath *et al.*, 1994). The transcription factors with the highest similarity to this family are USF1 and USF2. Our special interest in MITF was due to the finding that mast cells are depleted in MITF mutated mast cells and because of its similar structure to USF2. Indeed, we found that IgE-Ag induction of mast cells results in USF2 binding to MITF (Nechushtan *et al.*, 1997). Since MITF was demonstrated to have a critical role in mast cell physiology (Kitamura *et al.*, 2002; Nechushtan and Razin, 2002), we decided to try and isolate the MITF binding proteins. We used the yeast two-hybrid assay as a screening method, utilizing a truncated MITF lacking both transactivation domains to probe a library from a mast cell line (Razin *et al.*, 1999) (Fig. 1.2).

Several proteins were found to bind to MITF with high affinity. We initially concentrated our efforts on the study of a protein known at that time as PKCI (PKC interacting protein), but now known as Hint-1 (Razin *et al.*, 1999). We demonstrated that this HIT family protein can bind and inhibit transcriptional activation by MITF. Interestingly, we also demonstrated that Hint-1 could be released from MITF following IgE-Ag stimulation of mast cells and that Hint-1 can bind to MITF in melanoma cells.

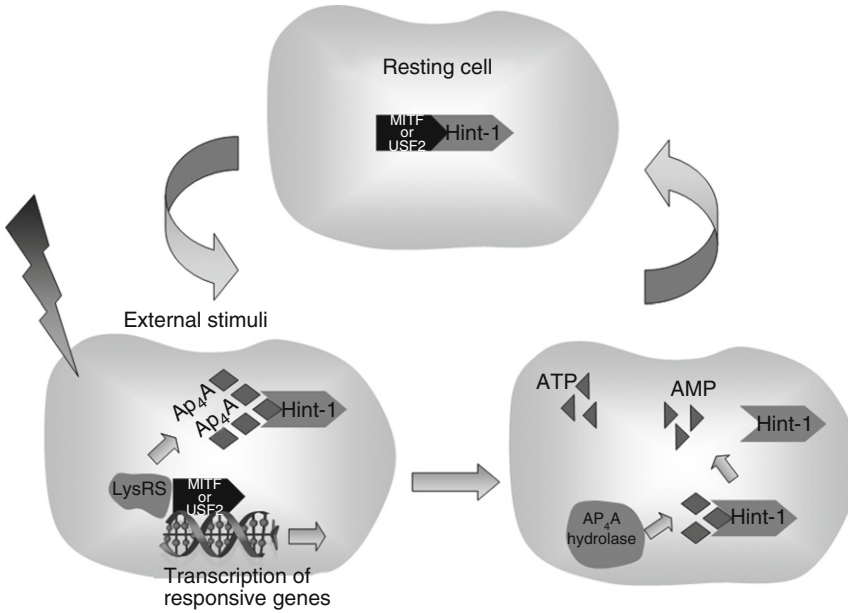

FIGURE 1.2 A model of LysRS/Ap_4A transcriptional regulation. We and others demonstrated that in resting cells, MITF is inhibited by Hint-1. In activated cells, LysRS starts to produce Ap_4A which can bind to Hint-1 and release it from MITF (the best studied example being IgE-Ag stimulated mast cells; similar findings in cardiomyocytes have been described). The initial increase in Ap_4A levels and activation of MITF is followed by degradation of Ap_4A by Ap_4A hydrolase, and decreased production of Ap_4A by LysRS, which leads to the unassociated HINT being available to bind to MITF again and so inhibit its transcriptional activity. This figure summarizes three articles on the role of Ap_4A in transcriptional regulation.

Weinstein and colleagues have recently presented preliminary data at the 2008 AACR meeting whereby they corroborate our results regarding the binding of Hint-1 to MITF and its ability to inhibit MITF (Genovese *et al.*, 2008). Their results were obtained with several types of human melanocytes and human melanoma cell lines and they seem to suggest that Hint-1 could modulate MITF activity in melanoma and might have a role in the progression of this kind of tumor (Genovese *et al.*, 2008).

One of the proteins found to be highly bound to MITF was LysRS (Razin *et al.*, 1999). We verified our observation from the yeast two-hybrid assay with coimmunoprecipitation utilizing extracts from mast cells (Lee *et al.*, 2004b). We noted that several earlier studies from the 1980s previously demonstrated that LysRS can produce the unique dinucleotide

Ap_4A. We found using Biacore that Hint-1 binds specifically to Ap_4A (Lee *et al.*, 2004b). We then demonstrated *in vitro* that MITF could be dissociated from Hint-1 by the application of Ap_4A but not by other dinucleotides, such as Ap_3A, and Ap_5A (Lee *et al.*, 2004b). We therefore hypothesized that the association of LysRS and MITF is not related to LysRS's role as a tRNA synthetase, but to one of its ''moonlight'' functions as a producer of Ap_4A (Lee *et al.*, 2004b). We proposed that Ap_4A produced by LysRS played a critical role in the regulation of several transcription factors through its ability to control gene expression. As an initial *in vivo* assay to test this hypothesis, we introduced Ap_4A into cultured mast cells using the rather crude ''cold shock'' method, and demonstrated that this introduction increased the expression of some MITF-regulated genes (Lee *et al.*, 2004b).

We later demonstrated that LysRS associates with USF2, which unlike MITF, is ubiquitously expressed in eukaryotic cells. In mast cells, we have found that similarly to MITF, USF2 is negatively regulated by Hint-1 and Ap_4A acts as a positive regulator of USF2 by a molecular mechanism similar to that described for MITF (Lee and Razin, 2005). This finding lent support to the notion that LysRS and Ap_4A may be involved in regulation of gene transcription in many cell types and not limited to those where MITF is expressed (Lee and Razin, 2005).

To elucidate the mechanisms involved in this transcriptional regulation pathway, we decided to study the regulation of both the synthesis of Ap_4A by LysRS and its degradation. Interestingly, we found that following IgE-Ag stimulation of mast cells, there is a transient two- to threefold increase of intracellular Ap_4A (Carmi-Levy *et al.*, 2008).

Several enzymes that can degrade Ap_4A have been described (Hankin *et al.*, 1997; Swarbrick *et al.*, 2005; Vollmayer *et al.*, 2003). However, only one of these, NUDT2, which is a member of the Nudix family, has been shown to reside intracellularly and has been proposed as the intracellular enzyme responsible for the degradation of Ap_4A (Abdelghany *et al.*, 2001; Hankin *et al.*, 1997). We downregulated rat NUDT2 by electroporating cultured rat mast cells with NUDT2 siRNA. This downregulation caused significantly prolonged elevation of Ap_4A following IgE-Ag stimulation of mast cells (Carmi-Levy *et al.*, 2008). This result was the first demonstration of a physiological role of NUDT2 in the regulation of the increase in intracellular Ap_4A levels in response to an immunological stimulus.

Following the initial characterization of Ap_4A, various studies demonstrated impressive increases in intracellular Ap_4A levels following a variety of external stimuli. However, most of these observations were performed before modern techniques were available, such as siRNA silencing of specific genes, and thus the enzymes responsible for the production and degradation of Ap_4A under various conditions have not yet been delineated.

As mentioned, we hypothesized that increased Ap_4A levels should allow the release of Hint-1 from MITF and so increase the transcriptional activity of MITF. By blocking NUDT2, we managed to increase intracellular Ap_4A levels in a more physiological manner than with the cold shock method (Carmi-Levy *et al.*, 2008). This increase in Ap_4A levels resulted in an increase in the expression of some, but not all, USF2- and MITF-regulated genes. The effect was not dramatic, with increases of up to two-fold in most genes (Carmi-Levy *et al.*, 2008). A limitation of this approach is that the mast cells were stimulated in a way that was chosen to achieve the maximal IgE-Ag response of the cells. Thus, inhibiting the endogenous hydrolase, which basically prolongs the time that endogenous Ap_4A levels are high, does not result in very large differences in IgE-Ag-stimulated Ap_4A peak levels. Such conditions might not be typical of endogenous situations since IgE-Ag stimulation probably happens at lower than optimal conditions *in vivo*.

It is important to note here that we did witness an increased release of MITF from Hint-1 and increases in the expression of luciferase driven by MITF responsive genes following inhibition of Ap_4A hydrolase expression (Carmi-Levy *et al.*, 2008). Our study lends further support to the notion that intracellularly produced Ap_4A has a role in the regulation of gene transcription.

3.4.4.2. LysRS and HIV tRNA is used as a primer to initiate the reverse transcriptase (RT)-catalyzed synthesis of the minus-strand in retroviruses. Different retroviruses use different tRNAs as a primer. Kleiman and colleagues defined the roles of lys tRNA and LysRS in retroviruses and more specifically in HIV. In lentiviruses, including human immunodeficiency virus type 1 (HIV-1), tRNA3Lys serves as the primer tRNA (Jiang *et al.*, 1993; Mak and Kleiman, 1997). In avian retroviruses and HIV-1, the primer tRNAs are selectively packaged, and the percentage of the tRNA population of the primer tRNA type increases in the virus. In HIV-1, the relative concentration of tRNALys increases from 5% to 6% in the cytoplasm to 50–60% in virus (Huang *et al.*, 1994). Importantly in HIV-1, increases in the concentration of primer tRNA3Lys in the viral population is correlated with an increase in tRNA3Lys annealing and viral infectivity (Gabor *et al.*, 2002). In addition to tRNA3Lys it was found out that LysRS is also selectively packaged into HIV-1 (Cen *et al.*, 2001). It seems that the main role of LysRS in HIV is the targeting of tRNALys for virion incorporation (Cen *et al.*, 2001; Gabor *et al.*, 2002; Kovaleski *et al.*, 2006). Gag alone is sufficient for the incorporation of LysRS into Gag virus-like particles (Cen *et al.*, 2001), whereas GagPol is required in addition for the incorporation of tRNALys (Huang *et al.*, 1994) (Fig. 1.3).

Overexpression of LysRS in the cell results in a near doubling of the incorporation of both tRNALys and LysRS into HIV-1 (Cen *et al.*, 2004).

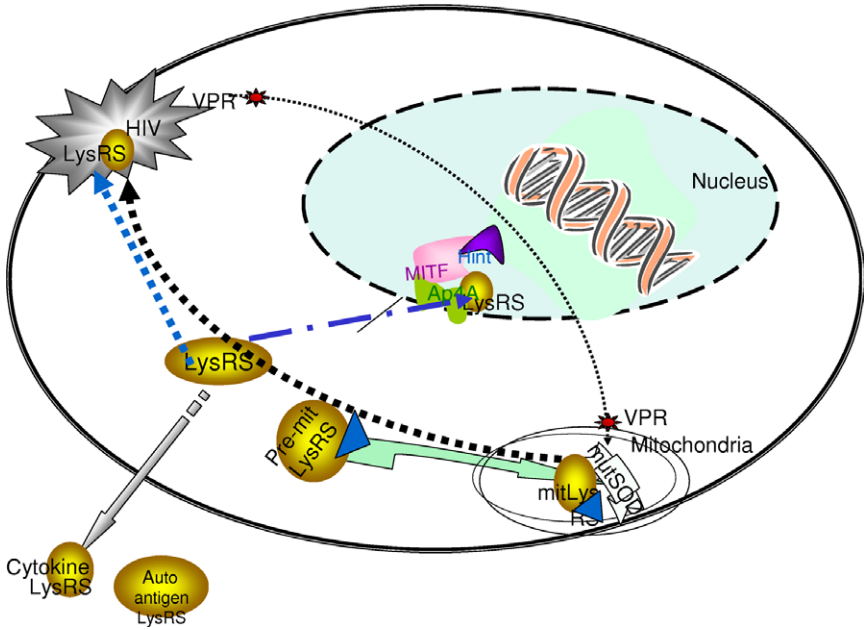

FIGURE 1.3 Roles of LysRS. LysRS is produced in two forms from the same gene—a mitochondrial form, harboring a mitochondrial localization sequence, and a regular form. The premitochondrial form of LysRS is cleaved once it enters the mitochondria and 17 amino acids are lost and mitochondrial LysRS (mitoLysRS) is obtained. Besides its role in translation, mitochondrial LysRS can specifically bind mutated superoxide dismutase (mutSOD), an interaction which is proposed to have a role in the pathogenesis of amyotropic lateral sclerosis. Mitochondrial LysRS has also been proposed as the LysRS used by the HIV virion. It has proposed that a protein released from the HIV virion, VPR leads to increased release of mitochondrial LysRS which can be incorporated into HIV. Alternatively, it has been proposed that newly synthesized LysRS can be incorporated into the HIV virions before incorporation into the multisynthetase complex. LysRS is also secreted and has cytokine-like properties. In addition, LysRS has been found to be an autoantigen in certain autoimmune disorders. LysRS has also been found in the nucleus. LysRS is a producer of Ap_4A, and can influence the dissociation of the transcriptional inhibitor Hint-1 from transcription factors such as MITF.

Thus, considering the huge clinical importance of HIV and the important role of LysRS in the biology of HIV, it was interesting to try and locate the source of LysRS in the virions. Regarding this question, there is currently a heated debate in the literature. Kleiman's group claim that the source for viral LysRS is newly synthesized cytoplasmatic LysRS that binds to the GAG molecule before it binds to the multisynthetase complex (Cen *et al.*, 2004). This claim is based on the use of truncated and tagged exogenous LysRS. In contrast, Mirande and colleagues claim that the source for HIV

LysRS is mitochondrial LysRS that is released in higher amounts from the mitochondria following HIV infection and specific mitochondrial degradation as a result of the action of HIV-derived VPR protein (Francin *et al.*, 2002). Their conclusions were based on the use of specific antibodies to the mitochondrial LysRS. Neither of these research groups studied activated immune cells, in which there might be substantial release of LysRS from the multisynthetase complex, and only used model cell lines *in vitro* for their studies. Therefore, it seems that while the critical role of LysRS in the life cycle of HIV is now supported by evidence from several laboratories, the source of LysRS in HIV and its specific form in the virus is still not clear.

4. CONCLUDING REMARKS

LysRS has several roles besides its function as a key enzyme involved in translation. In the immune system, it may function both as an extracellular cytokine-like molecule and a signal-transduction protein in a signal-transduction pathway ultimately regulating gene expression. It has a critical role in HIV viral genesis. LysRS has been implicated as having a role both in autoimmune disease and in amyotrophic lateral sclerosis. It is found in cells in the cytoplasm mainly as part of the multisynthetase complex, in mitochondria and in some cases has been found to be nuclear or membrane bound. Studies of the regulation of LysRS function inside the cells should provide us with critical insights as to the basic mechanisms of LysRS function in its various noncanonical roles. It seems that much more work is needed to understand the complex regulation of LysRS function and the involvement of LysRS in such seemingly unrelated processes such as HIV genesis and the response to external stimuli.

REFERENCES

Abdelghany, H. M., Gasmi, L., Cartwright, J. L., Bailey, S., Rafferty, J. B., and McLennan, A. G. (2001). Cloning, characterisation and crystallisation of a diadenosine 5′,5‴-P(1),P(4)-tetraphosphate pyrophosphohydrolase from *Caenorhabditis elegans*. *Biochim. Biophys. Acta* **1550,** 27–36.

Ataide, S. F., and Ibba, M. (2004). Discrimination of cognate and noncognate substrates at the active site of class II lysyl-tRNA synthetase. *Biochemistry* **43,** 11836–11841.

Bandyopadhyay, A. K., and Deutscher, M. P. (1971). Complex of aminoacyl-transfer RNA synthetases. *J. Mol. Biol.* **60,** 113–122.

Boukerche, H., Su, Z. Z., Emdad, L., Baril, P., Balme, B., Thomas, L., Randolph, A., Valerie, K., Sarkar, D., and Fisher, P. B. (2005). mda-9/Syntenin: A positive regulator of melanoma metastasis. *Cancer Res* **65,** 10901–10911.

Brevet, A., Plateau, P., Cirakoglu, B., Pailliez, J. P., and Blanquet, S. (1982). Zinc-dependent synthesis of 5′,5′-diadenosine tetraphosphate by sheep liver lysyl- and phenylalanyl-tRNA synthetases. *J. Biol. Chem.* **257,** 14613–14615.

Carmi-Levy, I., Yannay-Cohen, N., Kay, G., Razin, E., and Nechushtan, H. (2008). Diadenosine tetraphosphate hydrolase is part of the transcriptional regulation network in immunologically activated mast cells. *Mol. Cell Biol.* **28,** 5777–5784.

Cen, S., Khorchid, A., Javanbakht, H., Gabor, J., Stello, T., Shiba, K., Musier-Forsyth, K., and Kleiman, L. (2001). Incorporation of lysyl-tRNA synthetase into human immunodeficiency virus type 1. *J. Virol.* **75,** 5043–5048.

Cen, S., Javanbakht, H., Niu, M., and Kleiman, L. (2004). Ability of wild-type and mutant lysyl-tRNA synthetase to facilitate tRNA(Lys) incorporation into human immunodeficiency virus type 1. *J. Virol.* **78,** 1595–1601.

Charlier, J., and Sanchez, R. (1987). Lysyl-tRNA synthetase from *Escherichia coli* K12. Chromatographic heterogeneity and the lysU-gene product. *Biochem. J.* **248,** 43–51.

Chou, T. F., and Wagner, C. R. (2007). Lysyl-tRNA synthetase-generated lysyl-adenylate is a substrate for histidine triad nucleotide binding proteins. *J. Biol. Chem.* **282,** 4719–4727.

Chou, T. F., Tikh, I. B., Horta, B. A., Ghosh, B., De Alencastro, R. B., and Wagner, C. R. (2007). Engineered monomeric human histidine triad nucleotide-binding protein 1 hydrolyzes fluorogenic acyl-adenylate and lysyl-tRNA synthetase-generated lysyl-adenylate. *J. Biol. Chem.* **282,** 15137–15147.

Cusack, S. (1995). Eleven down and nine to go. *Nat. Struct. Biol.* **2,** 824–831.

Deutscher, M. P., and Ni, R. C. (1982). Purification of a low molecular weight form of rat liver arginyl-tRNA synthetase. *J. Biol. Chem.* **257,** 6003–6006.

Fabre, S., Reynaud, C., and Jalinot, P. (2000). Identification of functional PDZ domain binding sites in several human proteins. *Mol. Biol. Rep.* **27,** 217–224.

Ferber, S., and Ciechanover, A. (1987). Role of arginine-tRNA in protein degradation by the ubiquitin pathway. *Nature* **326,** 808–811.

Francin, M., and Mirande, M. (2003). Functional dissection of the eukaryotic-specific tRNA-interacting factor of lysyl-tRNA synthetase. *J. Biol. Chem.* **278,** 1472–1479.

Francin, M., Kaminska, M., Kerjan, P., and Mirande, M. (2002). The N-terminal domain of mammalian Lysyl-tRNA synthetase is a functional tRNA-binding domain. *J. Biol. Chem.* **277,** 1762–1769.

Freist, W., and Gauss, D. H. (1995). Lysyl-tRNA synthetase. *Biol. Chem. Hoppe Seyler.* **376,** 451–472.

Gabor, J., Cen, S., Javanbakht, H., Niu, M., and Kleiman, L. (2002). Effect of altering the tRNA (Lys)(3) concentration in human immunodeficiency virus type 1 upon its annealing to viral RNA, GagPol incorporation, and viral infectivity. *J. Virol.* **76,** 9096–9102.

Gelpi, C., Kanterewicz, E., Gratacos, J., Targoff, I. N., and Rodriguez-Sanchez, J. L. (1996). Coexistence of two antisynthetases in a patient with the antisynthetase syndrome. *Arthritis Rheum.* **39,** 692–697.

Genovese, G., Li, H., Sgambato, A., Cittadini, A., and Weinstein, I. B. (2008). The tumor suppressor HINT1 regulates MITF transcriptional activity in melanoma cells. *AACR Meet. Abstr* **8732008**.

Giritch, A., Herbik, A., Balzer, H. J., Ganal, M., Stephan, U. W., and Baumlein, H. (1997). A root-specific iron-regulated gene of tomato encodes a lysyl-tRNA-synthetase-like protein. *Eur. J. Biochem.* **244,** 310–317.

Grootjans, J. J., Zimmermann, P., Reekmans, G., Smets, A., Degeest, G., Durr, J., and David, G. (1997). Syntenin, a PDZ protein that binds syndecan cytoplasmic domains. *Proc. Natl. Acad. Sci. USA* **94,** 13683–13688.

Guo, M., Ignatov, M., Musier-Forsyth, K., Schimmel, P., and Yang, X. L. (2008). Crystal structure of tetrameric form of human lysyl-tRNA synthetase: Implications for multisynthetase complex formation. *Proc. Natl. Acad. Sci. USA* **105,** 2331–2336.

Guzzo, C. M., and Yang, D. C. (2008). Lysyl-tRNA synthetase interacts with EF1alpha, aspartyl-tRNA synthetase and p38 *in vitro*. *Biochem. Biophys. Res. Commun.* **365,** 718–723.

Han, J. M., Lee, M. J., Park, S. G., Lee, S. H., Razin, E., Choi, E. C., and Kim, S. (2006). Hierarchical network between the components of the multi-tRNA synthetase complex: Implications for complex formation. *J. Biol. Chem.* **281,** 38663–38667.

Han, J. M., Park, B. J., Park, S. G., Oh, Y. S., Choi, S. J., Lee, S. W., Hwang, S. K., Chang, S. H., Cho, M. H., and Kim, S. (2008). AIMP2/p38, the scaffold for the multi-tRNA synthetase complex, responds to genotoxic stresses via p53. *Proc. Natl. Acad. Sci. USA* **105,** 11206–11211.

Hankin, S., Wintero, A. K., and McLennan, A. G. (1997). Molecular cloning of diadenosine tetraphosphatase from pig small intestinal mucosa and identification of sequence blocks common to diadenosine polyphosphate hydrolases and phosphorylases. *Int. J. Biochem. Cell Biol.* **29,** 317–323.

Hausmann, C. D., and Ibba, M. (2008a). Aminoacyl-tRNA synthetase complexes: Molecular multitasking revealed. *FEMS Microbiol. Rev.* **32,** 705–721.

Hausmann, C. D., and Ibba, M. (2008b). Structural and functional mapping of the archaeal multi-aminoacyl-tRNA synthetase complex. *FEBS Lett.* **582,** 2178–2182.

Hausmann, C. D., Praetorius-Ibba, M., and Ibba, M. (2007). An aminoacyl-tRNA synthetase: Elongation factor complex for substrate channeling in archaeal translation. *Nucleic Acids Res.* **35,** 6094–6102.

Hemesath, T. J., Steingrimsson, E., McGill, G., Hansen, M. J., Vaught, J., Hodgkinson, C. A., Arnheiter, H., Copeland, N. G., Jenkins, N. A., and Fisher, D. E. (1994). Microphthalmia, a critical factor in melanocyte development, defines a discrete transcription factor family. *Genes Dev.* **8,** 2770–2780.

Hilderman, R. H., and Ortwerth, B. J. (1987). A preferential role for lysyl-tRNA4 in the synthesis of diadenosine 5′,5‴-P1,P4-tetraphosphate by an arginyl-tRNA synthetase-lysyl-tRNA synthetase complex from rat liver. *Biochemistry* **26,** 1586–1591.

Hodgkinson, C. A., Moore, K. J., Nakayama, A., Steingrimsson, E., Copeland, N. G., Jenkins, N. A., and Arnheiter, H. (1993). Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. *Cell* **74,** 395–404.

Huang, Y., Mak, J., Cao, Q., Li, Z., Wainberg, M. A., and Kleiman, L. (1994). Incorporation of excess wild-type and mutant tRNA(3Lys) into human immunodeficiency virus type 1. *J. Virol.* **68,** 7676–7683.

Jia, J., Arif, A., Ray, P. S., and Fox, P. L. (2008). WHEP domains direct noncanonical function of glutamyl-Prolyl tRNA synthetase in translational control of gene expression. *Mol. Cell* **29,** 679–690.

Jiang, M., Mak, J., Ladha, A., Cohen, E., Klein, M., Rovinski, B., and Kleiman, L. (1993). Identification of tRNAs incorporated into wild-type and mutant human immunodeficiency virus type 1. *J. Virol.* **67,** 3246–3253.

Kawamata, H., Magrane, J., Kunst, C., King, M. P., and Manfredi, G. (2008). Lysyl-tRNA synthetase is a target for mutant SOD1 toxicity in mitochondria. *J. Biol. Chem.* **283,** 28321–28328.

Kim, Y., Shin, J., Li, R., Cheong, C., Kim, K., and Kim, S. (2000). A novel anti-tumor cytokine contains an RNA binding motif present in aminoacyl-tRNA synthetases. *J. Biol. Chem.* **275,** 27062–27068.

Kim, M. J., Park, B. J., Kang, Y. S., Kim, H. J., Park, J. H., Kang, J. W., Lee, S. W., Han, J. M., Lee, H. W., and Kim, S. (2003). Downregulation of FUSE-binding protein and c-myc by tRNA synthetase cofactor p38 is required for lung cell differentiation. *Nat. Genet.* **34,** 330–336.

Kim, E., Kim, S. H., Kim, S., Cho, D., and Kim, T. S. (2008a). AIMP1/p43 protein induces the maturation of bone marrow-derived dendritic cells with T helper type 1-polarizing ability. *J. Immunol.* **180,** 2894–2902.

Kim, K. J., Park, M. C., Choi, S. J., Oh, Y. S., Choi, E. C., Cho, H. J., Kim, M. H., Kim, S. H., Kim, D. W., Kim, S., and Kang, B. S. (2008b). Determination of three-dimensional structure and residues of the novel tumor suppressor AIMP3/p18 required for the interaction with ATM. *J. Biol. Chem.* **283,** 14032–14040.

Kitamura, Y., Morii, E., Jippo, T., and Ito, A. (2002). Effect of MITF on mast cell differentiation. *Mol. Immunol.* **38,** 1173–1176.

Kleeman, T. A., Wei, D., Simpson, K. L., and First, E. A. (1997). Human tyrosyl-tRNA synthetase shares amino acid sequence homology with a putative cytokine. *J. Biol. Chem.* **272,** 14420–14425.

Ko, Y. G., Kang, Y. S., Park, H., Seol, W., Kim, J., Kim, T., Park, H. S., Choi, E. J., and Kim, S. (2001). Apoptosis signal-regulating kinase 1 controls the proapoptotic function of death-associated protein (Daxx) in the cytoplasm. *J. Biol. Chem.* **276,** 39103–39106.

Koo, T. H., Lee, J. J., Kim, E. M., Kim, K. W., Kim, H. D., and Lee, J. H. (2002). Syntenin is overexpressed and promotes cell migration in metastatic human breast and gastric cancer cell lines. *Oncogene* **21,** 4080–4088.

Kovaleski, B. J., Kennedy, R., Hong, M. K., Datta, S. A., Kleiman, L., Rein, A., and Musier-Forsyth, K. (2006). *In vitro* characterization of the interaction between HIV-1 Gag and human lysyl-tRNA synthetase. *J. Biol. Chem.* **281,** 19449–19456.

Kunst, C. B., Mezey, E., Brownstein, M. J., and Patterson, D. (1997). Mutations in SOD1 associated with amyotrophic lateral sclerosis cause novel protein interactions. *Nat. Genet.* **15,** 91–94.

Kyriacou, S. V., and Deutscher, M. P. (2008). An important role for the multienzyme aminoacyl-tRNA synthetase complex in mammalian translation and cell growth. *Mol. Cell* **29,** 419–427.

Lee, Y. N., and Razin, E. (2005). Nonconventional involvement of LysRS in the molecular mechanism of USF2 transcriptional activity in FcepsilonRI-activated mast cells. *Mol. Cell Biol.* **25,** 8904–8912.

Lee, S. W., Cho, B. H., Park, S. G., and Kim, S. (2004a). Aminoacyl-tRNA synthetase complexes: Beyond translation. *J. Cell Sci.* **117,** 3725–3734.

Lee, Y. N., Nechushtan, H., Figov, N., and Razin, E. (2004b). The function of lysyl-tRNA synthetase and Ap_4A as signaling regulators of MITF activity in FcεRI-activated mast cells. *Immunity* **20,** 145–151.

Lee, Y. S., Han, J. M., Son, S. H., Choi, J. W., Jeon, E. J., Bae, S. C., Park, Y. I., and Kim, S. (2008). AIMP1/p43 downregulates TGF-beta signaling via stabilization of smurf2. *Biochem. Biophys. Res. Commun.* **371,** 395–400.

Lewin, I., Nechushtan, H., Ke, Q., and Razin, E. (1993). Regulation of AP-1 expression and activity in antigen-stimulated mast cells: The role played by protein kinase C and the possible involvement of Fos interacting protein. *Blood* **82,** 3745–3751.

Lin, J. J., Jiang, H., and Fisher, P. B. (1998). Melanoma differentiation associated gene-9, mda-9, is a human gamma interferon responsive gene. *Gene* **207,** 105–110.

Linke, A. T., Marchant, B., Marsh, P., Frampton, G., Murphy, J., and Rose, M. L. (2001). Screening of a HUVEC cDNA library with transplant-associated coronary artery disease sera identifies RPL7 as a candidate autoantigen associated with this disease. *Clin. Exp. Immunol.* **126,** 173–179.

Mak, J., and Kleiman, L. (1997). Primer tRNAs for reverse transcription. *J. Virol.* **71,** 8087–8095.

Mansilla, F., Hansen, L. L., Jakobsen, H., Kjeldgaard, N. O., Clark, B. F., and Knudsen, C. R. (2005). Deconstructing PTI-1: PTI-1 is a truncated, but not mutated, form of translation elongatin factor 1A1, eEF1A1. *Biochim. Biophys. Acta* **1727,** 116–124.

Mazumder, B., Sampath, P., Seshadri, V., Maitra, R. K., DiCorleto, P. E., and Fox, P. L. (2003). Regulated release of L13a from the 60S ribosomal subunit as a mechanism of transcript-specific translational control. *Cell* **115,** 187–198.

Meerschaert, K., Remue, E., De Ganck, A., Staes, A., Boucherie, C., Gevaert, K., Vandekerckhove, J., Kleiman, L., and Gettemans, J. (2008). The tandem PDZ protein Syntenin interacts with the aminoacyl tRNA synthetase complex in a lysyl-tRNA synthetase-dependent manner. *J. Proteome Res.* **7,** 4962–4973.

Mirande, M., Le Corre, D., and Waller, J. P. (1985). A complex from cultured Chinese hamster ovary cells containing nine aminoacyl-tRNA synthetases. Thermolabile leucyl-tRNA synthetase from the tsH1 mutant cell line is an integral component of this complex. *Eur. J. Biochem.* **147,** 281–289.

Mukhopadhyay, R., Ray, P. S., Arif, A., Brady, A. K., Kinter, M., and Fox, P. L. (2008). DAPK-ZIPK-L13a axis constitutes a negative-feedback module regulating inflammatory gene expression. *Mol. Cell* **32,** 371–382.

Nagaraju, K., Raben, N., Loeffler, L., Parker, T., Rochon, P. J., Lee, E., Danning, C., Wada, R., Thompson, C., Bahtiyar, G., Craft, J., Hooft Van Huijsduijnen, R., *et al*. (2000). Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies. *Proc. Natl. Acad. Sci. USA* **97,** 9209–9214.

Nechushtan, H., and Razin, E. (1998). Deciphering the early-response transcription factor networks in mast cells. *Immunol. Today* **19,** 441–444.

Nechushtan, H., and Razin, E. (2002). The function of MITF and associated proteins in mast cells. *Mol. Immunol.* **38,** 1177.

Nechushtan, H., Zhang, Z., and Razin, E. (1997). Microphthalmia (mi) in murine mast cells: Regulation of its stimuli-mediated expression on the translational level. *Blood* **89,** 2999–3008.

Nourry, C., Grant, S. G., and Borg, J. P. (2003). PDZ domain proteins: Plug and play!. *Sci. STKE* **2003,** RE7.

Park, B. J., Kang, J. W., Lee, S. W., Choi, S. J., Shin, Y. K., Ahn, Y. H., Choi, Y. H., Choi, D., Lee, K. S., and Kim, S. (2005a). The haploinsufficient tumor suppressor p18 upregulates p53 via interactions with ATM/ATR. *Cell* **120,** 209–221.

Park, S. G., Kim, H. J., Min, Y. H., Choi, E. C., Shin, Y. K., Park, B. J., Lee, S. W., and Kim, S. (2005b). Human lysyl-tRNA synthetase is secreted to trigger proinflammatory response. *Proc. Natl. Acad. Sci. USA* **102,** 6356–6361.

Park, B. J., Oh, Y. S., Park, S. Y., Choi, S. J., Rudolph, C., Schlegelberger, B., and Kim, S. (2006a). AIMP3 haploinsufficiency disrupts oncogene-induced p53 activation and genomic stability. *Cancer Res.* **66,** 6913–6918.

Park, S. G., Kang, Y. S., Kim, J. Y., Lee, C. S., Ko, Y. G., Lee, W. J., Lee, K. U., Yeom, Y. I., and Kim, S. (2006b). Hormonal activity of AIMP1/p43 for glucose homeostasis. *Proc. Natl. Acad. Sci. USA* **103,** 14913–14918.

Park, S. G., Schimmel, P., and Kim, S. (2008). Aminoacyl tRNA synthetases and their connections to disease. *Proc. Natl. Acad. Sci. USA* **105,** 11043–11049.

Pezo, V., Metzgar, D., Hendrickson, T. L., Waas, W. F., Hazebrouck, S., Doring, V., Marliere, P., Schimmel, P., and De Crecy-Lagard, V. (2004). Artificially ambiguous genetic code confers growth yield advantage. *Proc. Natl. Acad. Sci. USA* **101,** 8593–8597.

Piatigorsky, J. (2007). "Gene Sharing and Evolution The Diversity of Protein Functions." Harvard University Press, Cambridge.

Piatigorsky, J., and Wistow, G. J. (1989). Enzyme/crystallins: Gene sharing as an evolutionary strategy. *Cell* **57,** 197–199.

Plotz, P. H. (2003). The autoantibody repertoire: Searching for order. *Nat. Rev. Immunol.* **3,** 73–78.

Putney, S. D., and Schimmel, P. (1981). An aminoacyl tRNA synthetase binds to a specific DNA sequence and regulates its gene transcription. *Nature* **291,** 632–635.

Quevillon, S., Robinson, J. C., Berthonneau, E., Siatecka, M., and Mirande, M. (1999). Macromolecular assemblage of aminoacyl-tRNA synthetases: Identification of protein–protein interactions and characterization of a core protein. *J. Mol. Biol.* **285,** 183–195.

Randerath, K., Janeway, C. M., Stephenson, M. L., and Zamecnik, P. C. (1966). Isolation and characterization of dinucleoside tetra- and tri-phosphates formed in the presence of lysyl-sRNA synthetase. *Biochem. Biophys. Res. Commun.* **24,** 98–105.

Ray, P. S., Arif, A., and Fox, P. L. (2007). Macromolecular complexes as depots for releasable regulatory proteins. *Trends Biochem. Sci.* **32,** 158–164.

Razin, E., Zhang, Z. C., Nechushtan, H., Frenkel, S., Lee, Y. N., Arudchandran, R., and Rivera, J. (1999). Suppression of microphthalmia transcriptional activity by its association with protein kinase C-interacting protein 1 in mast cells. *J. Biol. Chem.* **274,** 34272–34276.

Reed, V. S., and Yang, D. C. (1994). Characterization of a novel N-terminal peptide in human aspartyl-tRNA synthetase. Roles in the transfer of aminoacyl-tRNA from aminoacyl-tRNA synthetase to the elongation factor 1 alpha. *J. Biol. Chem.* **269,** 32937–32941.

Ribas de Pouplana, L., and Geslain, R. (2008). Not just because it is there: Aminoacyl-tRNA synthetases gain control of the cell. *Mol. Cell* **30,** 3–4.

Ribas de Pouplana, L., and Schimmel, P. (2001). Aminoacyl-tRNA synthetases: Potential markers of genetic code development. *Trends Biochem. Sci.* **26,** 591–596.

Rousset, R., Fabre, S., Desbois, C., Bantignies, F., and Jalinot, P. (1998). The C-terminus of the HTLV-1 Tax oncoprotein mediates interaction with the PDZ domain of cellular proteins. *Oncogene* **16,** 643–654.

Sampath, P., Mazumder, B., Seshadri, V., Gerber, C. A., Chavatte, L., Kinter, M., Ting, S. M., Dignam, J. D., Kim, S., Driscoll, D. M., and Fox, P. L. (2004). Noncanonical function of glutamyl-prolyl-tRNA synthetase: Gene-specific silencing of translation. *Cell* **119,** 195–208.

Schimmel, P., and Ribas de Pouplana, L. (1995). Transfer RNA: From minihelix to genetic code. *Cell* **81,** 983–986.

Schimmel, P., and Ribas de Pouplana, L. (2001). Formation of two classes of tRNA synthetases in relation to editing functions and genetic code. *Cold Spring Harb. Symp. Quant. Biol.* **66,** 161–166.

Stathopoulos, C., Ahel, I., Ali, K., Ambrogelly, A., Becker, H., Bunjun, S., Feng, L., Herring, S., Jacquin-Becker, C., Kobayashi, H., Korencic, D., Krett, B., *et al*. (2001). Aminoacyl-tRNA synthesis: A postgenomic perspective. *Cold Spring Harb. Symp. Quant. Biol.* **66,** 175–183.

Swarbrick, J. D., Buyya, S., Gunawardana, D., Gayler, K. R., McLennan, A. G., and Gooley, P. R. (2005). Structure and substrate-binding mechanism of human Ap4A hydrolase. *J. Biol. Chem.* **280,** 8471–8481.

Tachibana, M., Perez-Jurado, L. A., Nakayama, A., Hodgkinson, C. A., Li, X., Schneider, M., Miki, T. J. F., Francke, U., and Arnheiter, H. (1994). Cloning of MITF, the human homolog of the mouse microphthalmia gene and assignment to chromosome 3p14.1–p12.3. *Hum. Mol. Genet.* **3,** 553–557.

Targoff, I. N., Trieu, E. P., and Miller, F. W. (1993). Reaction of anti-OJ autoantibodies with components of the multi-enzyme complex of aminoacyl-tRNA synthetases in addition to isoleucyl-tRNA synthetase. *J. Clin. Invest.* **91,** 2556–2564.

Tolkunova, E., Park, H., Xia, J., King, M. P., and Davidson, E. (2000). The human lysyl-tRNA synthetase gene encodes both the cytoplasmic and mitochondrial enzymes by means of an unusual alternative splicing of the primary transcript. *J. Biol. Chem.* **275,** 35063–35069.

Tolstrup, A. B., Bejder, A., Fleckner, J., and Justesen, J. (1995). Transcriptional regulation of the interferon-gamma-inducible tryptophanyl-tRNA synthetase includes alternative splicing. *J. Biol. Chem.* **270,** 397–403.

Vellekamp, G., Sihag, R. K., and Deutscher, M. P. (1985). Comparison of the complexed and free forms of rat liver arginyl-tRNA synthetase and origin of the free form. *J. Biol. Chem.* **260,** 9843–9847.

Vollmayer, P., Clair, T., Goding, J. W., Sano, K., Servos, J., and Zimmermann, H. (2003). Hydrolysis of diadenosine polyphosphates by nucleotide pyrophosphatases/phosphodiesterases. *Eur. J. Biochem.* **270,** 2971–2978.

Wahab, S. Z., and Yang, D. C. (1985a). Influence of supramolecular structure on the enzyme mechanisms of rat liver lysyl-tRNA synthetase-catalyzed reactions. Synthesis of P1,P4-bis (5′-adenosyl)tetraphosphate. *J. Biol. Chem.* **260,** 12735–12739.

Wahab, S. Z., and Yang, D. C. (1985b). Synthesis of diadenosine 5′,5‴-P1,P4-tetraphosphate by lysyl-tRNA synthetase and a multienzyme complex of aminoacyl-tRNA synthetases from rat liver. *J. Biol. Chem.* **260,** 5286–5289.

Wakasugi, K., and Schimmel, P. (1999). Two distinct cytokines released from a human aminoacyl-tRNA synthetase. *Science* **284,** 147–151.

Wakasugi, K., Slike, B. M., Hood, J., Otani, A., Ewalt, K. L., Friedlander, M., Cheresh, D. A., and Schimmel, P. (2002). A human aminoacyl-tRNA synthetase as a regulator of angiogenesis. *Proc. Natl. Acad. Sci. USA* **99,** 173–177.

Wistow, G. J., Mulders, J. W., and de Jong, W. W. (1987). The enzyme lactate dehydrogenase as a structural protein in avian and crocodilian lenses. *Nature* **326,** 622–624.

Wolfson, A., and Knight, R. (2005). Occurrence of the aminoacyl-tRNA synthetases in high-molecular weight complexes correlates with the size of substrate amino acids. *FEBS Lett.* **579,** 3467–3472.

Zamecnik, P. C., Stephenson, M. L., Janeway, C. M., and Randerath, K. (1966). Enzymatic synthesis of diadenosine tetraphosphate and diadenosine triphosphate with a purified lysyl-sRNA synthetase. *Biochem. Biophys. Res. Commun.* **24,** 91–97.

Zimmermann, P. (2006). The prevalence and significance of PDZ domain-phosphoinositide interactions. *Biochim. Biophys. Acta* **1761,** 947–956.

CHAPTER 2

Kill the Bacteria . . . and Also Their Messengers?

Robert Munford,[*,1] **Mingfang Lu,**[*,1] and **Alan Varley**[*]

Contents

Abstract

We consider here a previously neglected aspect of recovery from infectious diseases: how animals dispose of the dead microbes in their tissues. For one of the most important disease-causing microorganisms, Gram-negative bacteria, there is now evidence

* Departments of Internal Medicine and Microbiology, UT-Southwestern Medical Center, Dallas, Texas, USA
[1] Laboratory of Clinical Infectious Diseases, NIAID/NIH, Bethesda, Maryland, USA

Advances in Immunology, Volume 103
ISSN 0065-2776, DOI: 10.1016/S0065-2776(09)03002-8

that the host catabolism of a key microbial molecule is essential for full recovery. As might be expected, it is the same bacterial molecule that animals sense to detect the presence of Gram-negative bacteria in their tissues, the cell wall lipopolysaccharide (LPS). Here, we discuss current knowledge about LPS sensing with emphasis on the host enzyme that inactivates this microbial "messenger" molecule. We also consider the possibility that the rate at which stimulatory microbial molecules undergo inactivation may influence the duration and severity of diseases caused by other infectious agents.

1. LIPOPOLYSACCHARIDE (LPS) SENSING BY MD-2–TLR4

All Gram-negative bacteria living in natural environments produce LPS, a complex glycolipid that contributes to outer membrane impermeability, confers resistance to detergents and cationic antimicrobial peptides, provides cell-surface diversity, and prevents complement-mediated cell death. Animals sense the lipid A moiety of LPS via MD-2–TLR4 receptors on phagocytes and other cells, and much evidence suggests that recognizing LPS in this way is essential for detecting the presence of Gram-negative bacteria in tissues and mobilizing antibacterial defenses. The structure of lipid A is not identical in different Gram-negative bacteria, however, and not all lipid As can trigger inflammatory responses via MD-2–TLR4. Extensive structure–activity studies have shown that a bisphosphorylated, hexaacyl lipid A structure (Fig. 2.1) is most stimulatory; removal or addition of a single acyl chain can diminish potency, as can the absence of either of the backbone phosphates. Although many Gram-negative bacteria make LPSs that are poorly recognized by MD-2–TLR4, with potentially important consequences for disease pathogenesis (Munford, 2008), the aerobic commensals and pathogens that colonize the mucosae of the upper respiratory and gastrointestinal tracts generally produce LPSs that have hexaacyl lipid A moieties and are readily sensed by cells bearing MD-2–TLR4 (Munford and Varley, 2006). It is these bacteria that animals are best equipped to defeat using TLR4-based inflammatory responses, and it is the LPSs from these bacteria that are most likely to translocate into the bloodstream to produce ''endotoxemia.'' These are also the LPSs that can be inactivated by the unusual host lipase, acyloxyacyl hydrolase (AOAH).

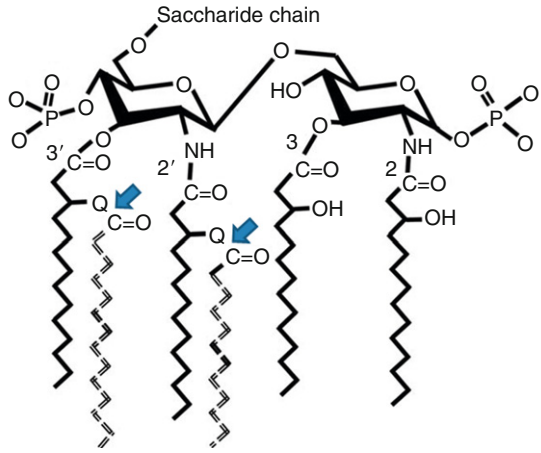

FIGURE 2.1 The structure of *Escherichia coli* lipid A. Arrows indicate the acyloxyacyl-linkages. The secondary acyl chains are shown as dashed lines.

2. ACYLOXYACYL HYDROLASE

AOAH was found during a search for human neutrophil enzymes that could deacylate LPS (Hall and Munford, 1983). The ''bait,'' a biosynthetically labeled LPS that had ^{14}C-glucosamine and ^{3}H-fatty acyl chains (Fig. 2.1), was opsonized with an anti-LPS antibody and fed to human neutrophils *in vitro*. The ^{14}C dpm, which marked the carbohydrate backbone, remained chloroform-insoluble during the next 6 h, whereas the ^{3}H in the LPS acyl chains gradually became chloroform-soluble and could be recovered in cellular phospholipids. Further analysis showed that only the nonhydroxylated fatty acids (myristate and laurate) were released from the LPS—unexpectedly, all of the 3-hydroxymyristoyl chains remained attached to the backbone, suggesting that neutrophils may lack the ability to degrade lipid A completely. Correct interpretation of the sites of enzymatic hydrolysis became possible when the existence of acyloxyacyl linkages (Wollenweber *et al.*, 1982) in lipid A and the first accurate lipid A structure (Takayama *et al.*, 1983) were published: the myristoyl and lauroyl chains are attached to the hydroxyl functions of glucosamine-linked 3-hydroxymyristoyl residues to form acyloxyacyl linkages (Fig. 2.1, arrowheads). Denis McGarry suggested that the enzymatic activity be named ''acyloxyacyl hydrolysis'' and the enzyme(s) ''*a*cyloxy*a*cyl *h*ydrolase(s)''. A literature review revealed that enzymatic release of nonhydroxylated fatty acids from LPSs had been reported previously in slime molds (*Dictyostelium discoideum*, Nigam *et al.*, 1970; *Physarum polycephalum*, Saddler *et al.*, 1979a) and a snail (*Helix pomatia*, Saddler *et al.*, 1979b).

In mammals, AOAH is produced by monocyte-macrophages and dendritic cells as well as neutrophils. Another prominent source, particularly in rodents, is the renal cortical epithelial (proximal tubule) cell (Feulner *et al.*, 2004). Evidence that AOAH can inactivate LPS followed partial purification of the protein (Munford and Hall, 1986). Table 2.1 summarizes the bioassays used to evaluate this point. Note that AOAH-treated LPS not only lacks potency in these assays but it can also inhibit the ability of untreated LPS to stimulate both human and murine cells. The species-specific inhibition reported for tetraacyl lipid A structures (such as lipid IVa) (Golenbock *et al.*, 1990; Hajjar *et al.*, 2002) does not necessarily apply to tetraacylated LPSs, such as those produced by AOAH treatment, in which tetraacyl lipid A is linked to a polysaccharide chain of variable length; these tetraacyl LPSs are poor agonists for both rodent and human cells. AOAH-treated LPS was the first lipid A derivative shown to inhibit LPS (Pohlman *et al.*, 1987); it and other tetraacylated lipid ''A''s can compete with hexaacylated lipid A or LPS for binding LBP, CD14 (Kitchens and Munford, 1995), and, most importantly, MD-2 (Kim *et al.*, 2007).

2.1. Structure, biosynthesis

Purification of the enzyme from HL-60 human promyelocytes revealed a low abundance, glycosylated protein of Mr = ~65 kDa that has two disulfide-linked subunits (Munford and Hall, 1989). The cDNA sequence indicated that the enzyme is produced as a single polypeptide chain; proteolytic cleavage is thus required to yield the two subunits (Hagen *et al.*, 1991) (Fig. 2.2). The larger subunit (50 kDa) has the GXSXG consensus motif that has been found in serine-active site enzymes, and mutating the Ser to Leu inactivated the enzyme (Staab *et al.*, 1994). This subunit is now considered a GDSL or SGNH lipase (Akoh *et al.*, 2004). The smaller subunit is a member of the saposin-like protein (SAPLIP) family (Munford *et al.*, 1995). It shares six Cys residues and other features with several small proteins that act as enzymes or cofactors for glycosphingolipid catabolism (the saposins, acid sphingomyelinase), form pores in membranes (amoebophore, NK-lysin), or act at lipid–air interphases in the lung (surfactant protein B). The enzymatically active AOAH large subunit is thus armed with a covalently linked ''cofactor,'' the saposin-like small subunit (Fig. 2.3). Without it, the enzyme did not localize in intracellular vacuoles and had lower affinity for LPS (Staab *et al.*, 1994). AOAH and a closely similar *Trypanosoma bruceii* inositol deacylase (Güther *et al.*, 2001) are the only known lipases that have this saposin–lipase combination, which has been very highly conserved in AOAHs from *D. discoideum* to *Homo sapiens* (Munford and Varley, 2006). Whereas saposin B may participate in NK-T cell activation by transferring glycolipid antigens to CD1d (Zhou *et al.*, 2004), several attempts identify a similar role for AOAH have been unsuccessful.

TABLE 2.1 AOAH inactivates LPS

Animal host	Assay	LPS used	Reduction in activity after AOAH treatment	References
A. Evidence that AOAH-treated LPS has decreased bioactivity *in vivo*				
Rabbit	Dermal Shwartzman reaction	*Salmonella typhimurium*	~100-fold	Munford and Hall (1986)
Rabbit	CSF leukocytosis	*Haemophilus influenzae* type b		Syrogiannopoulos *et al*. (1988)
Mouse	Hepatomegaly	*Escherichia coli*	>10-fold	Shao *et al*. (2007)
Mouse	Polyclonal antibody production	*E. coli*, *Neisseria meningitidis*	~100-fold	Lu *et al*. (2005)

Cell source	Cell type	Assay	Reduction in activity after AOAH treatment	References
B. Evidence that AOAH-treated LPS has decreased bioactivity *in vitro*				
Human	Umbilical vein endothelial cells	Leukocyte adhesion	~90%	Pohlman *et al*. (1987)
Human	Umbilical vein endothelial cells	Production of prostanoids, plasminogen activator inhibitor-1	~90%	Riedo *et al*. (1990)
Human	Neutrophils	Adherence, superoxide production, secondary granule protein release, CD11b expression	>95%	Dal Nogare and Yarbrough (1990)

(*continued*)

TABLE 2.1 (*continued*)

Cell source	Cell type	Assay	Reduction in activity after AOAH treatment	References
Human	Monocyte (THP-1 cell)	IL-1 production, NF-κB activation	>95%	Kitchens *et al.* (1992)
Mouse	Splenocytes	Proliferation	10–500-fold	Erwin *et al.* (1991), Lu *et al.* (2005), Munford and Hall (1986)
Mouse	Macrophages, dendritic cells	Cytokine production	50-fold or greater	Lu *et al.* (2003, 2005)

Cell source	Cell type	Assay	Inhibition by equimolar concentration of dLPS (%)	References
C. Evidence that AOAH-treated LPS can inhibit LPS-induced cell responses *in vitro*				
Human	Endothelial cells	Neutrophil adhesion	~30%	Kovach *et al.* (1990), Pohlman *et al.* (1987)
Human	Endothelial cells	Prostanoid, plasminogen activator inhibitor-1 production	~60%	Riedo *et al.* (1990)
Human	Monocytes (THP-1 cell line)	NF-κB activation	66%	Kitchens and Munford (1995), Kitchens *et al.* (1992)
Mouse	Splenocytes	Proliferation, antibody production	20–40%	Erwin *et al.* (1991), Lu *et al.* (2005)

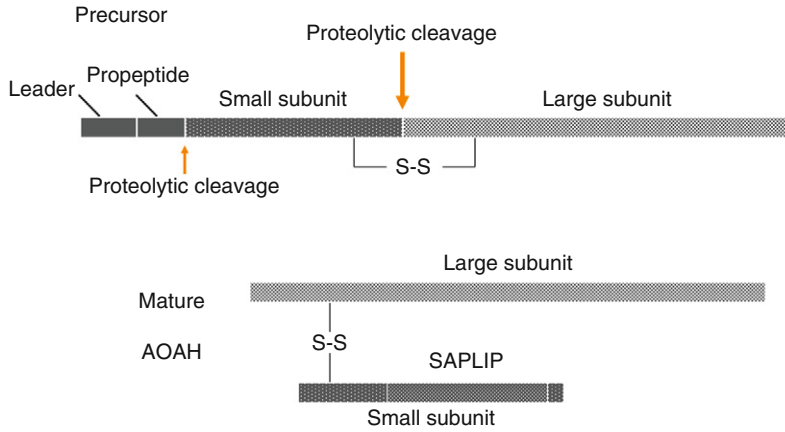

FIGURE 2.2 AOAH structure. The precursor (top) undergoes proteolytic processing to yield the mature protein (bottom). SAPLIP, sphingolipid activator protein-like peptide.

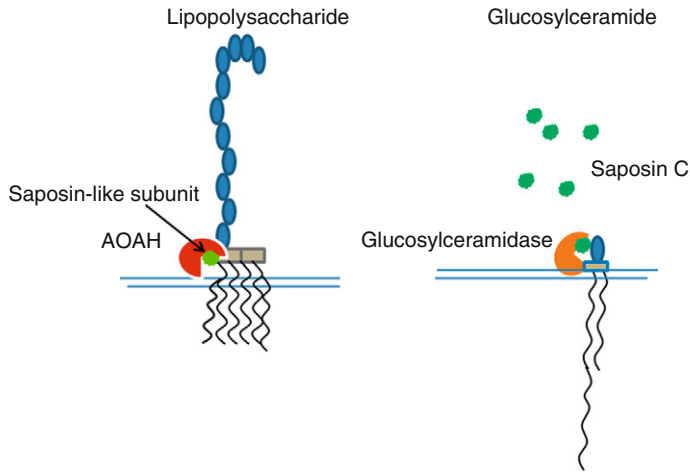

FIGURE 2.3 AOAH (left), with its covalently linked saposin-like subunit (SAPLIP), acts on the bacterial glycolipid, LPS, whereas glucosylceramidase (right), requires saposin C to cleave its eukaryotic glycolipid substrate, glucosylceramide.

The human and murine AOAH genes have 21 small exons on chromosome 7p14-p12 and 13, respectively. In both species, the gene extends over ~200 kb of genomic DNA. To prevent synthesis of AOAH in mice, the starting ATG and the downstream 126 bp of the first exon were replaced with a neomycin resistance cassette. Mice carrying this construct do not produce AOAH protein (Shao *et al.*, 2007) or have LPS-deacylating activity (Lu *et al.*, 2003; Shao *et al.*, 2007).

2.2. Enzymatic activity

As noted, AOAH removes secondary (piggyback, acyloxyacyl-linked) chains from different positions on the diglucosamine lipid A backbone without attacking any of the primary glucosamine-linked chains (Erwin and Munford, 1990). Immunoadsorption of AOAH from leukocyte lysates removed all enzymatic activity toward LPS, indicating that AOAH was the only LPS-deacylating enzyme in these cells, and recombinant AOAH can remove both of the secondary acyl chains from the LPS backbone. It can also be a phospholipase A_1/B, diglyceride lipase, and acyl transferase *in vitro*, and it has a preference for cleaving saturated (or mid-length) acyl chains from both phospholipid and LPS substrates (Munford and Hunter, 1992). AOAH can remove acyl chains that are attached to different positions on the diglucosamine and glycerol backbones of LPS and glycerolipids, respectively (Erwin and Munford, 1990; Munford and Hunter, 1992). It thus has specificity for acyl chain character, not backbone position. Transfected fibroblasts secrete the precursor, which can be taken up by the same or different cells and proteolytically processed into the mature enzyme (Feulner *et al.*, 2004). The AOAH precursor peptide has enzymatic activity toward both LPS and phosphatidylcholine (PC), yet cleaving the precursor to form the mature enzyme increased its activity toward LPS more than 10-fold without altering its ability to act on PC (Staab *et al.*, 1994).

3. LPS DEACYLATION *IN VIVO*

The double-radiolabeled LPS substrate can also be used to quantitate the rate and extent of LPS deacylation *in vivo*. Whether the LPS is injected subcutaneously (footpad, skin site), intraperitoneally or intravenously, deacylation occurs over many hours (Lu *et al.*, 2005; Shao *et al.*, 2007). Despite this seemingly sluggish performance, AOAH-mediated deacylation completely inactivated almost 80% of a subcutaneous dose of LPS before the LPS could travel to draining lymph nodes (Lu *et al.*, 2005). No loss of the LPS secondary acyl chains was detected in $Aoah^{-/-}$ mice, and in neither $Aoah^{-/-}$ nor $Aoah^{+/+}$ animals was there loss of primary (3-hydroxymyristoyl) acyl chains from the backbone. LPS deacylation *in vivo* is thus remarkably selective and limited. Of the various LPS-catabolizing enzymes produced by *D. discoideum* (Verret *et al.*, 1982a,b), which eat bacteria as a foodstuff, only AOAH has been conserved during animal evolution.

One important unresolved issue is the extent to which LPS deacylation occurs inside and outside host cells. An intracellular site was suggested by the enzyme's acid pH optimum, its location in an intracellular vacuole

(Staab *et al.*, 1994), and the apparent colocalization of AOAH and deacylated LPS in neutrophils (Luchi and Munford, 1993). Both rabbit macrophages and murine dendritic cells deacylate the LPS in phagocytosed *Escherichia coli* in an AOAH-dependent fashion (Katz *et al.*, 1999; Lu *et al.*, 2003). Moreover, AOAH does not act on LPS in buffered salt solutions in the absence of a nonionic detergent such as Triton X-100, suggesting that it may require an intracellular environment or factor(s) to do its job. The enzyme can be secreted by rabbit neutrophils and monocytes (Erwin and Munford, 1991), however, and extracellular deacylation has been demonstrated in rabbit peritoneal exudate fluid (Katz *et al.*, 1999), a rich mixture of extravasated plasma, leukocyte products, and other molecules. The AOAH secreted into the urine by renal cortical epithelial cells can also deacylate LPS (Feulner *et al.*, 2004). Gioannini *et al.* recently reported that CD14 and LBP can bind LPS in a way that allows AOAH to deacylate its lipid A moiety (Gioannini *et al.*, 2007), providing an attractive mechanism for extracellular LPS deacylation (Weinrauch *et al.*, 1999). The relative contributions of intra- and extracellular deacylation to LPS inactivation *in vivo* remain uncertain.

Very little is known about how AOAH activity is regulated *in vivo*. In part this reflects the enzyme's low abundance, which has hindered quantitative detection of both AOAH protein and mRNA. In addition, it has not been possible to detect AOAH activity or protein in human plasma or serum. Since the enzyme is easily measured in rodent and rabbit serum, most studies of AOAH regulation have been performed in these animals. In rabbits, plasma AOAH levels rise dramatically within a few minutes of an intravenous injection of LPS and remain elevated for many hours (Erwin and Munford, 1991). The increase in AOAH activity was significantly less in animals that had been given methchlorethamine to induce leukopenia, suggesting that much of the extracellular enzyme is produced and released by neutrophils or monocytes. Indeed, rabbit leukocytes released AOAH in response to stimulation by LPS *ex vivo* (Erwin and Munford, 1991). In studies performed in mice, Cody *et al.* found that AOAH mRNA and activity in liver and lung increased several-fold following intraperitoneal treatment with LPS (Cody *et al.*, 1997). In addition, low concentrations of LPS and interferon-γ induced greater than 10-fold increases in AOAH mRNA in cultured murine macrophages. Neither IL-10 nor dexamethasone prevented AOAH mRNA accumulation in response to LPS, in keeping with the discovery, many years later, that AOAH participates in the anti-inflammatory (recovery) phase of local infection. Indeed, Mages *et al.* (2008) found an approximately sixfold increase in AOAH mRNA abundance in LPS-primed (tolerant) murine macrophages relative to unstimulated controls. Immature murine dendritic cells also produce AOAH; cytokine-induced maturation was associated with diminished LPS-deacylating ability,

whereas exposure to LPS, CpG oligonucleotides, or staphylococci was stimulatory (Lu *et al.*, 2003). Unfortunately, it is uncertain that mice are useful models for human AOAH regulation, since the tissue-specific expression of the enzyme differs substantially (mice produce much more AOAH in the kidney than do humans, and less in myeloid cells). DeLeo and colleagues found that AOAH mRNA abundance decreased approximately twofold in human neutrophils during the 6 h following phagocytosis of latex beads, a time when many of the neutrophils were undergoing apoptosis (Kobayashi *et al.*, 2003). Further study of AOAH regulation in human phagocytes is needed.

4. INACTIVATING LPS *IN VIVO*

Animals have several mechanisms for inactivating LPS (Munford, 2005), including lipid A-neutralizing proteins (bactericidal permeability-increasing protein, lactoferrin, lysozyme, collectins, etc. (Chaby, 2004)), specific and cross-reactive anti-LPS antibodies, and sequestration of the lipid A moiety within lipoprotein micelles. Although intestinal alkaline phosphatase can inactivate LPS in zebrafish (Bates *et al.*, 2007), a role for endogenous alkaline phosphatase in LPS inactivation in mammals has not been established. At present, AOAH is the only endogenous enzyme known to inactivate LPS in tissues.

Early expectations that AOAH would protect animals from LPS-induced inflammation met with disappointment when it was learned that $Aoah^{-/-}$ and $Aoah^{+/+}$ mice had similar acute inflammatory responses to LPS and indistinguishable survival outcomes following LPS or Gram-negative bacterial challenge (Fig. 2.4A). In addition, macrophages and dendritic cells from wild-type and AOAH-deficient animals had similar responses to LPS *in vitro*. On the other hand, the $Aoah^{+/+}$ mice that survived a *Neisseria meningitidis* challenge recovered more rapidly than did the surviving $Aoah^{-/-}$ mice (Fig. 2.4B). Suspecting that the enzyme's role might be discernable only when late responses to LPS and Gram-negative bacteria were studied, we began to look for long-term abnormalities in LPS-treated mice. Three AOAH-dependent phenotypes have now been identified, each of which reflects the ability of persistently active (fully acylated) LPS to stimulate cells *in vivo* for long periods of time. It seems that the enzyme's low abundance and slow deacylation rate are useful for a host defense that responds rapidly and vigorously to LPS but then needs to inactivate this microbial ''messenger'' so as to avoid prolonged cell activation and possible immunosuppression.

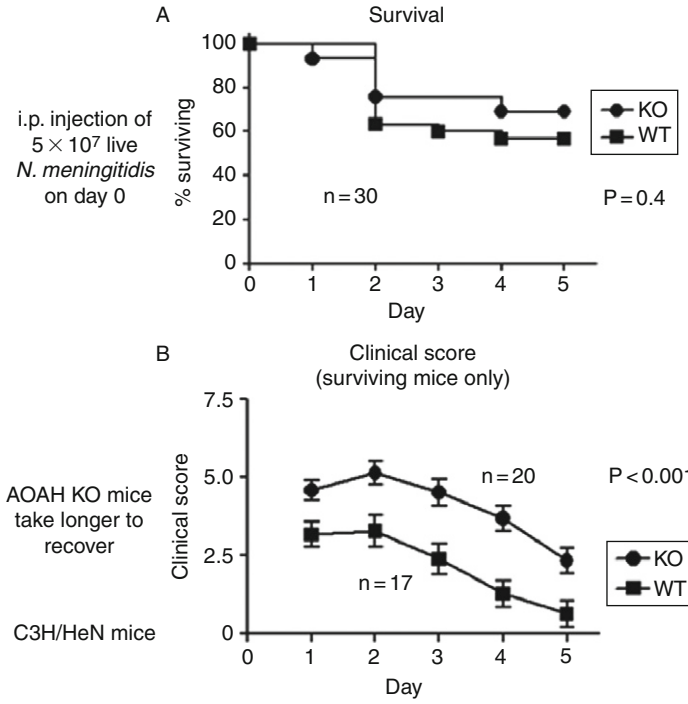

FIGURE 2.4 (A) *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ C3H/HeN mice had similar survival time-courses following intraperitoneal challenge with *Neisseria meningitidis* (5×10^7 colony-forming units), yet (B) the surviving *Aoah*$^{+/+}$ mice recovered more rapidly than did the *Aoah*$^{-/-}$ mice. Recovery was measured using a clinical scoring system that included piloerection, diarrhea, ocular exudate, immobility, and body weight.

4.1. AOAH-dependent phenotypes

4.1.1. Prolonged elevations in polyclonal plasma IgM and IgG_3 antibodies

LPS is a B cell mitogen in mice, which express TLR4 on B cells. B cell proliferation and polyclonal antibody production are thus quantitative indices of LPS stimulation. Subcutaneous LPS inoculation elicits much greater IgM and IgG_3 responses in *Aoah*$^{-/-}$ mice than in wild-type mice, suggesting that AOAH normally exerts a braking influence on B cell stimulation by LPS *in vivo* (Lu *et al.*, 2005) (Fig. 2.5A). Indeed, the presence of the enzyme also prevents impressive, prolonged enlargement of the lymph nodes that drain the site of inoculation. *Tlr4*$^{-/-}$, *Aoah*$^{-/-}$ mice developed neither lymphadenopathy nor elevated antibody titers, in keeping with TLR4's essential role in LPS signaling.

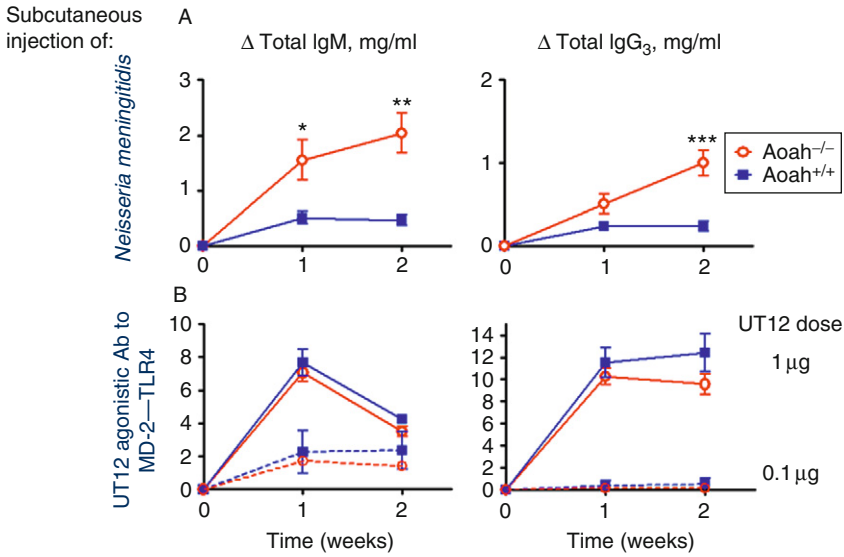

FIGURE 2.5 (A) Antibody responses of *Aoah*$^{+/+}$ (closed boxes) and *Aoah*$^{-/-}$ (open circles) mice to a single subcutaneous dose of 10^7 cfu live *Neisseria meningitidis*. (B) Antibody responses to a single injection of UT12 agonistic antibody to MD-2–TLR4. Note that the responses of *Aoah*$^{+/+}$ and *Aoah*$^{-/-}$ mice were different only when LPS was used to activate MD-2–TLR4. $N = 3$–5 mice/group. The data in (A) were published in Lu *et al.* (2005).

4.1.2. Persistent hepatomegaly

Much of the LPS that enters the bloodstream from the gastrointestinal tract travels via the portal venous system to the liver. Hepatic macrophages (Kupffer cells), which take up a large fraction of this LPS, are also the major AOAH-producing cells in the liver (Shao *et al.*, 2007). Small intravenous doses of LPS induce prolonged, possibly irreversible, hepatomegaly in *Aoah*$^{-/-}$ mice. This phenomenon was first noticed during experiments performed to define the time-course of LPS deacylation in the liver. In wild-type mice, the liver weight/body weight ratio increased transiently, peaking 3 days after i.v. injection and returning to baseline by day 7. In contrast, in *Aoah*$^{-/-}$ mice the liver continued to enlarge, reaching 30–50% above baseline within 1 week of i.v. injection and remaining enlarged for at least 3 weeks (Shao *et al.*, 2007). Although the basis for this striking phenomenon remains uncertain, it is associated with the retention of fully acylated LPS by Kupffer cells and sinusoidal engorgement with blood that contains neutrophils, B cells, CD4 and CD8 T cells, and monocytes ((Shao *et al.*, 2007); B.M. Shao, unpublished results). LPS also induces impressive splenomegaly in *Aoah*$^{-/-}$ mice but this response is transient, resolving within 2 weeks (Shao *et al.*, 2007).

4.1.3. Prolonged tolerance and immunosuppression

Another remarkable consequence of AOAH deficiency is the development of prolonged endotoxin tolerance and immunosuppression following exposure to very small amounts of LPS. In this context, tolerance refers to the ability of a small priming dose of LPS to induce a state of cellular reprogramming in which responses to subsequent, larger doses of LPS and several other microbial agonists are altered. Endotoxin tolerance is known to occur in many animals, including humans; it is usually considered an adaptation to prevent excessive inflammatory reactions to invading microbes—as others have suggested, it may prevent ''friendly fire'' while animals recover from infection (Cross, 2002; Medvedev *et al.*, 2006). The duration of the tolerant period is influenced by several factors, including LPS dose, the route of administration, and the animal's ability to deacylate the LPS. Whereas $Aoah^{+/+}$ mice recover from the tolerant state within 5–10 days after intraperitoneal exposure to a small dose of LPS or *E. coli*, $Aoah^{-/-}$ mice remain tolerant for at least 4 months! Moreover, LPS-primed $Aoah^{-/-}$ mice were exquisitely sensitive to challenge with a virulent *E. coli* strain; susceptibility was associated with delayed production of TNF and IL-6 and massive bacterial growth during the first 24 h after inoculation (Lu *et al.*, 2008). Initial analyses of mRNA expression by LPS-primed $Aoah^{+/+}$ and $Aoah^{-/-}$ peritoneal macrophages suggest that LPS exposure induces low-grade, persistent activation in AOAH-deficient animals, largely in keeping with the reprogramming phenomenon observed previously in macrophages induced to develop tolerance *in vitro* (Foster *et al.*, 2007; Mages *et al.*, 2008) but differing from those studies in many of the individual mRNAs that are up- and downregulated during the tolerant period (A. Varley, M. Lu, unpublished results). As with the other two phenotypes, prolonged tolerance in $Aoah^{-/-}$ mice is associated with the presence of fully acylated LPS in cells for long periods of time (Lu *et al.*, 2008).

4.1.4. Do AOAH-dependent phenotypes require exposure to LPS?

If these AOAH-dependent phenotypes result from the enzyme's ability to deacylate LPS, they should not occur in $Aoah^{-/-}$ animals when the MD-2–TLR4 signaling pathway is activated by a non-LPS agonist. We tested this hypothesis using UT12, an agonistic monoclonal antibody to MD-2–TLR4 that was developed by Shoichiro Ohta and colleagues (Ohta *et al.*, 2006). For each of the phenotypes discussed above, $Aoah^{-/-}$ and $Aoah^{+/+}$ mice had indistinguishable responses to UT12; an example is shown in Fig. 2.5B, which should be compared with Fig. 2.5A. AOAH's ability to act on LPS, and not other potential substrates, is thus likely to account for the prolonged LPS-induced responses observed in $Aoah^{-/-}$ mice.

4.2. AOAH-dependent immunomodulation: Only *in vivo*?

Whereas LPS-injected *Aoah*$^{+/+}$ mice produce significantly less antibody than do *Aoah*$^{-/-}$ mice, naïve splenocytes from *Aoah*$^{-/-}$ and *Aoah*$^{+/+}$ animals proliferate and produce antibody to the same extent when they are exposed to LPS *in vitro* (Lu *et al.*, 2005). AOAH thus has a strikingly different influence on the *in vivo* and *in vitro* responses of splenocytes to LPS. Similarly, whereas LPS-primed *Aoah*$^{-/-}$ peritoneal macrophages retain their tolerant (reprogrammed) phenotype when they are removed from the peritoneal cavity and grown for several days *ex vivo*, naïve *Aoah*$^{-/-}$ and *Aoah*$^{+/+}$ macrophages recover from tolerance at the same rate when they are first exposed to LPS *in vitro*. Again, the *in vivo* phenotype cannot be modeled *in vitro*. It should be interesting to define the properties of the *in vivo* environment that allow LPS inactivation to have such an important impact on the duration and nature of host responses to LPS.

4.3. Providing AOAH prevents prolonged responses to LPS *in vivo*

The three AOAH-dependent phenotypes have been observed on two widely different murine strain backgrounds, C3H/HeN and C57Bl/6, indicating that these responses to LPS are not strain specific. Another way to show that the observations in *Aoah*$^{-/-}$ animals are truly the result of AOAH deficiency is to prevent or ameliorate them by providing AOAH. We found that intravenous doses of recombinant human AOAH, given prior to LPS injection, can prevent LPS-induced hepatomegaly (Shao *et al.*, 2007) and that producing AOAH *in vivo* using a gutted adenoviral vector can prevent prolonged LPS-induced tolerance (Lu *et al.*, 2008). Recombinant adenoviruses produce their cargo proteins largely in hepatocytes, whereas AOAH is made by phagocytic cells and renal cortical epithelial cells. To study the effects of overproducing AOAH in cells that normally make it, we also engineered mice that produce large amounts of AOAH in macrophages and dendritic cells. These mice express AOAH from the human CD68 promoter in a cassette developed by David Greaves at the University of Oxford (Gough *et al.*, 2001). The transgenic mice recovered from LPS challenge more rapidly than did wildtype mice and they were protected from LPS-induced hepatosplenomegaly (N. Ojogun *et al.*, in press). The transgenic animals were also less likely to succumb to an *E. coli* challenge, confirming the important role that LPS plays in Gram-negative bacterial toxicity *in vivo* and raising the possibility that increasing AOAH levels might ameliorate harmful responses to Gram-negatives in other animals, including humans (Munford, 2008). For example, early studies suggested that AOAH might play a protective role in bovine coliform mastitis (Dosogne *et al.*, 1998; McDermott *et al.*, 1991). Engineering transgenic cattle to

overproduce AOAH in myeloid cells or milk might thus be advantageous for the dairy industry.

5. POTENTIAL CLINICAL CONNECTIONS

5.1. AOAH deficiency

Large-scale screens for AOAH-deficient humans have not been performed. One group has reported an association between a particular AOAH haplotype and risk of asthma (Barnes *et al.*, 2006); whether or not this haplotype is associated with altered AOAH production or activity is not known. Screening for AOAH deficiency might also be fruitful in individuals with viral, alcoholic or nonalcoholic steatohepatitis, for which gut-derived LPS may be a contributing factor (Tilg and Diehl, 2000); severe sepsis induced by Gram-negative bacteria that make hexaacyl LPS (see Section 1); prolonged recovery from Gram-negative bacterial diseases; autoimmune diseases in which a role for LPS has been suggested (such as Guillain–Barre syndrome following exposure to *Campylobacter jejuni* (Ang *et al.*, 2002)); or patients with xanthogranulomatous pyelonephritis or malakoplakia, rare complications of bacterial infection in which macrophages accumulate lipid and polysaccharides (Gregg *et al.*, 1999). A role for AOAH-mediated LPS inactivation might also be sought in patients with HIV/AIDS, in whom immune dysfunction has been related to the absorption of bacterial molecules, including LPS, from the gastrointestinal tract into portal blood (Brenchley *et al.*, 2006; Jiang *et al.*, 2009).

5.2. Host inactivation of microbial agonists other than LPS

Greater understanding of how animals inactivate other microbial molecules could also have important consequences for patient care. For example, some individuals who become infected with *Borrelia burgdorferi* remain symptomatic despite having received effective antibiotic therapy; it has not been possible to grow *B. burgdorferi* or detect their DNA in inflamed tissues from such patients (Marques, 2008). Perhaps their persistent symptoms are related to an inability to inactivate stimulatory bacterial lipoproteins, which activate host cells via TLR2, or too-rapid deacylation of the Borrelia glycolipid that stimulates protective NK-T cells (Tupin *et al.*, 2008). The same question might be raised regarding recovery from staphylococcal and streptococcal diseases, which can be very prolonged despite negative cultures; lipoproteins are the most potent known immunostimulatory staphylococcal molecules. Recovery from certain viral infections may also take a long time (Didierlaurent *et al.*, 2008), raising the possibility that hosts might differ in their ability to degrade stimulatory viral nucleic acids or proteins. If they shorten the

time to full recovery, measures that enhance the host's ability to inactivate stimulatory microbial molecules would be a useful adjunct to antimicrobial chemotherapy. Since recovery from infectious diseases can take many weeks, with increased risk of another infection during that period (Yende *et al.*, 2008), further investigation of this possibility is clearly warranted.

6. CONCLUSION

Studies of LPS inactivation by an unusual host lipase have revealed that killing Gram-negative bacteria does not prepare an animal to confront another microbial invader: the major microbial ''messenger'' molecule must also be inactivated. Although it seems likely that persistence of microbial agonists other than LPS could also have long-term consequences, much more work is required to test this notion and to identify the important catabolic pathways. If the LPS-primed *Aoah*$^{-/-}$ mouse is a fruitful model, eliminating bioactive microbial molecules should hasten recovery from infection-induced immunosuppression (tolerance) and possibly prevent other lingering symptoms and signs of disease.

ACKNOWLEDGMENTS

We thank our many helpful colleagues and collaborators. The authors' laboratory at the University of Texas Southwestern Medical School is supported by grant AI18188 from the National Institute for Allergy and Infectious Diseases, NIH, and by the Bromberg Chair in Internal Medicine.

REFERENCES

Akoh, C. C., Lee, G. C., Liaw, Y. C., Huang, T. H., and Shaw, J. F. (2004). GDSL family of serine esterases/lipases. *Prog. Lipid Res.* **43,** 534–552.

Ang, C. W., Laman, J. D., Willison, H. J., Wagner, E. R., Endtz, H. P., De Klerk, M. A., Tio-Gillen, A. P., Van Den, B. N., Jacobs, B. C., and Doorn, P. A. (2002). Structure of *Campylobacter jejuni* lipopolysaccharides determines antiganglioside specificity and clinical features of Guillain-Barre and Miller Fisher patients. *Infect. Immun.* **70,** 1202–1208.

Barnes, K. C., Grant, A., Gao, P., Baltadjieva, D., Berg, T., Chi, P., Zhang, S., Zambelli-Weiner, A., Ehrlich, E., Zardkoohi, O., Brummet, M. E., Stockton, M., *et al.* (2006). Polymorphisms in the novel gene acyloxyacyl hydroxylase (AOAH) are associated with asthma and associated phenotypes. *J. Allergy Clin. Immunol.* **118,** 70–77.

Bates, J. M., Akerlund, J., Mittge, E., and Guillemin, K. (2007). Intestinal alkaline phosphatase detoxifies lipopolysaccharide and prevents inflammation in zebrafish in response to the gut microbiota. *Cell Host Microbe* **2,** 371–382.

Brenchley, J. M., Price, D. A., Schacker, T. W., Asher, T. E., Silvestri, G., Rao, S., Kazzaz, Z., Bornstein, E., Lambotte, O., Altmann, D., Blazar, B. R., Rodriguez, B., *et al.* (2006). Microbial translocation is a cause of systemic immune activation in chronic HIV infection. *Nat. Med.* **12,** 1365–1371.

Chaby, R. (2004). Lipopolysaccharide-binding molecules: Transporters, blockers and sensors. *Cell Mol. Life Sci.* **61,** 1697–1713.

Cody, M. J., Salkowski, C. A., Henricson, B. E., Detore, G. R., Munford, R. S., and Vogel, S. N. (1997). Effect of inflammatory and anti-inflammatory stimuli on acyloxyacyl hydrolase gene expression and enzymatic activity in murine macrophages. *J. Endotoxin Res.* **4,** 371–379.

Cross, A. S. (2002). Endotoxin tolerance—Current concepts in historical perspective. *J. Endotoxin Res.* **8,** 83–98.

Dal Nogare, A. R., and Yarbrough, W. C., Jr. (1990). A comparison of the effects of intact and deacylated lipopolysaccharide on human polymorphonuclear leukocytes. *J. Immunol.* **144,** 1404–1410.

Didierlaurent, A., Goulding, J., Patel, S., Snelgrove, R., Low, L., Bebien, M., Lawrence, T., van Rijt, L. S., Lambrecht, B. N., Sirard, J. C., and Hussell, T. (2008). Sustained desensitization to bacterial Toll-like receptor ligands after resolution of respiratory influenza infection. *J. Exp. Med.* **205,** 323–329.

Dosogne, H., Capuco, A. V., Paape, M. J., Roets, E., Burvenich, C., and Fenwick, B. (1998). Reduction of acyloxyacyl hydrolase activity in circulating neutrophils from cows after parturition. *J. Dairy Sci.* **81,** 672–677.

Erwin, A. L., and Munford, R. S. (1990). Deacylation of structurally diverse lipopolysaccharides by human acyloxyacyl hydrolase. *J. Biol. Chem.* **265,** 16444–16449.

Erwin, A. L., and Munford, R. S. (1991). Plasma lipopolysaccharide-deacylating activity (acyloxyacyl hydrolase) increases following lipopolysaccharide administration to rabbits. *Lab. Invest.* **65,** 138–144.

Erwin, A. L., Mandrell, R. E., and Munford, R. S. (1991). Enzymatically deacylated *Neisseria* LPS inhibits murine splenocyte mitogenesis induced by LPS. *Infect. Immun.* **59,** 1881–1887.

Feulner, J. A., Lu, M., Shelton, J. M., Zhang, M., Richardson, J. A., and Munford, R. S. (2004). Identification of acyloxyacyl hydrolase, a lipopolysaccharide-detoxifying enzyme, in the murine urinary tract. *Infect. Immun.* **72,** 3171–3178.

Foster, S. L., Hargreaves, D. C., and Medzhitov, R. (2007). Gene-specific control of inflammation by TLR-induced chromatin modifications. *Nature* **447,** 972–978.

Gioannini, T. L., Teghanemt, A., Zhang, D., Prohinar, P., Levis, E. N., Munford, R. S., and Weiss, J. P. (2007). Endotoxin-binding proteins modulate the susceptibility of bacterial endotoxin to deacylation by acyloxyacyl hydrolase. *J. Biol. Chem.* **282,** 7877–7884.

Golenbock, D. T., Hampton, R. Y., and Raetz, C. R. H. (1990). Lipopolysaccharide antagonism by lipid A precursor lipid IV_A is species dependent. *FASEB J.* **4,** A2055.

Gough, P. J., Gordon, S., and Greaves, D. R. (2001). The use of human CD68 transcriptional regulatory sequences to direct high-level expression of class A scavenger receptor in macrophages *in vitro* and *in vivo*. *Immunology* **103,** 351–361.

Gregg, C. R., Rogers, T. E., and Munford, R. S. (1999). Xanthogranulomatous pyelonephritis. *Curr. Clin. Top. Infect. Dis.* **19,** 287–304.

Güther, M. L. S., Leal, S., Morrice, N. A., Cross, G. A. M., and Ferguson, M. A. (2001). Purification, cloning and characterization of a GPI inositol deacylase from *Trypanosoma brucei*. *EMBO J.* **20,** 4923–4934.

Hagen, F. S., Grant, F. J., Kuijper, J. L., Slaughter, C. A., Moomaw, C. R., Orth, K., O'Hara, P. J., and Munford, R. S. (1991). Expression and characterization of recombinant human acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides. *Biochemistry* **30,** 8415–8423.

Hajjar, A. M., Ernst, R. K., Tsai, J. H., Wilson, C. B., and Miller, S. I. (2002). Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat. Immunol.* **3,** 354–359.

Hall, C. L., and Munford, R. S. (1983). Enzymatic deacylation of the lipid A moiety of *Salmonella typhimurium* lipopolysaccharides by human neutrophils. *Proc. Natl. Acad. Sci. USA* **80,** 6671–6675.

Jiang, W., Lederman, M., Hunt, P., Sieg, S., Haley, K., Rodriguez, B., Landay, A., Martin, J., Sinclair, E., Asher, A., Deeks, S., Douek, D., *et al*. (2009). Plasma levels of bacterial DNA correlate with immune activation and the magnitude of immune restoration in persons with antiretroviral-treated HIV infection. *J. Infect. Dis.* **199,** 1177–1185.

Katz, S. S., Weinrauch, Y., Munford, R. S., Elsbach, P., and Weiss, J. (1999). Deacylation of lipopolysaccharide in whole *Escherichia coli* during destruction by cellular and extracellular components of a rabbit inflammatory peritoneal exudate. *J. Biol. Chem.* **274,** 36579–36584.

Kim, H. M., Park, B. S., Kim, J. I., Kim, S. E., Lee, J., Oh, S. C., Enkhbayar, P., Matsushima, N., Lee, H., Yoo, O. J., and Lee, J. O. (2007). Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritoran. *Cell* **130,** 906–917.

Kitchens, R. L., and Munford, R. S. (1995). Enzymatically deacylated lipopolysaccharide (LPS) can antagonize LPS at multiple sites in the LPS recognition pathway. *J. Biol. Chem.* **270,** 9904–9910.

Kitchens, R. L., Ulevitch, R. J., and Munford, R. S. (1992). Lipopolysaccharide (LPS) partial structures inhibit responses to LPS in a human macrophage cell line without inhibiting LPS uptake by a CD14-mediated pathway. *J. Exp. Med.* **1760,** 485–494.

Kobayashi, S. D., Voyich, J. M., Somerville, G. A., Braughton, K. R., Malech, H. L., Musser, J. M., and DeLeo, F. R. (2003). An apoptosis-differentiation program in human polymorphonuclear leukocytes facilitates resolution of inflammation. *J. Leukoc. Biol.* **73,** 315–322.

Kovach, N. L., Yee, E., Munford, R. S., Raetz, C. R. H., and Harlan, J. M. (1990). Lipid IVA inhibits synthesis and release of tumor necrosis factor induced by LPS in human whole blood *ex vivo*. *J. Exp. Med.* **172,** 77–84.

Lu, M., Zhang, M., Kitchens, R. L., Fosmire, S., Takashima, A., and Munford, R. S. (2003). Stimulus-dependent deacylation of bacterial lipopolysaccharide by dendritic cells. *J. Exp. Med.* **197,** 1745–1754.

Lu, M., Zhang, M., Takashima, A., Weiss, J., Apicella, M. A., Li, X. H., Yuan, D., and Munford, R. S. (2005). Lipopolysaccharide deacylation by an endogenous lipase controls innate antibody responses to Gram-negative bacteria. *Nat. Immunol.* **6,** 989–994.

Lu, M., Varley, A. W., Ohta, S., Hardwick, J., and Munford, R. S. (2008). Host inactivation of bacterial lipopolysaccharide prevents prolonged tolerance following gram-negative bacterial infection. *Cell Host Microbe* **4,** 293–302.

Luchi, M., and Munford, R. S. (1993). Binding, internalization, and deacylation of bacterial lipopolysaccharides by human neutrophils. *J. Immunol.* **151,** 959–969.

Mages, J. R., Dietrich, H., and Lang, R. (2008). A genome-wide analysis of LPS tolerance in macrophages. *Immunobiology* **212,** 723–737.

Marques, A. (2008). Chronic Lyme disease: A review. *Infect. Dis. Clin. North Am.* **22,** 341–348.

McDermott, C. M., Morrill, J. L., and Fenwick, B. W. (1991). Deacylation of endotoxin during natural cases of bovine mastitis. *J. Dairy Sci.* **74,** 1227–1234.

Medvedev, A. E., Sabroe, I., Hasday, J. D., and Vogel, S. N. (2006). Tolerance to microbial TLR ligands: Molecular mechanisms and relevance to disease. *J. Endotoxin Res.* **12,** 133–150.

Munford, R. S. (2005). Detoxifying endotoxin: Time, place, person. *J. Endotoxin Res.* **11,** 69–84.

Munford, R. S. (2008). Sensing Gram-negative bacterial lipopolysaccharides: A human disease determinant? *Infect. Immun.* **76,** 454–465.

Munford, R. S., and Hall, C. L. (1986). Detoxification of bacterial lipopolysaccharides (endotoxins) by a human neutrophil enzyme. *Science* **234,** 203–205.

Munford, R. S., and Hall, C. L. (1989). Purification of acyloxyacyl hydrolase, a leukocyte enzyme that removes secondary acyl chains from bacterial lipopolysaccharides. *J. Biol. Chem.* **264,** 15613–15619.

Munford, R. S., and Hunter, J. P. (1992). Acyloxyacyl hydrolase, a leukocyte enzyme that deacylates bacterial lipopolysaccharides, has phospholipase, lysophospholipase, diacylglycerollipase, and acyltransferase activities *in vitro*. *J. Biol. Chem.* **267,** 10116–10121.

Munford, R. S., and Varley, A. W. (2006). Shield as signal: Lipopolysaccharides and the evolution of immunity to Gram-negative bacteria. *PLoS Pathog.* **2,** e67.

Munford, R. S., Sheppard, P. O., and O'Hara, P. J. (1995). Saposin-like proteins (SAPLIP) carry out diverse functions on a common backbone structure. *J. Lipid Res.* **36,** 1653–1663.

Nigam, V. N., Malchow, D., Rietschel, E. Th., Lüderitz, O., and Westphal, O. (1970). Die enzymatische abspaltung langkettiger fettsäuren aus bakteriellen lipopolysacchariden mittels extrakten aus der amöbe von *Dictyostelium discoideum*. *Hoppe-Seyler's Z. Physiol. Chem.* **351,** 1123–1132.

Ohta, S., Bahrun, U., Shimazu, R., Matsushita, H., Fukudome, K., and Kimoto, M. (2006). Induction of long-term lipopolysaccharide tolerance by an agonistic monoclonal antibody to the toll-like receptor 4/MD-2 complex. *Clin. Vaccine Immunol.* **13,** 1131–1136.

Pohlman, T. H., Munford, R. S., and Harlan, J. M. (1987). Deacylated lipopolysaccharide inhibits neutrophil adherence to endothelium induced by lipopolysaccharide *in vitro*. *J. Exp. Med.* **165,** 1393–1402.

Riedo, F. X., Munford, R. S., Campbell, W. B., Reisch, J. S., Chien, K. R., and Gerard, R. D. (1990). Deacylated lipopolysaccharide inhibits plasminogen activator inhibitor-1, prostacyclin, and prostaglandin E2 induction by lipopolysaccharide but not by tumor necrosis factor-alpha. *J. Immunol.* **144,** 3506–3512.

Saddler, J. N., Coote, J. G., and Wardlaw, A. C. (1979). Degradation of bacterial lipopolysaccharide by the slime mould *Physarum polycephalum*. *Can. J. Microbiol.* **25,** 124–128.

Saddler, J. N., Patron, R., and Wardlaw, A. C. (1979). Degradation of bacterial lipopolysaccharide by gut juice of the snail *Helix pomatia*. *Specialia* **35,** 494–495.

Shao, B., Lu, M., Katz, S. C., Varley, A. W., Hardwick, J., Rogers, T. E., Ojogun, N., Rockey, D. C., DeMatteo, R. P., and Munford, R. S. (2007). A host lipase detoxifies bacterial lipopolysaccharides in the liver and spleen. *J. Biol. Chem.* **282,** 13726–13735.

Staab, J. F., Ginkel, D. L., Rosenberg, G. B., and Munford, R. S. (1994). A saposin-like domain influences the intracellular localization, stability, and catalytic activity of human acyloxyacyl hydrolase. *J. Biol. Chem.* **269,** 23736–23742.

Syrogiannopoulos, G. A., Hansen, E. J., Erwin, A. L., Munford, R. S., Rutledge, J., Reisch, J. S., and McCracken, G. H., Jr. (1988). *Haemophilus influenzae* type b lipooligosaccharide induces meningeal inflammation. *J. Infect. Dis.* **157,** 237–244.

Takayama, K., Qureshi, N., and Mascagni, P. (1983). Complete structure of lipid A obtained from the lipopolysaccharides of the heptoseless mutant of *Salmonella typhimurium*. *J. Biol. Chem.* **258,** 12801–12803.

Tilg, H., and Diehl, A. M. (2000). Cytokines in alcoholic and nonalcoholic steatohepatitis. *N. Engl. J. Med.* **343,** 1467–1476.

Tupin, E., Benhnia, M. R.-E.-I., Kinjo, Y., Patsey, R., Lena, C. J., Haller, M. C., Caimano, M. J., Imamura, M., Wong, C. H., Crotty, S., Radolf, J. D., Sellati, T. J., *et al*. (2008). NKT cells prevent chronic joint inflammation after infection with *Borrelia burgdorferi*. *Proc. Natl. Acad. Sci. USA* **105,** 19863–19868.

Verret, C. R., Rosner, M. R., and Khorana, H. G. (1982). Fatty acyl amidases from *Dictyostelium discoideum* that act on lipopolysaccharide and derivatives. II. Aspects of substrate specificity. *J. Biol. Chem.* **257,** 10228–10234.

Verret, C. R., Rosner, M. R., and Khorana, H. G. (1982). Fatty acyl amidases from *Dictyostelium discoideum* that act on lipopolysaccharide and derivatives. I. Partial purification and properties. *J. Biol. Chem.* **257,** 10222–10227.

Weinrauch, Y., Katz, S. S., Munford, R. S., Elsbach, P., and Weiss, J. (1999). Deacylation of purified LPS by cellular and extracellular components of a sterile rabbit peritoneal inflammatory exudate. *Infect. Immun.* **67,** 3376–3382.

Wollenweber, H.-W., Broady, K. W., Luderitz, O., and Rietschel, E. Th. (1982). The chemical structure of lipid A. Demonstration of amide-linked 3-acyloxyacyl residues in *Salmonella minnesota* Re lipopolysaccharide. *Eur. J. Biochem.* **124,** 191–198.

Yende, S., D'Angelo, G., Kellum, J. A., Weissfeld, L., Fine, J., Welch, R. D., Kong, L., Carter, M., and Angus, D. C. (2008). Inflammatory markers at hospital discharge predict subsequent mortality after pneumonia and sepsis. *Am. J. Respir. Crit. Care Med.* **177,** 1242–1247.

Zhou, D., Mattner, J., Cantu, C., III, Schrantz, N., Yin, N., Gao, Y., Sagiv, Y., Hudspeth, K., Wu, Y. P., Yamashita, T., Teneberg, S., Wang, D., *et al.* (2004). Lysosomal glycosphingolipid recognition by NKT cells. *Science* **306,** 1786–1789.

CHAPTER 3

Role of SOCS in Allergic and Innate Immune Responses

Suzanne L. Cassel and **Paul B. Rothman**

Contents

Abstract

Cytokines are powerful mediators of the immune response that, following initial release by components of the innate system, drive effector functions as well as stimulate the additional arms of the response. Their individual functions are diverse, with stimulatory and inhibitory actions, with the resultant systemic immune response a summation of these actions. The frequently opposing effects of cytokines determine that the blockade of one results in the functional augmentation of the other. Thus, the differential regulation of cytokines profoundly influences the character of the immune response. The suppressor of cytokine signaling proteins are

Department of Internal Medicine, Carver College of Medicine, University of Iowa, Iowa City, Iowa, USA

Advances in Immunology, Volume 103
ISSN 0065-2776, DOI: 10.1016/S0065-2776(09)03003-X

a family of molecules pivotal to this critical regulation. In this review, we will discuss their structural components and functions and our understanding of their impact on the systemic immune response.

The immune system must mount an exponentially expanding attack to counter the advances of pathogenic infections, which is coordinated by an integrated response by the innate and adaptive immune systems. Danger signals, such as pathogen-associated molecular patterns (PAMPs) on the surface of invading organisms are sensed by pattern recognition receptors (PRRs) on the surface of innate immune cells. The recognition of these danger signals by innate immune cells triggers an inflammatory cascade and, via the consequent interaction with cells of the adaptive immune system, drives the generation of the specialized adaptive immune response. An important method of communication between cells involves the secretion of cytokines, which have a well described and pivotal role in the defensive response. However, such a powerful tool is not without danger to the host, as it is well known that if unfettered the immune response is equally toxic to host tissues. To prevent excessive pathology, the immune system has mechanisms in place to precisely regulate and extinguish the cytokine-driven response. These systems are finely balanced to achieve protection from intruders while avoiding excessive injury to the host.

The immune system has several regulatory systems that function to limit cytokine responses. Regulation can occur by the action of protein tyrosine phosphatases such as Src-homology-2 (SH2) containing phosphatase (SHP)-1 and SHP-2, which remove activating phosphate groups from signaling molecules (Shuai and Liu, 2003) and thereby extinguish downstream responses. Incomplete, nonfunctional versions of signaling proteins can be produced that can be integrated into nascent signaling complexes and act in a dominant negative manner to interrupt transmission of inflammatory signals (Hendry and John, 2004). Protein inhibitors of activated signal transducer and activator of transcription (STAT) (PIAS) are a family of proteins that contain E3 small ubiquitin-related modifier (SUMO)-ligase activity. PIAS proteins ligate SUMO to target signaling molecules, labeling the signaling molecules for degradation. It has been postulated that PIAS proteins interfere with inflammatory signal transduction in two additional ways: they may directly block binding of signaling complexes to DNA and may also recruit separate repressor proteins to the signaling complex (Lee *et al.*, 2007). Additionally, a family of molecules called the suppressor of cytokine signaling (SOCS) family has been identified as the critical regulators of this system. In this chapter, we will review aspects of the SOCS family with a particular focus on their role in regulating atopic immune responses and aspects of the innate immune system.

1. ATOPIC IMMUNE RESPONSES

The careful regulation of immune responses prevents damage to the organism from over exuberant as well as inappropriate inflammatory responses. Allergic diseases, such as those responsible for atopic forms of asthma, are an example of inappropriate inflammatory responses directed at otherwise innocuous antigens. This inflammation results in the clinical features characteristic of the allergic response. In asthma patients develop shortness of breath and wheezing while pathological samples reveal eosinophilic infiltration, mast cell degranulation, and elevated mucus secretion. IgE antibodies specific to environmental antigens are present in the blood as evidence of a systemic response. The allergic immune response is dominated by antigen-specific $CD4^+$ T helper cells that produce type 2 cytokines, such as IL-4, 5, and 13. These T helper 2 (Th2) cells drive an antigen-specific response that involves a variety of inflammatory cells including B lymphocytes, mast cells, and eosinophils (Corrigan and Kay, 1992; Wills-Karp, 1999).

The marked increased incidence of asthma and atopic diseases over the past several decades has been well documented (Eder *et al.*, 2006) as has the profound cost to society. Asthma has become a major source of disability and absenteeism from work, and asthma-related illnesses cost an estimated 10 billion dollars per year in the US alone (Gergen, 2001). Moreover, asthma is the most common childhood disease, afflicting 7–10% of children in this country (Bloom and Dey, 2006), and is a major cause for school absenteeism (Dougherty and Fahy, 2009). Thus, it is apparent that understanding the underlying mechanisms leading to asthma and atopic disease with a goal of development of more effective therapies or even prevention is of substantial interest.

Years of research have successfully elucidated many of the critical cellular events key to the development of the allergic response. The response begins, in the case of asthma, with inhalation of an allergen. Most allergens are relatively small, highly soluble proteins that are inhaled in desiccated particles such as pollen grains or dust mite feces. The allergen is encountered and phagocytosed by antigen processing cells (APCs) such as dendritic cells (DCs). The APC processes the antigen and migrates to the lymph node, where it presents the antigen to T cells. Antigens must be associated with MHC on the surface of an APC for T cells recognition to occur. It is this direct recognition and specific binding of the T cell, through its T cell receptor (TCR), to the complexed peptide–MHC on the APC along with the second stimulatory signal provided by costimulatory molecules that initiates T cell activation. Mature APCs provide more than contact dependent stimulation to the T cell; they secrete specific cytokines that have the pivotal role of driving

the T cell along a particular differentiation pathway. When activated in the presence of Th1 skewing cytokines such as IL-12, naïve T cells preferentially become Th1 effectors, while the Th2 cytokine IL-4 provides the stimulation for the T cells to become Th2 effectors. On subsequent exposure to the allergen, these effector Th2 cells in turn release the specific cytokines that are critical to drive the activation of other cells associated with allergic inflammation (reviewed in Eisenbarth *et al.*, 2004).

Studies of the specific functions of the individual Th2 cytokines, IL-4, IL-5, and IL-13, have revealed separate as well as overlapping roles. IL-4 is key to the initiation of the Th2 response, while in humans both IL-4 and IL-13 induce immunoglobulin class switching to IgE. These cytokines are also important in the induction of cell adhesion molecules and chemokines by endothelial and epithelial cells. IL-13 is important for the propogation and the effector phase of atopic immune responses (Wills-Karp *et al.*, 1998), IL-5 plays an important role in eosinophil development and maintenance. It is clear that the regulation of these cytokines and their signaling is of paramount importance to generation and thus control of allergic disease.

Cytokine signal transduction is a multistep process and can be regulated at numerous different points. Signaling begins with the cytokine binding the receptor, which triggers the assembly of the receptor subunits. The receptor for IL-4 in hematopoietic cells is composed of a heterodimer of the IL-4-specific α chain (IL-4Rα) and the common γ chain. IL-13 is also a heterodimer, sharing IL-4Rα but has a unique second component, IL-13Rα. This complex may also play a role in binding IL-4 in cells that lack the common γ chain. The receptors themselves do not have enzymatic activity but are associated with a specific cytoplasmic tyrosine kinase of the Janus kinase family (JAK), either JAK1, JAK2, JAK3, or Tyk2. The common γ chain is associated with JAK3, IL-4Rα is associated with JAK1, and the IL-13Rα is associated with Tyk2. The receptor chains undergo phosphorylation upon activation of the respective receptor by binding of the cognate ligand. The phosphorylation of conserved tyrosine residues in the cytoplasmic tail of the receptor recruits a member of the STAT family of molecules via their SH-2 domains. The association of differing STAT molecules allows individual cytokines their specific transcriptional responses. In the case of IL-4 and IL-13, the STAT family member recruited to the receptor is STAT6, which is also phosphorylated by the JAK kinase. STAT6 dimerizes and translocates to the nucleus where it regulates the transcription of numerous genes involved in the Th2 response, including the Th2-specific transcription factor GATA3 and the Iε promoter, which is required for B cell immunoglobulin class switch to IgE (Coffman *et al.*, 1986; Nelms *et al.*, 1999). The importance of IL-4 and STAT6 in the generation of the Th2 response has been supported by the analysis of responses in mice with genetic modifications in these

pathways. IL-12 signaling involves similar specificity, activating JAK2 and Tyk2 which in turn drive STAT4 phosphorylation (Gately *et al.*, 1998).

2. SOCS FAMILY

The SOCS family consists of eight members, SOCS1–SOCS7 and CIS (cytokine-inducible SH2-containing protein) that share structural and functional homology. CIS, the first family member identified, was identified based on its differential expression following IL-3 and erythropoietin (EPO) exposure (Yoshimura *et al.*, 1995). SOCS1, the next described family member was identified simultaneously by three separate groups for its inhibition of IL-6 (Starr *et al.*, 1997), its binding to JAK2 in a yeast two-hybrid screen (Endo *et al.*, 1997), and by its homology to the SH2 region of STAT3 (Naka *et al.*, 1997). The remaining family members were identified by their similar structural elements (Hilton *et al.*, 1998; Minamoto *et al.*, 1997; Starr *et al.*, 1997). They are expressed in response to different inducers and function to inhibit specific signaling pathways. The different inducers, pathways, and binding targets are shown in Table 3.1.

The structure of each family member consists of three regions, a variable amino terminal region, a central SH2 domain and a SOCS box at the carboxyl terminus (Fig. 3.1). The SH2 domain binds to phosphorylated tyrosines on subject proteins. While this domain is not responsible for the actual degradation of the subject protein, it is responsible for the specificity of the degradation. Binding of the SH2 domain to its specific target brings the other domains in proximity to the target protein, keeping their initiation of degradation directed at the appropriate protein. The specific targets of the SH2 domains are shown in Table 3.1. The SH2 domain of SOCS1 and SOCS3 contains an extension of the N-terminus called the N-terminal extended SH2 subdomain (N-ESS). Deletion of the N-ESS interrupts the function of SOCS1 and SOCS3, thus while its role is not clear it is known to be necessary for function (Sasaki *et al.*, 2000; Yasukawa *et al.*, 1999). In some family members, the SH2 domain has additional functions; in SOCS1, the SH2 domain binds to a specific phosphorylated tyrosine in the activation loop of JAK kinases (e.g., JAK2) (Yasukawa *et al.*, 1999) that is required for JAK function (Zhou *et al.*, 1997).

The SOCS box is a 40-amino acid motif found not only in the eight SOCS family members but also in more than 50 other proteins. The SOCS box is a three-α-helical structure that functions to target proteins for degradation by recruitment of the components of an E3 ubiquitin ligase that in turn covalently binds ubiquitin to lysines in the target (reviewed in Kile *et al.*, 2002). The N-terminus of the SOCS box contains the B/C box subdomain, also found in Von Hippel-Landau (VHL) tumor suppressor protein (Spruck and Strohmaier, 2002; Zhang *et al.*, 1999). The B/C box

TABLE 3.1 Each SOCS family member is coupled with its best defined inducers and the primary signaling pathways each is known to regulate

	Inducers	Regulates (primary)	SH2 target
CIS	IL-3, EPO, GH	IL-3, EPO, prolactin, GM-CSF	STAT5 recruitment sites on receptors
SOCS1	IFN-α, IFN-γ, IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-13, IL-15, LPS, TNF-α, GH, CpG DNA	IFN-α, IFN-γ, IL-2, IL-3, IL-4, IL-6, IL-7, IL-12, IL-13, IL-15, prolactin, Epo, OSM, TSLP, TNF-α, Tpo, LIF, LPS	Binds the activation loop of JAK kinases, phosphorylated tyrosines on IFN-α and IFN-γ receptors
SOCS2	GH	GH, IGF1	Phosphorylated tyrosines on receptors
SOCS3	IL-1, TGF-β, IL-6, IL-10, GH, LPS, epidermal growth factor (EGF)	IL-1, TGF-β, IL-6, IL-11, IL-12, IL-27, G-CSF, leptin, leukemic-inhibitory factor (LIF), ocostatin-M (OSM) cardiotrophin-1 (CT-1), ciliary neutrophic factor (CNTF) cardiotrophin-like cytokine (CLC)	Phosphorylated tyrosine of gp130-related cytokine receptors
SOCS4	EGF	EGFR	EGFR phosphorylated tyrosine
SOCS5	EGF	EGFR	?
SOCS6	Insulin	P85PI3K, IRS, INSR	IRS
SOCS7	Insulin, IGF	IRS, INSR, IGF	IRS, INSR

The specific target of each SH2 domain is shown as well.

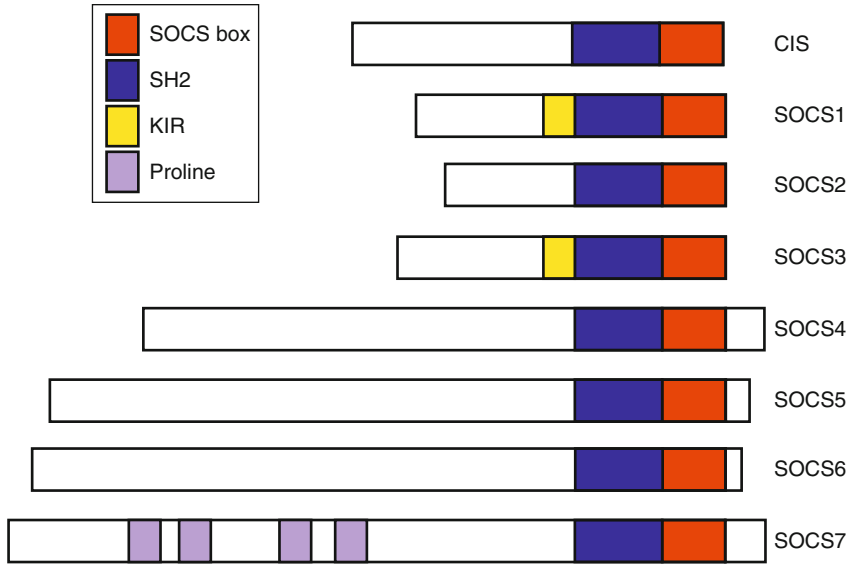

FIGURE 3.1 Structural organization of SOCS family members.

serves to interact with Elongin C, which in turn is bound by Elongin B. Together, the Elongin complex and a Cullin box motif of the SOCS box associate with a Cullin scaffold protein that recruits Rbx1, completing the Elongin C-Cullin-SOCS box (ESC)-type ubiquitin ligase. The carboxy terminus of the SOCS box contains a Cul5 box that directs an association with Cullin 5, in turn recruiting the stabilizing RING finger protein Rbx.

SOCS1 has been shown to be capable of driving the ubiquitination of specific proteins including JAK2 (Ungureanu *et al.*, 2002). Targeted deletion of the SOCS box of SOCS1 results in partial loss of SOCS1 function, supporting its importance in SOCS1 function (Zhang *et al.*, 2001).

The third common domain of the SOCS family of molecules is the N-terminal region. These regions are of variable length and sequence, with SOCS5, SOCS6, and SOCS7 having extended regions, the N-terminus of CIS, SOCS1, SOCS2, and SOCS3 are comparatively truncated while the terminus of SOCS4 is intermediate.

In SOCS1 and SOCS3, the N-terminal region also contains a kinase-inhibitory region (KIR) of 12 amino acids. This region is required for inhibition of JAK kinases, perhaps functioning as a pseudosubstrate via a conserved tyrosine. Removal of this tyrosine does not affect binding of SOCS1 or SOCS3 to JAK but does prevent the inhibition of the kinase (Sasaki *et al.*, 2000). In addition, treatment of JAK2 with a KIR peptide results in inhibition of kinase action (Flowers *et al.*, 2004;

Waiboci *et al.*, 2007). The role of the N-terminus in the other SOCS family members has yet to be elucidated.

Thus, the structural analysis of the SOCS family members reveal they are able to influence signaling by a number of mechanisms. This is accomplished by blocking the binding of signaling molecules to the receptor through competition for binding sites, inhibition of activity of the signaling molecules as well as interruption of signaling by labeling the signaling molecules for degradation (Fig. 3.2).

Crystal structure of SOCS family members using SOCS4 and SOCS2 has allowed increased insight into the function of these molecules. The crystal structures have been analyzed in the context of Elongin B/C binding, which have been shown to be strongly conserved between family members (Bullock *et al.*, 2006, 2007). Crystal structures have also revealed significant differences between the family members, dividing the family

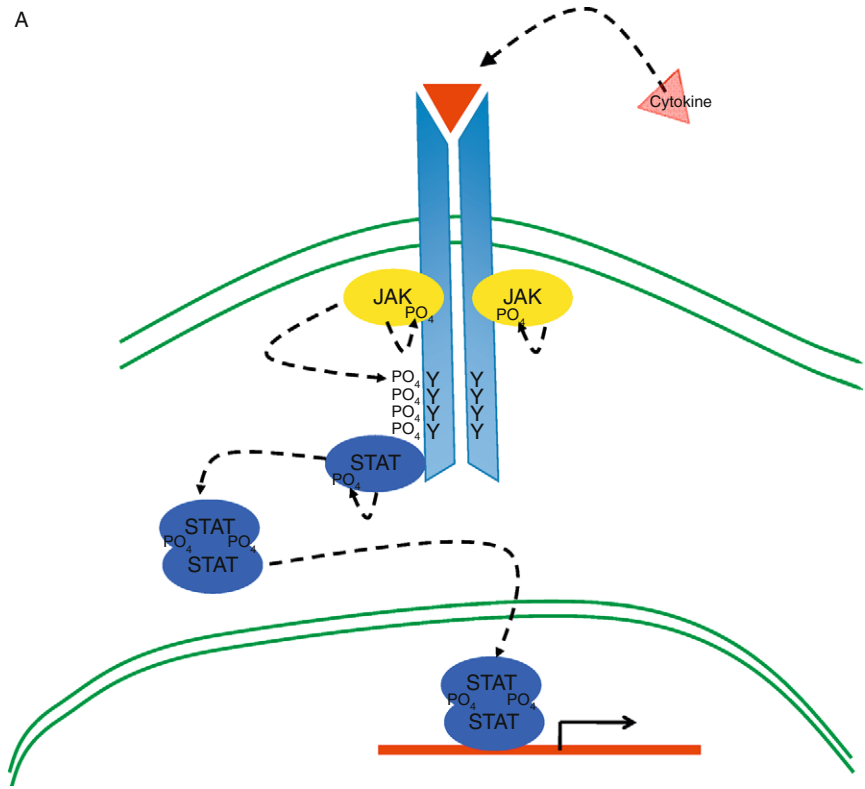

FIGURE 3.2 (Continued)

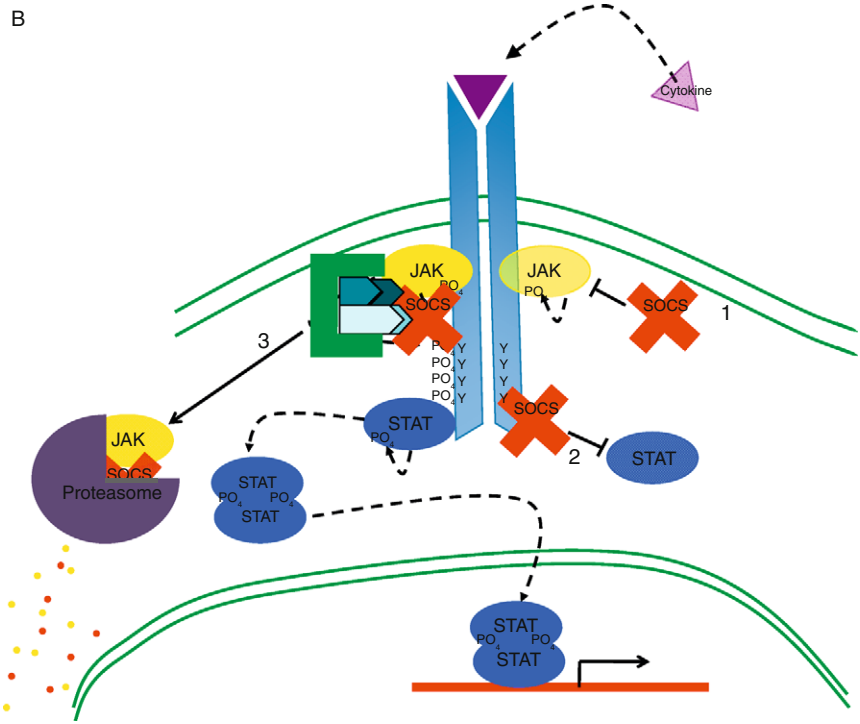

FIGURE 3.2 (A) Cytokine signaling occurs in a multistep process. Binding of the cytokine to the receptor triggers its oligomerization as well as activation of associated JAK kinases. Tyrosines (Y) in the cytoplasmic tail of the receptor are phosphorylated and recruit STAT molecules, in turn phosphorylated by the JAK kinase. STAT molecules dimerize and translocate to the nucleus where they act as transcriptional regulators. (B) SOCS family members can block signaling at several steps. (1) SOCS molecules interfere with JAK activation. (2) SOCS bind to phosphorylated tyrosines, blocking association by STAT molecules. (3) SOCS molecules facilitate ubiquitination and degradation of specific signaling molecules.

into two groups: the first group contains CIS and SOCS1 through SOCS3 and the second group is SOCS4 through SOCS7. In the first group, the short C-terminus is internal and is proposed to stabilize the SH2 and SOCS box domains (Bullock *et al.*, 2006). This organization of the protein leaves the N-terminus, in particular the KIR domains of SOCS1 and SOCS3, exposed and accessible for interactions. In contrast, the crystal structure of SOCS4, representative of SOCS4-7, reveals the extended

C-terminus is not internalized and rather the N-terminal seems to serve the role of stabilizer for SH2 and the SOCS box (Bullock *et al.*, 2007).

3. SOCS GENES

3.1. Evolution

Mammalian SOCS genes exhibit similarities in sequence and function, implying an underlying redundancy. The strongest protein identities are found in pairwise clusters between Socs1/Socs3, Cis/Socs2, Socs4/Socs5, and Socs6/Socs7 (Rawlings *et al.*, 2004) (Fig. 3.3). The sequencing of the *Drosophila* genome revealed three SOCS genes, one homologous to *SOCS4* and *SOCS5* and two homologous to *SOCS6* and *SOCS7*. The lone *SOCS* gene identified in the *Caenorhabditis elegans* genome (CeSOCSa) is similar to *SOCS6* and *SOCS7*. *Drosophila* has a single cytokine receptor, with a homolog of Jak and Stat proteins; in contrast, *C. elegans* lack both a cytokine receptor and a JAK homolog but do have at least one STAT molecule (reviewed in Zeidler *et al.*, 2000).

3.2. SOCS1

The best studied of the family members, SOCS1, also called JAK-binding protein (JAB)-1 or STAT-induced STAT inhibitor (SSI)-1, was simultaneously described by three groups (Endo *et al.*, 1997; Naka *et al.*, 1997; Starr *et al.*, 1997). Its structure contains the conserved SOCS box and SH2 domain, but in addition has a KIR domain also critical for its function (Fig. 3.1). The importance of the SH2 domain for function has been examined in SOCS1 via mutational analysis, demonstrating that both the SH2 domain and the 24 amino acids N-terminal to the SH2 domain are required for suppression of cytokine signaling (Narazaki *et al.*, 1998).

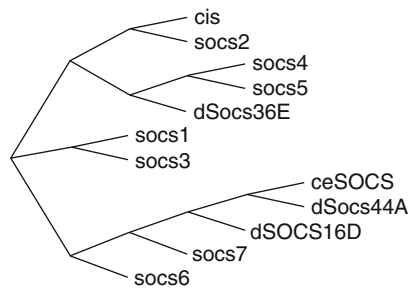

FIGURE 3.3 Protein sequence comparison of *Drosophila* and mouse SOCS. Based on the protein alignments, the neighbor-joining method was used to construct a phylogenetic tree of SOCS proteins.

SOCS1 expression is induced by many cytokines (Table 3.1), most notably IFN-γ (Starr *et al.*, 1997). In overexpression studies, SOCS1 has been shown to interact with all four JAK kinases through its SH2 domain as well as to inhibit their tyrosine kinase activity *in vitro* (Endo *et al.*, 1997; Naka *et al.*, 1997; Yasukawa *et al.*, 1999). SOCS1 overexpression in cell lines inhibits STAT activation by multiple cytokines (Endo *et al.*, 1997; Hansen *et al.*, 1999; Isaksen *et al.*, 1999; Losman *et al.*, 1999; Pezet *et al.*, 1999; Song and Shuai, 1998; Starr *et al.*, 1997). Further overexpression studies have shown that SOCS1 can also inhibit the activity of tyrosine kinase Tec and the adaptor protein Vav (Yasukawa *et al.*, 2000). Specific overexpression of SOCS1 in T cells results in impaired T cell development, suggesting a role for SOCS1 in the normal generation of thymocytes (Fujimoto *et al.*, 2000).

3.2.1. Roles of SOCS1 domains

The precise function of the SOCS box in SOCS1 remains unclear, but studies suggest this domain may play an important role in signal transduction. Deletion of the SOCS box was first reported to result in significantly lower expression of SOCS1 in M1 cells (Narazaki *et al.*, 1998). Expression levels of the SOCS1 mutant were restored by treatment with proteasome inhibitors (Narazaki *et al.*, 1998). A subsequent study found that proteasome-dependent degradation of SOCS1 is blocked by interaction with the Elongin B/C complex (Kamura *et al.*, 1998). Together, these studies suggest a role for the SOCS box in stabilizing SOCS1. However, in a third study the interaction of the SOCS box of SOCS1 with Elongin B/C was observed to accelerate SOCS1 degradation (Zhang *et al.*, 1999). Consistent with this observation, a recent report suggests that phosphorylation of SOCS1 by Pim kinases stabilizes SOCS1 protein by decreasing interaction with Elongin B/C (Chen *et al.*, 2002). Considered together, these findings have led to the model in which signaling proteins that become associated with the SOCS box of SOCS1 are ubiquitinated and thereby targeted for proteasome-mediated degradation.

The KIR domain of SOCS1 (also found in SOCS3) is a 12-amino acid region between the variable amino region and the SH2 domain (Fig. 3.1) that serves to inhibit JAK kinase ability. It may do so by binding the catalytic cleft of the kinase and thus preventing access for the true substrate. This was suggested by point mutational studies disrupting the SOCS1 KIR that showed loss of JAK inhibition despite preservation of binding through the SH2 domain (Sasaki *et al.*, 1999; Yasukawa *et al.*, 1999).

3.2.2. Knockout mouse studies

While *in vitro* studies have provided insights regarding SOCS1 function, the generation of SOCS1-deficient mice has helped clarify its true physiologic role. Although normal at birth, SOCS1$^{-/-}$ display stunted

growth with a multiorgan disease that is characterized by lymphopenia, fatty degeneration of the liver and macrophage infiltration of various tissues followed by death before 3 weeks of age is reached (Alexander *et al.*, 1999; Marine *et al.*, 1999b; Starr *et al.*, 1998; Yasukawa *et al.*, 2000). These defects resemble those seen in wild-type mice given IFN-γ as neonates (Gresser *et al.*, 1981). Interestingly, macrophages derived from SOCS1$^{-/-}$ bone marrow are hyperresponsive to IFN-γ; furthermore, the injection of neutralizing anti-IFN-γ antibody twice weekly from birth can prevent disease in SOCS1$^{-/-}$ mice, thus demonstrating a requirement for IFN-γ in the observed perinatal lethality (Alexander *et al.*, 1999). Consistent with this observation, mice that are doubly deficient for SOCS1 and IFN-γ do not suffer perinatal lethality, further implicating excessive IFN-γ signaling in the disease observed in SOCS1$^{-/-}$ animals (Alexander *et al.*, 1999).

The lethality in SOCS1$^{-/-}$ mice is also significantly delayed in the RAG2$^{-/-}$, STAT1$^{-/-}$, and STAT6$^{-/-}$ backgrounds, thus implicating SOCS1 as a critical regulator of both the IFN-γ and IL-4 signaling pathways (Alexander and Hilton, 2004). Many of the phenotypes associated with SOCS1 deficiency can be reconstituted by transferring SOCS1$^{-/-}$ bone marrow into JAK3$^{-/-}$ mice, suggesting that the pathology observed is mediated by hematopoietic cells (Cornish *et al.*, 2003a,b). However, specific deletion of SOCS1 in the thymocyte/T/NKT cell compartment is not sufficient to induce the lethal multiorgan disease, although it does cause abnormalities that include increased numbers of CD8^{+} T cells and increased sensitivity to common γ-chain cytokines (Naka *et al.*, 2001). Consistent with the observation that specific deletion of SOCS1 in the thymocyte/T/NKT cell compartment is not sufficient to cause the disease, SOCS1$^{-/-}$ DCs demonstrate increased IFN-γ- and IL-4-induced responses, suggesting an important role for SOCS1 in nonlymphoid cell function. Furthermore, altered transcriptional activity of SOCS1 may have important ramifications for the cytokine unresponsiveness demonstrated by many tumors, as it has been shown to be silenced by CpG methylation in hepatocellular carcinoma and in multiple myeloma (Depil *et al.*, 2003; Galm *et al.*, 2003; Yoshikawa *et al.*, 2001). In addition, the ability of SOCS1 to inhibit signaling is reduced by the ABL oncogene (Chen *et al.*, 2008).

As described earlier, the SOCS box appears to be important for recruitment of the ubiquitin transferase apparatus that targets SOCS1-interacting proteins for proteosomal-mediated degradation. Zhang *et al.* (2001) generated mice in which sequences in the SOCS1 gene that encode the SOCS box were deleted. Studies using these mice demonstrated the *in vivo* importance of the SOCS box for inhibition of IFN-γ signaling by SOCS1 (Zhang *et al.*, 2001). The SOCS1-SOCS box deletion mutant retains the ability to bind JAK1, thus explaining why the phenotype of these mice

is not as severe as that of SOCS1-deficient mice: the onset of disease is later and its severity decreased in mice lacking the SOCS1-SOCS box relative to mice lacking the entire SOCS1 protein. Nevertheless, like SOCS1-deficient mice, SOCS box deletion mutant mice die prematurely and suffer from reduced body weight (Zhang *et al.*, 2001). Inflammatory lesions are observed in skeletal and heart muscle, cornea, pancreas, and dermis in these mice. On a molecular level, IFN-γ-mediated STAT1 activation in the livers of mice lacking the SOCS1-SOCS box is prolonged. This phenotype is intermediate to that observed in wild-type or SOCS1-deficient mice. Interestingly, the SOCS box deletion mutant protein is expressed at much lower levels than the wild-type SOCS1 protein in heterozygote mice, supporting a role for the SOCS box, and, perhaps its interaction with the Elongin B/C complex in SOCS1 protein stabilization. It has recently been shown that the von Hippel-Lindau protein is protected from proteasomal degradation by its association with the Elongin B/C complex and proteasomal degradation (Schoenfeld *et al.*, 2000).

3.2.3. SOCS1 in innate immunity

SOCS1 has been associated with regulation of immune responses that occur in the innate immune system as well. The toll-like receptors (TLRs), the best characterized family of PRRs, bind PAMPs associated with infectious invasion. TLR4 binds lipopolysaccharide (LPS) found on the surface of Gram-negative bacteria and TLR9 binds CpG DNA found in bacterial infections. These molecules signal through intracellular adaptors; for most TLRs, this is the molecule myeloid differentiation factor 88 (MyD88). TLR4 signals through MyD88 as well as TIR domain-containing protein inducing IFN-β (TRIF), which TLR3 uses exclusively. The association of TLR2 and TLR4 with MyD88 requires a secondary adaptor, MyD88-adaptor-like protein (Mal). MyD88 signaling activates interleukin-1 receptor-associated kinase (IRAK), which in turn lead to activation of the MAP kinase pathway as well activation of nuclear factor κB (NFκB). TRIF signaling activates interferon regulatory factor (IRF)-3 and results in IFN-β production (reviewed in O'Neill, 2006). SOCS1 is induced by these pathways and also regulates them. SOCS1 is induced in cells following treatment with LPS (Stoiber *et al.*, 1999) and similarly, SOCS1 (as well as SOCS3) is expressed following TLR3 activation by CpG DNA (Dalpke *et al.*, 2001). Given the known induction of SOCS1 by inflammatory cytokines, it was considered this induction might not be directly related to TLR engagement but secondary to the inflammation generated by TLR activation. However, the direct association of SOCS1 expression and TLR signaling was supported by a number of studies, including a transwell experiment showing soluble factors were not solely responsible for the effects observed (Baetz *et al.*, 2004).

The inhibition of TLR signaling by SOCS1 was described in studies of LPS response in SOCS1$^{-/-}$ mice that found they were profoundly sensitive to LPS, showing increased lethality and generating elevated amounts of the proinflammatory cytokines TNF-α and IL-12 (Kinjyo *et al.*, 2002; Nakagawa *et al.*, 2002). Studies defining the mechanism of this inhibition have shown a number of ways by which SOCS1 downregulates the inflammatory response to TLR activation, including direct interference with the signaling cascade and blockade of the signaling by cytokines generated in response to TLR stimulation.

The first step in the TLR signaling pathway proposed to be targeted by SOCS1 is the adaptor Mal. Mal is phosphorylated by Brutons tyrosine kinase (BTK) after TLR stimulation, allowing binding by the SH2 domain of SOCS1. It is believed this binding makes Mal accessible to the SOCS box of SOCS1, as the association of SOCS1 with Mal results in its ubiquitination and subsequent degradation (Mansell *et al.*, 2006), blocking the progression of the TLR signal to MyD88.

The protein IRAK that serves to bridge the activation signal from MyD88 to NFκB is also a potential target of SOCS1. SOCS1 has been shown to bind IRAK through its SH2 domain in cotransfection studies (Nakagawa *et al.*, 2002) and thus may do so *in vivo* as well. Binding to the SH2 domain brings IRAK into range of the SOCS box and could result in enhanced ubiquitination and proteasomal degradation. This blockade of signaling beyond IRAK prevents activation of NFκB, effectively halting the generation of inflammatory cytokines.

The final known interference of SOCS1 with TLR signaling occurs at the level of NFκB activation. If the TLR signal proceeds through IRAK it leads to removal of the inhibitory IκB from the p50 and p65 subunits of NFκB, generating active NFκB which translocates to the nucleus and drives the expression of proinflammatory cytokines. SOCS1 has been shown to bind *in vitro* to the p65 subunit, leading to its polyubiquitination and degradation by the p65 proteasome (Ryo *et al.*, 2003). Thus, SOCS1 directly negatively regulates TLR signaling at multiple steps in the pathway.

The TRIF pathway is also potentially a target of SOCS1, but indirectly via the blockade of the response to the inflammatory cytokines produced. TRIF stimulation culminates in release of type I interferons which act on the same cell as well as neighboring cells via the IFN-α/-β receptor. This activates STAT1 and results in upregulation of costimulatory markers such as CD40 and release of numerous proinflammatory molecules. In studies overexpressing SOCS1, this activation of STAT1 is inhibited as shown by impaired phosphorylation (Nakagawa *et al.*, 2002). In addition, there is inhibition of the upregulation of CD40 on the surface of macrophages, further supporting blockade of the STAT1 pathway (Baetz *et al.*, 2004; Qin *et al.*, 2006).

3.2.4. SOCS1 in adaptive immune responses

The generation of the adaptive immune response is dependent on specific cytokines driving the differentiation of naïve T cells into the varied subsets of effector T cells. As noted earlier, IL-4 binding its IL-4 receptor on CD4 cells is critical to the generation of a Th2 response, much as IL-12 release by innate immune cells skews a CD4 cell to a Th1 response. Presence of the Th1 cytokine IFN-γ inhibits differentiation to Th2 cells while IL-4 likewise blocks Th1 development. SOCS1 has been shown to regulate all these cytokines, thus its ability to block generation of these cytokines as well as to block their functions is critical in determining the type of CD4 response that is generated. The role of SOCS1 in adaptive immune responses has been studied using *in vivo* infection models. $Socs1^{+/+}$ and $Socs1^{+/-}$ mice were infected with the Th1 skewing bacteria *Listeria monocytogenes* or the parasite *Nippostrongylus braziliensis* that drives the adaptive immune system to a Th2 response. Upon restimulation, CD4 cells from $Socs1^{+/-}$ mice infected with *L. monocytogenes* produced significantly higher levels of IFN-γ than CD4 cells from similarly treated $Socs^{+/+}$ mice, while IL-4 from $Socs^{+/-}$ mice after infection with *N. braziliensis* was significantly elevated in comparison to $Socs^{+/+}$ mice (Fujimoto *et al.*, 2002). This suggests that the adaptive immune response, whether Th1 or Th2, is enhanced if triggered in the context of lower levels of SOCS1. The application of this finding to human atopic disease is intriguing; an environmental trigger generating low levels of IL-4 in an individual with normal SOCS1 may be insignificant. However, if that individual lacked full SOCS1 function the IL-4 response may be less controlled, perhaps predisposing to atopic disease.

The association of the function of SOCS molecules in the allergic response has been supported by human studies analyzing the association of polymorphisms in SOCS genes with allergic disease in people. Specifically, an association of a promoter polymorphism in SOCS1 has been linked to adult asthma. The linked polymorphism leads to a promoter with modified activity *in vitro*, suggesting changes in expression of SOCS1 can have appreciable effects on disease manifestations in patients (Harada *et al.*, 2007).

3.3. CIS

CIS was the first family member described. It was identified as induced by IL-3 and found, via overexpression studies, to inhibit the response to IL-3. The CIS SH2 domain is similar to the SH2 domain of STAT5 and thus it may act by binding the tyrosine phosphorylated cytoplasmic tail of the activated cytokine receptor, competitively inhibiting the binding by STAT5 (Yoshimura *et al.*, 1995). This is supported by studies

overexpressing CIS in mice that recreate a phenotype quite similar to STAT5-deficient mice with defects in natural killer, natural killer T cell and T cell development. CD4 cells from mice overexpressing CIS preferentially differentiated into Th2 cells, suggesting a role for CIS in regulating adaptive immune responses (Matsumoto *et al.*, 1999). Intriguingly, mice lacking CIS do not display any overt abnormalities, making the analysis of CIS as a critical regulator less straightforward (Marine *et al.*, 1999a).

3.4. SOCS2

SOCS2 shares structural homology with CIS and like CIS (as well as SOCS1 and SOCS3) is induced by growth hormone (GH). In overexpression studies, all GH-induced SOCS family members are capable of binding the GH receptor, but only SOCS2 seems to exert a regulatory effect on GH signaling *in vivo* (Greenhalgh *et al.*, 2002). This is supported by knockout studies as mice lacking SOCS2 develop accelerated growth with gigantism, elevated levels of insulin-like growth factor (IGF)-1 mRNA which is upregulated by GH and enhanced responses to exogenous GH (Greenhalgh *et al.*, 2005; Metcalf *et al.*, 2000). Enhanced GH responses in mice lacking SOCS2 suggest the normal function of SOCS2 is to curtail GH response. The contradictory finding that SOCS2 overexpressing mice do not have impaired GH responses was unexpected and makes the role of SOCS2 in regulation of GH less clear (Greenhalgh CJ 2002 JBC). There have been no studies demonstrating a role for SOCS2 in the regulation of atopic immune responses.

3.5. SOCS3

SOCS3 has similarity in structure and function to SOCS1, and contains a comparable KIR domain (Fig. 3.1). Additionally, the SH2 domain of SOCS3 contains a 35-amino acid insertion with proline, glutamate, serine, and threonine, or a PEST sequence. PEST sequences are believed to mark proteins for accelerated proteolysis and thus deletion of the PEST insertion of SOCS3 stabilizes the protein (Babon *et al.*, 2006; Rogers *et al.*, 1986). The SH2 domain of SOCS3 binds specifically to phosphorylated tyrosines in gp130, the common signaling subunit of the IL-6 receptor family (Table 3.1).

Studies of SOCS3 have been hampered by the fact that SOCS3-deficient embryos die by gestational day 16 due to anomalies in placental and embryonic vessel formation (Roberts *et al.*, 2001). Generation of SOCS3-deficient embryos with SOCS3 sufficient placental tissues resulted in the live birth of SOCS3-deficient mice, but all succumbed by 25 days with cardiac defects unless the mice were also deficient in leukemia-inhibitory

factor (LIF) (Robb *et al.*, 2005; Takahashi *et al.*, 2003). More recent studies using tissue-specific knockout mice have greatly assisted our understanding of the functions of SOCS3 *in vivo*.

Similar to SOCS1, SOCS3 has a role in regulating the immune response to LPS. The mechanism of action of the two family members differs as SOCS1 binds activated JAK kinases and inhibits them directly, SOCS3 acts by binding the activated cytokine receptor and indirectly inhibiting JAK kinases (Nicholson *et al.*, 2000). This mechanistic difference may explain how these proteins affect different components of signaling pathways. For example, SOCS1 primarily regulates the signaling cascade for LPS and TLR4, SOCS3 regulates the cytokines generated by the LPS response (Table 3.1). The regulation by SOCS3 has been studied in detail for IL-10. IL-10 acts to inhibit the inflammatory response to LPS; its importance is shown by the exuberant inflammatory response to LPS seen in mice lacking IL-10 (Rennick *et al.*, 1997). While it had been known a portion of the anti-inflammatory effect seen with IL-10 was due to inhibition of the production of inflammatory cytokines, the mechanism by which this occurred was not. It has now been suggested SOCS3 mediates this effect as overexpression of SOCS3 in macrophages blocked the production of TNF-α, GM-CSF, and IL-6 in macrophages in response to LPS at a level similar to that seen in normal cells treated with LPS and IL-10 (Berlato *et al.*, 2002).

An intriguing study by Yasukawa *et al.* suggests the inhibitory IL-10 effect is due to the sustained duration of STAT3 activation rather than an IL-10-specific effect. They postulate STAT3 activation in response to signaling by the IL-10 receptor is prolonged in duration, in contrast to only brief and transient STAT3 activation following IL-6 binding the IL-6 receptor. The IL-6 receptor contains a gp130 site, absent in the IL-10 receptor and allowing the IL-6 receptor to be bound and inhibited by SOCS3, resulting in a pulse of STAT3 activation. Without gp130, SOCS3 does not bind the IL-10 receptor and thus STAT3 activation following IL-10 stimulation is not downregulated and is sustained, resulting in the inhibition ascribed to IL-10 (Yasukawa *et al.*, 2003).

3.5.1. SOCS3 in dendritic cells

DCs are key to transmitting the initial innate response to the adaptive system. They perform this role by presenting peptides in class II MHC molecules to naïve CD4 T cells. The associated expression of costimulatory markers and release of specific cytokines results in differentiation of the CD4 cell into distinct lineages of effector cells. The specific class of resultant effector, Th1, Th2, Th17, or Treg, is dependent on precise regulation of the cytokines and costimulatory molecules; modification in these factors results in a profoundly variant adaptive immune response.

SOCS3 has an intriguing role in the regulation of the DC. DCs with constitutively expressed SOCS3 have reduced expression of MHC class II molecules as well as impaired expression of costimulatory molecules. These DCs drive CD4 cells to become Th2 effectors that in turn are capable of suppressing the Th17-dependent mouse model of multiple sclerosis, experimental autoimmune encephalitis (EAE) (Li *et al.*, 2006). Additional studies of the role of SOCS3 in DCs have used SOCS3-deficient cells. Interestingly, these cells drive the generation of regulatory T cells, suggesting that the regulation by SOCS3 in DCs has a critical role in determining whether the adaptive response is inflammatory or tolerogenic (Matsumura *et al.*, 2007).

3.5.2. SOCS3 in adaptive immunity

While it is apparent the expression of SOCS3 in DCs drives the differentiation of Th2 cells, it has been suggested SOCS3 expression is important in CD4 cells. Analysis of *in vitro* generated Th2 and Th1 cells revealed elevated SOCS3 mRNA in the Th2 cells. To assess if this applies in humans, $CD3^+$ cells were isolated from peripheral blood of patients with or without asthma and were analyzed for SOCS mRNA. Asthmatics had higher levels of SOCS3 than nonasthmatics and more severely affected asthmatics had higher SOCS3 than those patients with milder asthma. A causative role for SOCS3 in T cells was supported by the overexpression of SOCS3 in naïve CD4 cells, which upon stimulation preferentially produced IL-4 (Seki *et al.*, 2003). However, generation of mice with selective loss of SOCS3 in T cells casts doubt on the requirement for SOCS3 in T cells, as these deficient T cells are fully capable of normal differentiation into Th2 cells (Chen *et al.*, 2006). Further investigation is needed to determine the true role of SOCS3 in T cells.

3.5.3. SOCS3 in cancer

Modified SOCS3 expression has been associated with carcinogenesis in a number of studies. For example, samples of squamous cell cancer of the head and neck have been found in the vast majority of cases to have epigenetic silencing of the *Socs3* gene (Niwa *et al.*, 2005; Weber *et al.*, 2005). Mice generated with a specific deletion of *Socs3* in hepatocytes have an increased susceptibility to induction to hepatocellular carcinoma (Riehle *et al.*, 2008). Numerous additional studies, though, suggest an opposite role for SOCS3 in the regulation of cancer. SOCS3 has been found to be constitutively expressed in infiltrating ductal breast carcinomas (Raccurt *et al.*, 2003) and stabilized in certain myeloproliferative disorders (Hookham *et al.*, 2007). These authors offer a possible explanation for this dichotomy; they note SOCS3, while stabilized, is also hyperphosphorylated. They suggest the increased phosphorylation, while prolonging SOCS3's half-life, also interferes with its regulatory ability. Thus, despite

increased levels of SOCS3 it is postulated there is in fact impaired SOCS3 function. While this theory awaits validation, it is an attractive explanation for seemingly discordant data.

3.6. SOCS4

SOCS4 was first identified through a database search for SOCS box-containing proteins (Hilton *et al.*, 1998). The structure of SOCS4 is quite homologous to SOCS5, particularly at their SH2 domains, suggesting similarities in binding targets. SOCS4 and SOCS5 are orthologs of *Drosophila* SOCS36E, which in overexpression studies suggest impair the function of the epidermal growth factor receptor (EGFR) (Callus and Mathey-Prevot, 2002; Rawlings *et al.*, 2004). Thus, SOCS4 was proposed as a regulator of EGFR signaling. This was supported by the induction of SOCS4 in response to EGFR signals. The exact mechanism by which SOCS4 could bind and thus regulate EGFR was unclear until additional structural information was gained. Solving the crystal structure revealed novel conformation of the SOCS box with a newly recognized binding face which was shown to bind specifically to phosphorylated tyrosine of EGFR, signaling its degradation (Bullock *et al.*, 2007; Kario *et al.*, 2005). The role of SOCS4 *in vivo*, or in immune responses, is not known.

3.7. SOCS5

SOCS5 is also expressed following EGFR signaling and, like SOCS4, has also been shown to facilitate the degradation of the EGFR. Unlike SOCS4, the interaction by SOCS5 seems to be independent of phosphorylation state of the receptor (Kario *et al.*, 2005; Nicholson *et al.*, 2005).

SOCS5 has been studied more extensively than SOCS4 and may have a role in adaptive immunity, as it has differential expression in T cells, with a higher level in Th1 cells in comparison to Th2 cells. In addition, studies of Th1 cells have shown SOCS5 is capable of binding to IL-4R and suppressing STAT6 phosphorylation, potentially inhibiting development along the Th2 pathway (Seki *et al.*, 2002). Overexpression studies with elevated SOCS5 specifically within T cells showed an enhanced Th1 response to a number of stimuli (Ozaki *et al.*, 2005; Seki *et al.*, 2002; Watanabe *et al.*, 2006). In contrast, mice overexpressing SOCS5 had enhanced Th2 responses to inhalant antigens (Ohshima *et al.*, 2007). Further confounding results are seen in mice deficient in SOCS5 that appear to have normal T cell development and differentiation to both Th1 and Th2 cells, which casts into doubt a critical role of SOCS5 for the development and function of CD4 T cells (Brender *et al.*, 2004; Seki *et al.*, 2002).

Recently, a role for SOCS4 and SOCS5 in pathogenesis of cancer has been postulated. Analyzed human hepatocarcinoma samples were more

likely to have inactivation of SOCS4 and SOCS5 than normal samples. In addition, the inactivation of either gene was associated with a worse prognosis (Calvisi *et al.*, 2007).

3.8. SOCS6

SOCS6 and SOCS7 have the highest level of overall amino acid homology of all the family members, with greater than 50% identity for both their SOCS boxes as well as SH2 domains. Thus, it is not surprising that at least some of their functions appear related; both have been implicated in regulation of the insulin receptor (INSR). *In vitro* analysis of SOCS6 shows it is capable of binding to INSR and the INSR substrate protein (IRS)-4 as well as inhibiting IRS-1. It also interacts with the p85 subunit of phosphoinositol-3 (PI3) kinase following exposure to insulin. Overexpression of SOCS6 results in inhibited insulin signaling and improvement in glucose tolerance, which is similar to the p85-deficient mouse that has normalized glucose metabolism despite impaired PI3 kinase function (Terauchi *et al.*, 1999). However, mice deficient in SOCS6 had no significant anomalies of insulin signaling, although demonstrated slight growth defects compared to wild-type controls (Krebs *et al.*, 2002; Li *et al.*, 2004; Mooney *et al.*, 2001). This suggested that SOCS6 may actually facilitate cell growth. A role for regulating immune responses has not been delineated for SOCS6.

3.9. SOCS7

SOCS7 is the largest human SOCS protein with 581 amino acids. It consists of a central SH2 domain, the common carboxyl SOCS box that contains a carboxyl terminal extension, as well as the longest amino terminus (Fig. 3.1). SOCS7 is unique among the SOCS proteins in that it contains multiple poly-proline regions that may allow association with SH3 domain-containing proteins. SOCS7-interacting molecules were identified both by mass spectrometry and in two independent yeast two-hybrid screens. Both two-hybrid studies found interactions with the adaptor molecule Nck and a single-hit identification of the signaling molecules Grb-2 and STAT-3, the tyrosine kinases EGFR, Lck, Hck, the lipase PLC-γ, and the multiple SH3-domain-containing protein vinexin (Martens *et al.*, 2004; Matuoka et al., 1997). In addition, using mass spectrometry a GST-fusion protein of the SOCS7 SH2 domain was found to associate with IRS4 and the regulatory subunit of PI3-kinase ($p85^{PI3K}$) (Krebs *et al.*, 2002). Several of these SOCS7-interacting proteins were also reported to bind other SOCS molecules. For example, Nck binds SOCS1 and SOCS3 through its SH3 and SH2 domains, respectively

(De Sepulveda *et al.*, 1999; Sitko *et al.*, 2004). SOCS1 can also bind to Grb2, $p85^{PI3K}$, and the EGFR (Xia *et al.*, 2002). Many of these proteins act in insulin or cytokine signaling pathways, thereby suggesting an interaction between SOCS7 and the insulin-signaling cascade.

A regulatory role for SOCS7 in insulin signaling is supported by knockout studies. SOCS7-deficient mice develop hypoglycemia and have enhanced glucose metabolism. The INSR is normally tightly regulated by multiple IRS proteins; loss of these regulators is associated with insulin resistance and the disease noninsulin-dependent diabetes mellitus (NIDDM). On a C57Bl6 background, loss of SOCS7 results in hydrocephalus and neonatal death (REF Hilton PNSA and Rothman JCI). However, in a mixed 129/C57Bl6 background, the mice survive and reproduce normally. In these mice lacking SOCS7, IRS levels are elevated and insulin action is enhanced (Banks AS JCI 2005 115:2462-71). Validation of the role of SOCS7 in glucose metabolism via overexpression studies will cast light on the relevance of SOCS7 levels to NIDDM.

A seemingly separate role for SOCS7 has been proposed in the regulation of allergic inflammatory disease. SOCS7-deficient mice have a propensity toward spontaneous development of cutaneous disease with infiltration of degranulated mast cells. *In vitro*, mast cells from these mice release elevated amounts of IL-6, TNF-α, and IL-13 upon IgE receptor ligation (Knisz *et al.*, 2009). Interestingly, these mast cells also show elevated mRNA expression for thymic stromal lymphopoietin (TSLP), the expression of which has been previously described as a critical switch for the initiation of allergic inflammation (Liu, 2006). These studies suggest SOCS7 serves to dampen the development of allergic disease.

4. CONCLUSION

The SOCS family of molecules plays a critical and divergent role in regulating immune responses as well as metabolic pathways. While they utilize a common paradigm to bind and inhibit signaling molecules their individual binding requirements result in fine specificity and, in some cases, limited redundancy. Further studies using mice that lack more than one SOCS gene may be important in further defining potential roles and the possible redundant roles these proteins play. In addition, conflicting results between studies relying on genetic deletion versus those utilizing overexpression of these molecules need to be resolved in order to understand how these proteins regulate the immune system and metabolic pathways. The clear applicability of these molecules in therapeutic regulation of the immune response remains to be explored.

REFERENCES

Alexander, W. S., and Hilton, D. J. (2004). The role of suppressors of cytokine signaling (SOCS) proteins in regulation of the immune response. *Annu. Rev. Immunol.* **22,** 503–529.

Alexander, W. S., Starr, R., Fenner, J. E., Scott, C. L., Handman, E., Sprigg, N. S., Corbin, J. E., Cornish, A. L., Darwiche, R., Owczarek, C. M., Kay, T. W., Nicola, N. A., *et al*. (1999). SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* **98**(5), 597–608.

Babon, J. J., McManus, E. J., Yao, S., DeSouza, D. P., Mielke, L. A., Sprigg, N. S., Willson, T. A., Hilton, D. J., Nicola, N. A., Baca, M., Nicholson, S. E., and Norton, R. S. (2006). The structure of SOCS3 reveals the basis of the extended SH2 domain function and identifies an unstructured insertion that regulates stability. *Mol. Cell* **22**(2), 205–216.

Baetz, A., Frey, M., Heeg, K., and Dalpke, A. H. (2004). Suppressor of cytokine signaling (SOCS) proteins indirectly regulate toll-like receptor signaling in innate immune cells. *J. Biol. Chem.* **279**(52), 54708–54715.

Berlato, C., Cassatella, M. A., Kinjyo, I., Gatto, L., Yoshimura, A., and Bazzoni, F. (2002). Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. *J. Immunol.* **168**(12), 6404–6411.

Bloom, B., and Dey, A. N. (2006). Summary health statistics for U.S. children: National Health Interview Survey, 2004. *Vital Health Stat.* **10**(227), 1–85.

Brender, C., Columbus, R., Metcalf, D., Handman, E., Starr, R., Huntington, N., Tarlinton, D., Odum, N., Nicholson, S. E., Nicola, N. A., Hilton, D. J., and Alexander, W. S. (2004). SOCS5 is expressed in primary B and T lymphoid cells but is dispensable for lymphocyte production and function. *Mol. Cell. Biol.* **24**(13), 6094–6103.

Bullock, A. N., Debreczeni, J. E., Edwards, A. M., Sundstrom, M., and Knapp, S. (2006). Crystal structure of the SOCS2-elongin C-elongin B complex defines a prototypical SOCS box ubiquitin ligase. *Proc. Natl. Acad. Sci. USA* **103**(20), 7637–7642.

Bullock, A. N., Rodriguez, M. C., Debreczeni, J. E., Songyang, Z., and Knapp, S. (2007). Structure of the SOCS4-ElonginB/C complex reveals a distinct SOCS box interface and the molecular basis for SOCS-dependent EGFR degradation. *Structure* **15**(11), 1493–1504.

Callus, B. A., and Mathey-Prevot, B. (2002). SOCS36E, a novel *Drosophila* SOCS protein, suppresses JAK/STAT and EGF-R signalling in the imaginal wing disc. *Oncogene* **21**(31), 4812–4821.

Calvisi, D. F., Ladu, S., Orden, A., Farina, M., Lee, J. S., Conner, E. A., Schroeder, I., Factor, V. M., and Thorgeirsson, S. S. (2007). Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. *J. Clin. Invest.* **117**(9), 2713–2722.

Chen, X. P., Losman, J. A., Cowan, S., Donahue, E., Fay, S., Vuong, B. Q., Nawijn, M. C., Capece, D., Cohan, V. L., and Rothman, P. (2002). Pim serine/threonine kinases regulate the stability of Socs-1 protein. *Proc. Natl. Acad. Sci. USA* **99**(4), 2175–2180.

Chen, Z., Laurence, A., Kanno, Y., Pacher-Zavisin, M., Zhu, B. M., Tato, C., Yoshimura, A., Hennighausen, L., and O'Shea, J. J. (2006). Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc. Natl. Acad. Sci. USA* **103**(21), 8137–8142.

Chen, J. L., Limnander, A., and Rothman, P. B. (2008). Pim-1 and Pim-2 kinases are required for efficient pre-B-cell transformation by v-Abl oncogene. *Blood* **111**(3), 1677–1685.

Coffman, R. L., Ohara, J., Bond, M. W., Carty, J., Zlotnik, A., and Paul, W. E. (1986). B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* **136**(12), 4538–4541.

Cornish, A. L., Chong, M. M., Davey, G. M., Darwiche, R., Nicola, N. A., Hilton, D. J., Kay, T. W., Starr, R., and Alexander, W. S. (2003). Suppressor of cytokine signaling-1 regulates signaling in response to interleukin-2 and other gamma c-dependent cytokines in peripheral T cells. *J. Biol. Chem.* **278**(25), 22755–22761.

Cornish, A. L., Davey, G. M., Metcalf, D., Purton, J. F., Corbin, J. E., Greenhalgh, C. J., Darwiche, R., Wu, L., Nicola, N. A., Godfrey, D. I., Heath, W. R., Hilton, D. J., *et al*. (2003). Suppressor of cytokine signaling-1 has IFN-gamma-independent actions in T cell homeostasis. *J. Immunol.* **170**(2), 878–886.

Corrigan, C. J., and Kay, A. B. (1992). T cells and eosinophils in the pathogenesis of asthma. *Immunol. Today* **13**(12), 501–507.

Dalpke, A. H., Opper, S., Zimmermann, S., and Heeg, K. (2001). Suppressors of cytokine signaling (SOCS)-1 and SOCS-3 are induced by CpG-DNA and modulate cytokine responses in APCs. *J. Immunol.* **166**(12), 7082–7089.

De Sepulveda, P., Okkenhaug, K., Rose, J. L., Hawley, R. G., Dubreuil, P., and Rottapel, R. (1999). Socs1 binds to multiple signalling proteins and suppresses steel factor-dependent proliferation. *EMBO J.* **18**(4), 904–915.

Depil, S., Saudemont, A., and Quesnel, B. (2003). SOCS-1 gene methylation is frequent but does not appear to have prognostic value in patients with multiple myeloma. *Leukemia* **17**(8), 1678–1679.

Dougherty, R. H., and Fahy, J. V. (2009). Acute exacerbations of asthma: Epidemiology, biology and the exacerbation-prone phenotype. *Clin. Exp. Allergy* **39**(2), 193–202.

Eder, W., Ege, M. J., and von Mutius, E. (2006). The asthma epidemic. *N. Engl. J. Med.* **355**(21), 2226–2235.

Eisenbarth, S. C., Cassel, S., and Bottomly, K. (2004). Understanding asthma pathogenesis: Linking innate and adaptive immunity. *Curr. Opin. Pediatr.* **16**(6), 659–666.

Endo, T. A., Masuhara, M., Yokouchi, M., Suzuki, R., Sakamoto, H., Mitsui, K., Matsumoto, A., Tanimura, S., Ohtsubo, M., Misawa, H., Miyazaki, T., Leonor, N., *et al*. (1997). A new protein containing an SH2 domain that inhibits JAK kinases. *Nature* **387**(6636), 921–924.

Flowers, L. O., Johnson, H. M., Mujtaba, M. G., Ellis, M. R., Haider, S. M., and Subramaniam, P. S. (2004). Characterization of a peptide inhibitor of Janus kinase 2 that mimics suppressor of cytokine signaling 1 function. *J. Immunol.* **172**(12), 7510–7518.

Fujimoto, M., Naka, T., Nakagawa, R., Kawazoe, Y., Morita, Y., Tateishi, A., Okumura, K., Narazaki, M., and Kishimoto, T. (2000). Defective thymocyte development and perturbed homeostasis of T cells in STAT-induced STAT inhibitor-1/suppressors of cytokine signaling-1 transgenic mice. *J. Immunol.* **165**(4), 1799–1806.

Fujimoto, M., Tsutsui, H., Yumikura-Futatsugi, S., Ueda, H., Xingshou, O., Abe, T., Kawase, I., Nakanishi, K., Kishimoto, T., and Naka, T. (2002). A regulatory role for suppressor of cytokine signaling-1 in T(h) polarization *in vivo*. *Int. Immunol.* **14**(11), 1343–1350.

Galm, O., Yoshikawa, H., Esteller, M., Osieka, R., and Herman, J. G. (2003). SOCS-1, a negative regulator of cytokine signaling, is frequently silenced by methylation in multiple myeloma. *Blood* **101**(7), 2784–2788.

Gately, M. K., Renzetti, L. M., Magram, J., Stern, A. S., Adorini, L., Gubler, U., and Presky, D. H. (1998). The interleukin-12/interleukin-12-receptor system: Role in normal and pathologic immune responses. *Annu. Rev. Immunol.* **16,** 495–521.

Gergen, P. J. (2001). Understanding the economic burden of asthma. *J. Allergy Clin. Immunol.* **107**(5 Suppl.), S445–S448.

Greenhalgh, C. J., Metcalf, D., Thaus, A. L., Corbin, J. E., Uren, R., Morgan, P. O., Fabri, L. J., Zhang, J. G., Martin, H. M., Willson, T. A., Billestrup, N., Nicola, N. A., *et al*. (2002). Biological evidence that SOCS-2 can act either as an enhancer or suppressor of growth hormone signaling. *J. Biol. Chem.* **277**(43), 40181–40184.

Greenhalgh, C. J., Rico-Bautista, E., Lorentzon, M., Thaus, A. L., Morgan, P. O., Willson, T. A., Zervoudakis, P., Metcalf, D., Street, I., Nicola, N. A., Nash, A. D., Fabri, L. J., *et al*. (2005). SOCS2 negatively regulates growth hormone action *in vitro* and *in vivo*. *J. Clin. Invest.* **115**(2), 397–406.

Gresser, I., Aguet, M., Morel-Maroger, L., Woodrow, D., Puvion-Dutilleul, F., Guillon, J. C., and Maury, C. (1981). Electrophoretically pure mouse interferon inhibits growth, induces liver and kidney lesions, and kills suckling mice. *Am. J. Pathol.* **102**(3), 396–402.

Harada, M., Nakashima, K., Hirota, T., Shimizu, M., Doi, S., Fujita, K., Shirakawa, T., Enomoto, T., Yoshikawa, M., Moriyama, H., Matsumoto, K., Saito, H., *et al.* (2007). Functional polymorphism in the suppressor of cytokine signaling 1 gene associated with adult asthma. *Am. J. Respir. Cell Mol. Biol.* **36,** 491–496.

Hansen, J. A., Lindberg, K., Hilton, D. J., Nielsen, J. H., and Billestrup, N. (1999). Mechanism of inhibition of growth hormone receptor signaling by suppressor of cytokine signaling proteins. *Mol. Endocrinol.* **13**(11), 1832–1843.

Hendry, L., and John, S. (2004). Regulation of STAT signalling by proteolytic processing. *Eur. J. Biochem.* **271**(23–24), 4613–4620.

Hilton, D. J., Richardson, R. T., Alexander, W. S., Viney, E. M., Willson, T. A., Sprigg, N. S., Starr, R., Nicholson, S. E., Metcalf, D., and Nicola, N. A. (1998). Twenty proteins containing a C-terminal SOCS box form five structural classes. *Proc. Natl. Acad. Sci. USA* **95**(1), 114–119.

Hookham, M. B., Elliott, J., Suessmuth, Y., Staerk, J., Ward, A. C., Vainchenker, W., Percy, M. J., and McMullin, M. F. (2007). The myeloproliferative disorder-associated JAK2 V617F mutant escapes negative regulation by suppressor of cytokine signaling 3. *Blood* **109**(11), 4924–4929.

Isaksen, D. E., Baumann, H., Trobridge, P. A., Farr, A. G., Levin, S. D., and Ziegler, S. F. (1999). Requirement for stat5 in thymic stromal lymphopoietin-mediated signal transduction. *J. Immunol.* **163**(11), 5971–5977.

Kamura, T., Sato, S., Haque, D., Liu, L., Kaelin, W. G., Jr., Conaway, R. C., and Conaway, J. W. (1998). The Elongin BC complex interacts with the conserved SOCS-box motif present in members of the SOCS, ras, WD-40 repeat, and ankyrin repeat families. *Genes Dev.* **12**(24), 3872–3881.

Kario, E., Marmor, M. D., Adamsky, K., Citri, A., Amit, I., Amariglio, N., Rechavi, G., and Yarden, Y. (2005). Suppressors of cytokine signaling 4 and 5 regulate epidermal growth factor receptor signaling. *J. Biol. Chem.* **280**(8), 7038–7048.

Kile, B. T., Schulman, B. A., Alexander, W. S., Nicola, N. A., Martin, H. M., and Hilton, D. J. (2002). The SOCS box: A tale of destruction and degradation. *Trends Biochem. Sci.* **27**(5), 235–241.

Kinjyo, I., Hanada, T., Inagaki-Ohara, K., Mori, H., Aki, D., Ohishi, M., Yoshida, H., Kubo, M., and Yoshimura, A. (2002). SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity* **17**(5), 583–591.

Knisz, J., Banks, A., McKeag, L., Metcalfe, D. D., Rothman, P. B., and Brown, J. M. (2009). Loss of SOCS7 in mice results in severe cutaneous disease and increased mast cell activation. *Clin. Immunol.* **132**(2), 277–284.

Krebs, D. L., Uren, R. T., Metcalf, D., Rakar, S., Zhang, J. G., Starr, R., De Souza, D. P., Hanzinikolas, K., Eyles, J., Connolly, L. M., Simpson, R. J., Nicola, N. A., *et al.* (2002). SOCS-6 binds to insulin receptor substrate 4, and mice lacking the SOCS-6 gene exhibit mild growth retardation. *Mol. Cell. Biol.* **22**(13), 4567–4578.

Lee, S. J., Lee, J. R., Hahn, H. S., Kim, Y. H., Ahn, J. H., Bae, C. D., Yang, J. M., and Hahn, M. J. (2007). PIAS1 interacts with the KRAB zinc finger protein, ZNF133, via zinc finger motifs and regulates its transcriptional activity. *Exp. Mol. Med.* **39**(4), 450–457.

Li, L., Gronning, L. M., Anderson, P. O., Li, S., Edvardsen, K., Johnston, J., Kioussis, D., Shepherd, P. R., and Wang, P. (2004). Insulin induces SOCS-6 expression and its binding to the p85 monomer of phosphoinositide 3-kinase, resulting in improvement in glucose metabolism. *J. Biol. Chem.* **279**(33), 34107–34114.

Li, Y., Chu, N., Rostami, A., and Zhang, G. X. (2006). Dendritic cells transduced with SOCS-3 exhibit a tolerogenic/DC2 phenotype that directs type 2 Th cell differentiation *in vitro* and *in vivo*. *J. Immunol.* **177**(3), 1679–1688.

Liu, Y. J. (2006). Thymic stromal lymphopoietin: Master switch for allergic inflammation. *J. Exp. Med.* **203**(2), 269–273.

Losman, J. A., Chen, X. P., Hilton, D., and Rothman, P. (1999). Cutting edge: SOCS-1 is a potent inhibitor of IL-4 signal transduction. *J. Immunol.* **162**(7), 3770–3774.

Mansell, A., Smith, R., Doyle, S. L., Gray, P., Fenner, J. E., Crack, P. J., Nicholson, S. E., Hilton, D. J., O'Neill, L. A., and Hertzog, P. J. (2006). Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. *Nat. Immunol.* **7**(2), 148–155.

Marine, J. C., McKay, C., Wang, D., Topham, D. J., Parganas, E., Nakajima, H., Pendeville, H., Yasukawa, H., Sasaki, A., Yoshimura, A., and Ihle, J. N. (1999). SOCS3 is essential in the regulation of fetal liver erythropoiesis. *Cell* **98**(5), 617–627.

Marine, J. C., Topham, D. J., McKay, C., Wang, D., Parganas, E., Stravopodis, D., Yoshimura, A., and Ihle, J. N. (1999). SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell* **98**(5), 609–616.

Martens, N., Wery, M., Wang, P., Braet, F., Gertler, A., Hooghe, R., Vandenhaute, J., and Hooghe-Peters, E. L. (2004). The suppressor of cytokine signaling (SOCS)-7 interacts with the actin cytoskeleton through vinexin. *Exp. Cell Res.* **298**(1), 239–248.

Matsumoto, A., Seki, Y., Kubo, M., Ohtsuka, S., Suzuki, A., Hayashi, I., Tsuji, K., Nakahata, T., Okabe, M., Yamada, S., and Yoshimura, A. (1999). Suppression of STAT5 functions in liver, mammary glands, and T cells in cytokine-inducible SH2-containing protein 1 transgenic mice. *Mol. Cell. Biol.* **19**(9), 6396–6407.

Matsumura, Y., Kobayashi, T., Ichiyama, K., Yoshida, R., Hashimoto, M., Takimoto, T., Tanaka, K., Chinen, T., Shichita, T., Wyss-Coray, T., Sato, K., and Yoshimura, A. (2007). Selective expansion of foxp3-positive regulatory T cells and immunosuppression by suppressors of cytokine signaling 3-deficient dendritic cells. *J. Immunol.* **179**(4), 2170–2179.

Matuoka, K., Miki, H., Takahashi, K., and Takenawa, T. (1997). A novel ligand for an SH3 domain of the adaptor protein Nck bears an SH2 domain and nuclear signaling motifs. *Biochem. Biophys. Res. Commun.* **239**(2), 488–492.

Metcalf, D., Greenhalgh, C. J., Viney, E., Willson, T. A., Starr, R., Nicola, N. A., Hilton, D. J., and Alexander, W. S. (2000). Gigantism in mice lacking suppressor of cytokine signalling-2. *Nature* **405**(6790), 1069–1073.

Minamoto, S., Ikegame, K., Ueno, K., Narazaki, M., Naka, T., Yamamoto, H., Matsumoto, T., Saito, H., Hosoe, S., and Kishimoto, T. (1997). Cloning and functional analysis of new members of STAT induced STAT inhibitor (SSI) family: SSI-2 and SSI-3. *Biochem. Biophys. Res. Commun.* **237**(1), 79–83.

Mooney, R. A., Senn, J., Cameron, S., Inamdar, N., Boivin, L. M., Shang, Y., and Furlanetto, R. W. (2001). Suppressors of cytokine signaling-1 and -6 associate with and inhibit the insulin receptor. A potential mechanism for cytokine-mediated insulin resistance. *J. Biol. Chem.* **276**(28), 25889–25893.

Naka, T., Narazaki, M., Hirata, M., Matsumoto, T., Minamoto, S., Aono, A., Nishimoto, N., Kajita, T., Taga, T., Yoshizaki, K., Akira, S., and Kishimoto, T. (1997). Structure and function of a new STAT-induced STAT inhibitor. *Nature* **387**(6636), 924–929.

Naka, T., Tsutsui, H., Fujimoto, M., Kawazoe, Y., Kohzaki, H., Morita, Y., Nakagawa, R., Narazaki, M., Adachi, K., Yoshimoto, T., Nakanishi, K., and Kishimoto, T. (2001). SOCS-1/SSI-1-deficient NKT cells participate in severe hepatitis through dysregulated cross-talk inhibition of IFN-gamma and IL-4 signaling *in vivo*. *Immunity* **14**(5), 535–545.

Nakagawa, R., Naka, T., Tsutsui, H., Fujimoto, M., Kimura, A., Abe, T., Seki, E., Sato, S., Takeuchi, O., Takeda, K., Akira, S., Yamanishi, K., *et al.* (2002). SOCS-1 participates in negative regulation of LPS responses. *Immunity* **17**(5), 677–687.

Narazaki, M., Fujimoto, M., Matsumoto, T., Morita, Y., Saito, H., Kajita, T., Yoshizaki, K., Naka, T., and Kishimoto, T. (1998). Three distinct domains of SSI-1/SOCS-1/JAB protein

are required for its suppression of interleukin 6 signaling. *Proc. Natl. Acad. Sci. USA* **95**(22), 13130–13134.

Nelms, K., Keegan, A. D., Zamorano, J., Ryan, J. J., and Paul, W. E. (1999). The IL-4 receptor: Signaling mechanisms and biologic functions. *Annu. Rev. Immunol.* **17,** 701–738.

Nicholson, S. E., De Souza, D., Fabri, L. J., Corbin, J., Willson, T. A., Zhang, J. G., Silva, A., Asimakis, M., Farley, A., Nash, A. D., Metcalf, D., Hilton, D. J., *et al*. (2000). Suppressor of cytokine signaling-3 preferentially binds to the SHP-2-binding site on the shared cytokine receptor subunit gp130. *Proc. Natl. Acad. Sci. USA* **97**(12), 6493–6498.

Nicholson, S. E., Metcalf, D., Sprigg, N. S., Columbus, R., Walker, F., Silva, A., Cary, D., Willson, T. A., Zhang, J. G., Hilton, D. J., Alexander, W. S., and Nicola, N. A. (2005). Suppressor of cytokine signaling (SOCS)-5 is a potential negative regulator of epidermal growth factor signaling. *Proc. Natl. Acad. Sci. USA* **102**(7), 2328–2333.

Niwa, Y., Kanda, H., Shikauchi, Y., Saiura, A., Matsubara, K., Kitagawa, T., Yamamoto, J., Kubo, T., and Yoshikawa, H. (2005). Methylation silencing of SOCS-3 promotes cell growth and migration by enhancing JAK/STAT and FAK signalings in human hepatocellular carcinoma. *Oncogene* **24**(42), 6406–6417.

Ohshima, M., Yokoyama, A., Ohnishi, H., Hamada, H., Kohno, N., Higaki, J., and Naka, T. (2007). Overexpression of suppressor of cytokine signalling-5 augments eosinophilic airway inflammation in mice. *Clin. Exp. Allergy* **37**(5), 735–742.

O'Neill, L. A. (2006). How toll-like receptors signal: What we know and what we don't know. *Curr. Opin. Immunol.* **18**(1), 3–9.

Ozaki, A., Seki, Y., Fukushima, A., and Kubo, M. (2005). The control of allergic conjunctivitis by suppressor of cytokine signaling (SOCS)3 and SOCS5 in a murine model. *J. Immunol.* **175**(8), 5489–5497.

Pezet, A., Favre, H., Kelly, P. A., and Edery, M. (1999). Inhibition and restoration of prolactin signal transduction by suppressors of cytokine signaling. *J. Biol. Chem.* **274**(35), 24497–24502.

Qin, H., Wilson, C. A., Roberts, K. L., Baker, B. J., Zhao, X., and Benveniste, E. N. (2006). IL-10 inhibits lipopolysaccharide-induced CD40 gene expression through induction of suppressor of cytokine signaling-3. *J. Immunol.* **177**(11), 7761–7771.

Raccurt, M., Tam, S. P., Lau, P., Mertani, H. C., Lambert, A., Garcia-Caballero, T., Li, H., Brown, R. J., McGuckin, M. A., Morel, G., and Waters, M. J. (2003). Suppressor of cytokine signalling gene expression is elevated in breast carcinoma. *Br. J. Cancer* **89**(3), 524–532.

Rawlings, J. S., Rennebeck, G., Harrison, S. M., Xi, R., and Harrison, D. A. (2004). Two *Drosophila* suppressors of cytokine signaling (SOCS) differentially regulate JAK and EGFR pathway activities. *BMC Cell Biol.* **5**(1), 38.

Rennick, D. M., Fort, M. M., and Davidson, N. J. (1997). Studies with IL-10$^{-/-}$ mice: An overview. *J. Leukoc. Biol.* **61**(4), 389–396.

Riehle, K. J., Campbell, J. S., McMahan, R. S., Johnson, M. M., Beyer, R. P., Bammler, T. K., and Fausto, N. (2008). Regulation of liver regeneration and hepatocarcinogenesis by suppressor of cytokine signaling 3. *J. Exp. Med.* **205**(1), 91–103.

Robb, L., Boyle, K., Rakar, S., Hartley, L., Lochland, J., Roberts, A. W., Alexander, W. S., and Metcalf, D. (2005). Genetic reduction of embryonic leukemia-inhibitory factor production rescues placentation in SOCS3-null embryos but does not prevent inflammatory disease. *Proc. Natl. Acad. Sci. USA* **102**(45), 16333–16338.

Roberts, A. W., Robb, L., Rakar, S., Hartley, L., Cluse, L., Nicola, N. A., Metcalf, D., Hilton, D. J., and Alexander, W. S. (2001). Placental defects and embryonic lethality in mice lacking suppressor of cytokine signaling 3. *Proc. Natl. Acad. Sci. USA* **98**(16), 9324–9329.

Rogers, S., Wells, R., and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science* **234**(4774), 364–368.

Ryo, A., Suizu, F., Yoshida, Y., Perrem, K., Liou, Y. C., Wulf, G., Rottapel, R., Yamaoka, S., and Lu, K. P. (2003). Regulation of NF-kappaB signaling by Pin1-dependent prolyl isomerization and ubiquitin-mediated proteolysis of p65/RelA. *Mol. Cell* **12**(6), 1413–1426.

Sasaki, A., Yasukawa, H., Suzuki, A., Kamizono, S., Syoda, T., Kinjyo, I., Sasaki, M., Johnston, J. A., and Yoshimura, A. (1999). Cytokine-inducible SH2 protein-3 (CIS3/SOCS3) inhibits Janus tyrosine kinase by binding through the N-terminal kinase inhibitory region as well as SH2 domain. *Genes Cells* **4**(6), 339–351.

Sasaki, A., Yasukawa, H., Shouda, T., Kitamura, T., Dikic, I., and Yoshimura, A. (2000). CIS3/SOCS-3 suppresses erythropoietin (EPO) signaling by binding the EPO receptor and JAK2. *J. Biol. Chem.* **275**(38), 29338–29347.

Schoenfeld, A. R., Davidowitz, E. J., and Burk, R. D. (2000). Elongin BC complex prevents degradation of von Hippel-Lindau tumor suppressor gene products. *Proc. Natl. Acad. Sci. USA* **97**(15), 8507–8512.

Seki, Y., Hayashi, K., Matsumoto, A., Seki, N., Tsukada, J., Ransom, J., Naka, T., Kishimoto, T., Yoshimura, A., and Kubo, M. (2002). Expression of the suppressor of cytokine signaling-5 (SOCS5) negatively regulates IL-4-dependent STAT6 activation and Th2 differentiation. *Proc. Natl. Acad. Sci. USA* **99**(20), 13003–13008.

Seki, Y., Inoue, H., Nagata, N., Hayashi, K., Fukuyama, S., Matsumoto, K., Komine, O., Hamano, S., Himeno, K., Inagaki-Ohara, K., Cacalano, N., O'Garra, A., *et al*. (2003). SOCS-3 regulates onset and maintenance of T(H)2-mediated allergic responses. *Nat. Med.* **9**(8), 1047–1054.

Shuai, K., and Liu, B. (2003). Regulation of JAK-STAT signalling in the immune system. *Nat. Rev. Immunol.* **3**(11), 900–911.

Sitko, J. C., Guevara, C. I., and Cacalano, N. A. (2004). Tyrosine-phosphorylated SOCS3 interacts with the Nck and Crk-L adapter proteins and regulates Nck activation. *J. Biol. Chem.* **279**(36), 37662–37669.

Song, M. M., and Shuai, K. (1998). The suppressor of cytokine signaling (SOCS) 1 and SOCS3 but not SOCS2 proteins inhibit interferon-mediated antiviral and antiproliferative activities. *J. Biol. Chem.* **273**(52), 35056–35062.

Spruck, C. H., and Strohmaier, H. M. (2002). Seek and destroy: SCF ubiquitin ligases in mammalian cell cycle control. *Cell Cycle* **1**(4), 250–254.

Starr, R., Willson, T. A., Viney, E. M., Murray, L. J., Rayner, J. R., Jenkins, B. J., Gonda, T. J., Alexander, W. S., Metcalf, D., Nicola, N. A., and Hilton, D. J. (1997). A family of cytokine-inducible inhibitors of signalling. *Nature* **387**(6636), 917–921.

Starr, R., Metcalf, D., Elefanty, A. G., Brysha, M., Willson, T. A., Nicola, N. A., Hilton, D. J., and Alexander, W. S. (1998). Liver degeneration and lymphoid deficiencies in mice lacking suppressor of cytokine signaling-1. *Proc. Natl. Acad. Sci. USA* **95**(24), 14395–14399.

Stoiber, D., Kovarik, P., Cohney, S., Johnston, J. A., Steinlein, P., and Decker, T. (1999). Lipopolysaccharide induces in macrophages the synthesis of the suppressor of cytokine signaling 3 and suppresses signal transduction in response to the activating factor IFN-gamma. *J. Immunol.* **163**(5), 2640–2647.

Takahashi, Y., Carpino, N., Cross, J. C., Torres, M., Parganas, E., and Ihle, J. N. (2003). SOCS3: An essential regulator of LIF receptor signaling in trophoblast giant cell differentiation. *EMBO J.* **22**(3), 372–384.

Terauchi, Y., Tsuji, Y., Satoh, S., Minoura, H., Murakami, K., Okuno, A., Inukai, K., Asano, T., Kaburagi, Y., Ueki, K., Nakajima, H., Hanafusa, T., *et al*. (1999). Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 alpha subunit of phosphoinositide 3-kinase. *Nat. Genet.* **21**(2), 230–235.

Ungureanu, D., Saharinen, P., Junttila, I., Hilton, D. J., and Silvennoinen, O. (2002). Regulation of Jak2 through the ubiquitin-proteasome pathway involves phosphorylation of Jak2 on Y1007 and interaction with SOCS-1. *Mol. Cell. Biol.* **22**(10), 3316–3326.

Waiboci, L. W., Ahmed, C. M., Mujtaba, M. G., Flowers, L. O., Martin, J. P., Haider, M. I., and Johnson, H. M. (2007). Both the suppressor of cytokine signaling 1 (SOCS-1) kinase inhibitory region and SOCS-1 mimetic bind to JAK2 autophosphorylation site: Implications for the development of a SOCS-1 antagonist. *J. Immunol.* **178**(8), 5058–5068.

Watanabe, H., Kubo, M., Numata, K., Takagi, K., Mizuta, H., Okada, S., Ito, T., and Matsukawa, A. (2006). Overexpression of suppressor of cytokine signaling-5 in T cells augments innate immunity during septic peritonitis. *J. Immunol.* **177**(12), 8650–8657.

Weber, A., Hengge, U. R., Bardenheuer, W., Tischoff, I., Sommerer, F., Markwarth, A., Dietz, A., Wittekind, C., and Tannapfel, A. (2005). SOCS-3 is frequently methylated in head and neck squamous cell carcinoma and its precursor lesions and causes growth inhibition. *Oncogene* **24**(44), 6699–6708.

Wills-Karp, M. (1999). Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* **17,** 255–281.

Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T. Y., Karp, C. L., and Donaldson, D. D. (1998). Interleukin-13: Central mediator of allergic asthma. *Science* **282**(5397), 2258–2261.

Xia, L., Wang, L., Chung, A. S., Ivanov, S. S., Ling, M. Y., Dragoi, A. M., Platt, A., Gilmer, T. M., Fu, X. Y., and Chin, Y. E. (2002). Identification of both positive and negative domains within the epidermal growth factor receptor COOH-terminal region for signal transducer and activator of transcription (STAT) activation. *J. Biol. Chem.* **277**(34), 30716–30723.

Yasukawa, H., Misawa, H., Sakamoto, H., Masuhara, M., Sasaki, A., Wakioka, T., Ohtsuka, S., Imaizumi, T., Matsuda, T., Ihle, J. N., and Yoshimura, A. (1999). The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. *EMBO J.* **18**(5), 1309–1320.

Yasukawa, H., Sasaki, A., and Yoshimura, A. (2000). Negative regulation of cytokine signaling pathways. *Annu. Rev. Immunol.* **18,** 143–164.

Yasukawa, H., Ohishi, M., Mori, H., Murakami, M., Chinen, T., Aki, D., Hanada, T., Takeda, K., Akira, S., Hoshijima, M., Hirano, T., Chien, K. R., *et al*. (2003). IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. *Nat. Immunol.* **4**(6), 551–556.

Yoshikawa, H., Matsubara, K., Qian, G. S., Jackson, P., Groopman, J. D., Manning, J. E., Harris, C. C., and Herman, J. G. (2001). SOCS-1, a negative regulator of the JAK/STAT pathway, is silenced by methylation in human hepatocellular carcinoma and shows growth-suppression activity. *Nat. Genet.* **28**(1), 29–35.

Yoshimura, A., Ohkubo, T., Kiguchi, T., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., Hara, T., and Miyajima, A. (1995). A novel cytokine-inducible gene CIS encodes an SH2-containing protein that binds to tyrosine-phosphorylated interleukin 3 and erythropoietin receptors. *EMBO J.* **14**(12), 2816–2826.

Zeidler, M. P., Bach, E. A., and Perrimon, N. (2000). The roles of the *Drosophila* JAK/STAT pathway. *Oncogene* **19**(21), 2598–2606.

Zhang, J. G., Farley, A., Nicholson, S. E., Willson, T. A., Zugaro, L. M., Simpson, R. J., Moritz, R. L., Cary, D., Richardson, R., Hausmann, G., Kile, B. J., Kent, S. B., *et al*. (1999). The conserved SOCS box motif in suppressors of cytokine signaling binds to elongins B and C and may couple bound proteins to proteasomal degradation. *Proc. Natl. Acad. Sci. USA* **96**(5), 2071–2076.

Zhang, J. G., Metcalf, D., Rakar, S., Asimakis, M., Greenhalgh, C. J., Willson, T. A., Starr, R., Nicholson, S. E., Carter, W., Alexander, W. S., Hilton, D. J., and Nicola, N. A. (2001). The SOCS box of suppressor of cytokine signaling-1 is important for inhibition of cytokine action *in vivo*. *Proc. Natl. Acad. Sci. USA* **98**(23), 13261–13265.

Zhou, Y. J., Hanson, E. P., Chen, Y. Q., Magnuson, K., Chen, M., Swann, P. G., Wange, R. L., Changelian, P. S., and O'Shea, J. J. (1997). Distinct tyrosine phosphorylation sites in JAK3 kinase domain positively and negatively regulate its enzymatic activity. *Proc. Natl. Acad. Sci. USA* **94**(25), 13850–13855.

CHAPTER 4

Multitasking by Exploitation of Intracellular Transport Functions: The Many Faces of FcRn

E. Sally Ward* and **Raimund J. Ober*,†**

Contents

* Department of Immunology, University of Texas Southwestern Medical Center, Dallas, Texas, USA
† University of Texas at Dallas, Richardson, Texas, USA

Advances in Immunology, Volume 103
ISSN 0065-2776, DOI: 10.1016/S0065-2776(09)03004-1

Abstract

The MHC Class I-related receptor, FcRn, transports antibodies of the immunoglobulin G (IgG) class within and across a diverse array of different cell types. Through this transport, FcRn serves multiple roles throughout adult life that extend well beyond its earlier defined function of transcytosing IgGs from mother to offspring. These roles include the maintenance of IgG levels and the delivery of antigen in the form of immune complexes to degradative compartments within cells. Recent studies have led to significant advances in knowledge of the intracellular trafficking of FcRn and (engineered) IgGs at both the molecular and cellular levels. The engineering of FcRn–IgG (or Fc) interactions to generate antibodies of increased longevity represents an area of active interest, particularly in the light of the expanding use of antibodies in therapy. The strict pH dependence of FcRn–IgG interactions, with binding at pH 6 that becomes essentially undetectable as near neutral pH is approached, is essential for efficient transport. The requirement for retention of low affinity at near neutral pH increases the complexity of engineering antibodies for increased half-life. Conversely, engineered IgGs that have gained significant binding for FcRn at this pH can be potent inhibitors of FcRn that lower endogenous IgG levels and have multiple potential uses as therapeutics. In addition, molecular studies of FcRn–IgG interactions indicate that mice have limitations as preclinical models for FcRn function, primarily due to cross-species differences in FcRn-binding specificity.

1. INTRODUCTION

The MHC Class I-related receptor, FcRn (n for neonatal), was originally identified as the receptor that transports maternal IgG in mother's milk across the neonatal rodent gut during the suckling period (Brambell, 1970; Rodewald and Abrahamson, 1982; Wallace and Rees, 1980). However, more recent studies have not only shown that this receptor serves to regulate IgG levels and distribution throughout adult life (Ghetie *et al.*, 1996; Israel *et al.*, 1996; Junghans and Anderson, 1996), but also that it has multiple other roles in diverse cell types and tissues (e.g., Akilesh *et al.*, 2008; Dickinson *et al.*, 1999; Kim *et al.*, 2008; Spiekermann *et al.*, 2002; Zhu *et al.*, 2001). FcRn orthologs have been isolated from many species, including mouse, rat, man, sheep, cow, possum, pig, and camel (Adamski *et al.*, 2000; Ahouse *et al.*, 1993; Kacskovics *et al.*, 2000, 2006; Kandil *et al.*, 1995; Mayer *et al.*, 2002; Schnulle and Hurley, 2003; Simister and Mostov, 1989; Story *et al.*, 1994), indicating that this receptor is present in essentially all mammalian species. The multiple functions of FcRn are dependent on its ability to sort IgG away from lysosomal degradation within cells and release bound cargo during exocytic events at the plasma membrane (Ober *et al.*, 2004a,b; Prabhat *et al.*, 2007). Consequently, this receptor transports IgG within and across cellular barriers for a diverse array of cell types (Antohe *et al.*, 2001; Claypool *et al.*, 2004; Dickinson *et al.*, 1999; Firan *et al.*, 2001; Haymann *et al.*, 2000; McCarthy *et al.*, 2000; Spiekermann *et al.*, 2002; Yoshida *et al.*, 2004). More recently, FcRn has also been shown to control albumin levels (Andersen *et al.*, 2006; Chaudhury *et al.*, 2003). How this receptor behaves at the subcellular level of intracellular trafficking, and what controls its intracellular routing are of fundamental relevance to understanding its function. In addition, given the potential for modulating IgG trafficking pathways and behavior *in vivo*, the earlier report of engineering of antibodies to increase their half-life in mice (Ghetie *et al.*, 1997) has expanded into an area of intense interest in the biopharma industry (Dall'Acqua *et al.*, 2006b; Hinton *et al.*, 2004, 2006; Shields *et al.*, 2001).

In the current review, we will describe the multiple functions of FcRn and the intracellular trafficking pathways of this receptor and its ligand. The modulation of FcRn–ligand interactions for the development of therapeutics will also be discussed, with a particular focus on how the complexity of the pH dependence of FcRn–IgG interactions and cross-species differences in behavior impact this area.

2. FcRn: A HISTORICAL PERSPECTIVE

Neonatal rodents acquire the major portion of their maternal IgG from mothers' milk during the suckling period (Brambell, 1970). An early model for the trafficking of IgG across the neonatal gut was originally

proposed in the absence of specific knowledge of the receptor involved (Brambell, 1970): IgGs are taken into enterocytes at the apical surface by receptor-mediated uptake at the acidic pH in the small intestine. These IgGs are then transcytosed across the cells and released at the basolateral membrane which is at near neutral pH. A central feature of this early model was that the unidentified receptor, which was later shown to be FcRn (Rodewald and Abrahamson, 1982; Wallace and Rees, 1980), is a salvage receptor which binds and transports IgG in intact form across cells.

FcRn was subsequently isolated from rodent gut as a heterodimer comprising 12 kDa and 40–45 kDa proteins (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985). Significantly, in these early studies, the FcRn–IgG interaction was shown to be highly pH dependent with relatively tight binding at acidic pH (6) and very weak, if not negligible, binding at near neutral pH (Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985). The cloning of the gene for rat FcRn in 1989 unexpectedly revealed that this receptor comprises an α-chain that is homologous to MHC Class I α-chains, and the 12 kDa component is β2-microglobulin (β2m) (Simister and Mostov, 1989). This was followed by the isolation of orthologous FcRn α-chains from mouse and man (Ahouse *et al.*, 1993; Kandil *et al.*, 1995; Story *et al.*, 1994), and subsequently from multiple other species (Adamski *et al.*, 2000; Kacskovics *et al.*, 2000, 2006; Mayer *et al.*, 2002; Schnulle and Hurley, 2003). Although FcRn orthologs share some similarities, there are cross-species differences at the level of binding specificity that can have functional effects (Ober *et al.*, 2001; Vaccaro *et al.*, 2006), in addition to variations in intracellular trafficking and subcellular distribution (Claypool *et al.*, 2002; Kuo *et al.*, 2009) (discussed further in Sections 5.5 and 8).

3. FcRn IS A MULTITASKING RECEPTOR

3.1. A role for FcRn in regulating IgG levels

At the time of the isolation of the gene encoding rat FcRn (Simister and Mostov, 1989), the primary function of this receptor was believed to be to deliver maternal IgG to offspring. Although Brambell and colleagues proposed in the 1960s that the cellular processes involved in transporting maternal IgG from mother to young and in regulating IgG levels throughout life might be related (Brambell, 1970; Brambell *et al.*, 1964), data to provide direct support for the involvement of FcRn in both of these processes were absent. However, in the mid-1990s, several observations

led to the conclusion that FcRn exploits its capability to transport IgG within and across cells to regulate IgG levels throughout adult life. First, we demonstrated that the same IgG residues (on both CH2 and CH3 domains of the Fc region) are involved in controlling the *in vivo* half-life of Fc fragments and their transport across the neonatal gut (Kim *et al.*, 1994a,b). Second, we observed that mice deficient in β2m that do not express functional FcRn are characterized by abnormally rapid clearance rates of IgG/Fc fragments (Ghetie *et al.*, 1996, see also Israel *et al.*, 1996; Junghans and Anderson, 1996). Third, FcRn expression is not restricted to the gestational or neonatal periods, but can be detected in multiple tissues/cell types throughout adult life (Ghetie *et al.*, 1996). Fourth, an engineered Fc fragment with higher affinity for FcRn at pH 6, but with retention of very low affinity at near neutral pH, was shown to have increased *in vivo* persistence in mice (Ghetie *et al.*, 1997).

The ubiquitous nature of FcRn expression leads to the question as to which cell types are most relevant for the regulation of IgG levels *in vivo*? Distribution studies of IgGs with different binding properties for FcRn indicated that the (micro)vasculature, primarily in skin and muscle with lesser amounts in liver and adipose tissue, contributes to IgG homeostasis (Borvak *et al.*, 1998), consistent with the earlier suggestion that this regulation occurs at diffuse sites throughout the body (Waldmann and Strober, 1969). More recent studies involving bone marrow transfers indicate that FcRn expression in hematopoietic cells such as dendritic cells, monocytes, and macrophages also contributes to the regulation of IgG levels (Akilesh *et al.*, 2007; Qiao *et al.*, 2008). To delineate the role of specific cell types in the maintenance of IgG concentrations *in vivo*, we have generated a mouse strain in which FcRn can be conditionally deleted (Perez-Montoyo *et al.*, 2009). This strain harbors FcRn alleles (exons 5–7) flanked by loxP sites, and in combination with Tie2-Cre mice which express Cre recombinase under the control of the Tie2 promoter in endothelial and hematopoietic cells (Kisanuki *et al.*, 2001) can be used to analyze the impact of site-specific deletion of FcRn in these cells. Analyses of the clearance rates of IgGs in these mice demonstrate that endothelial and hematopoietic cells are the primary sites responsible for FcRn-mediated homeostasis of IgG (Perez-Montoyo *et al.*, 2009).

Although targeted deletion of human FcRn is clearly not possible, the analysis of archived human blood samples from patients with a deficiency in β2m expression has provided a naturally occurring human knockout for FcRn (Wani *et al.*, 2006). These patients have abnormally low IgG levels. Taken together with correlations between FcRn-binding properties of an IgG and *in vivo* persistence in nonhuman primates (discussed further in Section 7.2), the available data therefore indicate that FcRn is also a major contributor to IgG homeostasis in humans.

3.2. FcRn-mediated transport of IgG across cellular barriers: Opportunities for drug delivery

In addition to the role of FcRn in transporting maternal IgG across the neonatal intestine (Rodewald and Abrahamson, 1982; Wallace and Rees, 1980), the central function of FcRn in transporting IgG across both the mouse yolk sac and human placenta during gestation has been demonstrated (Firan *et al.*, 2001; Medesan *et al.*, 1996). More recently, it has become apparent that FcRn serves to deliver IgGs across cellular barriers throughout life. Extensive analyses of FcRn-mediated trafficking of IgGs and IgG–antigen complexes across epithelial cells in cell lines and in adult mice/nonhuman primates provide insight into these transport processes (Bitonti and Dumont, 2006; Bitonti *et al.*, 2004; Dickinson *et al.*, 1999; Haymann *et al.*, 2000; Kobayashi *et al.*, 2002; Sakagami *et al.*, 2006; Spiekermann *et al.*, 2002; Yoshida *et al.*, 2004). For example, in transgenic mice that are engineered to express mouse FcRn in adult intestinal epithelium, FcRn can transport antigen bound to IgG from the intestinal lumen into the lamina propria to elicit $CD4^+$ T cell responses against bacteria (Yoshida *et al.*, 2006). Thus, FcRn can function as a scavenger of luminal antigens in the gut, indicating that it can play an important role in mucosal immunity.

Trans-epithelial transfer offers opportunities for the delivery of therapeutic proteins, and consistent with this, erythropoeitin–Fc fusions can be transferred in an FcRn-dependent mode across the lung epithelium of adult mice and nonhuman primates (Bitonti *et al.*, 2004; Spiekermann *et al.*, 2002). Interestingly, a ''monomeric'' Epo–Fc fusion comprising a single Epo molecule connected to one arm of the dimeric Fc molecule was transported more efficiently than an Epo–Fc dimer containing two Epo molecules per Fc (Bitonti *et al.*, 2004). This enhanced transport was shown to be due in part to an increased affinity for binding of the monomer to FcRn, but in addition, size reduction and/or a change in charge might be contributing properties (Bitonti and Dumont, 2006). Surfaces such as lung epithelium that are bathed in mucus may be particularly susceptible to such effects, indicating that it will be advantageous to design molecules with optimized properties such as charge, size, and minimization of steric hindrance on FcRn binding for a given delivery route. For lung delivery, high potency of the biologic is also important since the volume of the vehicle is, by necessity, relatively low (Wang *et al.*, 2008). Although transport across the intestine avoids this potential limitation, a major challenge is to generate recombinant proteins that are resistant to the hostile proteolytic and acidic environment of this locale. The targeting of FcRn with Fc-fusion proteins to deliver therapeutics *in utero* is also attractive and promise for this approach in a mouse model of the lysosomal storage disease, mucopolysaccharidosis, has been demonstrated

(Grubb *et al.*, 2008). Collectively, these studies indicate that the use of FcRn as a drug delivery vehicle has multiple possible applications.

3.3. FcRn can deliver antigen for presentation

The earlier observation that FcRn is expressed in monocyte/macrophages and dendritic cells led to the suggestion that this receptor might play a role in antigen presentation (Zhu *et al.*, 2001). More recent analyses have shown that FcRn can direct immune complexes (ICs) into lysosomes in dendritic cells, which in turn can enhance antigen presentation (Qiao *et al.*, 2008). Although FcRn was originally shown not to be expressed in B cell lines and primary B cells (Akilesh *et al.*, 2007; Ghetie *et al.*, 1996; Zhu *et al.*, 2001), this receptor is present in splenic B cells in mice (Mi *et al.*, 2008; Perez-Montoyo *et al.*, 2009). This extends the expression of FcRn to all major subsets of professional antigen presenting cells (APCs). Taken together with the report that invariant chain, for which the expression is generally restricted to APCs, can associate with FcRn and direct it into lysosomes (Ye *et al.*, 2008), this suggests that the intracellular trafficking pathways in these cells can be modulated to optimize antigen presentation (discussed further in Section 5.7). Interestingly, phagocytosis of opsonized bacteria by human neutrophils is also increased by FcRn expression, leading to the suggestion that the nascent phagocytic cup is acidified to facilitate FcRn–IgG interactions during uptake (Vidarsson *et al.*, 2006). This might provide an explanation for the higher phagocytic activity of monocytes relative to NK cells that do not express FcRn. Taken together, the data therefore indicate that FcRn cannot only enhance phagocytic uptake, but can also redirect antigen complexed with antibodies into degradative compartments that are associated with the loading of antigenic peptides onto MHC Class II molecules within cells.

3.4. Possible functions of FcRn in specialized cell types

FcRn expression in highly specialized cells such as podocytes in the kidney plays an important role in removing IgG from the glomerular basement membrane (Akilesh *et al.*, 2008). Indeed, blocking of FcRn in mice leads to serum-induced nephritis, suggesting that impaired function of this clearance process could predispose toward glomerular disease. This raises questions concerning whether FcRn (dys)function might contribute to the pathology of diseases such as systemic lupus erythematosus, in which IC-mediated kidney damage is common.

FcRn expression has also been demonstrated in multiple ocular tissues, including the cornea, retina, conjunctiva, and the blood–ocular barrier (Kim *et al.*, 2008). The function of FcRn at these sites is currently unknown, but may be related to the immune-privileged status of the eye.

Similarly, FcRn is expressed at the blood–brain barrier (BBB) in both the brain microvasculature and the choroid plexus epithelium (Schlachetzki *et al.*, 2002) where it might be important for maintaining low levels of potentially inflammatory antibodies in the CNS. Consistent with this, several studies demonstrate that IgG is transported by FcRn in the brain-to-blood direction (Deane *et al.*, 2005; Zhang and Pardridge, 2001). This directional transport has specific relevance to the clearance of amyloid β peptide (Aβ) from the brain by Aβ-specific IgG, which results in a reduction of symptoms of Alzheimer's disease in a mouse model (Deane *et al.*, 2005). Such studies indicate that it could be fruitful to explore this pathway further for Aβ-directed immunotherapy. However, others have reported that the brain-to-blood exposure ratios for IgG are the same in both wild-type and FcRn-deficient mice (Wang *et al.*, 2008), indicating that the role of FcRn at this barrier requires further investigation.

4. THE MOLECULAR NATURE OF FcRn–IgG INTERACTIONS

4.1. The interaction site for FcRn on IgG

The molecular details of FcRn–IgG interactions have been extensively analyzed. For example, site-directed mutagenesis of recombinant IgG or Fc fragments has been used to identify residues that are involved in the mouse FcRn–IgG interaction for both human and mouse IgG1 (Kim *et al.*, 1994b, 1999; Medesan *et al.*, 1997). These studies have involved a combination of *in vitro* binding analyses and *in vivo* assays in mice, and demonstrate that His310, Ile253, and His435 of IgG play a central role in these interactions (Fig. 4.1). These same residues are involved in the human FcRn–human IgG1 (Firan *et al.*, 2001; Shields *et al.*, 2001) and rat FcRn–IgG (mouse, rat or human) interactions (Martin *et al.*, 2001; Raghavan *et al.*, 1995). Residue 436 (His in mouse IgG1, Tyr in human IgG1) plays a minor role in the binding of IgG to FcRn (Medesan *et al.*, 1997; Shields *et al.*, 2001). The high-resolution X-ray crystallographic structure of rat FcRn complexed with rat IgG2a clearly shows the direct involvement of residues 253, 310, 435, and 436 of IgG in binding (Martin *et al.*, 2001). These four residues are relatively well conserved across species and are located at the CH2–CH3 domain interface of IgG (Deisenhofer, 1981) (Fig. 4.1). The role of the highly conserved His433 of IgG in the interaction across species is more uncertain: in some systems it has been proposed to play a role (Martin *et al.*, 2001; Raghavan *et al.*, 1995; Shields *et al.*, 2001), whereas in others not (Kim *et al.*, 1999; Medesan *et al.*, 1997). Nevertheless, the involvement of several histidines on IgG in complex formation that interact with acidic residues on FcRn provides an explanation for the marked

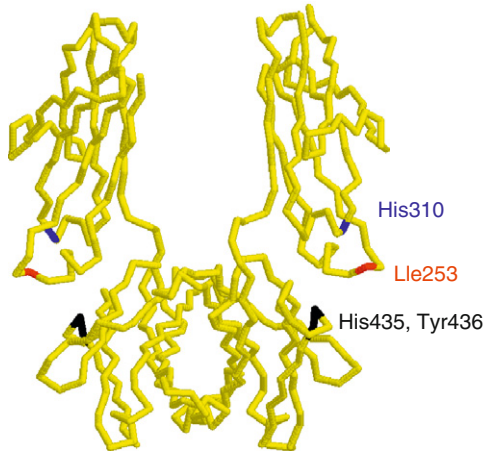

FIGURE 4.1 Structure (α-carbon trace) of the Fc region of human IgG1 (Deisenhofer, 1981) with the location of the key residues that are involved in binding to mouse or human FcRn indicated. The same residues of mouse IgG1 are also involved in FcRn binding, except that Tyr436 is replaced by histidine. The structure was drawn using Rasmol (courtesy of Roger Sayle, Bioinformatics Research Institute, University of Edinburgh).

pH dependence of FcRn–IgG binding, with binding at pH 6–6.5 which for most IgGs becomes progressively weaker as pH 7.4 is approached (Popov *et al.*, 1996; Raghavan *et al.*, 1995; Rodewald, 1976; Wallace and Rees, 1980). This pH dependence is essential for FcRn to function as an IgG transporter (see Section 7).

4.2. The interaction site for IgG on FcRn

Structure–function studies in the Bjorkman laboratory have identified FcRn residues that are involved in the rat FcRn–rat IgG2a interaction (Vaughn *et al.*, 1997), and the results of these analyses have been confirmed by the solution of the high-resolution structure of this complex (Martin *et al.*, 2001). To date, structural studies of human FcRn in complex with IgG have not been reported. Although the X-ray crystallographic structure of human FcRn in the absence of ligand indicates that it is structurally similar to rat FcRn, there are also some differences (West and Bjorkman, 2000). Rat FcRn residues that interact with IgG(2a) include Ile1 of β2m and Glu117, Glu118, Glu132, Trp133, Glu135, and Asp137 of the FcRn α-chain (Fig. 4.2). These amino acids are generally well conserved across species, although some notable exceptions exist. For example, Asp or Glu137 of rodent FcRn is replaced by leucine in human FcRn (Ahouse *et al.*, 1993; Simister and Mostov, 1989; Story *et al.*, 1994)

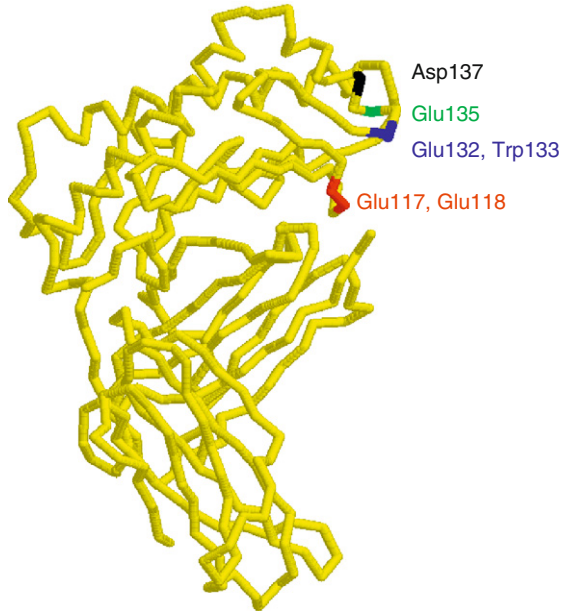

FIGURE 4.2 Structure (α-carbon trace) of rat FcRn with the location of the key residues that are involved in binding to rat IgG2a indicated (Martin *et al.*, 2001). The structure was drawn using Rasmol (courtesy of Roger Sayle, Bioinformatics Research Institute, University of Edinburgh).

(note that the numbering used throughout this review for human FcRn, which is two residues shorter than mouse/rat FcRn, ignores this two residue deletion and is based on the homology alignment of human and rodent FcRn). This sequence variation accounts, in part at least, for cross-species differences in binding specificity between rodent and human FcRn (Zhou *et al.*, 2003, 2005) (discussed in Section 8).

The ectodomains of FcRn also bear one or more potential glycosylation sites, raising the question as to whether this might contribute to IgG binding. It has been demonstrated that this is the case for rat FcRn, since carbohydrate attached to an N-linked glycosylation site at residue 128 of the receptor makes contacts with Val348, His433, Asn434, and Lys439 of rat IgG2a (Martin *et al.*, 2001). However, the relevance of an analogous interaction for the mouse FcRn–mouse IgG1 or mouse FcRn–human IgG1 complex is made unlikely by our observation that mutation of His433 or Asn434 individually to alanine in IgG1-derived Fc fragments does not affect activity in mouse FcRn-mediated functions (Kim *et al.*, 1999; Medesan *et al.*, 1997). Furthermore, human FcRn functions effectively in binding to IgG without a potential glycosylation site at residue 128,

suggesting that there may be differences at this level between rat and human FcRn. It is, however, interesting to note that differences in glycosylation between human and rat FcRn lead to variations in intracellular trafficking (Kuo *et al.*, 2009) and this is discussed further in Section 5.5.

4.3. The stoichiometry of the FcRn–IgG interaction

The presence of two possible binding sites for FcRn on IgG (or Fc) raises questions concerning the stoichiometry of the interaction. By generating a ''hybrid'' Fc comprising one CH2–CH3 polypeptide with a defective FcRn interaction site complexed with a wild-type CH2–CH3 polypeptide, two functional sites per Fc (mouse IgG1- or rat IgG2a-derived) have been shown to be essential for full activity *in vivo* in mice (Kim *et al.*, 1994b,c) and *in vitro* transport across rat FcRn-transfected epithelial cells (Tesar *et al.*, 2006). On the other hand, interaction analyses with soluble, recombinant FcRn demonstrated that the stoichiometry can be 2 FcRn:1 IgG or 1:1 (Martin and Bjorkman, 1999; Popov *et al.*, 1996; Sanchez *et al.*, 1999; Schuck *et al.*, 1999). This apparent discrepancy can be resolved by the demonstration that two possible binding sites on IgG (or Fc) are not equivalent (Sanchez *et al.*, 1999; Schuck *et al.*, 1999; Weng *et al.*, 1998), consistent with the concept that binding of FcRn to one site may reduce the affinity for the second site, that is, negative cooperativity (Ghetie and Ward, 1997). Whether this asymmetry is due to steric effects and/or some longer range conformational changes at the CH2–CH3 domain junction is currently unknown. However, the segmental flexibility of the IgG molecule (Nezlin, 1990; Oi *et al.*, 1978), together with the observation that a hinge-less Fc has lower activity in FcRn-mediated functions (Kim *et al.*, 1995), would be consistent with conformational alterations.

5. THE INTRACELLULAR TRAFFICKING OF FcRn

5.1. A model for FcRn trafficking

The pH dependence of FcRn interactions with the majority of naturally occurring IgGs is central to its function (Popov *et al.*, 1996; Raghavan *et al.*, 1995; Rodewald and Kraehenbuhl, 1984; Simister and Rees, 1985; Zhou *et al.*, 2005). Earlier models for how FcRn traffics within cells suggested that in most cell types, IgG is taken up primarily by fluid-phase processes (Brambell *et al.*, 1964; Ghetie and Ward, 1997), since the pH at most cell surfaces is not favorable for binding. However, it is possible that for cells such as those of epithelial origin, for which Na^+/H^+ exchanger activity results in acidification of the local environment (Hattori *et al.*, 2001), or in the acidic environments of tumors or inflammatory sites (Edlow and

Sheldon, 1971; Gerweck and Seetharaman, 1996; Tannock and Rotin, 1989; Ward and Steigbigel, 1978), significant levels of uptake by receptor-mediated processes can also occur. Whatever the route of uptake, entry of IgG into cells is followed by accumulation in early endosomes for which the acidic pH is permissive for binding (Fig. 4.3). If binding of the IgG to FcRn occurs, then the IgG is recycled or transcytosed (Ober *et al.*, 2004b). By contrast, IgGs that do not bind to FcRn enter late endosomes and are subsequently delivered to lysosomes (Ober *et al.*, 2004b). The predictions of this model are consistent with experimental observations: first, IgGs that have reduced affinity for binding to FcRn have shorter *in vivo* half-lives and are transported across cellular barriers less effectively (Firan *et al.*, 2001; Kim *et al.*, 1999; Medesan *et al.*, 1997; Spiekermann *et al.*, 2002). Second, reduced expression of FcRn within cells results in increased degradation of IgG (Ghetie *et al.*, 1996; Israel *et al.*, 1996; Junghans and Anderson, 1996; Roopenian *et al.*, 2003). Third, engineered IgGs that bind to FcRn with increased affinity at near neutral pH are taken into cells by receptor-mediated uptake and not released efficiently at the cell surface following recycling or transcytosis (Vaccaro *et al.*, 2005, 2006).

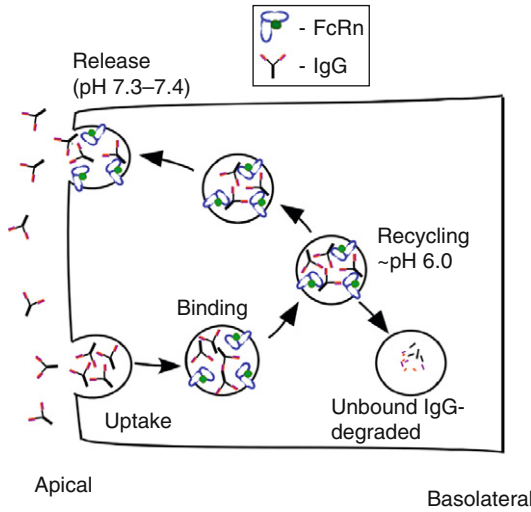

FIGURE 4.3 Schematic representation of FcRn-mediated recycling of IgG in a polarized cell such as an endothelial cell. IgGs are taken into the cell by fluid phase and enter early endosomes. The pH of the early endosome is permissive for FcRn binding, and binding of the IgG to FcRn results in recycling (or transcytosis, not shown) and salvage from lysosomal degradation. Conversely, unbound IgG enters the lysosome and is degraded.

5.2. Endosomal sorting of IgGs within endothelial cells

Live-cell-fluorescence imaging has been used to analyze several facets of FcRn-mediated trafficking of IgGs in human endothelial cells (Ober *et al.*, 2004a,b; Prabhat *et al.*, 2007; Ram *et al.*, 2008; Gan *et al.*, 2009). For example, the intracellular trafficking of fluorescently labeled IgGs that have different binding properties for FcRn have been compared to address the question as to where and how IgGs are sorted within cells. These IgGs include wild-type human IgG1 and a mutated derivative (H435A, His435 to Ala) that does not bind detectably to human FcRn (Firan *et al.*, 2001; Ober *et al.*, 2004b). Treatment of human FcRn–GFP-transfected endothelial cells with these two IgGs in fluorescently labeled form, followed by live-cell imaging, has led to a dynamic picture as to how IgGs with distinct binding properties for FcRn are sorted within cells. The wild-type IgG1 leaves sorting endosomes in FcRn-positive tubules and vesicles that are also involved in transferrin recycling (Ober *et al.*, 2004b) (Fig. 4.4). Recently, tubulovesicular, $FcRn^+$ transport containers (TCs) have been visualized using electron tomography of rat jejunal sections by Bjorkman and colleagues (He *et al.*, 2008) that are most likely analogous to the TCs

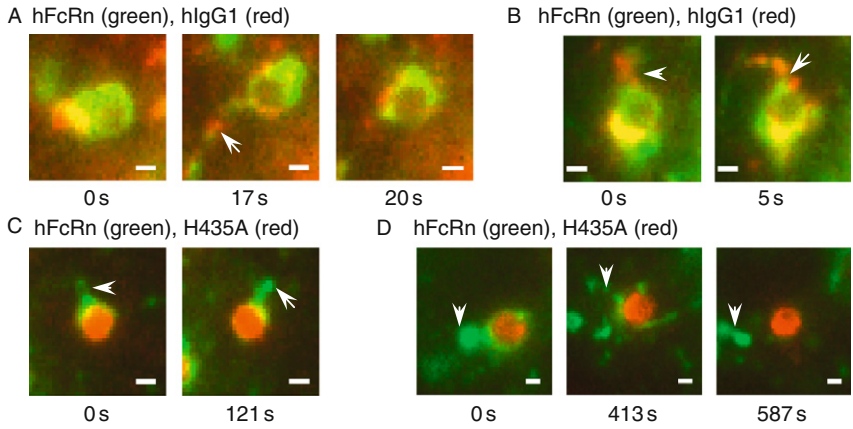

FIGURE 4.4 Individual frames from live-cell imaging of sorting endosomes in human FcRn (hFcRn)–GFP-transfected endothelial (HMEC-1) cells pulse-chased with (A, B) Alexa 546-labeled human IgG1 (hIgG1) or (C, D) Alexa 546-labeled H435A (His435 to Ala) mutant that binds with immeasurably low affinity to human FcRn (Firan *et al.*, 2001). Cells were pulsed with labeled IgG and subsequently chased in medium at 37 °C. Images of live cells were acquired and processed as described in Ober *et al.* (2004b). Arrowheads indicate tubules ($FcRn^+IgG^+$ for A, B, $FcRn^+$ only for C, D) and in (A) the tubule separates from the endosome at ~20 s. The first frame for each dataset is arbitrarily labeled 0 s, although the frames shown were taken at different times after the start of the chase period. Bar = 1 μm.

observed in transfected endothelial cells. By contrast with wild-type IgG1, in endothelial cells the H435A mutant persists in the ''vacuole'' of sorting endosomes while FcRn-positive tubules and vesicles segregate from these compartments to enter the recycling/transcytotic pathway (Ober *et al.*, 2004b). Ultimately, the H435A mutant can be detected in the lysosomes of these cells, through delivery processes that most likely involve different types of fusion events of late endosomes and lysosomes (Gan *et al.*, 2009; Luzio *et al.*, 2003). Thus, the intracellular trafficking behavior of the two IgGs correlates with their *in vivo* properties: the IgG1 molecule has a long persistence and is transported across cellular barriers, whereas the half-life of the H435A mutant is short and its delivery across cells is at background levels (Firan *et al.*, 2001; Kim *et al.*, 1999).

5.3. Exocytic processes that result in IgG release from endothelial cells

The question as to how IgG molecules are released from cells during exocytosis has also been addressed using total internal fluorescence microscopy (TIRFM) combined with single molecule imaging in live cells (Ober *et al.*, 2004a). It is interesting to note that in most cell types the steady state, cell-surface expression levels of FcRn are low (Antohe *et al.*, 2001; Dickinson *et al.*, 1999; Ghetie *et al.*, 1996; Kristoffersen and Matre, 1996; Ober *et al.*, 2004b; Roberts *et al.*, 1990). This raises the question as to whether FcRn ''cohorts'' bound IgG to the cell surface during exocytosis or whether bifurcation of FcRn and ligand occurs prior to fusion of exocytic compartments with the plasma membrane. Our live-cell imaging data demonstrated that FcRn is delivered to the plasma membrane during exocytic events (Ober *et al.*, 2004a). Using electron tomographic analyses, clathrin has been shown to be associated with both exo- and endocytic processes involving FcRn (He *et al.*, 2008). This association provides a molecular mechanism by which FcRn can be rapidly retrieved following exocytic fusion, which in turn results in low steady-state levels on the plasma membrane.

In addition to the classic type of full fusion exocytic event, using TIRFM, we observed processes in which IgG was released at the plasma membrane of endothelial cells over relatively long time periods (up to several minutes) in bursts of release, in a process that we named prolonged release (Ober *et al.*, 2004a). Multiple other types of exocytic events were also visualized, suggesting that exocytosis can occur via different processes that fall on a continuum ranging from full fusion to prolonged release. The molecular components that determine the type of exocytic event are currently unknown, but most likely relate to the local concentrations of fusion and fission effectors at the exocytic sites. Importantly, different types of exocytic processes can be observed for an individual cell

(Ober *et al.*, 2004a), indicating that these events are not predetermined by the physiological state of the cell. The biological significance of these exocytic pathways remains to be determined, and will need to be preceded by an analysis of the molecular components that regulate, for example, prolonged release versus full fusion.

The implementation of single molecule analyses of FcRn and IgG during exocytosis in endothelial cells has also allowed the behavior of individual IgG and FcRn molecules, rather than bulk populations, to be studied (Ober *et al.*, 2004a). This led to the observation of retrograde movement of IgG and FcRn molecules back to the exocytic site following exocytosis, generating insight into the molecular nature of these processes.

5.4. Imaging FcRn trafficking in three dimensions using multifocal plane microscopy

The observation of multiple different types of exocytic events using TIRFM at the plasma membrane (Ober *et al.*, 2004a) leads to the question as to which intracellular trafficking processes precede different types of release mechanisms? To address this and other questions, we have developed a multifocal plane microscopy (''MUM'') set up that allows simultaneous visualization of multiple planes within the cell combined with TIRFM imaging at the plasma membrane (Prabhat *et al.*, 2004, 2007). This approach has to date given insight into the intracellular events that precede exocytosis: for example, the recycling tubulovesicular TCs that leave sorting endosomes can be categorized into pathways of direct and indirect recycling processes. In the most direct type of recycling, tubules extend from sorting endosomes and undergo exocytosis while remaining connected (Prabhat *et al.*, 2007). By contrast, for less direct pathways, TCs accumulate in ''holding zones'' in proximity to the plasma membrane prior to exocytosis.

More recently, we have also used MUM to visualize endocytic events involving FcRn and its IgG ligand (Ram *et al.*, 2008). In these analyses, we have utilized an engineered IgG–FcRn pair of high affinity to enable receptor-mediated uptake at near neutral pH (Vaccaro *et al.*, 2005; Zhou *et al.*, 2005). Reminiscent of the analyses of exocytic processes, these studies demonstrate that endocytic processes can be broadly categorized into two classes: ''direct'' in which the endocytic TC moves rapidly toward a sorting endosome and fuses and ''indirect'' in which more circuitous itineraries are taken within the cell prior to endosomal fusion. Collectively, these studies of endo- and exocytosis have implications for understanding the dynamics of FcRn-mediated trafficking and IgG homeostasis, and may relate to the fast and slow recycling processes that have been described for transferrin and its receptor (Sheff *et al.*, 1999).

5.5. FcRn trafficking in polarized epithelial cells

In addition to studies of endothelial cells, much information concerning the intracellular trafficking of FcRn has been gleaned from analyses of Madin–Darby canine kidney (MDCK) cells transfected with human or rat FcRn (Claypool *et al.*, 2004; Tesar *et al.*, 2006), or with rat-derived inner medullary collecting duct (IMCD) cells transfected with rat FcRn (McCarthy *et al.*, 2000). Using these cells as polarized monolayers, the polarity of subcellular trafficking events such as transcytosis and recycling, together with the molecular mechanisms, have been investigated. IMCD cells express rat β2m, whereas it is essential to cotransfect human/rat β2m into (canine) MDCK cells to analyze the trafficking of human/rat FcRn in this heterologous system (Claypool *et al.*, 2002; Praetor and Hunziker, 2002; Tesar *et al.*, 2006; Zhu *et al.*, 2002). Comparison of the distribution of human and rat FcRn in transfected, polarized cells has shown that the distribution of the human receptor is strongly polarized toward the basolateral surface, whereas this bias is reversed for rat FcRn (Claypool *et al.*, 2004; McCarthy *et al.*, 2000). The distribution of human FcRn is also biased toward the basolateral surface of untransfected Caco-2 and T84 cells (both intestinal epithelial cells), indicating that its basolateral bias in MDCK cells is not due to overexpression and/or transfection (Claypool *et al.*, 2004). Indeed, the cross-species difference in basolateral bias has recently been demonstrated to be due to the presence of four potential glycosylation sites in the ectodomains of rat (mouse) FcRn, whereas human FcRn has only one such site (Kuo *et al.*, 2009). Engineering of the three additional glycosylation sites of mouse/rat FcRn into human FcRn results in increased apical localization in transfected MDCK cells (Kuo *et al.*, 2009), consistent with earlier analyses in which carbohydrate was shown to function as an apical targeting signal (Scheiffele *et al.*, 1995). The relative levels of apical and basolateral localization of FcRn impact the directionality of transcytosis. Specifically, although bidirectional transcytosis of FcRn in both transfected IMCD and MDCK cells occurs, for human FcRn more basolateral to apical transcytosis is observed relative to apical to basolateral transport, whereas this is reversed for rat FcRn (Claypool *et al.*, 2004; Kim *et al.*, 2004; McCarthy *et al.*, 2000; Tesar *et al.*, 2006). Consistent with the redistribution of a ''rodentized'' variant of human FcRn with four potential glycosylation sites to the apical surface, this FcRn mutant shows the directional bias observed for rodent FcRn, that is, preferential transcytosis of IgG in the apical to basolateral direction (Kuo *et al.*, 2009). However, in the human trophoblast cell line, BeWo, greater transcytosis by endogenous FcRn in the apical to basolateral direction occurs (Leitner *et al.*, 2006), suggesting that there may be fundamental differences in the regulation of transcytosis between different cell types. In the case of BeWo cells, this

directionality would be consistent with the role of FcRn in delivering maternal IgG across the placenta (Firan *et al.*, 2001; Story *et al.*, 1994).

5.6. Molecular determinants and effectors of FcRn trafficking

The cytosolic tail motifs of rat FcRn that regulate endocytosis and basolateral targeting have been identified by analyses of mutated FcRn variants in transfected IMCD cells (Newton *et al.*, 2005; Wernick *et al.*, 2005; Wu and Simister, 2001). Both tryptophan (W311; with tryptophan replacing the more common tyrosine in the YXXtheta motif) and dileucine (Leu322Leu323) motifs have been shown to play partially redundant roles in endocytosis (Wu and Simister, 2001). Biochemical studies have shown that the tryptophan motif directly interacts with the μ subunit of AP-2 (Wernick *et al.*, 2005). Taken together with the knowledge that dileucine motifs interact with σ and γ subunits of the adaptor protein AP-2, this has led to the suggestion that two subunits of AP-2 can bind simultaneously to the two cytosolic tail motifs (Wernick *et al.*, 2005). Both tryptophan and dileucine motifs also play a role in basolateral targeting of rat FcRn (Newton *et al.*, 2005). The tryptophan and dileucine motifs are conserved across species that range from camels to humans (Fig. 4.5), suggesting that additional differences such as variations in glycosylation patterns (Kuo *et al.*, 2009) account for cross-species variability in trafficking. However, it is interesting to note that some species (e.g., possum, cows, sheep, dromedaries, pigs, and dogs) have cytosolic tails that are 10 residues shorter than those of other species (e.g., humans, macaques, orangutans,

```
Human       RRMRSGLPAPWISLR--GDDTGVLLPTPGEAQDADLKDVNVIPATA
Orangutan   RRMRSGLPAPWISLR--GDDTGALLPTPGEAQDADSKDVNVIPATA
Macaque     RRMRSGLPAPWISLR--GDDTGSLLPTPGEAQDADSKDINVIPATA
Ovine       RRMRKGLPASWISFR--GEDVGALLPTPGLSKDGES----------
Bovine      RRMRKGLPAPWISFR--GEDVGALLPTPGLSKDGES----------
Dromedary   RR-RKGLPAPWIAFR--GDDIGALLPTPGLSKDAES----------
Swine       RRMRKGLPAPWISFH--GDDVGALLPTPDLAKDAES----------
Canine      RRMRKGLPAPWMSLR--GDDVGALLPTPGVPKDADS----------
Rat         NRMRSGLPAPWLSLS--GDDSGDLLPGGNLPPEAEPQGVNAFPATS
Mouse       GRMRSGLPAPWLSLS--GDDSGDLLPGGNLPPEAEPQGANAFPATS
Possum      SRKRGARPAPWIFRRRAGDDVGSLLSAPASAQDSSP----------
Rabbit      RRRR-GLPAPWVFLR--GDDIRTLLP-----QDEGPQDVSAFPATA
Consensus   -R-R---PA-W------G-D---LL----------------------
```

FIGURE 4.5 Cytosolic tail sequences of FcRn from different species (Adamski *et al.*, 2000; Ahouse *et al.*, 1993; Kacskovics *et al.*, 2000, 2006; Kandil *et al.*, 1995; Mayer *et al.*, 2002; Schnulle and Hurley, 2003; Simister and Mostov, 1989; Story *et al.*, 1994). Identity is indicated by red, and the first residue of the sequence corresponds to residue 301 of mouse/rat FcRn. The consensus sequence is also shown, with the tryptophan (W311) and dileucine (L322, L323) motifs that are important for intracellular trafficking indicated in blue.

rats, and mice) (Fig. 4.5). In addition, the cytosolic tail of possum has a two-residue insertion, whereas rabbit has a five amino acid deletion (Fig. 4.5). Whether these differences are functionally relevant remains to be tested.

Recent studies have identified a motif in the cytosolic tail of human FcRn encompassing Arg301, Arg302 (Fig. 4.5) that binds to calmodulin (Dickinson *et al.*, 2008). Ablation of this interaction by mutagenesis of FcRn results in reduced transcytosis and decreased stability of this receptor (Dickinson *et al.*, 2008). Since calmodulin binding to FcRn would mask a putative amphipathic α-helix that in other proteins can insert into the membrane and induce or sense curvature (Ford *et al.*, 2002; Lee *et al.*, 2005; McMahon and Gallop, 2005), this might provide a mechanism through which calmodulin can affect endosomal sorting. Together with the knowledge that calmodulin function is highly regulatable, this could constitute an important pathway for the control of FcRn trafficking.

In the context of possible regulators of the intracellular pathways taken by FcRn, several studies indicate that Rab proteins play a role (Tzaban *et al.*, 2009; Ward *et al.*, 2005). These small Ras-like GTPases are known to play regulatory functions in endocytic and exocytic trafficking (Miaczynska and Zerial, 2002; Somsel and Wandinger-Ness, 2000). The activity of this class of GTPases is controlled by GTP–GDP exchange cycles, and such proteins exist in either membrane-bound or cytosolic forms. In combination with proteins such as soluble NSF attachment protein receptors (SNAREs) that usually exist as transmembrane receptors (Jahn *et al.*, 2003), Rabs are key regulators of fusion events between different membranous compartments (Grosshans *et al.*, 2006; Miaczynska and Zerial, 2002; Somsel and Wandinger-Ness, 2000). Due to the pivotal role that Rabs play in intracellular trafficking, it is therefore of interest to understand which of these proteins are associated with FcRn.

Using fluorescence imaging, we observed that Rab4(a), Rab5(a), and Rab11(a) are all present on $FcRn^+$ endosomes (Ward *et al.*, 2005). Rab4 and Rab11 are known to be involved in recycling cargo from sorting endosomes to the plasma membrane (Daro *et al.*, 1996; Green *et al.*, 1997; Sönnichsen *et al.*, 2000; Ullrich *et al.*, 1996; van der Sluijs *et al.*, 1992), whereas Rab5 is an early endosomal marker (Christoforidis *et al.*, 1999; Simonsen *et al.*, 1998). Although FcRn can be sorted into tubulovesicular TCs in $Rab4^+Rab11^+$ or $Rab11^+$ compartments, only Rab11 but not Rab4 is associated with FcRn during exocytic events at the plasma membrane (Ward *et al.*, 2005). Rab4 depletion from these TCs occurs via the formation of discrete $Rab4^+$ domains that can subsequently separate. The distribution of Rab5, Rab11 and the late endosomal markers Rab7 and Rab 9 (Bucci *et al.*, 2000; Soldati *et al.*, 1995) with tubulovesicular TCs that transport IgG/Fc in the neonatal rodent gut has also been analyzed using electron tomography (He *et al.*, 2008). These studies demonstrate that

compartments on the endolysosomal pathway cannot be segregated into groups based on their Rab associations. Rather, there is overlap between the Rabs that are associated with different compartments, consistent with models of ''Rab conversion'' in which Rabs are gradually lost and replaced by different Rab proteins as endosomes mature (Rink *et al.*, 2005).

Recently, we have analyzed the intracellular trafficking pathways, including Rab GTPases, involved in the constitutive degradation of FcRn in endothelial cells (Gan *et al.*, 2009). Transfer of FcRn from late endosomes to lysosomes occurs via kiss-and-linger-like processes (Bright *et al.*, 2005; Gandhi and Stevens, 2003; Ryan, 2003; Storrie and Desjardins, 1996) that frequently involve tubular extensions, whereas full fusion of late endosomes and lysosomes is rarely observed (Gan *et al.*, 2009). Unexpectedly, in our studies, the ''early endosomal'' marker Rab5 persists on the limiting membrane of late endosomes until a relatively late stage in maturation. This suggests that (late) endosomes have functional plasticity due to the presence of both Rab5 and Rab7, allowing FcRn to leave these compartments to enter the recycling or lysosomal pathways. Consequently, this prolongs the time window during which FcRn (or other receptors) can be sorted into distinct pathways during endosomal maturation, and might provide a mechanism by which increased fidelity in sorting can be achieved.

Given the potential of FcRn as a drug delivery vehicle, it is of considerable interest to understand the molecular effectors that regulate recycling versus transcytosis in polarized cells. Insight into this has recently been generated by the observation that Rab25, a Rab GTPase that is known to be involved in the transcytosis of IgA by pIgR (Casanova *et al.*, 1999; Wang *et al.*, 2000), also regulates the transcytosis of human FcRn in polarized epithelial (MDCK) cells (Tzaban *et al.*, 2009). By contrast, Rab11a is not involved in transcytosis but is an important player in recycling to the basolateral, but not apical, membrane of polarized MDCK cells (Tzaban *et al.*, 2009). These observations are consistent with the concept that in epithelial cells, at least, there are endosomal compartments that have functional plasticity, insofar as sorting into both transcytotic and recycling pathways can occur from the same common endosome (Casanova *et al.*, 1999; Thompson *et al.*, 2007; Tzaban *et al.*, 2009; Wang *et al.*, 2000). These studies have significant potential for regulating the directionality of FcRn-mediated transport.

5.7. Effects of ligand valency on intracellular trafficking

To date, the majority of studies of the cell biology of FcRn and its IgG ligand have been carried out using monomeric IgG that has two possible interaction sites for FcRn. Indeed, two active binding sites per IgG or Fc

molecule have been shown to be important for activity in FcRn-mediated functions that include transcytosis, recycling, and *in vivo* half-life (Kim *et al.*, 1994b,c; Tesar *et al.*, 2006). It is interesting that a hybrid Fc with only one functional FcRn interaction site is transported more efficiently into lysosomes in rat FcRn-transfected MDCK cells relative to wild-type Fc that has two possible interaction sites (Tesar *et al.*, 2006). Whether this effect is due to a higher off-rate of the Fc from FcRn in endosomes and/or a difference in trafficking induced by FcRn dimerization is currently unknown.

The ligand for FcRn can also be highly multimeric when IgGs form ICs with cognate antigen. It is, therefore, of interest to compare the intracellular trafficking of monomeric IgGs with that of multivalent ICs. It has recently been shown that ICs with the propensity to cross-link FcRn preferentially traffic into lysosomes, thereby enhancing antigen presentation in dendritic cells (Qiao *et al.*, 2008). It remains to be demonstrated whether this trafficking pathway is specific for APCs. This might be the case, since a recent report demonstrated that invariant chain, which is expressed in professional APCs, directs the transport of FcRn into lysosomes (Ye *et al.*, 2008). Such a process results in a pathway for the enhancement of T cell responses by ICs, thereby providing an additional link between humoral and cellular immunity. By contrast, the transport of ICs in intact form across cells such as epithelial barriers (Yoshida *et al.*, 2004) might be enabled by the lack of invariant chain in these cells, at least under steady-state, noninflammatory conditions.

6. REGULATION OF FcRn EXPRESSION

FcRn represents a receptor that is subject to both tissue-specific and developmental regulation. For example, following the suckling period of neonatal rodents, a dramatic decrease in FcRn expression in intestine occurs (Ghetie *et al.*, 1996; Martin *et al.*, 1997). FcRn levels are down-regulated by hormones such as corticosteroids and thyroxine that are known to affect gastrointestinal adaptation during the neonatal period (Capano *et al.*, 1994; Martin *et al.*, 1993; Morris and Morris, 1976). The promoter regions for human and rodent FcRn have been analyzed and indicate that the regulation of expression at the transcriptional level is complex with sites for Sp-like transcription factors, AP-1, Ets, or NF-IL6 (Jiang *et al.*, 2004; Kandil *et al.*, 1995; Tiwari and Junghans, 2005). Given the immunological relevance of FcRn, it is plausible that modulation of expression and/or activity by inflammatory (or anti-inflammatory) mediators such as cytokines might occur. In this context, recent studies have shown that the expression levels of human FcRn in *in vitro* cell lines can be regulated by cytokines such as TNF-α and IFN-γ (Liu *et al.*, 2007b, 2008).

Although these cytokines are classically associated with proinflammatory effects, much data supports the concept that they can also be anti-inflammatory (Chu *et al.*, 2000; Cope *et al.*, 1997; Isomaki *et al.*, 2001; Kassiotis and Kollias, 2001; Liu *et al.*, 1998; Willenborg *et al.*, 1999a,b). It is, therefore, interesting that while TNF-α and IL-1β upregulate the transcription of FcRn through NFκB binding to intronic sequences of FcRn (Liu *et al.*, 2007b), IFN-γ has the reverse effect by activating JAK/STAT-1 signaling (Liu *et al.*, 2008). Consequently, the factors that control the expression of FcRn and MHC Class I molecules are distinct, since IFN-γ is known to upregulate the levels of the latter. How FcRn expression and function might be modulated by both anti- and proinflammatory cytokines and possibly other immune mediators such as chemokines has broad implications for understanding the factors that regulate inflammatory responses. This area offers multiple possibilities for further exploration.

7. THE COMPLEXITY OF ENGINEERING FcRn–IgG INTERACTIONS

7.1. Antibody engineering: From variable to constant regions

Much of antibody engineering over the past two decades has been directed toward the manipulation of antibody variable regions for both targeting and blocking effects (Souriau and Hudson, 2003; Weiner and Carter, 2005). By contrast, the modification of Fc regions to alter their interactions with Fc receptors, particularly to impact FcRn function, is relatively underdeveloped. Fc engineering has obvious implications for the application of therapeutic antibodies (Carter, 2006; Ghetie *et al.*, 1997), and interest in this area is currently expanding (Dall'Acqua *et al.*, 2006a; Hinton *et al.*, 2004; Lazar *et al.*, 2006; Shields *et al.*, 2001; Vaccaro *et al.*, 2005, 2006). Although of considerable importance, recent studies describing the engineering of Fc regions for the enhancement of FcγR binding (e.g., Lazar *et al.*, 2006; Shields *et al.*, 2001) fall outside the scope of the current review and will not be discussed further. However, it is important to point out that the sites for FcRn and FcγR interactions on IgG are distinct (Duncan *et al.*, 1988; Jefferis *et al.*, 1998; Kim *et al.*, 1994b; Shields *et al.*, 2001), so that in general mutations that impact FcRn binding do not affect function in FcγR-dependent assays and vice versa. In the cases where effects on both functionalities are observed (e.g., Shields *et al.*, 2001), this is most likely due to longer range conformational perturbations.

We will first describe how FcRn–IgG interactions can be modified to generate antibodies with altered pharmacokinetics and transport properties, and subsequently discuss how FcRn itself can be targeted to modulate IgG levels *in vivo*. The knowledge that albumin is dependent on

FcRn for *in vivo* persistence (Andersen *et al.*, 2006; Chaudhury *et al.*, 2003) can also be exploited by using therapeutic reagents fused to albumin binding peptides or Ig domains with the aim of generating longer lived therapeutics (Dennis *et al.*, 2002; Holt *et al.*, 2008; Nguyen *et al.*, 2006; Stork *et al.*, 2007), but will not be discussed further here.

7.2. Modulating the pharmacokinetic properties of IgG: The importance of pH dependence

The knowledge that FcRn regulates serum IgG levels (Ghetie *et al.*, 1996; Israel *et al.*, 1996; Junghans and Anderson, 1996), together with structure–function studies of FcRn–IgG interactions, presents possibilities for the modulation of the *in vivo* persistence and/or transcellular transport of (therapeutic) antibodies. The approach of ''tuning'' antibody half-lives by altering FcRn–IgG interactions has obvious relevance to the successful use of therapeutic and diagnostic antibodies. Mouse IgG1-derived Fc fragments that are engineered and selected to have increased affinity for FcRn at pH 6, but with retention of low affinity at near neutral pH, persist for longer in the circulation of mice (Ghetie *et al.*, 1997). This approach has subsequently been used to generate engineered human IgGs that have longer half-lives in primates (Dall'Acqua *et al.*, 2006b; Hinton *et al.*, 2004, 2006) and are transported more efficiently across the *ex vivo* human placenta (Vaccaro *et al.*, 2006).

Although several reports describe a correlation between FcRn-binding properties of engineered IgGs and *in vivo* persistence/transport (Dall'Acqua *et al.*, 2006b; Ghetie *et al.*, 1997; Hinton *et al.*, 2004, 2006; Vaccaro *et al.*, 2006), other studies would appear to contradict this (Datta-Mannan *et al.*, 2007a,b; Gurbaxani and Morrison, 2006; Gurbaxani *et al.*, 2006). This apparently discordant data can be explained in several cases by increased binding of engineered antibodies to FcRn at near neutral pH, which in general occurs as the affinity at pH 6 is improved (Dall'Acqua *et al.*, 2002; Vaccaro *et al.*, 2006). In this context, FcRn–IgG interactions can be distinguished from the majority of other protein–protein interactions by their marked pH dependence. Consequently, there is not a linear relationship between increase in affinity and activity. Gain of significant binding activity at near neutral pH results in reduced release during exocytosis at the plasma membrane and enhanced trafficking of the antibody into lysosomes (Gan *et al.*, 2009). Furthermore, such engineered IgGs accumulate very efficiently in cells since they are taken up by receptor (FcRn)-mediated processes (Mi *et al.*, 2008; Vaccaro *et al.*, 2005, 2006). As the affinity at pH 6 is increased, the concomitant improvement in binding at near neutral pH therefore mitigates the factors such as elevated recycling that lead to longer half-life. The difficulty in separating enhancement in affinities at pH 6 and 7.4 during the engineering of FcRn–

IgG interactions therefore limits the increase in *in vivo* persistence that is achievable (discussed in Vaccaro *et al.*, 2006), and this presents a significant challenge in Fc engineering. It is also important to note that some cases of apparent discrepancies between binding data and *in vivo* half-lives could be due to the interaction models for FcRn–IgG complexes that are used and/or the introduction of valency effects induced by immobilization of FcRn on the sensor chip during SPR analyses (Datta-Mannan *et al.*, 2007a; Gurbaxani and Morrison, 2006).

In addition to the detrimental effect of gain of binding at near neutral pH on *in vivo* persistence, shorter lived antibodies can alternatively be generated by engineering IgGs or Fc fragments so that they do not bind detectably to FcRn at any pH (Kim *et al.*, 1994a; Medesan *et al.*, 1997). Such ''FcRn-null'' antibodies also function poorly in other FcRn-mediated functions such as transport across cellular barriers (Firan *et al.*, 2001; Spiekermann *et al.*, 2002). Although in general not useful in therapeutic settings, FcRn-null antibodies have uses in applications such as tumor imaging where short persistence is desirable to minimize background signal (Kenanova *et al.*, 2005; Olafsen *et al.*, 2006).

7.3. Generation of inhibitors of FcRn function to lower endogenous IgG levels

A prediction of the model shown in Fig. 4.3 is that inhibition of FcRn function will lead to enhanced degradation of IgGs and a reduction in IgG transport. FcRn inhibition can be achieved by injecting relatively large quantities of intravenous immunoglobulin (IVIG) (Akilesh *et al.*, 2004; Hansen and Balthasar, 2002; Jin and Balthasar, 2005). The IgG in these high doses of IVIG competes with endogenous IgG for binding and can reduce pathology in IgG-mediated disease (Akilesh *et al.*, 2004; Hansen and Balthasar, 2002; Jin and Balthasar, 2005; Masson, 1993). IVIG can also be used following the delivery of radiolabeled antitumor antibodies to increase their therapeutic and diagnostic efficacy (Jaggi *et al.*, 2007), resulting in enhancement of whole body clearance of radiolabeled IgG and less nonspecific radiation damage.

In many applications in which IVIG is currently used to enhance the clearance of endogenous IgGs, FcRn blockers that have higher affinity for FcRn relative to endogenous wild-type IgGs could be used at substantially lower doses. For example, anti-FcRn or anti-β2m antibodies that block Fc/IgG binding to FcRn through variable region binding have been shown to be effective in treating ITP and myasthenia gravis, respectively, in rodent models by lowering the levels of pathogenic IgGs (Getman and Balthasar, 2005; Liu *et al.*, 2007a). We have also generated engineered IgGs (''MST-HN'' and ''HN'') derived from human IgG1 that bind through their Fc regions to FcRn with increased affinity (~200-fold at pH 6 relative

to mouse IgG1) and reduced pH dependence (Vaccaro *et al.*, 2005, 2006). These engineered IgGs act as competitive inhibitors with wild-type IgGs for FcRn binding and can enhance the clearance of endogenous IgGs in mice (Vaccaro *et al.*, 2005, 2006) (Fig. 4.6). Such engineered antibodies (Abdegs, for *antibodies* that enhance IgG *deg*radation) have potential uses in modulating endogenous IgG levels. In support of this, a human IgG1 variant (Thr307 to Ala/Glu380 to Ala/Asn434 to Ala) with increased affinity for mouse/human FcRn at both pH 6 and 7.4 has been shown to be effective in treating disease in a serum transfer model of arthritis (Petkova *et al.*, 2006). However, the relatively high doses needed in this study were most likely due to retention of significant pH dependence for binding to FcRn of this antibody, that is, low affinity at near neutral pH (Petkova *et al.*, 2006), which results in poor competitive activity (Vaccaro *et al.*, 2006).

The effects of Abdegs on endogenous IgG levels can be regarded to be ''extrinsic,'' in contrast to ''intrinsic'' effects that impact the half-life of the engineered IgG itself. In this context, due to the loss of pH-dependent binding to FcRn, both Abdegs and anti-FcRn antibodies (that bind to FcRn

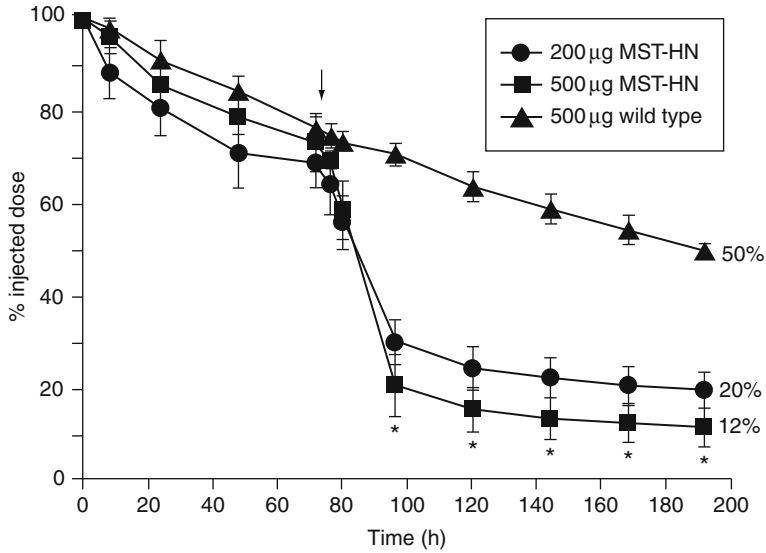

FIGURE 4.6 Enhancement of clearance of injected wild-type IgG by an Abdeg (Vaccaro *et al.*, 2005). Mice were injected with ^{125}I-labeled wild-type human IgG1, and injected with 500 µg wild-type human IgG1, 200 or 500 µg Abdeg (MST-HN mutant) 72 h later (indicated by arrow). Levels of remaining ^{125}I labeled IgG were determined at the indicated times. Error bars indicate standard deviations. * Indicates that data for these time points for mice treated with 500 or 200 µg Abdeg are significantly different.

through their V regions) have short *in vivo* half-lives (Dall'Acqua *et al.*, 2002; Getman and Balthasar, 2005; Vaccaro *et al.*, 2006). Consistent with this, delivery of Abdegs results in a reduction of serum IgG levels that lasts for several days prior to a rebound of IgG concentrations to their original levels (Vaccaro *et al.*, 2005). The ''intrinsic'' consequences of an Fc mutation on the *in vivo* half-life of an IgG or Fc fragment itself will impact the ''extrinsic'' effects of this engineered IgG/Fc on the lowering of endogenous IgG levels, since they will determine the *in vivo* longevity of a potential Abdeg.

Insight at the quantitative level as to how a change in pH dependence of an IgG–FcRn interaction impacts *in vivo* persistence has been obtained by comparing the properties of two engineered human IgG1 molecules, HN and MST-HN (HN, His433 to Lys/Asn434 to Phe; MST-HN, Met252 to Tyr/Ser254 to Thr/Thr256 to Glu/His433 to Lys/Asn434 to Phe) (our unpublished data). These two mutants have similar affinities for mouse FcRn at pH 6, whereas the affinity of the HN mutant is about 10-fold lower at pH 7.2 (Table 4.1). This has allowed the impact of differences in pH dependence on intrinsic (*in vivo* half-life) and extrinsic (lowering of endogenous IgG levels) properties to be assessed in mice. The HN mutant is less effective in lowering endogenous IgG levels than MST-HN, but the HN mutant has a longer *in vivo* persistence (Vaccaro *et al.*, 2005, 2006) (Table 4.1). Thus, there is a trade-off between activity as an FcRn inhibitor and *in vivo* half-life. This indicates that, dependent on the situation, these extrinsic and intrinsic properties need to be counterbalanced to optimize the effect. For example, if a ''one-off'' rapid clearance of endogenous IgG is needed, then an Abdeg with high affinities for FcRn in the range pH 6–7.4 is expected to be optimal. Conversely, if treatment of an IgG-mediated, chronic disease is required, then a balance between reduced half-life and inhibitory activity needs to be achieved.

Synthetic peptides that block the binding of endogenous IgGs to FcRn in nonhuman primates have also been described (Mezo *et al.*, 2008). One

TABLE 4.1 The impact of pH dependence on *in vivo* half-life in mice of engineered variants of human IgG1

	Dissociation constant, K_D (nM)[a]		
Human IgG1 (mutant)	pH 6	pH 7.2	β-Phase half-life (h)
Wild type	32	N.D.[b]	250.6 ± 15.3[a]
MST-HN	1.2	7.4	35.6 ± 1.1
HN	1.5	82	62.8 ± 2.7[a]

[a] Described previously in Vaccaro *et al.* (2005, 2006).
[b] N.D., not determined because affinity is too low to accurately estimate a dissociation constant.

such peptide has been used to make a dimer that is active in reducing serum IgG levels in cynomolgus monkeys (Mezo *et al.*, 2008). The antibody levels rebound in peptide-treated monkeys, consistent with the clearance of the peptide. FcRn-binding peptides, or engineered IgGs/Fc fragments with increased affinity for FcRn, have multiple potential uses and offer an alternative to the use of IVIG which needs to be delivered in relatively high doses for efficacy. In addition, the sources of IVIG are limited and its expense is high. However, in addition to FcRn blockade, IVIG has multiple other possible modes of action that include FcγR-mediated effects (discussed in Clynes, 2007). For example, in mice the monomeric IgG component of IVIG can induce the upregulation of FcγRIIB expression (Bruhns *et al.*, 2003; Samuelsson *et al.*, 2001; Siragam *et al.*, 2005). IVIG treatment can also result in signaling by ICs through the activating receptor, FcγRIII, to inhibit IFN-γ responses or regulate dendritic cell activity in mice (Park-Min *et al.*, 2007; Siragam *et al.*, 2006). Recent studies have shown that monomeric IgGs with sialylated core oligosaccharides, that constitute about 1–2% of IVIG, are responsible for the upregulation of FcγRIIB expression through a mechanism that involves binding to SIGN-R1 (in mice) or DC-SIGN (in humans) (Anthony *et al.*, 2008; Kaneko *et al.*, 2006). For the treatment of IgG-mediated, inflammatory diseases, it is therefore possible that due to the induction of additional anti-inflammatory effects, the use of (engineered) antibodies might be preferable over the use of FcRn-binding peptides that solely target FcRn.

8. CROSS-SPECIES DIFFERENCES IN FcRn-BINDING SPECIFICITY AND IMPLICATIONS FOR PRECLINICAL MODELS

Despite the similarities of human and mouse FcRn at the sequence level (Ahouse *et al.*, 1993; Story *et al.*, 1994), in addition to the conservation of several key interaction residues on IgG across species, the binding specificity of human and mouse FcRn are distinct (Ober *et al.*, 2001). For example, mouse FcRn binds promiscuously to IgGs from multiple species, whereas human FcRn is much more selective. Most notably, although human FcRn interacts with relatively low affinity with mouse IgG2b, it does not bind detectably to mouse IgG1, IgG2a, or rat IgGs. This lack of binding provides a molecular explanation for the short *in vivo* persistence of (therapeutic) mouse IgGs in humans (Frodin *et al.*, 1990; Saleh *et al.*, 1992).

Using the earlier crystallographic structure of the rat FcRn–rat IgG2a complex (Martin *et al.*, 2001) as a guide, we have used site-directed mutagenesis combined with interaction analyses to transfer the binding

properties of mouse FcRn onto human FcRn (Zhou *et al.*, 2003, 2005). With this approach, several regions of sequence variation are responsible for the specificity differences between mouse and human FcRn: first, residues 132–147, encompassing the nonconserved residue 137 (Leu in human FcRn, Glu in mouse FcRn, Asp in rat FcRn) play a central role (Zhou *et al.*, 2003, 2005). The important role of residue 137, in particular, is consistent with crystallographic and structure–function studies for rat FcRn (Martin and Bjorkman, 2001; Vaughn *et al.*, 1997). Second, residues 79–89 (which in human FcRn encompass a two-residue deletion) have a lesser contribution to the difference in specificity and may modulate the overall orientation of the interaction (Zhou *et al.*, 2005). Residues 79–89 also contain a potential glycosylation site in rodent, but not human, FcRn, leading to the possibility that this might contribute to the cross-species difference in binding properties. However, recent studies (Kuo *et al.*, 2009) have shown that glycosylation at position 87 (numbering based on homology alignment with rodent FcRn) of human FcRn, by mutation of Lys to Asn, does not confer the binding properties of mouse/rat FcRn for mouse IgG1 on the human ortholog (Kuo *et al.*, 2009). It is interesting to note that species such as pigs, sheep, camels, and cows have arginine at position 137 (Kacskovics *et al.*, 2000, 2006; Mayer *et al.*, 2002; Schnulle and Hurley, 2003), whereas dog, rat, and mouse have glutamic/aspartic acid (Ahouse *et al.*, 1993; Kacskovics *et al.*, 2006; Simister and Mostov, 1989) and possum has the same residue as humans (leucine) (Adamski *et al.*, 2000). Given the central role of residue 137 in FcRn–IgG interactions, this leads to the speculation that binding specificities might fall into three or more clades.

In general, the affinities of mouse FcRn for IgGs of multiple different species such as human, rat, mouse, and rabbit are higher than the corresponding human FcRn interactions (Ober *et al.*, 2001). This is of relevance when considering the preclinical analysis of human IgGs in murine models since, for example, the affinity of mouse FcRn for human IgG1 is about 10-fold higher than that of the corresponding human FcRn interaction (Zhou *et al.*, 2005). Consequently, although the mouse FcRn–wild-type human IgG1 interaction retains sufficient pH dependence for this IgG1 to have a relatively long half-life in mice, this is not the case for multiple variants of human IgG(1) that have been engineered to have higher affinity for FcRn (Dall'Acqua *et al.*, 2002; Vaccaro *et al.*, 2006). Specifically, a higher affinity IgG mutant can acquire significant binding to mouse FcRn at near neutral pH while retaining the necessary low affinity for human FcRn to allow efficient recycling in human systems (Vaccaro *et al.*, 2006). Consequently, such IgGs have shortened *in vivo* half-lives and inhibit FcRn function in mice (Dall'Acqua *et al.*, 2002; Vaccaro *et al.*, 2005), whereas analyses in nonhuman primates (Dall'Acqua *et al.*, 2006b) or the human placental transfer model (Vaccaro *et al.*, 2006) are predictive of longer half-lives in humans.

Several engineered IgGs of this class have to date been described (Dall'Acqua *et al.*, 2002, 2006b; Datta-Mannan *et al.*, 2007a,b; Vaccaro *et al.*, 2006), indicating the severe limitations of mice as models. Consequently, there is a need for improved preclinical models that can recapitulate human FcRn function. Although nonhuman primates represent good models, their high cost makes them inaccessible for routine screens. Alternatively, mice that transgenically express human FcRn (Chaudhury *et al.*, 2003; Petkova *et al.*, 2006) are a step toward a suitable preclinical model, but have low endogenous IgG levels due to poor binding of mouse IgGs to human FcRn. Such mice combined with transgenic mice expressing human IgGs (Jakobovits *et al.*, 2007; Scott, 2007) might therefore provide an attractive model.

9. CONCLUDING REMARKS

Much has been learnt about FcRn function during the past two decades. Perhaps most importantly, a diverse array of activities at different body sites can be attributed to this multitasking receptor. Furthermore, FcRn impacts both the humoral and cellular arms of the immune response. Consequently, understanding the molecular and cellular mechanisms by which this receptor functions, combined with the engineering of FcRn–IgG interactions, has relevance to fundamental aspects of the immune system in addition to providing possible therapeutic routes for multiple diseases.

ACKNOWLEDGMENTS

We thank our many colleagues and coworkers who have contributed to our FcRn-related studies.

The research of the authors reported herein was supported in part by grants from the NIH to E.S.W. (RO1 AI039167, RO1 AI055556, and RO1 AR056478) and R.J.O. (RO1 GM071048 and RO1 GM085575).

REFERENCES

Adamski, F. M., King, A. T., and Demmer, J. (2000). Expression of the Fc receptor in the mammary gland during lactation in the marsupial *Trichosurus vulpecula* (brushtail possum). *Mol. Immunol.* **37,** 435–444.

Ahouse, J. J., Hagerman, C. L., Mittal, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Simister, N. E. (1993). Mouse MHC class I-like Fc receptor encoded outside the MHC. *J. Immunol.* **151,** 6076–6088.

Akilesh, S., Petkova, S., Sproule, T. J., Shaffer, D. J., Christianson, G. J., and Roopenian, D. (2004). The MHC class I-like Fc receptor promotes humorally mediated autoimmune disease. *J. Clin. Invest.* **113,** 1328–1333.

Akilesh, S., Christianson, G. J., Roopenian, D. C., and Shaw, A. S. (2007). Neonatal FcR expression in bone marrow-derived cells functions to protect serum IgG from catabolism. *J. Immunol.* **179,** 4580–4588.

Akilesh, S., Huber, T. B., Wu, H., Wang, G., Hartleben, B., Kopp, J. B., Miner, J. H., Roopenian, D. C., Unanue, E. R., and Shaw, A. S. (2008). Podocytes use FcRn to clear IgG from the glomerular basement membrane. *Proc. Natl. Acad. Sci. USA* **105,** 967–972.

Andersen, J. T., Dee, Q. J., and Sandlie, I. (2006). The conserved histidine 166 residue of the human neonatal Fc receptor heavy chain is critical for the pH-dependent binding to albumin. *Eur. J. Immunol.* **36,** 3044–3051.

Anthony, R. M., Wermeling, F., Karlsson, M. C., and Ravetch, J. V. (2008). Identification of a receptor required for the anti-inflammatory activity of IVIG. *Proc. Natl. Acad. Sci. USA* **105,** 19571–19578.

Antohe, F., Radulescu, L., Gafencu, A., Ghetie, V., and Simionescu, M. (2001). Expression of functionally active FcRn and the differentiated bidirectional transport of IgG in human placental endothelial cells. *Hum. Immunol.* **62,** 93–105.

Bitonti, A. J., and Dumont, J. A. (2006). Pulmonary administration of therapeutic proteins using an immunoglobulin transport pathway. *Adv. Drug Deliv. Rev.* **58,** 1106–1118.

Bitonti, A. J., Dumont, J. A., Low, S. C., Peters, R. T., Kropp, K. E., Palombella, V. J., Stattel, J. M., Lu, Y., Tan, C. A., Song, J. J., Garcia, A. M., Simister, N. E., *et al*. (2004). Pulmonary delivery of an erythropoietin Fc fusion protein in non-human primates through an immunoglobulin transport pathway. *Proc. Natl. Acad. Sci. USA* **101,** 9763–9768.

Borvak, J., Richardson, J., Medesan, C., Antohe, F., Radu, C., Simionescu, M., Ghetie, V., and Ward, E. S. (1998). Functional expression of the MHC class I-related receptor, FcRn, in endothelial cells of mice. *Int. Immunol.* **10,** 1289–1298.

Brambell, F. W. R. (1970). The Transmission of Passive Immunity from Mother to Young. North Holland Publishing Corp., Amsterdam.

Brambell, F. W. R., Hemmings, W. A., and Morris, I. G. (1964). A Theoretical Model of γ-globulin catabolism. *Nature* **203,** 1352–1355.

Bright, N. A., Gratian, M. J., and Luzio, J. P. (2005). Endocytic delivery to lysosomes mediated by concurrent fusion and kissing events in living cells. *Curr. Biol.* **15,** 360–365.

Bruhns, P., Samuelsson, A., Pollard, J. W., and Ravetch, J. V. (2003). Colony-stimulating factor-1-dependent macrophages are responsible for IVIG protection in antibody-induced autoimmune disease. *Immunity* **18,** 573–581.

Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J., and van Deurs, B. (2000). Rab7: A key to lysosome biogenesis. *Mol. Biol. Cell* **11,** 467–480.

Capano, G., Bloch, K. J., Schiffrin, E. J., Dascoli, J. A., Israel, E. J., and Harmatz, P. R. (1994). Influence of the polyamine, spermidine, on intestinal maturation and dietary antigen uptake in the neonatal rat. *J. Pediatr. Gastroenterol. Nutr.* **19,** 34–42.

Carter, P. J. (2006). Potent antibody therapeutics by design. *Nat. Rev. Immunol.* **6,** 343–357.

Casanova, J. E., Wang, X., Kumar, R., Bhartur, S. G., Navarre, J., Woodrum, J. E., Altschuler, Y., Ray, G. S., and Goldenring, J. R. (1999). Association of Rab25 and Rab11a with the apical recycling system of polarized Madin–Darby canine kidney cells. *Mol. Biol. Cell* **10,** 47–61.

Chaudhury, C., Mehnaz, S., Robinson, J. M., Hayton, W. L., Pearl, D. K., Roopenian, D. C., and Anderson, C. L. (2003). The major histocompatibility complex-related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan. *J. Exp. Med.* **197,** 315–322.

Christoforidis, S., McBride, H. M., Burgoyne, R. D., and Zerial, M. (1999). The Rab5 effector EEA1 is a core component of endosome docking. *Nature* **397,** 621–625.

Chu, C. Q., Wittmer, S., and Dalton, D. K. (2000). Failure to suppress the expansion of the activated CD4 T cell population in interferon gamma-deficient mice leads to exacerbation of experimental autoimmune encephalomyelitis. *J. Exp. Med.* **192,** 123–128.

Claypool, S. M., Dickinson, B. L., Yoshida, M., Lencer, W. I., and Blumberg, R. S. (2002). Functional reconstitution of human FcRn in Madin–Darby canine kidney cells requires co-expressed human beta 2-microglobulin. *J. Biol. Chem.* **277,** 28038–28050.

Claypool, S. M., Dickinson, B. L., Wagner, J. S., Johansen, F.-E., Venu, N., Borawski, J. A., Lencer, W. I., and Blumberg, R. S. (2004). Bidirectional transepithelial IgG transport by a strongly polarized basolateral membrane Fc-γ receptor. *Mol. Biol. Cell* **15,** 1746–1759.

Clynes, R. (2007). IVIG therapy: Interfering with interferon-gamma. *Immunity* **26,** 4–6.

Cope, A. P., Liblau, R. S., Yang, X. D., Congia, M., Laudanna, C., Schreiber, R. D., Probert, L., Kollias, G., and McDevitt, H. O. (1997). Chronic tumor necrosis factor alters T cell responses by attenuating T cell receptor signaling. *J. Exp. Med.* **185,** 1573–1584.

Dall'Acqua, W., Woods, R. M., Ward, E. S., Palaszynski, S. R., Patel, N. K., Brewah, Y. A., Wu, H., Kiener, P. A., and Langermann, S. (2002). Increasing the affinity of a human IgG1 to the neonatal Fc receptor: Biological consequences. *J. Immunol.* **169,** 5171–5180.

Dall'Acqua, W. F., Cook, K. E., Damschroder, M. M., Woods, R. M., and Wu, H. (2006a). Modulation of the effector functions of a human IgG1 through engineering of its hinge region. *J. Immunol.* **177,** 1129–1138.

Dall'Acqua, W. F., Kiener, P. A., and Wu, H. (2006b). Properties of human IgG1s engineered for enhanced binding to the neonatal Fc receptor (FcRn). *J. Biol. Chem.* **281,** 23514–23524.

Daro, E., van der Sluijs, P., Galli, T., and Mellman, I. (1996). Rab4 and cellubrevin define different early endosome populations on the pathway of transferrin receptor recycling. *Proc. Natl. Acad. Sci. USA* **93,** 9559–9564.

Datta-Mannan, A., Witcher, D. R., Tang, Y., Watkins, J., Jiang, W., and Wroblewski, V. J. (2007a). Humanized IgG1 variants with differential binding properties to the neonatal Fc receptor: Relationship to pharmacokinetics in mice and primates. *Drug Metab. Dispos.* **35,** 86–94.

Datta-Mannan, A., Witcher, D. R., Tang, Y., Watkins, J., and Wroblewski, V. J. (2007b). Monoclonal antibody clearance. Impact of modulating the interaction of IgG with the neonatal Fc receptor. *J. Biol. Chem.* **282,** 1709–1717.

Deane, R., Sagare, A., Hamm, K., Parisi, M., LaRue, B., Guo, H., Wu, Z., Holtzman, D. M., and Zlokovic, B. V. (2005). IgG-assisted age-dependent clearance of Alzheimer's amyloid beta peptide by the blood–brain barrier neonatal Fc receptor. *J. Neurosci.* **25,** 11495–11503.

Deisenhofer, J. (1981). Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-Å resolution. *Biochemistry* **20,** 2361–2370.

Dennis, M. S., Zhang, M., Meng, Y. G., Kadkhodayan, M., Kirchhofer, D., Combs, D., and Damico, L. A. (2002). Albumin binding as a general strategy for improving the pharmacokinetics of proteins. *J. Biol. Chem.* **277,** 35035–35043.

Dickinson, B. L., Badizadegan, K., Wu, Z., Ahouse, J. C., Zhu, X., Simister, N. E., Blumberg, R. S., and Lencer, W. I. (1999). Bidirectional FcRn-dependent IgG transport in a polarized human intestinal epithelial cell line. *J. Clin. Invest.* **104,** 903–911.

Dickinson, B. L., Claypool, S. M., D'Angelo, J. A., Aiken, M. L., Venu, N., Yen, E. H., Wagner, J. S., Borawski, J. A., Pierce, A. T., Hershberg, R., Blumberg, R. S., and Lencer, W. I. (2008). Ca^{2+}-dependent calmodulin binding to FcRn affects immunoglobulin G transport in the transcytotic pathway. *Mol. Biol. Cell* **19,** 414–423.

Duncan, A. R., Woof, J. M., Partridge, L. J., Burton, D. R., and Winter, G. (1988). Localization of the binding site for the human high-affinity Fc receptor on IgG. *Nature* **332,** 563–564.

Edlow, D. W., and Sheldon, W. H. (1971). The pH of inflammatory exudates. *Proc. Soc. Exp. Biol. Med.* **137,** 1328–1332.

Firan, M., Bawdon, R., Radu, C., Ober, R. J., Eaken, D., Antohe, F., Ghetie, V., and Ward, E. S. (2001). The MHC class I related receptor, FcRn, plays an essential role in the maternofetal transfer of gammaglobulin in humans. *Int. Immunol.* **13,** 993–1002.

Ford, M. G., Mills, I. G., Peter, B. J., Vallis, Y., Praefcke, G. J., Evans, P. R., and McMahon, H. T. (2002). Curvature of clathrin-coated pits driven by epsin. *Nature* **419,** 361–366.

Frodin, J. E., Lefvert, A. K., and Mellstedt, H. (1990). Pharmacokinetics of the mouse monoclonal antibody 17–1A in cancer patients receiving various treatment schedules. *Cancer Res.* **50,** 4866–4871.

Gan, Z., Ram, S., Vaccaro, C., Ober, R. J., and Ward, E. S. (2009). Analyses of the recycling receptor, FcRn, in live cells reveal novel pathways for lysosomal delivery. *Traffic* **10,** 600–614.

Gandhi, S. P., and Stevens, C. F. (2003). Three modes of synaptic vesicular recycling revealed by single-vesicle imaging. *Nature* **423,** 607–613.

Gerweck, L. E., and Seetharaman, K. (1996). Cellular pH gradient in tumor versus normal tissue: Potential exploitation for the treatment of cancer. *Cancer Res.* **56,** 1194–1198.

Getman, K. E., and Balthasar, J. P. (2005). Pharmacokinetic effects of 4C9, an anti-FcRn antibody, in rats: Implications for the use of FcRn inhibitors for the treatment of humoral autoimmune and alloimmune conditions. *J. Pharm. Sci.* **94,** 718–729.

Ghetie, V., and Ward, E. S. (1997). FcRn: The MHC class I-related receptor that is more than an IgG transporter. *Immunol. Today* **18,** 592–598.

Ghetie, V., Hubbard, J. G., Kim, J. K., Tsen, M. F., Lee, Y., and Ward, E. S. (1996). Abnormally short serum half-lives of IgG in beta 2-microglobulin-deficient mice. *Eur. J. Immunol.* **26,** 690–696.

Ghetie, V., Popov, S., Borvak, J., Radu, C., Matesoi, D., Medesan, C., Ober, R. J., and Ward, E. S. (1997). Increasing the serum persistence of an IgG fragment by random mutagenesis. *Nat. Biotechnol.* **15,** 637–640.

Green, E. G., Ramm, E., Riley, N. M., Spiro, D. J., Goldenring, J. R., and Wessling-Resnick, M. (1997). Rab11 is associated with transferrin-containing recycling compartments in K562 cells. *Biochem. Biophys. Res. Commun.* **239,** 612–616.

Grosshans, B. L., Ortiz, D., and Novick, P. (2006). Rabs and their effectors: Achieving specificity in membrane traffic. *Proc. Natl. Acad. Sci. USA* **103,** 11821–11827.

Grubb, J. H., Vogler, C., Tan, Y., Shah, G. N., MacRae, A. F., and Sly, W. S. (2008). Infused Fc-tagged beta-glucuronidase crosses the placenta and produces clearance of storage *in utero* in mucopolysaccharidosis VII mice. *Proc. Natl. Acad. Sci. USA* **105,** 8375–8380.

Gurbaxani, B. M., and Morrison, S. L. (2006). Development of new models for the analysis of Fc–FcRn interactions. *Mol. Immunol.* **43,** 1379–1389.

Gurbaxani, B., de la Cruz, L. L., Chintalacharuvu, K., and Morrison, S. L. (2006). Analysis of a family of antibodies with different half-lives in mice fails to find a correlation between affinity for FcRn and serum half-life. *Mol. Immunol.* **43,** 1462–1473.

Hansen, R. J., and Balthasar, J. P. (2002). Effects of intravenous immunoglobulin on platelet count and antiplatelet antibody disposition in a rat model of immune thrombocytopenia. *Blood* **100,** 2087–2093.

Hattori, R., Otani, H., Moriguchi, Y., Matsubara, H., Yamamura, T., Nakao, Y., Omiya, H., Osako, M., and Imamura, H. (2001). NHE and ICAM-1 expression in hypoxic/reoxygenated coronary microvascular endothelial cells. *Am. J. Physiol. Heart Circ. Physiol.* **280,** H2796–H2803.

Haymann, J. P., Levraud, J. P., Bouet, S., Kappes, V., Hagege, J., Nguyen, G., Xu, Y., Rondeau, E., and Sraer, J. D. (2000). Characterization and localization of the neonatal Fc receptor in adult human kidney. *J. Am. Soc. Nephrol.* **11,** 632–639.

He, W., Ladinsky, M. S., Huey-Tubman, K. E., Jensen, G. J., McIntosh, J. R., and Bjorkman, P. J. (2008). FcRn-mediated antibody transport across epithelial cells revealed by electron tomography. *Nature* **455,** 542–546.

Hinton, P. R., Johlfs, M. G., Xiong, J. M., Hanestad, K., Ong, K. C., Bullock, C., Keller, S., Tang, M. T., Tso, J. Y., Vasquez, M., and Tsurushita, N. (2004). Engineered human IgG antibodies with longer serum half-lives in primates. *J. Biol. Chem.* **279,** 6213–6216.

Hinton, P. R., Xiong, J. M., Johlfs, M. G., Tang, M. T., Keller, S., and Tsurushita, N. (2006). An engineered human IgG1 antibody with longer serum half-life. *J. Immunol.* **176,** 346–356.

Holt, L. J., Basran, A., Jones, K., Chorlton, J., Jespers, L. S., Brewis, N. D., and Tomlinson, I. M. (2008). Anti-serum albumin domain antibodies for extending the half-lives of short lived drugs. *Protein Eng. Des. Sel.* **21,** 283–288.

Isomaki, P., Panesar, M., Annenkov, A., Clark, J. M., Foxwell, B. M., Chernajovsky, Y., and Cope, A. P. (2001). Prolonged exposure of T cells to TNF down-regulates TCR zeta and expression of the TCR/CD3 complex at the cell surface. *J. Immunol.* **166,** 5495–5507.

Israel, E. J., Wilsker, D. F., Hayes, K. C., Schoenfeld, D., and Simister, N. E. (1996). Increased clearance of IgG in mice that lack beta 2-microglobulin: Possible protective role of FcRn. *Immunology* **89,** 573–578.

Jaggi, J. S., Carrasquillo, J. A., Seshan, S. V., Zanzonico, P., Henke, E., Nagel, A., Schwartz, J., Beattie, B., Kappel, B. J., Chattopadhyay, D., Xiao, J., Sgouros, G., *et al*. (2007). Improved tumor imaging and therapy via i.v. IgG-mediated time-sequential modulation of neonatal Fc receptor. *J. Clin. Invest.* **117,** 2422–2430.

Jahn, R., Lang, T., and Südhof, T. C. (2003). Membrane fusion. *Cell* **112,** 519–533.

Jakobovits, A., Amado, R. G., Yang, X., Roskos, L., and Schwab, G. (2007). From XenoMouse technology to panitumumab, the first fully human antibody product from transgenic mice. *Nat. Biotechnol.* **25,** 1134–1143.

Jefferis, R., Lund, J., and Pound, J. D. (1998). IgG-Fc-mediated effector functions: Molecular definition of interaction sites for effector ligands and the role of glycosylation. *Immunol. Rev.* **163,** 59–76.

Jiang, L., Wang, J., Solorzano-Vargas, R. S., Tsai, H. V., Gutierrez, E. M., Ontiveros, L. O., Kiela, P. R., Wu, S. V., and Martin, M. G. (2004). Characterization of the rat intestinal Fc receptor (FcRn) promoter: Transcriptional regulation of FcRn gene by the Sp family of transcription factors. *Am. J. Physiol. Gastrointest. Liver Physiol.* **286,** G922–G931.

Jin, F., and Balthasar, J. P. (2005). Mechanisms of intravenous immunoglobulin action in immune thrombocytopenic purpura. *Hum. Immunol.* **66,** 403–410.

Junghans, R. P., and Anderson, C. L. (1996). The protection receptor for IgG catabolism is the beta2-microglobulin-containing neonatal intestinal transport receptor. *Proc. Natl. Acad. Sci. USA* **93,** 5512–5516.

Kacskovics, I., Wu, Z., Simister, N. E., Frenyo, L. V., and Hammarström, L. (2000). Cloning and characterization of the bovine MHC class I-like Fc receptor. *J. Immunol.* **164,** 1889–1897.

Kacskovics, I., Mayer, B., Kis, Z., Frenyo, L. V., Zhao, Y., Muyldermans, S., and Hammarström, L. (2006). Cloning and characterization of the dromedary (*Camelus dromedarius*) neonatal Fc receptor (drFcRn). *Dev. Comp. Immunol.* **30,** 1203–1215.

Kandil, E., Noguchi, M., Ishibashi, T., and Kasahara, M. (1995). Structural and phylogenetic analysis of the MHC class I-like Fc receptor gene. *J. Immunol.* **154,** 5907–5918.

Kaneko, Y., Nimmerjahn, F., and Ravetch, J. V. (2006). Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. *Science* **313,** 670–673.

Kassiotis, G., and Kollias, G. (2001). Uncoupling the proinflammatory from the immunosuppressive properties of tumor necrosis factor (TNF) at the p55 TNF receptor level: Implications for pathogenesis and therapy of autoimmune demyelination. *J. Exp. Med.* **193,** 427–434.

Kenanova, V., Olafsen, T., Crow, D. M., Sundaresan, G., Subbarayan, M., Carter, N. H., Ikle, D. N., Yazaki, P. J., Chatziioannou, A. F., Gambhir, S. S., Williams, L. E., Shively, J. E., *et al*. (2005). Tailoring the pharmacokinetics and positron emission tomography imaging properties of anti-carcinoembryonic antigen single-chain Fv-Fc antibody fragments. *Cancer Res.* **65,** 622–631.

Kim, J. K., Tsen, M. F., Ghetie, V., and Ward, E. S. (1994a). Identifying amino acid residues that influence plasma clearance of murine IgG1 fragments by site-directed mutagenesis. *Eur. J. Immunol.* **24,** 542–548.

Kim, J. K., Tsen, M. F., Ghetie, V., and Ward, E. S. (1994b). Localization of the site of the murine IgG1 molecule that is involved in binding to the murine intestinal Fc receptor. *Eur. J. Immunol.* **24,** 2429–2434.

Kim, J. K., Tsen, M. F., Ghetie, V., and Ward, E. S. (1994c). Catabolism of the murine IgG1 molecule: evidence that both CH2–CH3 domain interfaces are required for persistence of IgG1 in the circulation of mice. *Scand. J. Immunol.* **40,** 457–465.

Kim, J. K., Tsen, M. F., Ghetie, V., and Ward, E. S. (1995). Evidence that the hinge region plays a role in maintaining serum levels of the murine IgG1 molecule. *Mol. Immunol.* **32,** 467–475.

Kim, J. K., Firan, M., Radu, C. G., Kim, C. H., Ghetie, V., and Ward, E. S. (1999). Mapping the site on human IgG for binding of the MHC class I-related receptor, FcRn. *Eur. J. Immunol.* **29,** 2819–2825.

Kim, K. J., Fandy, T. E., Lee, V. H., Ann, D. K., Borok, Z., and Crandall, E. D. (2004). Net absorption of IgG via FcRn-mediated transcytosis across rat alveolar epithelial cell monolayers. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287,** L616–L622.

Kim, H., Fariss, R. N., Zhang, C., Robinson, S. B., Thill, M., and Csaky, K. G. (2008). Mapping of the neonatal Fc receptor in the rodent eye. *Invest. Ophthalmol. Vis. Sci.* **49,** 2025–2029.

Kisanuki, Y. Y., Hammer, R. E., Miyazaki, J., Williams, S. C., Richardson, J. A., and Yanagisawa, M. (2001). Tie2-Cre transgenic mice: A new model for endothelial cell-lineage analysis *in vivo*. *Dev. Biol.* **230,** 230–242.

Kobayashi, N., Suzuki, Y., Tsuge, T., Okumura, K., Ra, C., and Tomino, Y. (2002). FcRn-mediated transcytosis of immunoglobulin G in human renal proximal tubular epithelial cells. *Am. J. Physiol. Renal Physiol.* **282,** F358–F365.

Kristoffersen, E. K., and Matre, R. (1996). Co-localization of the neonatal Fc gamma receptor and IgG in human placental term syncytiotrophoblasts. *Eur. J. Immunol.* **26,** 1668–1671.

Kuo, T. T., de Muinck, E. J., Claypool, S. M., Yoshida, M., Nagaishi, T., Aveson, V. G., Lencer, W. I., and Blumberg, R. S. (2009). *N*-Glycan moieties in neonatal Fc receptor determine steady-state membrane distribution and directional transport of IgG. *J. Biol. Chem.* **284,** 8292–8300.

Lazar, G. A., Dang, W., Karki, S., Vafa, O., Peng, J. S., Hyun, L., Chan, C., Chung, H. S., Eivazi, A., Yoder, S. C., Vielmetter, J., Carmichael, D. F., *et al*. (2006). Engineered antibody Fc variants with enhanced effector function. *Proc. Natl. Acad. Sci. USA* **103,** 4005–4010.

Lee, M. C., Orci, L., Hamamoto, S., Futai, E., Ravazzola, M., and Schekman, R. (2005). Sar1p N-terminal helix initiates membrane curvature and completes the fission of a COPII vesicle. *Cell* **122,** 605–617.

Leitner, K., Ellinger, I., Grill, M., Brabec, M., and Fuchs, R. (2006). Efficient apical IgG recycling and apical-to-basolateral transcytosis in polarized BeWo cells overexpressing hFcRn. *Placenta* **27,** 799–811.

Liu, J., Marino, M. W., Wong, G., Grail, D., Dunn, A., Bettadapura, J., Slavin, A. J., Old, L., and Bernard, C. C. (1998). TNF is a potent anti-inflammatory cytokine in autoimmune-mediated demyelination. *Nat. Med.* **4,** 78–83.

Liu, L., Garcia, A. M., Santoro, H., Zhang, Y., McDonnell, K., Dumont, J., and Bitonti, A. (2007a). Amelioration of experimental autoimmune myasthenia gravis in rats by neonatal FcR blockade. *J. Immunol.* **178,** 5390–5398.

Liu, X., Ye, L., Christianson, G. J., Yang, J. Q., Roopenian, D. C., and Zhu, X. (2007b). NF-kappaB signaling regulates functional expression of the MHC class I-related neonatal Fc receptor for IgG via intronic binding sequences. *J. Immunol.* **179,** 2999–3011.

Liu, X., Ye, L., Bai, Y., Mojidi, H., Simister, N. E., and Zhu, X. (2008). Activation of the JAK/STAT-1 signaling pathway by IFN-gamma can down-regulate functional expression of the MHC class I-related neonatal Fc receptor for IgG. *J. Immunol.* **181,** 449–463.

Luzio, J. P., Poupon, V., Lindsay, M. R., Mullock, B. M., Piper, R. C., and Pryor, P. R. (2003). Membrane dynamics and the biogenesis of lysosomes. *Mol. Membr. Biol.* **20,** 141–154.

Martin, W. L., and Bjorkman, P. J. (1999). Characterization of the 2:1 complex between the class I MHC-related Fc receptor and its Fc ligand in solution. *Biochemistry* **38,** 12639–12647.

Martin, M. G., Wu, S. V., and Walsh, J. H. (1993). Hormonal control of intestinal Fc receptor gene expression and immunoglobulin transport in suckling rats. *J. Clin. Invest.* **91,** 2844–2849.

Martin, M. G., Wu, S. V., and Walsh, J. H. (1997). Ontogenetic development and distribution of antibody transport and Fc receptor mRNA expression in rat intestine. *Dig. Dis. Sci.* **42,** 1062–1069.

Martin, W. L., West, A. P. J., Gan, L., and Bjorkman, P. J. (2001). Crystal structure at 2.8 Å of an FcRn/heterodimeric Fc complex: Mechanism of pH dependent binding. *Mol. Cell* **7,** 867–877.

Masson, P. L. (1993). Elimination of infectious antigens and increase of IgG catabolism as possible modes of action of IVIg. *J. Autoimmun.* **6,** 683–689.

Mayer, B., Zolnai, A., Frenyo, L. V., Jancsik, V., Szentirmay, Z., Hammarstrom, L., and Kacskovics, I. (2002). Redistribution of the sheep neonatal Fc receptor in the mammary gland around the time of parturition in ewes and its localization in the small intestine of neonatal lambs. *Immunology* **107,** 288–296.

McCarthy, K. M., Yoong, Y., and Simister, N. E. (2000). Bidirectional transcytosis of IgG by the rat neonatal Fc receptor expressed in a rat kidney cell line: A system to study protein transport across epithelia. *J. Cell Sci.* **113,** 1277–1285.

McMahon, H. T., and Gallop, J. L. (2005). Membrane curvature and mechanisms of dynamic cell membrane remodelling. *Nature* **438,** 590–596.

Medesan, C., Radu, C., Kim, J. K., Ghetie, V., and Ward, E. S. (1996). Localization of the site of the IgG molecule that regulates maternofetal transmission in mice. *Eur. J. Immunol.* **26,** 2533–2536.

Medesan, C., Matesoi, D., Radu, C., Ghetie, V., and Ward, E. S. (1997). Delineation of the amino acid residues involved in transcytosis and catabolism of mouse IgG1. *J. Immunol.* **158,** 2211–2217.

Mezo, A. R., McDonnell, K. A., Hehir, C. A., Low, S. C., Palombella, V. J., Stattel, J. M., Kamphaus, G. D., Fraley, C., Zhang, Y., Dumont, J. A., and Bitonti, A. J. (2008). Reduction of IgG in nonhuman primates by a peptide antagonist of the neonatal Fc receptor FcRn. *Proc. Natl. Acad. Sci. USA* **105,** 2337–2342.

Mi, W., Wanjie, S., Lo, S. T., Gan, Z., Pickl-Herk, B., Ober, R. J., and Ward, E. S. (2008). Targeting the neonatal Fc receptor for antigen delivery using engineered Fc fragments. *J. Immunol.* **181,** 7550–7561.

Miaczynska, M., and Zerial, M. (2002). Mosaic organization of the endocytic pathway. *Exp. Cell Res.* **272,** 8–14.

Morris, B., and Morris, R. (1976). The effects of corticosterone and cortisone on the uptake of polyvinyl pyrrolidone and the transmission of immunoglobulin G by the small intestine in young rats. *J. Physiol.* **254,** 389–403.

Newton, E. E., Wu, Z., and Simister, N. E. (2005). Characterization of basolateral-targeting signals in the neonatal Fc receptor. *J. Cell Sci.* **118,** 2461–2469.

Nezlin, R. (1990). Internal movements in immunoglobulin molecules. *Adv. Immunol.* **48**(1–40), 1–40.

Nguyen, A., Reyes, A. E., Zhang, M., McDonald, P., Wong, W. L., Damico, L. A., and Dennis, M. S. (2006). The pharmacokinetics of an albumin-binding Fab (AB.Fab) can be modulated as a function of affinity for albumin. *Protein Eng. Des. Sel.* **19,** 291–297.

Ober, R. J., Radu, C. G., Ghetie, V., and Ward, E. S. (2001). Differences in promiscuity for antibody–FcRn interactions across species: Implications for therapeutic antibodies. *Int. Immunol.* **13,** 1551–1559.

Ober, R. J., Martinez, C., Lai, X., Zhou, J., and Ward, E. S. (2004a). Exocytosis of IgG as mediated by the receptor, FcRn: An analysis at the single-molecule level. *Proc. Natl. Acad. Sci. USA* **101,** 11076–11081.

Ober, R. J., Martinez, C., Vaccaro, C., Zhou, J., and Ward, E. S. (2004b). Visualizing the site and dynamics of IgG salvage by the MHC class I-related receptor, FcRn. *J. Immunol.* **172,** 2021–2029.

Oi, V. T., Jones, P. P., Goding, J. W., and Herzenberg, L. A. (1978). Properties of monoclonal antibodies to mouse Ig allotypes, H-2 and Ia antigens. *Curr. Top. Microbiol. Immunol.* **81,** 115–120.

Olafsen, T., Kenanova, V. E., and Wu, A. M. (2006). Tunable pharmacokinetics: Modifying the *in vivo* half-life of antibodies by directed mutagenesis of the Fc fragment. *Nat. Protoc.* **1,** 2048–2060.

Park-Min, K. H., Serbina, N. V., Yang, W., Ma, X., Krystal, G., Neel, B. G., Nutt, S. L., Hu, X., and Ivashkiv, L. B. (2007). FcγRIII-dependent inhibition of interferon-gamma responses mediates suppressive effects of intravenous immune globulin. *Immunity* **26,** 67–78.

Perez-Montoyo, H., Vaccaro, C., Hafner, M., Ober, R. J., Mueller, W., and Ward, E. S. (2009). Conditional deletion of the MHC Class I-related receptor, FcRn, reveals the sites of IgG homeostasis in mice. *Proc. Natl. Acad. Sci. USA* **106,** 2788–2793.

Petkova, S. B., Akilesh, S., Sproule, T. J., Christianson, G. J., Al, K. H., Brown, A. C., Presta, L. G., Meng, Y. G., and Roopenian, D. C. (2006). Enhanced half-life of genetically engineered human IgG1 antibodies in a humanized FcRn mouse model: Potential application in humorally mediated autoimmune disease. *Int. Immunol.* **18,** 1759–1769.

Popov, S., Hubbard, J. G., Kim, J., Ober, B., Ghetie, V., and Ward, E. S. (1996). The stoichiometry and affinity of the interaction of murine Fc fragments with the MHC class I-related receptor, FcRn. *Mol. Immunol.* **33,** 521–530.

Prabhat, P., Ram, S., Ward, E. S., and Ober, R. J. (2004). Simultaneous imaging of different focal planes in fluorescence microscopy for the study of cellular dynamics in three dimensions. *IEEE Trans. Nanobioscience* **3,** 237–242.

Prabhat, P., Gan, Z., Chao, J., Ram, S., Vaccaro, C., Gibbons, S., Ober, R. J., and Ward, E. S. (2007). Elucidation of intracellular recycling pathways leading to exocytosis of the Fc receptor, FcRn, by using multifocal plane microscopy. *Proc. Natl. Acad. Sci. USA* **104,** 5889–5894.

Praetor, A., and Hunziker, W. (2002). beta(2)-Microglobulin is important for cell surface expression and pH-dependent IgG binding of human FcRn. *J. Cell Sci.* **115,** 2389–2397.

Qiao, S. W., Kobayashi, K., Johansen, F. E., Sollid, L. M., Andersen, J. T., Milford, E., Roopenian, D. C., Lencer, W. I., and Blumberg, R. S. (2008). Dependence of antibody-mediated presentation of antigen on FcRn. *Proc. Natl. Acad. Sci. USA* **105,** 9337–9342.

Raghavan, M., Bonagura, V. R., Morrison, S. L., and Bjorkman, P. J. (1995). Analysis of the pH dependence of the neonatal Fc receptor/immunoglobulin G interaction using antibody and receptor variants. *Biochemistry* **34,** 14649–14657.

Ram, S., Prabhat, P., Chao, J., Ward, E. S., and Ober, R. J. (2008). High accuracy 3D quantum dot tracking with multifocal plane microscopy for the study of fast intracellular dynamics in live cells. *Biophys. J.* **95,** 6025–6043.

Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell* **122,** 735–749.

Roberts, D. M., Guenthert, M., and Rodewald, R. (1990). Isolation and characterization of the Fc receptor from the fetal yolk sac of the rat. *J. Cell Biol.* **111,** 1867–1876.

Rodewald, R. (1976). pH-dependent binding of immunoglobulins to intestinal cells of the neonatal rat. *J. Cell Biol.* **71,** 666–669.

Rodewald, R., and Abrahamson, D.R (1982). Receptor-mediated transport of IgG across the intestinal epithelium of the neonatal rat. *Ciba Found. Symp.* 209–232.

Rodewald, R., and Kraehenbuhl, J. P. (1984). Receptor-mediated transport of IgG. *J. Cell Biol.* **99,** 159s–164s.

Roopenian, D. C., Christianson, G. J., Sproule, T. J., Brown, A. C., Akilesh, S., Jung, N., Petkova, S., Avanessian, L., Choi, E. Y., Shaffer, D. J., Eden, P. A., and Anderson, C. L. (2003). The MHC class I-like IgG receptor controls perinatal IgG transport, IgG homeostasis, and fate of IgG-Fc-coupled drugs. *J. Immunol.* **170,** 3528–3533.

Ryan, T. A. (2003). Kiss-and-run, fuse-pinch-and-linger, fuse-and-collapse: The life and times of a neurosecretory granule. *Proc. Natl. Acad. Sci. USA* **100,** 2171–2173.

Sakagami, M., Omidi, Y., Campbell, L., Kandalaft, L. E., Morris, C. J., Barar, J., and Gumbleton, M. (2006). Expression and transport functionality of FcRn within rat alveolar epithelium: A study in primary cell culture and in the isolated perfused lung. *Pharm. Res.* **23,** 270–279.

Saleh, M. N., Khazaeli, M. B., Wheeler, R. H., Dropcho, E., Liu, T., Urist, M., Miller, D. M., Lawson, S., Dixon, P., and Russell, C. H. (1992). Phase I trial of the murine monoclonal anti-GD2 antibody 14G2a in metastatic melanoma. *Cancer Res.* **52,** 4342–4347.

Samuelsson, A., Towers, T. L., and Ravetch, J. V. (2001). Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. *Science* **291,** 484–486.

Sanchez, L. M., Penny, D. M., and Bjorkman, P. J. (1999). Stoichiometry of the interaction between the major histocompatibility complex-related Fc receptor and its Fc ligand. *Biochemistry* **38,** 9471–9476.

Scheiffele, P., Peranen, J., and Simons, K. (1995). N-glycans as apical sorting signals in epithelial cells. *Nature* **378,** 96–98.

Schlachetzki, F., Zhu, C., and Pardridge, W. M. (2002). Expression of the neonatal Fc receptor (FcRn) at the blood–brain barrier. *J. Neurochem.* **81,** 203–206.

Schnulle, P. M., and Hurley, W. L. (2003). Sequence and expression of the FcRn in the porcine mammary gland. *Vet. Immunol. Immunopathol.* **91,** 227–231.

Schuck, P., Radu, C. G., and Ward, E. S. (1999). Sedimentation equilibrium analysis of recombinant mouse FcRn with murine IgG1. *Mol. Immunol.* **36,** 1117–1125.

Scott, C. T. (2007). Mice with a human touch. *Nat. Biotechnol.* **25,** 1075–1077.

Sheff, D. R., Daro, E. A., Hull, M., and Mellman, I. (1999). The receptor recycling pathway contains two distinct populations of early endosomes with different sorting functions. *J. Cell Biol.* **145,** 123–139.

Shields, R. L., Namenuk, A. K., Hong, K., Meng, Y. G., Rae, J., Briggs, J., Xie, D., Lai, J., Stadlen, A., Li, B., Fox, J. A., and Presta, L. G. (2001). High resolution mapping of the binding site on human IgG1 for FcγRI, FcγRII, FcγRIII, and FcRn and design of IgG1 variants with improved binding to the FcγR. *J. Biol. Chem.* **276,** 6591–6604.

Simister, N. E., and Mostov, K. E. (1989). An Fc receptor structurally related to MHC class I antigens. *Nature* **337,** 184–187.

Simister, N. E., and Rees, A. R. (1985). Isolation and characterization of an Fc receptor from neonatal rat small intestine. *Eur. J. Immunol.* **15,** 733–738.

Simonsen, A., Lippe, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M., and Stenmark, H. (1998). EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. *Nature* **394,** 494–498.

Siragam, V., Brinc, D., Crow, A. R., Song, S., Freedman, J., and Lazarus, A. H. (2005). Can antibodies with specificity for soluble antigens mimic the therapeutic effects of intravenous IgG in the treatment of autoimmune disease? *J. Clin. Invest.* **115,** 155–160.

Siragam, V., Crow, A. R., Brinc, D., Song, S., Freedman, J., and Lazarus, A. H. (2006). Intravenous immunoglobulin ameliorates ITP via activating Fcγ receptors on dendritic cells. *Nat. Med.* **12,** 688–692.

Soldati, T., Rancano, C., Geissler, H., and Pfeffer, S. R. (1995). Rab7 and Rab9 are recruited onto late endosomes by biochemically distinguishable processes. *J. Biol. Chem.* **270,** 25541–25548.

Somsel, R. J., and Wandinger-Ness, A. (2000). Rab GTPases coordinate endocytosis. *J. Cell Sci.* **113**(Pt. 2), 183–192.

Sönnichsen, B., de Renzis, S., Nielsen, E., Rietdorf, J., and Zerial, M. (2000). Distinct membrane domains on endosomes in the recycling pathway visualized by multicolor imaging of Rab4, Rab5, and Rab11. *J. Cell Biol.* **149,** 901–914.

Souriau, C., and Hudson, P. J. (2003). Recombinant antibodies for cancer diagnosis and therapy. *Expert Opin. Biol. Ther.* **3,** 305–318.

Spiekermann, G. M., Finn, P. W., Ward, E. S., Dumont, J., Dickinson, B. L., Blumberg, R. S., and Lencer, W. I. (2002). Receptor-mediated immunoglobulin G transport across mucosal barriers in adult life: Functional expression of FcRn in the mammalian lung. *J. Exp. Med.* **196,** 303–310.

Stork, R., Müller, D., and Kontermann, R. E. (2007). A novel tri-functional antibody fusion protein with improved pharmacokinetic properties generated by fusing a bispecific single-chain diabody with an albumin-binding domain from streptococcal protein G. *Protein Eng. Des. Sel.* **20,** 569–576.

Storrie, B., and Desjardins, M. (1996). The biogenesis of lysosomes: Is it a kiss and run, continuous fusion and fission process? *Bioessays* **18,** 895–903.

Story, C. M., Mikulska, J. E., and Simister, N. E. (1994). A major histocompatibility complex class I-like Fc receptor cloned from human placenta: Possible role in transfer of immunoglobulin G from mother to fetus. *J. Exp. Med.* **180,** 2377–2381.

Tannock, I. F., and Rotin, D. (1989). Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res.* **49,** 4373–4384.

Tesar, D. B., Tiangco, N. E., and Bjorkman, P. J. (2006). Ligand valency affects transcytosis, recycling and intracellular trafficking mediated by the neonatal Fc receptor. *Traffic* **7,** 1–16.

Thompson, A., Nessler, R., Wisco, D., Anderson, E., Winckler, B., and Sheff, D. (2007). Recycling endosomes of polarized epithelial cells actively sort apical and basolateral cargos into separate subdomains. *Mol. Biol. Cell* **18,** 2687–2697.

Tiwari, B., and Junghans, R. P. (2005). Functional analysis of the mouse Fcgrt 5′ proximal promoter. *Biochim. Biophys. Acta* **1681,** 88–98.

Tzaban, S., Massol, R. H., Yen, E., Hamman, W., Frank, S. R., Lapierre, L. A., Hansen, S. H., Goldenring, J. R., Blumberg, R. S., and Lencer, W. I. (2009). The recycling and transcytotic pathways for IgG transport by FcRn are distinct and display an inherent polarity. *J. Cell Biol.* **185,** 673–684.

Ullrich, O., Reinsch, S., Urbe, S., Zerial, M., and Parton, R. G. (1996). Rab11 regulates recycling through the pericentriolar recycling endosome. *J. Cell Biol.* **135,** 913–924.

Vaccaro, C., Zhou, J., Ober, R. J., and Ward, E. S. (2005). Engineering the Fc region of immunoglobulin G to modulate *in vivo* antibody levels. *Nat. Biotechnol.* **23,** 1283–1288.

Vaccaro, C., Bawdon, R., Wanjie, S., Ober, R. J., and Ward, E. S. (2006). Divergent activities of an engineered antibody in murine and human systems have implications for therapeutic antibodies. *Proc. Natl. Acad. Sci. USA* **103,** 18709–18714.

van der Sluijs, P., Hull, M., Webster, P., Male, P., Goud, B., and Mellman, I. (1992). The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway. *Cell* **70,** 729–740.

Vaughn, D. E., Milburn, C. M., Penny, D. M., Martin, W. L., Johnson, J. L., and Bjorkman, P. J. (1997). Identification of critical IgG binding epitopes on the neonatal Fc receptor. *J. Mol. Biol.* **274,** 597–607.

Vidarsson, G., Stemerding, A. M., Stapleton, N. M., Spliethoff, S. E., Janssen, H., Rebers, F. E., de Haas, M., and van de Winkel, J. G. (2006). FcRn: An IgG receptor on phagocytes with a novel role in phagocytosis. *Blood* **108,** 3573–3579.

Waldmann, T. A., and Strober, W. (1969). Metabolism of immunoglobulins. *Prog. Allergy* **13,** 1–110.

Wallace, K. H., and Rees, A. R. (1980). Studies on the immunoglobulin-G Fc-fragment receptor from neonatal rat small intestine. *Biochem. J.* **188,** 9–16.

Wang, X., Kumar, R., Navarre, J., Casanova, J. E., and Goldenring, J. R. (2000). Regulation of vesicle trafficking in Madin–Darby canine kidney cells by Rab11a and Rab25. *J. Biol. Chem.* **275,** 29138–29146.

Wang, W., Wang, E. Q., and Balthasar, J. P. (2008). Monoclonal antibody pharmacokinetics and pharmacodynamics. *Clin. Pharmacol. Ther.* **84,** 548–558.

Wani, M. A., Haynes, L. D., Kim, J., Bronson, C. L., Chaudhury, C., Mohanty, S., Waldmann, T. A., Robinson, J. M., and Anderson, C. L. (2006). Familial hypercatabolic hypoproteinemia caused by deficiency of the neonatal Fc receptor, FcRn, due to a mutant beta2-microglobulin gene. *Proc. Natl. Acad. Sci. USA* **103,** 5084–5089.

Ward, T. T., and Steigbigel, R. T. (1978). Acidosis of synovial fluid correlates with synovial fluid leukocytosis. *Am. J. Med.* **64,** 933–936.

Ward, E. S., Martinez, C., Vaccaro, C., Zhou, J., Tang, Q., and Ober, R. J. (2005). From sorting endosomes to exocytosis: Association of Rab4 and Rab11 GTPases with the Fc receptor, FcRn, during recycling. *Mol. Biol. Cell* **16,** 2028–2038.

Weiner, L. M., and Carter, P. (2005). Tunable antibodies. *Nat. Biotechnol.* **23,** 556–557.

Weng, Z., Gulukota, K., Vaughn, D. E., Bjorkman, P. J., and DeLisi, C. (1998). Computational determination of the structure of rat Fc bound to the neonatal Fc receptor. *J. Mol. Biol.* **282,** 217–225.

Wernick, N. L., Haucke, V., and Simister, N. E. (2005). Recognition of the tryptophan-based endocytosis signal in the neonatal Fc Receptor by the mu subunit of adaptor protein-2. *J. Biol. Chem.* **280,** 7309–7316.

West, A. P. J., and Bjorkman, P. J. (2000). Crystal structure and immunoglobulin G binding properties of the human major histocompatibility complex-related Fc receptor. *Biochemistry* **39,** 9698–9708.

Willenborg, D. O., Fordham, S. A., Staykova, M. A., Ramshaw, I. A., and Cowden, W. B. (1999a). IFN-gamma is critical to the control of murine autoimmune encephalomyelitis and regulates both in the periphery and in the target tissue: A possible role for nitric oxide. *J. Immunol.* **163,** 5278–5286.

Willenborg, D. O., Staykova, M. A., and Cowden, W. B. (1999b). Our shifting understanding of the role of nitric oxide in autoimmune encephalomyelitis: A review. *J. Neuroimmunol.* **100,** 21–35.

Wu, Z., and Simister, N. E. (2001). Tryptophan- and dileucine-based endocytosis signals in the neonatal Fc receptor. *J. Biol. Chem.* **276,** 5240–5247.

Ye, L., Liu, X., Rout, S. N., Li, Z., Yan, Y., Lu, L., Kamala, T., Nanda, N. K., Song, W., Samal, S. K., and Zhu, X. (2008). The MHC class II-associated invariant chain interacts with the neonatal Fc gamma receptor and modulates its trafficking to endosomal/lysosomal compartments. *J. Immunol.* **181,** 2572–2585.

Yoshida, M., Claypool, S. M., Wagner, J. S., Mizoguchi, E., Mizoguchi, A., Roopenian, D. C., Lencer, W. I., and Blumberg, R. S. (2004). Human neonatal Fc receptor mediates transport of IgG into luminal secretions for delivery of antigens to mucosal dendritic cells. *Immunity* **20,** 769–783.

Yoshida, M., Kobayashi, K., Kuo, T. T., Bry, L., Glickman, J. N., Claypool, S. M., Kaser, A., Nagaishi, T., Higgins, D. E., Mizoguchi, E., Wakatsuki, Y., Roopenian, D. C., *et al.* (2006). Neonatal Fc receptor for IgG regulates mucosal immune responses to luminal bacteria. *J. Clin. Invest.* **116,** 2142–2151.

Zhang, Y., and Pardridge, W. M. (2001). Mediated efflux of IgG molecules from brain to blood across the blood–brain barrier. *J. Neuroimmunol.* **114,** 168–172.

Zhou, J., Johnson, J. E., Ghetie, V., Ober, R. J., and Ward, E. S. (2003). Generation of mutated variants of the human form of the MHC class I-related receptor, FcRn, with increased affinity for mouse immunoglobulin G. *J. Mol. Biol.* **332,** 901–913.

Zhou, J., Mateos, F., Ober, R. J., and Ward, E. S. (2005). Conferring the binding properties of the mouse MHC class I-related receptor, FcRn, onto the human ortholog by sequential rounds of site-directed mutagenesis. *J. Mol. Biol.* **345,** 1071–1081.

Zhu, X., Meng, G., Dickinson, B. L., Li, X., Mizoguchi, E., Miao, L., Wang, Y., Robert, C., Wu, B., Smith, P. D., Lencer, W. I., and Blumberg, R. S. (2001). MHC class I-related neonatal Fc receptor for IgG is functionally expressed in monocytes, intestinal macrophages, and dendritic cells. *J. Immunol.* **166,** 3266–3276.

Zhu, X., Peng, J., Raychowdhury, R., Nakajima, A., Lencer, W. I., and Blumberg, R. S. (2002). The heavy chain of neonatal Fc receptor for IgG is sequestered in endoplasmic reticulum by forming oligomers in the absence of beta2-microglobulin association. *Biochem. J.* **367,** 703–714.

SUBJECT INDEX

CONTENTS OF RECENT VOLUMES

Volume 85

Volume 86

Volume 87

Volume 88

Volume 89

Volume 90

Volume 91

Volume 92

Volume 93

Volume 94

Volume 95

Volume 96

Volume 97

Volume 98

Volume 99

Volume 100

Volume 101

Volume 102