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Surendran Mahalingam

Chemokines in Viral Infections

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Dedication

To my wife, Helen and children, Kieren and Nathan,
for their love and support.

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FOREWORD

Viral infection in the 21st century is still a leading cause of human and animal disease. From historically well known scourges such as influenza to the growing list of emerging viruses and to the relatively recent intractable threat posed by human immunodeficiency virus (HIV), viruses continue to exercise the intellect of scientists, clinicians and public health experts.

The continuing problems presented by viral infection can only be addressed by vigorous integrated research from the molecular to population level. At the molecular and cellular levels of enquiry, the last twenty years have achieved much on understanding the language of the host inflammatory and immune response to infection via the cytokine network. Within this sphere comes the chemokines and their multiple and fascinating role in defence from infection.

Chemokines were initially recognised for their ability to recruit cells from one body/tissue compartment to another, but further study has uncovered additional functions of paramount importance to host defence. To this end, the effectiveness of the threat of chemokines to the survival of an invading virus has been vindicated by discoveries of viral mimics or homologs which function to subvert or confuse chemokine action mediated by the infected host. Therefore, in the quest to move towards more effective interventions for viral disease, the position of the host chemokine and viral countermeasure must be seen as a key factor in the continually evolving virus-host relationship.

This book highlights such questions with many examples of the activity and action of chemokines and their receptors across several disease-associated virus families, and with contemplation of chemokines in the broad theme of viruses and their hosts.

Brett A. Lidbury Ph.D.

PREFACE

Viruses are capable of driving the evolutionary process with great plasticity and at the same time enabling them to evolve in new directions.Their interactions with cells positions them to mediate subtle, cumulative evolutionary changes in their hosts as well.

In the past six years, a large number of new chemokines and chemokine receptors have been discovered and characterized. This is mainly attributed to the rapid progress in the area of bioinformatics and expressed sequence tag (EST) databases. It is now clear that chemokine function is not just restricted to cell attraction. Chemokines are also important in mediating the development and functional aspects of leukocytes. Recent discoveries provide convincing and compelling evidences supporting the roles of these messengers and their receptors in the resolution of viral infections and virus survival.

This book is the first to provide a comprehensive write up on the various evidences available to date on the interactions between host chemokine systems and viral chemokines. This book intends to unravel the chemokine constellation in the context of viral infections, a versatility that was not fully understood almost a decade ago.

Finally, I would like to express my sincere gratitute to all contributors for making the completion of this book possible.

Surendran Mahalingam, Ph.D.

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I am extremely grateful to all the authors who have contributed chapters to this book. I value the time and effort that they have contributed in making the completion of this book a success. I am also grateful to R.G. Landes and Cynthia Conomos for their support and patience over the past year in getting the book together.

CHAPTER 1

The Chemokines:

What Are They and What Do They Do?

Shaun R. McColl

Introduction

Whole body coverage by leukocytes is a critical feature of immunity. Various leukocyte populations flow through the peripheral blood, and depending on whether they encounter appropriate signals, they may move into inflamed tissues to deal with infections as is the case with myeloid cells, or into various regional lymph nodes or the spleen in order to screen for the presence of foreign antigen, as is the case with lymphocytes.

Movement of leukocytes to extravascular sites of inflammation and infection or into lymph nodes requires the coordinated action of several different classes of molecules, including cytokines, adhesion molecules, proteases and chemotactic factors.[1-3] While there are several important differences between myeloid and lymphoid cells with respect to extravasation, particularly regarding naïve lymphocytes, for ease, a general model will be presented. Leukocytes moving close to the luminal surface of the vasculature are held in a loose fashion by adhesion molecules known as selectins (P, E, and L-selectin). Firm adhesion to the endothelial cells will only occur if the leukocytes come into contact with chemotactic factors which will activate adhesion molecules on their surface (β2 integrins), and the counter receptors for integrins such as intercellular adhesion molecule-1 (ICAM-1) which is expressed on the endothelial cell surface in response to inflammatory cytokine production. Extravasation occurs, by an as yet undefined process which is believed to involve degradation of the basement membrane by proteases and the specialized adhesion molecule PECAM-1 which appears to be required for transendothelial migration but not leukocyte adhesion to the luminal surface of endothelial cells.[4,5] Once leukocytes have entered the tissue, chemotactic factors such as chemokines are also critical for the continued movement of the leukocytes towards the source of such factors.[6-8] In this way, chemokines regulate precise spatial positioning of leukocyte within tissues.

The Chemokine Gene Superfamily

In general, chemokines are low molecular weight cytokines. There are only two exceptions to this rule, CXCL16 and CX_3CL1, which will be discussed below. The term chemokine is a contraction of the words chemotactic cytokine, a name that reflects the major recognized role of all the chemokines cloned to date. The gene superfamily presently consists of over 40 members, all of which are chemotactic for various leukocyte subsets. However, while the major collective biological activity of these molecules is clearly chemotaxis, considerable data is emerging that various members of the chemokine gene superfamily exert other biological effects, including in development, angiogenesis and haematopoiesis.

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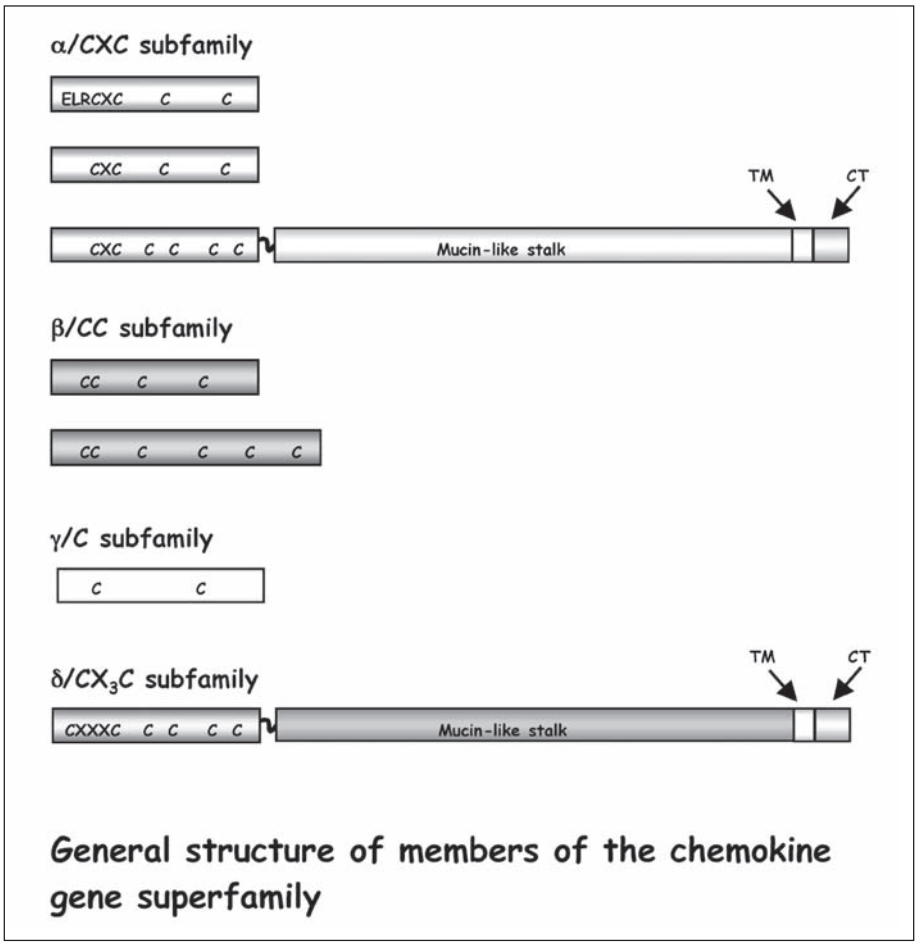

Figure 1. The structural subclassification of chemokines: TM, transmembrane segment; CT, cytoplasmic tail.

Chemokine superfamily members are highly homologous at the predicted primary amino acid level, with the single major characteristic being the presence of a cysteine signature motif. Based on the arrangement of this motif, members can be further divided into four structurally distinct branches, the CXC, CC, C and CX_3C chemokines (also known as the α, β, γ and δ chemokines, respectively) (see Fig. 1). The CXC chemokine family can be divided into two further subfamilies depending on whether the primary amino acid structure possesses a signature ELR motif prior to the CXC motif. The ELR-containing CXC chemokines attract neutrophils whereas the non-ELR-containing CXC chemokines attract lymphocytes. The second branch or the CC chemokines comprise the majority of chemokines, and are chemoattractants for a range of cells including monocytes, dendritic cells, granulocytes other than neutrophils and various subpopulations of lymphocytes, including NK cells. The only C chemokine cloned to date, lymphotactin, has been reported to selectively attract CD8+ T lymphocytes, while fractalkine, the only CX_3C chemokine cloned, has been reported to act as a chemottractant for T cells, NK cells and monocytes. Most chemokines cloned thus far attract overlapping as well

as distinct leukocyte populations. In summary, collectively all of the members cloned thus far are chemotactic for all of the circulating leukocyte subpopulations.[6-8]

Unlike all other chemokines that are exclusively secreted, CXCL16 and CX_3CL1 are believed to exert a dual function.[9-12] These two chemokines have a unique structure in that they are large proteins, with a chemokine module positioned at the N-terminus, a mucin-like stalk, a transmembrane domain and a cytoplasmic tail. Studies to date indicate that these chemokines exist in two states—membrane-bound or secreted. In the membrane-bound form, they act as adhesion molecules, whereas the secreted molecules that are released from the cell surface following peptidase-mediated cleavage,[13-15] function as soluble chemotactic factors. The soluble form may also inhibit the adhesive function via competition for receptors on target cells, in much the same way as soluble ICAM-1 and VCAM-1 inhibit integrin-dependent adhesion. The TNFα-converting enzyme (TACE/ADAM-17) has recently been shown to cleave CX_3CL1 from the surface of epithelial cells, fibroblasts and endothelial cells.[16,17] The enzyme(s) responsible for release of CXCL16 from the cell surface has not yet been identified.

The nomenclature for the chemokines has recently been restructured, with a more systematic approach being taken. The process whereby this occurred was initiated at the 1998 Gordon Research Conference on Chemotactic Cytokines, where a committee was formed to propose a new standardized chemokine ligand nomenclature.[18] This new system that essentially follows the same nomenclature as already existed for chemokine receptors is presented in Table 1, along with the original names.

Chemokine Receptors

Chemokines exert their biological effects upon binding to cell surface receptors. All of the chemokine receptors cloned to date are serpentine or seven transmembrane guanine nucleotide-binding protein receptors.[6-8,19-21] Ligation of chemokines to their receptors activates signal transduction cascades that lead not only to direction-specific movement (chemotaxis), but also a wide range of functions required for host defence, including adhesion, the respiratory burst, degranulation, and inflammatory lipid mediator synthesis in leukocytes.

The most remarkable feature of the chemokine receptor superfamily, however, is their promiscuity as far as ligand binding is concerned. Details of the known chemokine receptors and their ligands are shown in Figure 2. Within the CXC and CC subfamilies, there is a great deal of promiscuous binding. Particular examples of promiscuous binding include CXCR2 that has 7 known high-affinity ligands, and CCR3 which has 4. In addition, most other receptors have multiple ligands. Understanding the biological reason for this apparent redundancy is of major importance, but it is likely that it has evolved to ensure robust immune responses against infectious agents.[22] Recent data have indicated a high degree of diversity with respect to temporal and spatial expression of ligands, suggesting that not all ligands for a given receptor will be produced in the same place at the same time.

Chemokines Are Involved in Leukocyte Homeostasis and Effector Trafficking

Until recently, the structural subclassification based on the arrangement of the cysteine motif was an adequate classification system for chemokines and their receptors. However, this classification based on structure did not provide any indication of the functional role of the various chemokines. Therefore, an alternative system that relates expression to function has recently been developed.[23,24] In this system, chemokines and chemokine receptors are classified according to their functional expression as either homeostatic/constitutive or inflammatory/inducible (Fig. 2). Homeostatic chemokines are expressed constitutively within lymphoid tissues and regulate movement of thymocytes through the thymus during selection[8,25,26] and

Table I. Chemokine ligand nomenclature (see appendix for full chemokine names)

Systematic Name	Original Names/s	
	Human	**Mouse**
CXCL1	MGSA, gro-α	KC
CXCL2	MIP-2α, groβ	MIP-2*
CXCL3	MIP-2β, groγ	
CXCL4	PF4	PF4
CXCL5	ENA-78	LIX
CXCL6	GCP-2	CKα-2
CXCL7	NAP-2	?
CXCL8	IL-8, NAF, NAP-1	?
CXCL9	Mig	Mig
CXCL10	IP-10	Crg-2
CXCL11	I-TAC	I-TAC
CXCL12	SDF-1α, SDF-1β	SDF-1
CXCL13	BLC, BCA-1	BLC, BCA-1
CXCL14	BRAK, bolekine	BRAK
CXCL15	?	Lungkine
CXCL16	CXCL16	CXCL16
CCL1	I-309	TCA-3
CCL2	MCP-1, MCAF	JE
CCL3	MIP-1α,LD78α	MIP-1α
CCL4	MIP-1β, LD78β	MIP-1β
CCL5	RANTES	RANTES
CCL6	?	C10, MRP-1
CCL7	MCP-3	MARC
CCL8	MCP-2	MCP-2
CCL9/10	?	MRP-2, CCF18, MIP-1γ
CCL11	Eotaxin	Eotaxin
CCL12	?	MCP-5
CCL13	MCP-4	?
CCL14	HCC-1	?
CCL15	HCC-2,Lkn-1,MIP-1δ	?
CCL16	HCC-4, LEC	LCC-1
CCL17	TARC	TARC
CCL18	DC-CK1, MIP-4, PARC, AMAC-1	?
CCL19	MIP-3β, ELC, exodus-3	MIP-3β, ELC, exodus-3
CCL20	MIP-3α, LARC, exodus-1	MIP-3α, LARC, exodus-1
CCL21	6Ckine, SLC, TCA-4, exodus-2	6Ckine, SLC, TCA-4, exodus-2
CCL22	MDC, STCP-1	ABCD-1
CCL23	MPIF-1	?
CCL24	MPIF-2, eotaxin-2	?
CCL25	TECK	TECK
CCL26	Eotaxin-3	?
CCL27	CTACK, ILC	ALP, CTACK, ILC, ESkine
CCL28	CCL28, MEC	CCL28
XCL1	Lymphoctactin, SCM-1α, SCM-1β, ATAC	Lymphotactin
CX_3CL1	Fractalkine	Neurotactin

The systematic nomenclature was recently introduced following discussions at the 1998 Gordon Research Conference on Chemokines. It involves following the same nomenclature as already existed for the chemokine receptors. In general, all of the common alternative names for human and mouse chemokines have been listed. "?" indicates yet to be identified. * - human MIP-2α and MIP-2β are products of two separate genes. There has only been one mouse MIP-2 gene identified.

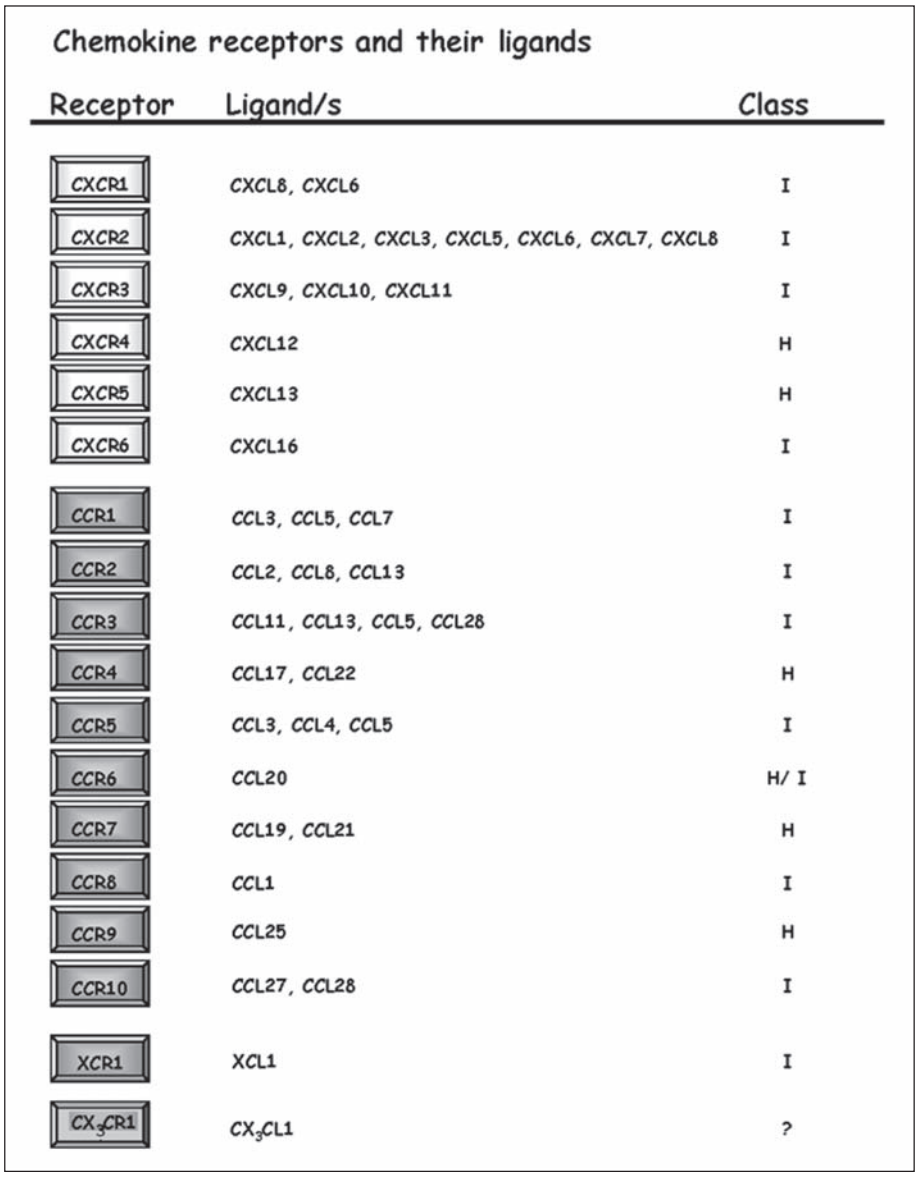

Figure 2. Chemokine receptors and their ligands: I, inflammatory; H, homeostatic.

the physiological trafficking of lymphocytes and mature antigen-loaded dendritic cells (DCs) into 2° lymphoid organs under normal conditions or during immune responses. Inflammatory chemokines are up-regulated at sites of inflammation and infection. These chemokines play a key role in the recruitment of effector leukocytes to peripheral tissues in response to immunological challenge.[6,7,27,28] There is also evidence that the maintenance of resident immune surveillance cells (for instance, macrophages and immature DCs) in peripheral tissues is regulated by low-level basal expression of some homeostatic and some inducible chemokines.

Not surprisingly, chemokine receptors also fit this functional pattern. This is particularly evident in the differential trafficking of T lymphocyte subsets and of DCs to lymph nodes. Immature DCs in peripheral tissues express receptors for chemokines that are expressed at low constitutive levels in the tissues. An example is CCR6, the receptor for CCL20, a chemokine that is constitutively expressed in some peripheral tissues including in the Peyer's patches and the skin.[29,30] Upon activation, and this may be through ligation of pattern recognition receptors on the DCs, the cells alter chemokine receptor expression. CCR6 is down-regulated, while CCR7, the receptor for the homoestatic chemokines CCL19 and CCL21 is upregulated.[31] The latter ligands are expressed in afferent lymphatic vessels and in the T cell zones in 2° lymphoid organs. This switch results in the recruitment of mature, antigen-loaded DCs to the T cell zones.

Naïve T lymphocytes express high levels of homeostatic chemokine receptors, particularly CCR7, that mediate recirculation through the 2° lymphoid organs.[32,33] However, upon clonal selection, the homeostatic receptors are downregulated, and inflammatory chemokine receptors are upregulated on the effector cells,[24,33-37] with specialization occurring at the level of type 1 and type 2 chemokine receptor profiles. This allows the effector cells to migrate into tissues where the ligands for the inflammatory receptors are being expressed as will be the case during an infection.

Chemokines in Infectious Diseases

Being a family of chemotactic factors, it is fairly easy to see roles for chemokines in defence against pathogens. Numerous studies have documented the upregulation of chemokines during host response to pathogens ranging from macroparasites to bacteria to viruses.[38-41] Importantly, as indicated above, specialization of T helper cells also occurs upon clonal selection during immune responses. Antigen-specific stimulation of these cells leads to significant upregulation of distinct chemokine receptors that can functionally define either type 1 or type 2 helper cells.[24,34] In this way, chemokine receptors play vital roles in tailoring the immune response towards intracellular or extracellular pathogens. Studies using gene knockout mice, chemokine receptor antagonists, or neutralizing antibodies have confirmed the importance of many chemokines and their receptors in host defence against infection. Of particular relevance are the data emerging that documents the interaction between the mammalian immune system and viruses. The single most significant observation in this area is the discovery that HIV uses several chemokine receptors as coreceptors for entry into CD4+ cells.[42-45] This has led to intense research into the development of chemokine receptor antagonists and anti-HIV agents, as well as driving strong research interactions between chemokine biologists and virologists.

Many viruses have adapted to the mammalian chemokine system using captured genes to mimic chemokines and chemokine receptors, presumably for ectopic expression during infection to disregulate the immune response.[38,46,47] For instance, several viruses also produce broad-spectrum chemokine receptor antagonists such as vMIP-II, and soluble chemokine-binding proteins such as an IFNγ receptor homolog, that directly neutralize endogenous chemokine activity.[38,46-48] The latter is particularly interesting given that the CXCR3 family of chemokines, CXCL9, CXCL10 and CXCL11, are crucial for anti-viral immunity and are tightly regulated by IFNγ. The work reviewed in this book highlight many of the latest findings in the area of chemokine and virus interactions.

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CHAPTER 2

Soluble Chemokine Binding Proteins Encoded by Viruses

Vincent P. Smith, Neil A. Bryant and Antonio Alcami

Modulation of the Chemokine System by Viruses

Chemokines are chemoattractant cytokines that regulate trafficking and effector functions of leukocytes, and play a key role in inflammation and host defence against invading pathogens.[1,2] For a detailed description of the chemokine family see chapter 1. The induction of particular chemokines together with differential expression of specific seven-transmembrane-domain G-protein-coupled chemokine receptors by leukocyte subsets determines the immune cells that migrate during inflammation.

Chemokines interact with both their specific receptors and with cell surface glycosaminoglycans (GAGs) via distinct binding sites.[3] Receptor binding is the means by which chemokines transduce their biological signals, while GAG binding is important for the formation of immobilized chemotactic chemokine gradients, along which immune cells travel across endothelial cell monolayers and into tissues. Disruption of either chemokine-receptor or chemokine-GAG complex formation might therefore inhibit chemokine biological activity.

It has become evident in recent years that viruses actively evade host immune defences.[4,5] Viral immune evasion strategies are diverse and depend on their replication strategy, ability to mutate, host cell and tissue tropism, transmission mechanism and genome coding capacity. Molecular mimicry is an immune evasion mechanism utilized by large DNA viruses (herpesviruses and poxviruses). The role of chemokines in anti-viral defence is emphasized by the finding of numerous viral proteins that mimic host chemokines and chemokine receptors, or modulate chemokine activity[6,7] (Fig. 1). Virus-encoded chemokine homologs (vCKs) function as agonists, binding to cellular receptors and transducing signals, or antagonists, preventing the activity of chemokines by occupying chemokine receptors. Viral seven-transmembrane-domain chemokine receptors (vCKRs) are expressed at the surface of infected cells and may transduce signals, sometimes in the absence of ligand. Some viruses encode secreted chemokine binding proteins (vCKBPs) with no host counterpart that are potent inhibitors of chemokine activity.

The primary function of vCK antagonists and vCKBPs is to neutralize ckemokines involved in immune responses. However, the function of vCK agonists and vCKRs may not be so obvious. vCK agonists may attract particular immune cells that drive the immune response in a direction that is beneficial for the virus. In addition, in the case of lymphotropic viruses, these viral proteins may induce the migration of immune cells that represent good targets for viral replication. The function of vCKRs is more complex. There is evidence that expression of vCKRs reduces chemokine levels in the vicinity of the infected cell and may downregulate

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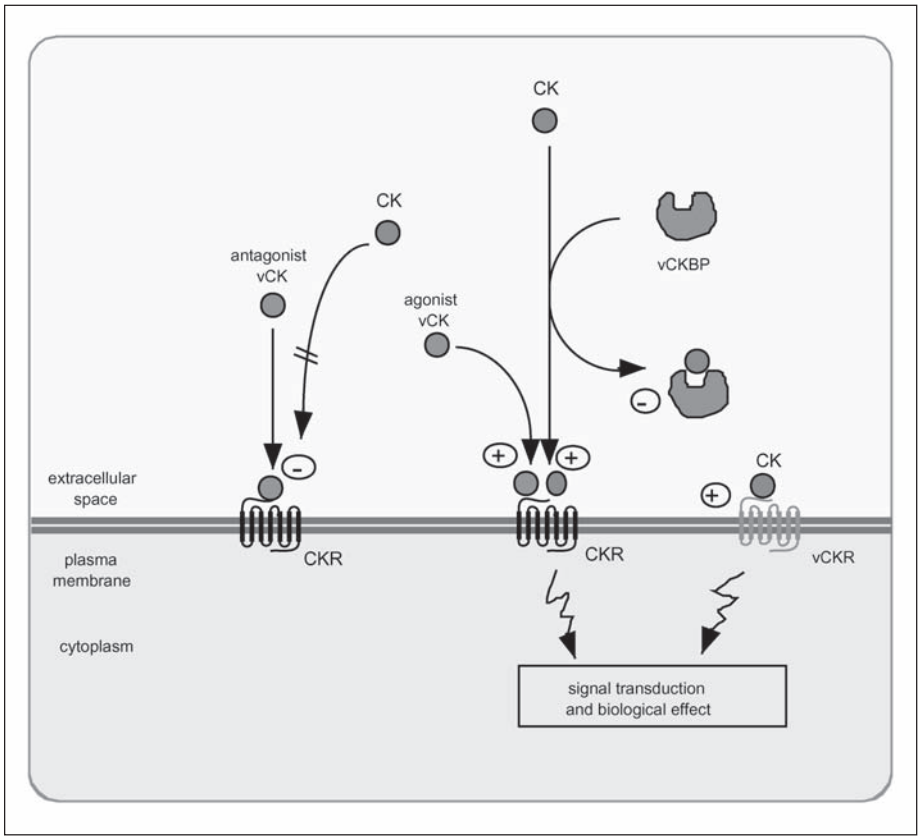

Figure 1. Modulation of the chemokine system by viral proteins. Chemokines (CKs) mediate their biological effect by interacting with specific seven-transmembrane-domain chemokine receptors (CKRs) at the surface of cells. Viruses encode chemokine homologs (vCKs) that function as agonists, binding to chemokine receptors and transducing signals, or as antagonists, occupying receptor binding sites and preventing the binding of host chemokines. Viruses also encode chemokine receptor homologs (vCKRs), expressed at the cell surface, and chemokine binding proteins (vCKBPs) that sequester chemokines in solution.

expression of host chemokines. vCKRs can influence the signaling status of the cell and have the potential to promote viral replication. Constitutive expression of some vCKRs induces cell proliferation, and this activity may be modulated by chemokine binding. Alternatively, expression of functional vCKRs may confer upon the infected cell the ability to migrate to other tissues in response to chemokines. Thus, some vCK agonists and vCKRs may promote viral replication rather than cause immunosuppresion.

Poxviruses

Poxviruses are a family of complex DNA viruses that normally cause acute infections and do not establish latency or persist in the infected host.[8-10] Poxviruses infect a wide variety of mammalian species, but only two species exclusively infect humans. These are the orthopoxvirus (OPV) variola virus (VaV), which caused smallpox before its eradication, and the distantly related molluscipoxvirus molluscum contagiosum virus (MCV), which causes papular tumors in the skin. Other species of OPV are: vaccinia virus (VV), the vaccine used for smallpox

eradication, which is of unknown origin and natural host; cowpox virus (CPV), probably a rodent virus that causes sporadic skin infections in cows, cats and humans; and ectromelia virus (EV), a highly virulent natural pathogen of mice that causes mousepox and has been isolated from outbreaks in laboratory mouse colonies. Myxoma virus (MV) is a leporipoxvirus that causes the lethal systemic disease myxomatosis in European rabbits.

Molecular Mimicry of Chemokines and Chemokine Receptors by Poxviruses

In contrast to herpesviruses, the number of genes encoding vCKs and vCKRs identified in poxviruses is limited (Table 1). Most of these poxvirus proteins have been recently identified by sequence analysis of viral genomes. Functional information is available for the MCV vCK MC148, but it has not been reported for the vCKs encoded by Fowlpox virus (FPV). No biological characterization of the 10 vCKRs identified in poxvirus genomes has been published yet. Like for herpesviruses, it is assumed that they act by sequestering chemokines and may transduce signals inside the cell. The finding that chemokine receptors are required for efficient replication of MV in specific cell types suggested that poxviruses may utilize chemokine receptors for entry or for events leading to appropriate viral gene expression.[11] Maybe some poxviruses encode their own vCKRs to induce intracellular chemokine signaling pathways necessary to render the cell fully susceptible for viral replication.

MCV Chemokine Homolog

MCV causes small benign skin tumors in humans that persists for months.[10] In immunocompromised individuals such as AIDS patients or those receiving immunosuppressive drugs, MCV infection can become recurrent and widespread. The tumors are located in the epidermal layer and are free of inflammatory cell infiltrates, initiating little or no immune response. MCV has proved difficult to grow in vitro and no in vivo model is available. This has complicated the molecular characterization of MCV until the recent determination of the MCV genome sequence.[12] Senkevich et al identified 182 MCV open reading frames (ORFs), 105 of which have counterparts in the OPVs and 16 of the ORFs have cellular homologs.[12,13]

The MC148 ORF was predicted to encode a 104 amino acid protein structurally related to CC chemokines. Comparison with macrophage inflammatory protein 1α (MIP-1α/CCL3) revealed that MC148 lacks an N-terminal five amino acid sequence involved in receptor activation, suggesting that it lacks agonistic activity[12] (Fig. 2). The MC148 coding sequence is highly conserved between subtypes 1 and 2 of MCV. MC148 is transcribed in vitro as an early gene, and in vivo its mRNA can be detected in MCV-infected tissue samples.[14] Expression of MC148 from VV showed that its gene product was secreted from infected cells as a 10 kDa polypeptide.

Predictions of MC148 vCK activity were confirmed when Krathwohl et al showed that purified MC148 proteins from MCV type 1 and 2 expressed in the baculovirus system were found to inhibit the chemotatic response of monocytes to CCL3.[15] In addition, like some host chemokines, both viral proteins inhibited the growth of human hematopoetic progenitor cells, suggesting that MC148 may activate some chemokine receptors.

A subsequent report by Damon et al suggested that MC148 interacts with the chemokine receptors CCR1 and/or CCR5, CCR2, CCR8, CXCR1 and/or CXCR2 and CXCR4.[16] Purified MC148 expressed in the VV system inhibited the migration of human monocytes in response to the CC chemokines monocyte chemoattractant protein 1 (MCP-1/CCL2), MCP-3/CCL7, CCL3, RANTES/CCL5 (regulated upon activation, normal T-cell expressed and secreted) or I-309/CCL1. MC148 also inhibited lymphocyte and monocyte chemotaxis in response to stromal cell-derived factor 1α (SDF-1α/CXCL12), and neutrophil chemotaxis in response to interleukin 8 (IL-8/CXCL8), both CXC chemokines. Consistent with its antagonistic activity, MC148 did

Table 1. Chemokines, chemokine receptors and chemokine binding proteins encoded by poxviruses

Viral Function	Gene/ Protein	Virus	Homology and Mechanism of Action	Refs.
vCK	MC148	MCV	Homolog of human CCL27, specific CCR8 antagonist, interference with monocyte function	15-17,19
	FPV060	FPV	CC chemokine homolog	23
	FPV061	FPV	CC chemokine homolog	23
	FPV116	FPV	CC chemokine homolog	23
	FPV121	FPV	CC chemokine homolog	23
vCKR	K2R	SPV	CXCL8 chemokine receptor homolog	20
	Q2/3L	CaPV strain KS-1	CC chemokine receptor homolog	21
	LSDV01	LSDV	CCR8 homolog	22
	FPV021	FPV	Monkey chemokine receptor GPR1 homolog	23
	FPV027	FPV	Monkey chemokine receptor GPR1 homolog	23
	FPV206	FPV	Epstein-Barr virus-induced chemokine receptor homolog	23
	M104L S104L	MV SFV	Truncated chemokine receptor encoding transmembrane domains 5 and 6, related to Ateline herpesvirus 3 ORF 74	25,26
	7L	YLDV	Related to human CCR8	24
	145R	YLDV	Related to human CCR8	24
vCKBP	vCKBP-1 M-T7 S-T7	 MV SFV	Secreted, binds C, CC and CXC chemokines through GAG binding domain, anti-inflammatory properties	29
	vCKBP-2 B29R vCCI/H5R G3R K2R M-T1 S-T1	 VV CPV VaV EV MV SFV	Secreted, binds CC chemokines, prevents interaction of CC chemokines with specific receptors, anti-inflammatory properties	30,32,33
	A41L A44L	VV VaV	Homolog of vCKBP-2 and orf virus GIF, ligand unknown	55

Abbreviations: CaPV, capripox virus; CCI, chemokine inhibitor; CPV, cowpox virus; EV, ectromelia virus; FPV, fowlpox virus; GAG, glycosaminoglycan; ILC, IL-11-receptor-alpha-locus chemokine; LSDV, Lumpy skin disease virus; MCV, molluscum contagiosum virus; MV, myxoma virus; ORF, open reading frame; SFV, Shope fibroma virus; SPV, swinepox virus; VaV, variola virus; vCK, viral chemokine homolog; vCKBP, viral chemokine binding protein; vCKR, viral chemokine receptor homolog; VV, vaccinia virus; YLDV, Yaba-like disease virus.

not induce signal transduction in monocytes or cells tranfected with CCR2B or CCR8, but prevented the intracellular calcium release induced by CCL2 and CCL1. MC148 interacts with chemokine receptors and blocks the binding of ^{125}I-CCL2 to CCR2B-transfected cells.

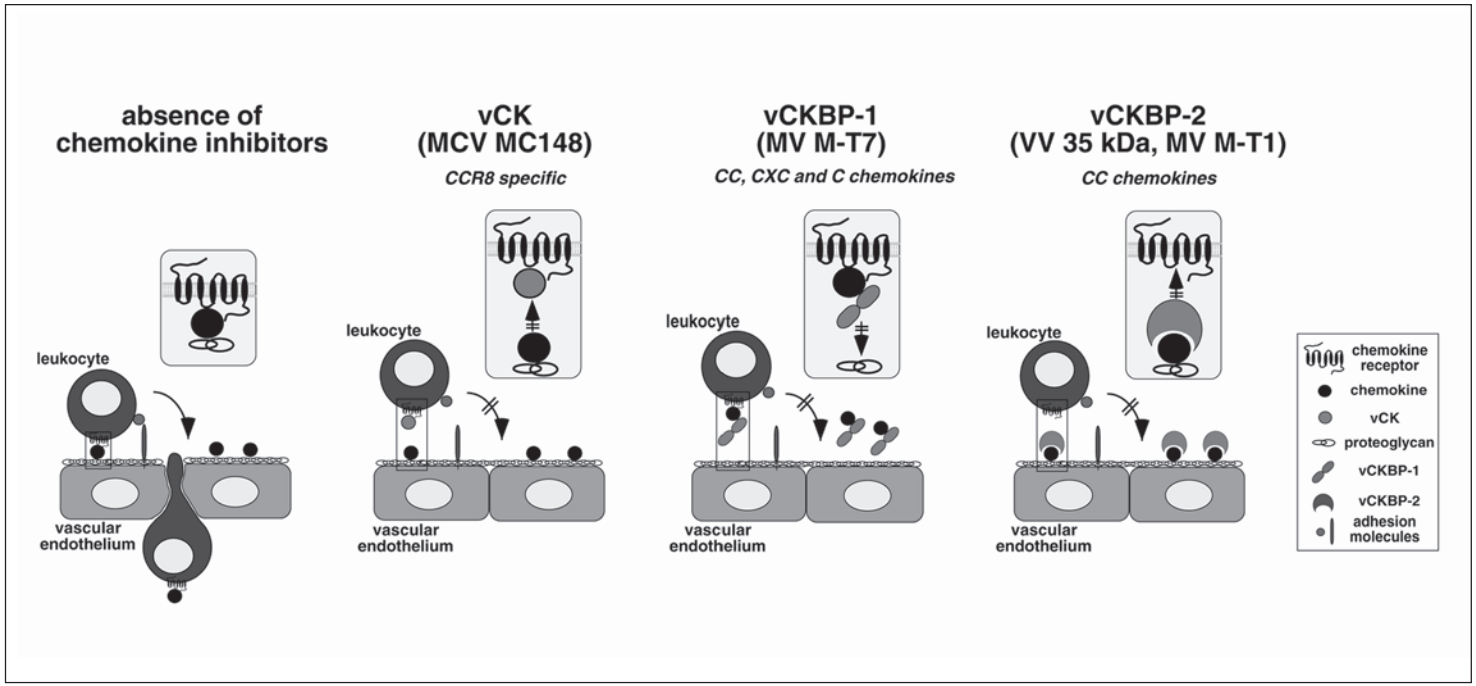

Figure 2. Blockade of chemokine activity by poxvirus-encoded vCKs an vCKBPs. Chemokines form a gradient by interacting with GAGs at the surface of endothelial cells and other surfaces, and binding of chemokines to chemokine receptors in leukocytes induce the expression of adhesion molecules and migration of leukocytes through the endothelium to areas of virus infection. Poxviruses have evolved three complementary mechanisms to block chemokine activity. First, the expression of the vCK MC148 by MCV that binds to chemokine receptors and prevents activation by host chemokines. Second, the soluble MV vCKBP-1, encoded by M-T7, that binds the GAG binding domain of a wide range of chemokines and may prevent the correct localization of chemokines at the surface of endothelium. Third, the secreted vCKBP-2 from MV (M-T1) and VV (35 kDa) binds chemokines with high affinity and blocks their interaction with cellular receptors.

Contradictory to the above studies, Luttichau et al showed that MC148 is a highly selective CCR8 antagonist.[17] This conclusion was based on work done with 16 different chemokine receptors transfected into cell lines and subsequently used in binding, calcium mobilization and chemotaxis assays. MC148 expressed in COS-7 cells only inhibited the binding of CCL1 to CCR8 and this specificity was corroborated in binding assays of ^{125}I-MC148 to cells. In calcium mobilization assays, MC148 had no effect on its own on any of the chemokine receptors, but blocked signal transduction of CCL1 on CCR8 without affecting signaling of any other receptor. Chemotaxis assays confirmed the specificity of MC148 for human CCR8. However, MC148 was unable to bind to and inhibit the murine CCR8 counterpart.[18] The reasons for the discrepancies with the above studies may be due, in part, to the fact that cells transfected with cloned chemokine receptors rather than primary cells expressing a multitude of receptors were used. Another reason may relate to the use of different expression systems imparting distinct properties to MC148.

Sequence database searches have identified a novel chemokine, interleukin-11 receptor alpha-locus chemokine (ILC/CCL27), as the closest MC148 homolog.[19] It was suggested that CCL27 and MC148 are most likely derived from the same ancestral gene. However, MC148 is unable to block activation of CCR10, the CCL27 receptor, and ^{125}I-MC148 does not bind to transfected COS-7 cells expressing CCR1O, CCR11, CXCR6, DARC, AJP or orphan receptors BOB, EBI-11, GRP17, HCR and RDC1.[18]

The role of MC148 in MCV immune evasion cannot be determined because there are no suitable animal models of infection. Nevertheless, the ability of MC148 to inhibit chemokine-mediated responses in vitro (Fig. 2) strongly suggests that this vCK plays a role in preventing an inflammatory response to MCV infection.[13]

Swinepox Virus (SPV) Chemokine Receptor Homolog

The K2R ORF of SPV, a poxvirus that causes mild infections in pigs, encodes a 370 amino acid protein with ~30% amino acid sequence identity to mammalian chemokine receptors.[20] A truncated and probably non-functional copy of the K2R ORF, designated C3L, is found at the other end of the viral genome.

Capripox Virus Chemokine Receptor Homolog

The capripoxvirus genus comprises the species of sheep pox, goat pox and Lumpy skin disease of cattle. Sequence analysis of the Kenya sheep isolate (strain KS-1) has identified the Q2/3L ORF encoding a 381 amino acid polypeptide with 38 % sequence identity to SPV K2R, and 34% and 29% identity to CC and CXC chemokine receptors, respectively.[21] Sequencing of the complete genome of Lumpy skin disease virus also revealed the presence of a CC chemokine receptor gene designated LSDV01. Its ORF is 43% identical to CCR8 at the amino acid level.[22]

FPV Chemokine and Chemokine Receptor Homologs

FPV, the prototipical member of the avipoxvirus genus, causes disease in chickens and turkeys with significant economical impact worldwide. The genomic sequence of a pathogenic strain of FPV showed that this avipoxvirus has the largest repertoire of ORFs encoding vCKs and vCKRs among poxviruses.[23] Four FPV ORFs, designated FPV060, FVP061, FVP116 and FVP121, encode homologs of CC chemokines. All but FVP061 have a predicted signal peptide at the amino terminus suggesting they may be secreted from infected cells. Three vCKRs were identified in FPV: FPV206 shows highest similarity to the Epstein-Barr virus-induced chemokine receptor, and FPV021 and FPV027 show highest similarity to GPRI, a monkey chemokine receptor.

Yaba-Like Disease Virus Chemokine Receptor Homologs

Yaba-like disease virus infects monkeys but can also be transmitted to humans where it causes a mild illness. Recent determination of the Yaba-like disease virus genome sequence has shown that it encodes two vCKRs, designated 7L and 145R, that are closely related to human CCR8.[24]

MV Chemokine Receptor Homolog

The genome sequence of MV contains an ORF, designated M104L, with significant sequence similarity to a region of ORF74, a vCKR found in gamma-herpesviruses.[25] A similar gene is also encoded by the leporipoxvirus Shope fibroma virus (SFV).[26] M104L is predicted to be a membrane protein of 53 amino acids with 42% sequence identity over a 40 amino acid region corresponding to the fifth and sixth transmembrane domains of ORF74. The fifth and sixth transmembrane domains and the intervening intracellular loop of chemokine receptors are thought to be involved in receptor signaling and dimerization. It has therefore been suggested that M104L could inhibit chemokine signaling by forming non-functional dimers with endogenous chemokine receptors or through the sequestration of downstream signaling molecules. However, M104L lacks the N-terminal signal peptide that directs chemokine receptors to the endoplasmic reticulum for transport to the plasma membrane, and is therefore unlikely to be expressed unless the fifth transmembrane domain could also function as a signal peptide.

The vCKBP-1 (M-T7) Encoded by MV

Discovery

Myxomatosis is associated with general immunosuppression of the host and thus MV was a prime candidate to encode immune evasion molecules. Initial work identified the MV M-T7 gene encoding a 37 kDa glycoprotein that is abundantly secreted from infected cells.[27] The M-T7 ORF showed significant similarity to the extracellular binding domain of human and mouse interferon-γ receptors (IFN-γRs), and was shown to bind to and inhibit the biological activity of rabbit IFN-γ in a species specific manner.[27,28]

Lalani et al reported later that M-T7 binds chemokines forming a complex that can be crosslinked and detected with specific antibodies.[29] M-T7 was therefore designated vCKBP-1. This finding was unexpected and is a unique property of the IFN-γR encoded by MV since the IFN-γR homolog encoded by VV does not bind chemokines.[30] M-T7 and its closely related counterpart from SFV, S-T7, are the only members of the vCKBP-1 family.

Binding Specificiy

Crosslinking studies showed that M-T7 interacts with a broad range of C, CXC and CC chemokines in a species non-specific manner. Studies with CXCL8 chemokine mutants indicated that chemokine binding to M-T7 is via the conserved C-terminal GAG binding domain found in a variety of chemokines.[29] Binding of CCL5 to M-T7 can be competed by rabbit IFN-γ suggesting that both ligands share M-T7 binding sites. However, heparin competes the interaction of M-T7 with CCL5 but not with rabbit IFN-γ. The interaction of CCL5 with M-T7 is of low affinity with a 50% inhibitory concentration of 900 nM.

Mechanism of Action

It is believed that under physiological conditions chemokines do not act in solution but are presented to chemokine receptors on leukocytes as ligands immobilized to a solid phase via interaction with GAGs. The interaction of M-T7 with the chemokine GAG binding domains led to the suggestion that M-T7 might prevent the correct localization of chemokines and the

formation of a chemokine gradient, rather than the blockade of chemokine binding to specific receptors.[29] However, this has not been formally demonstrated in chemokine binding assays to cells or GAGs.

Role in Viral Pathogenesis

Infection of rabbits with a MV mutant in which the M-T7 gene was inactivated by insertion of a LacZ cassette showed that vCKBP-1 is critical for MV pathogenesis in European rabbits.[31] Marked differences were seen in the size and progression of skin lesions, the onset and severity of secondary bacterial infections and clearance of the virus. Rabbits infected with the M-T7 mutant showed none of the severe symptoms associated with wild type MV infection and a reduced virus dissemination to secondary sites. M-T7 was implicated in the control of migration of inflammatory cells to sites of infection, which was increased in infections with the virus M-T7 mutant. These results are difficult to interpret because M-T7 targets IFN-γ and chemokines, and both have important roles in inflammatory immune responses. In addition, reliable analysis of M-T7 on virus virulence requires the generation of a revertant virus with restored M-T7 activity, not used in this study, to control for mutations introduced inadvertantly elsewhere in the genome during the generation of the original virus mutant.

The vCKBP-2 (MV M-T1/VV 35kDa) Encoded by Poxviruses

Discovery

Poxvirus genomes encode a second class of vCKBPs, known as vCKBP-2, that inhibit CC chemokines by a different mechanism from that of the MV M-T7 protein. Their identification and initial characterization was reported independently by three groups.[30,32,33] Two different strategies were used to identify the chemokine binding activities and map them to a specific viral gene.

In the first approach, Graham et al[33] and Alcami et al[30] noted that poxvirus genomes encode secreted proteins that bind to host cytokines.[4,5] Speculating that these viruses might employ a similar strategy to block chemokine activities, the two groups screened media from poxvirus-infected cultures and discovered that soluble, secreted viral proteins that bound to the human CC chemokines ^{125}I-CCL5 and ^{125}I-CCL3 were produced by MV, SFV, raccoonpox virus, CPV, camelpox virus and certain strains of VV, but not by SPV. Less consistent results were reported with CXC chemokines. Graham et al[33] readily detected binding between the viral factor and human ^{125}I-CXCL8. In contrast, Alcami et al[30] found that ^{125}I-CXCL8 and ^{125}I-CXCL1 gave weak or negative results.

Both groups identified the viral ORF encoding this vCKBP by exploiting the fact that it was not expressed by the VV strains Western Reserve (WR) and Copenhagen, but was expressed by VVs Lister and Rabbitpox (RPV), among others. This expression profile matched that of a 35-kDa protein of unknown function which is secreted from VV-infected cells at early and late times post-infection.[34,35] The gene encoding this protein lies within the inverted terminal repeats at the ends of the VV genome. In VV Copenhagen its ORF is truncated prematurely by a frameshift mutation within the N-terminal signal peptide,[36] while in VV WR it produces a 7.5-kDa protein unrelated to the 35-kDa species.[34,37]

This 35-kDa protein was confirmed as the vCKBP by performing the same assays with two recombinant VVs, VV RPVΔ35[35] and VV Lister Δ35K,[34] from which the fully functional gene had been inactivated by insertion of the LacZ gene. Neither of these viruses produced a secreted protein that bound to ^{125}I-chemokine. Furthermore, when the VV Lister 35-kDa ORF was expressed with a C-terminal 6xHis-tag in the baculovirus system, or as an Fc fusion protein from stably transfected mammalian cells, binding of the recombinant products to

^{125}I-CCL5 could be detected. The MV M-T1 and SFV ST-1 ORFs show 70% amino acid identity to each other and 40% identity to the VV Lister and RPV 35-kDa gene products. Expression of the M-T1 ORF from VV WR, which encodes no vCKBP activity, demonstrated that M-T1 encodes the leporipoxvirus vCKBP-2.[33]

In the second approach, Smith et al[32] presumed that the 35-kDa gene of VV and related viruses encodes a virulence factor that binds host immunoregulatory factors. They therefore searched for host ligands using surface plasmon resonance technology. The 35-kDa homolog from CPV Brighton Red (BR) was expressed as an Fc fusion protein, called p32/Fc, and immobilized on a sensor chip. Conditioned media from 164 cell lines were passed over the chip and one, from the murine thymic epithelium cell line TE71, gave a strong specific resonance signal. Immunoprecipitation with ^{35}S-methionine labelled TE71 conditioned medium and p32/Fc identified two proteins that bound to the viral species. Affinity purification using a p32/Fc column, followed by SDS-PAGE and partial amino acid sequencing identified them as differentially glycosylated isoforms of murine CCL2, a CC chemokine. Thus the CPV BR homolog of the VV 35-kDa gene encodes a vCKBP which was named viral CC chemokine inhibitor (vCCI). Parallel experiments with the VaV (India 1967) homolog confirmed that the virus which caused smallpox in humans also expressed vCKBP-2. Later work also established that the OPV EV expresses a vCKBP-2 family member.[38]

The leporipoxvirus vCKBP-1 was originally identified as a soluble IFN-γR. Since OPVs encode a related gene, designated B8R in VV, it was of relevance to determine that the chemokine binding activity was not encoded by the OPV B8R gene. This was formally demonstrated by using recombinant VV B8R and 35-kDa proteins in cross-linking experiments with ^{125}I-CCL5 and ^{125}I-IFN-γ, by cross-competition experiments, and by showing that the VV 35 kDa mutant retained its ability to bind IFN-γ.[30]

Binding Specificity

The binding of members of the vCKBP-2 family to numerous chemokines has been demonstrated and quantified using a variety of direct and competition binding assays including chemical crosslinking, scintillation proximity assays, plate binding assays and surface plasmon resonance.[30,32,33,38] This work has demonstrated that vCKBP-2 binds to almost all human, mouse and rat CC chemokines with high affinity. The low-affinity binding of vCKBP-2 to the CXC chemokines human CXCL8 and CXCL1 has also been detected, but it has not been possible to demonstrate binding to a range of other CXC, C or CX_3C chemokines.

Measurements of the affinity of vCKBP-2 for CC chemokines produced variable results for different vCKBP-2 family members, which may be accounted for by variability in reagent quality and binding assay sensitivity. Measurements with M-T1 and ^{125}I-CCL5 produced a K_D of 73 nM, comparable to that of CC chemokine binding to cellular receptors.[33] Lower K_Ds, indicating higher affinities, ranging between 103 pM for human CCL3 and 15 nM for human CCL2 were determined for a recombinant VV Lister vCKBP-2/Fc fusion protein using a scintillation proximity assay.[30] An assay of inhibition of ^{125}I-CCL2 binding to an immobilized CPV BR p32/Fc fusion protein was used to determine K_Ds for numerous CC chemokines.[32] These ranged from 2.4 nM for human CCL2 to 5 pM for rat CCL3. The affinity for human CCL5 determined using this method differed from that determined for M-T1[33] by a factor of almost 1000. Binding of the human CXC chemokine CXCL8 was also determined, using this method, to have a K_D of about 50 nM.

The high-affinity binding by vCKBP-2 of all CC, but not CXC, C or CX_3C chemokines, is a unique property that distinguishes it from cellular CC chemokine receptors, which bind only a subset of CC chemokines with high affinity. The amino acid residues of human CCL2 that are required for high affinity interaction with the VV 35 kDa vCKBP-2 have been recently

identified by using a panel of deletion and site-directed CCL2 mutants.[39] The general conclusion is that similar CCL2 binding epitopes are used for recognition of both VV 35-kDa protein and CCR2b. The N-terminal amino acids of CCL2 do not participate in binding to the VV 35-kDa protein, in spite of the N-terminal region of some chemokines having been implicated in receptor binding. The VV 35-kDa protein recognizes CCL2 epitopes relatively conserved amongst the CC chemokines, providing a structural basis for the ability of vCKBP-2 to promiscuously recognize CC chemokines.

Mechanism of Action

Disruption of either chemokine-receptor or chemokine-GAG complex formation might inhibit chemokine biological activity. It was of interest to determine whether the binding of CC chemokines by vCKBP-2 precluded their interaction with cellular receptors, GAGs or both.

Preincubation of ^{125}I-CCL3 with various concentrations of the GAGs heparin and heparan sulphate, up to 100 μg/ml, prior to binding to the VV Lister vCKBP-2 revealed that GAGs did not interfere with the vCKBP-chemokine interaction.[30] In contrast, pretreatment of human ^{125}I-CCL3, human ^{125}I-CCL5 or mouse ^{125}I-CCL2 with vCKBP-2 from CPV BR, VV Lister, RPV and camelpox virus inhibited the binding of chemokine to receptors on human U937 and THP-1 cells.[30,32] The EV vCKBP-2 also inhibited the interaction of ^{125}I-CCL5 with receptors on U937 cells.[38] In contrast, the binding of ^{125}I-CXCL1 to receptors on U937 cells was not inhibited by vCKBP-2 from CPV BR, VV Lister, RPV or camelpox virus.[30]

It therefore seems that the mechanism of vCKBP-2 action is competitive inhibition of CC-chemokine binding to cellular receptors. This distinguishes vCKBP-2 from vCKBP-1, the MV IFN-γR that interacts with chemokines via their GAG binding domains.[29] However, recent work has demonstrated that the M-T1 protein of MV has the unique ability to directly interact with GAGs via a GAG binding domain that is not present in other vCKBP-2 family members.[40] This domain is distinct from the M-T1 chemokine binding domain, since the vCKBP could interact with both GAGs and CC chemokine simultaneously, and was mapped to the C-terminus of M-T1. However, high concentrations of M-T1 could displace ^{125}I-CCL2 from complexes with cellular GAGs, presumably owing to the occupation of all available GAG sites by M-T1 itself. This unique property of M-T1 would retain the protein in the vicinity of infected cells and may enhance its ability to protect the sites of infection from chemokine-mediated anti-viral responses.

Evidence of chemokine biological activity is provided by two well-established indices of chemokine receptor activation upon binding of ligand: the induction of transient increases in cytoplasmic calcium concentrations and the migration of cells along chemotactic gradients. The ability of vCKBP-2 to inhibit the induction of these two phenomena by CC chemokines has been conclusively demonstrated. Migration of primary human monocytes and U937 cells in response to CCL3 or CCL2 is inhibited by vCKBP-2 from several poxviruses.[30,32,41] Similarly, chemokine-induced mobilization of intracellular calcium stores is inhibited in THP-1 cells, human eosinophils, human neutrophils and differentiated HL-60 cells by vCKBP-2.[30,32,41]

Role in Viral Pathogenesis

The expression at early and late times post-infection of an abundantly secreted vCKBP-2 might be expected to make a significant contribution to virus virulence in vivo by inhibiting CC-chemokine-mediated host inflammatory responses. Surprisingly, the experimental evidence currently available indicates that vCKBP-2 has only marginal effects on poxvirus virulence.

In the earliest study,[35] intranasal inoculation of BALB/c mice with a mutant RPV Δ35 in which the vCKBP-2 gene has been replaced by the LacZ gene had little difference in disease progression or mortality as compared to wild type RPV. Inoculation of rabbits with 500 PFU

of either RPV or RPV Δ35 resulted in no differences in the primary cutaneous lesions produced, or in the recovery of either virus from the lungs of systemically infected rabbits. A subsequent study[33] using larger doses of these viruses in the rabbit intradermal model found significant differences in the histopathology of primary cutaneous lesions that they produced at day 3 post-infection. RPV Δ35-infected rabbits had lesions with a marked increase in infiltrating lymphocytes and leukocytes, as compared with RPV. The natural conclusion of this study, that the absence of vCKBP-2 permits increased inflammation of the primary lesion, must be drawn with caution. RPV Δ35 was generated by the transfection of VV Lister DNA into VV RPV-infected cells.[35] Therefore, mutations in the VV Lister genome flanking the vCKBP-2 gene may have been incorporated into the RPV genome. The construction of a revertant virus from RPV Δ35 with restored vCKBP-2 activity and in vivo phenotype is necessary to support the conclusions.

More convincing data on the role of vCKBP-2 in viral pathogenesis has been obtained using MV.[42] Deletion mutant and revertant viruses in the M-T1 gene were constructed and compared with wild-type virus in the rabbit intradermal model of infection. All three viruses produced a similar pathogenic profile, but the M-T1 deletion mutant produced larger primary lesions in the first 3 days post-infection. Histopathology revealed that these lesions displayed a marked increase in the number of infiltrating mononuclear cells although virus levels in lesions were similar for all three viruses. These data do indeed suggest that vCKBP-2 inhibits the chemokine mediated infiltration of immune cells into primary sites of infection, but has little influence on the overall progression of disease.

There are two possible reasons to explain the minor effects that inactivation of vCKBP-2 had on viral pathogenesis. One is that other viral virulence factors have activities that compensate for or overlap with those of vCKBP-2. Therefore, studies with viruses lacking two or more virulence genes might be useful. The second is that the appropriate model of infection has not been used. The natural host of MV is the South American, but not the European, rabbit and VV RPV is not a natural pathogen of rabbits or mice. Perhaps EV, a natural mouse pathogen that causes mousepox, may provide a good alternative model.[43] EV expresses a CKBP-2 that binds to mouse chemokines and is highly conserved among different virus isolates.[38]

Structure

The unique biological properties of vCKBP-2 make it imperative that the mechanism of its interaction with chemokines is characterized at the molecular level. The structure of the CPV BR vCKBP-2 has been determined by X-ray crystallography[44] (Fig. 3). Unfortunately, no structure of any vCKBP-2-CC-chemokine complex is available. vCKBP-2 is a compact globular protein composed of two large parallel β-sheets, two short α-helices and several large connecting loops. The β sandwich topology of the protein is thought to be unique. The protein forms a crystallographic homodimer in which the two monomers are connected via β sheet extension between β sheet II and a large acidic loop which extends outwards from the same surface in the other monomer.

By looking for exposed charged residues that have no apparent structural role and which are conserved among different members of the vCKBP-2 family, it has been possible to suggest potential binding sites for chemokine on the vCKBP-2 molecule. On this basis there are two strong candidate chemokine binding regions. One is on the exposed face of β sheet II where 3 conserved acidic residues and one tyrosine residue form a large exposed surface. The second is at the edge of β sheet II between the conserved acidic residues D168 and E206. Further crystallographic and mutagenesis studies will be required to establish whether either surface actually plays a role in high affinity binding of CC chemokines.

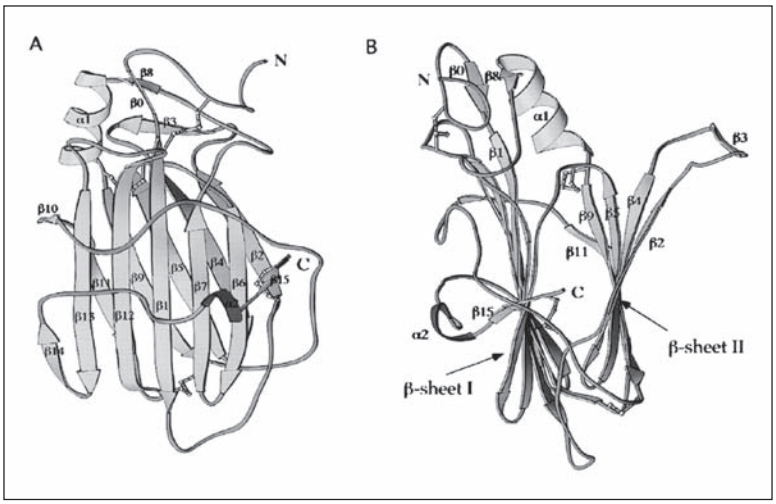

Figure 3. Structure of the CPV vCKBP-2. Ribbon diagrams. A and B are related by 90° rotation. Reproduced from reference 44 with permission.

The vCKBP-3 Encoded by Gammaherpesvirus

Discovery

The discovery of vCKBPs in the poxviruses raised an important question: is the production of a secreted vCKBP an immune evasion strategy unique to the poxviruses, or is it also exploited by other mammalian viruses? The herpesviruses are an obvious virus family in which to search for novel activities since they, like the poxviruses, have large DNA genomes encoding numerous genes involved in immune evasion.

Sequence analysis of several herpesvirus genomes has not, unlike in poxviruses, revealed the existence of many gene products with homology to cellular cytokine receptors or to soluble cytokine or chemokine binding proteins. Rather, these viruses encode cytokine and chemokine homologs, and vCKRs. The lack of herpesvirus gene products with sequence similarity to other cytokine or chemokine binding proteins does not preclude the existence of such factors. This viewpoint has recently been validated by the independent identification, by two groups, of a secreted herpesvirus CKBP, now named vCKBP-3, encoded by murine gammaherpesvirus 68 (MHV-68).[45,46]

MHV-68 is a pathogen of wild rodents. Genomic analysis indicates that it is related to the primate gammaherpesviruses Epstein-Barr virus, Kaposi's sarcoma associated herpesvirus and Herpesvirus Saimiri.[47] MHV-68 provides a useful mouse model of gammaherpesvirus pathogenesis. The MHV-68 genome encodes 80 ORFS, most of which are also present in other gammaherpesviruses. However, 16 ORFs, designated M1 to M14, are unique to MHV-68.[47]

A chemokine-binding activity secreted from MHV-68-infected cells was identified by covalently crosslinking culture supernatants to chemokines. In the first published report, Parry et al[46] detected 45-kDa crosslinked complexes between human ^{125}I-CCL3 and ^{125}I-CCL5 (CC chemokines), ^{125}I-CXCL8 (CXC chemokine) and ^{125}I-CX_3CL1 (CX_3C chemokine) and a secreted viral factor. This factor was identified as the product of the MHV-68 M3 gene by showing that a recombinant virus in which the M3 gene was disrupted by the insertion of a LacZ gene did not express chemokine binding activity. As a control, a revertant virus in which the M3 ORF is restored gave results identical to wild-type virus. Recombinant M3 produced

in the baculovirus system could also be crosslinked to labelled chemokine. In the second report, van Berkel et al[45] performed a similar assay with unlabelled human CCL5, CXCL8 and CCL2 but detected M3-chemokine complexes as supershifted bands by immunoblotting using an anti-M3 antiserum. Complexes between M3 and human ^{125}I-CCL5 could be immunoprecipitated using this antiserum. Thus, M3 was identified as the first herpesvirus and third viral CKBP, and named vCKBP-3.

The M3 gene is unique to MHV-68. It encodes a 406 amino acid polypeptide which is abundantly secreted from infected cells as a soluble protein that is not specifically associated with the cell surface or virions.[48] The M3 product is translated from an abundant, unspliced 1.4 kb mRNA detectable at both early and late times post-infection. The N-terminal 24 amino acids of the protein encode a secretory signal peptide that is cleaved from the mature protein. M3 has no significant amino acid similarity to any other protein of known function, including other known vCKBPs. Its only significant sequence similarity is with the MHV-68 M1 gene product which itself shares some amino acid similarity with poxvirus serpins, but whose function is unknown.[47] M3 itself is not homologous to these or any other poxvirus proteins. The M1 and M3 genes probably arose by a gene duplication event and have since diverged to encode distinct immune evasion functions.

Binding Specificity

Unlike vCKBP-2, which is specific for CC chemokines, vCKBP-3 is able to bind to CC, CXC, C and CX_3C chemokines. Competition experiments in which vCKBP-3 was crosslinked to human ^{125}I-CCL3 or ^{125}I-CXCL8 in the presence of numerous unlabelled chemokines revealed that vCKBP-3 bound all 7 of the CC chemokines tested, along with human lymphotactin and human fractalkine.[46] Of the 10 CXC chemokines investigated in this way, human CXCL8, CXCL1, CXCL10 and granulocyte chemotactic protein 2 (GCP-2/CXCL6) and mouse B-cell attracting chemokine 1 (BCA-1/CXCL13)were found to bind the viral protein,while murine KC/CXCL1,MIP-2/CXCL2,LPS-induced CXC chemokine (LIX/CXCL5),BCA-1/CXCL13 and human CXCL12 did not. Therefore although vCKBP-3 binds to chemokines of all four classes, it is not likely to be an effective inhibitor of all CXC chemokines. Estimates of vCKBP-3 affinity for several chemokines obtained using an immunoprecipitation assay with ^{125}I-CCL5 as the labelled ligand produced results that agreed with those from crosslinking experiments.[45] Human CCL5and CXCL8 and mouse CCL2, CCL3, XCL1 and CX_3CL1 were bound with K_Ds in the low nanomolar range, while K_Ds for the interaction with nine mouse CXC chemokines were in excess of 1 μM. It is surprising that vCKBP-3 binds CXCL8 with high affinity but the other ELR+ CXC chemokines GRO-α/CXCL1, CXCL6, KC/CXCL1, CXCL2, CXCL5 with low affinity, since all of these chemokines are ligands for CXCR-2, and CXCL6 is, like CXCL8, a ligand for both CXCR-1 and CXCR-2. Analysis of the interaction of recombinant purified M3 with chemokines using a scintillation proximity assay indicates very high affinities of M3 to various chemokines, with K_D values in the 10-100 pM range (R. Fallon, C.J. Bunce and A. Alcami, unpublished). The discrepancy with the published affinity data may reflect the use of a more sensitive binding assay.

Mechanism of Action

The mechanism by which vCKBP-3 inhibits chemokines appears to be similar to that of vCKBP-2. Both proteins bind free chemokine with high affinity in a manner which prevents their ligation of cellular chemokine receptors. The interaction between chemokine and vCKBP-3 was not inhibited by the addition of a large excess of the GAGs heparin or heparan sulphate, suggesting that vCKBP-3 does not interact with the GAG-binding domain of chemokines. vCKBP-3 blocked the binding of human ^{125}I-CXCL8 and ^{125}I-CCL5 to receptors on human U937 cells.[46] Furthermore, prevention of receptor-ligand interactions by vCKBP-3 results in a

blockade of chemokine biological activity. Addition of vCKBP-3 prevented the induction of transient increases in cytoplasmic calcium concentration in relevant cell types by the CC chemokines human CCL5, mouse CCL3 and mouse CCL2, the CXC chemokine human CXCL8 and the CX_3C chemokine mouse CX_3CL1.[45,46] Consistent with the binding data, vCKBP-3 failed to inhibit mouse CSCL1 or CXCL12 in similar assays.

Role in Viral Pathogenesis

The role of vCKBP-3 in MHV-68 pathogenesis has been very recently reported.[49] After intranasal infection, MHV-68 replicates transiently in respiratory epithelial cells and spreads to lymphoid tissue where latency is established in B lymphocytes, macrophages and dendritic cells. Targeted disruption of the M3 gene had surprisingly little effect on lytic virus replication in the respiratory tract or the initial spread of virus to lymphoid tissues after intranasal inoculation. However, the mutant virus failed to establish normal levels of latency in splenic B cells. Interestingly, in vivo CD8+ T cell depletion largely reversed the phenotype, suggesting that chemokine neutralization by M3 may function to block CD8+ T cell recruitment into lymphoid tissue and to enable the establishment of MHV-68 latency. These results suggest that expression of potent chemokine inhibitors may be a requirement for effective host colonization by lymphotropic herpesviruses.

vCKBPs: Their Evolutionary Origin and Potential Therapeutic Applications

Poxviruses have evolved three different mechanisms to block the activity of chemokines (Fig. 2): i) MCV encodes a homolog of host chemokines with antagonistic activity; ii) MV encodes vCKBP-1, a soluble vIFN-γR that also prevents the interaction of chemokines with GAGs and their correct localization; and iii) OPVs and MV encode vCKBP-2, a secreted protein that blocks the interaction of chemokines with specific receptors and the induction of biological activity. In addition, the herpesvirus MHV-68 encodes vCKBP-3 with no sequence similarity to vCKBPs but which, like the poxvirus vCKBP-2, also binds chemokines with high affinity and prevents their interaction with cellular receptors. The vCKBPs identified in viral genomes were not predicted from sequence analysis to bind chemokines, and it is possible that the secretion of CKBPs of unrelated structure is a strategy used by other viruses or maybe other pathogens such as parasites.

An interesting question derived from these studies is why viruses utilize different strategies to modulate chemokine activity. While vCKBPs have been identified mainly in poxviruses, herpesviruses frequently encode vCKs and vCKRs.[6,7] This may reflect the need to modulate different aspects of chemokine biology as a result of diverse viral replication mechanisms.

The unique structure of the vCKBPs encoded by poxviruses and herpesviruses raises an important question: did these viral anti-chemokine activity arise via the acquisition of a mammalian immunomoregulatory gene with a similar function, as is the case with many other virus virulence factors? Although analysis of the human genome sequence has not identified so far homologs of these vCKBPs, human soluble CKBPs of different structure may exist. This would be particularly relevant if soluble CKBPs are to be used as therapeutic reagents in the future.

The production by mammals of a soluble chemokine inhibitor that regulates the potent pro-inflammatory activity of chemokines is an attractive hypothesis, and one with precedent in biology. For example, IL-18 binding activity is downregulated in mice and humans by the production of a secreted IL-18 binding protein unrelated to cellular IL-18 receptors.[50] Indeed, the OPVs and MCV encode homologs of the mammalian protein that inhibit IL-18 responses.[51-53] However, in the case of chemokines, there is no formal requirement for such a soluble chemokine binding factor as other mechanisms by which the host regulates chemokine

responses have been identified. For example, the anti-inflammatory cytokine IL-10 decouples chemokine receptors from intracellular signaling processes, thereby allowing them to sequester chemokines without inducing biological responses.[54]

Alternatively, vCKBPs may result from evolutionary modification of an ancestral viral gene with an unrelated activity. This is illustrated by the OPV vCKBP-2 which shares some primary amino acid sequence similarity to VV A41L, another secreted virulence factor.[55] Most notably, eight cysteine residues are conserved between the two proteins, indicating that they share a similar overall fold. A weak interaction of the CXCR3 ligands CXCL9, CXCL10 and CXCL11 with A41L protein has been demonstrated using surface plasmon resonance technology, but A41L does not inhibit the biological activities of these or other known chemokines.[55] The gene is conserved in VV and VaV, suggesting evolutionary pressure for its retention by these viruses. However, to date, the biochemical basis of its function remains unknown. Interestingly, A41L, but not vCKBP-2, shares some sequence similarity with the orf virus GIF gene product, which binds to and inhibits granulocyte macrophage-colony stimulating factor and IL-2.[56] However, A41L itself does not interact with either of these cytokines.[55] Maybe a family of viral proteins related to vCKBP-2 has evolved to interact with a variety of host cytokines.

The identification of three vCKBPs with no amino acid sequence similarity among themselves or to cellular receptors is providing us with novel protein structures capable of neutralizing chemokines in solution. Elucidation of the interaction of these vCKBPs with chemokines at the molecular level will provide some of the most interesting findings in this area of research.

The chemokine network is a major target for the development of drugs useful in the control of inflammatory diseases.[57] vCKBPs have great potential in this area as they, or peptide mimetics designed using them, may be useful general inhibitors of chemokine-mediated inflammatory disease processes. The effectiveness of vCKBP-2 as an inhibitor of local inflammation in vivo was established by showing that vCKBP-2 from VV Lister completely inhibited the CCL11-mediated infiltration of eosinophils into guinea pig skin when present in only 3-fold molar excess over the chemokine.[30] Another study examined the effect of administering vCKBP-2 from CPV BR on the symptoms observed in a mouse model of allergen–induced asthma.[58] vCKBP treatment significantly reduced inflammation of the airway and lung parenchyma, and improved the physiological function of the lungs during airway hyperreactivity. The reduced inflammation may be directly attributable to a decrease in chemokine-mediated cellular infiltration, while the physiological effects may be due to a reduced infiltration of cells that produce physiological mediators of bronchoconstriction.

Two other viral chemokine inhibitors have been tested as potential therapeutic reagents. Transfer of plasmid DNA encoding the MCV vCK MC148 into a cardiac allograft in mice considerably prolonged allograft survival, decreased donor specific cytotoxic T cells infiltrating the grafts and inhibited antibody production.[59] In another study, a single injection of purified MV M-T7 given to rats or rabbits caused a significant attenuation of restenosis, a response to vascular injury that leads to recurrent atherosclerotic plaque growth. Results observed in rats and rabbits were very similar suggesting that the non-species specific chemokine binding activity of M-T7, and not its rabbit-specific IFN-γR activity, had a dominant role in the anti-atherogenic effects.[60]

Future studies will no doubt further assess the utility of viral chemokine inhibitors in other models of inflammation. It is interesting that products derived from pathogenic viruses hold substantial promise for the treatment of human inflammatory diseases.

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CHAPTER 3

Herpesvirus Encoded Chemokines and Chemokine Receptors

Thomas N. Kledal

Introduction

Herpesviruses and poxviruses have pirated components of the host chemokine system and optimized these proteins to increase their success during infection. Both the β-herpesviruses, e.g., human cytomegalovirus (HCMV), and the γ-herpesviruses, e.g., Kaposi's sarcoma associated herpesvirus (KSHV) devote a significant portion of their genomes to immune-modulatory gene homologs that have prominent and potent chemokine and chemokine receptor activities. HCMV encodes two chemokine homologs, vCXCL1(UL146) and vCXCL2(UL147), a constitutively active, broad spectrum CC/CX_3C-chemokine receptor, US28, and three orphan seven trans-membrane-spanning (7TM) G-protein coupled receptors (GPCRs), UL33, UL78 and US27. KSHV encodes three chemokine homologs, vCCL1 (vMIP-I/1α), vCCL2 (vMIP-II/1β) and vCCL3 (vMIP-III/BCK) and one constitutively active, broad-spectrum CXC-chemokine receptor, ORF74. By manipulating the host chemokine system, herpesviruses subvert the host immune response, not only to undermine the effectiveness of antiviral immunity, but also to directly benefit the virus by establishing a foothold and promoting dissemination in the host. Future studies on the interactions between the chemokine system and the leukotropic herpesviruses are likely to provide important insights into the biology of herpesviruses as well as into the regulation of the mammalian immune system.

Herpesvirus Encoded Chemokines and Chemokine Receptors

Mammalian and avian herpesviruses have been classified into three subfamilies based on biological and molecular criteria, α-, β- and γ-herpesviruses. The α-herpesvirus subfamily includes human herpesvirus (HHV) -1 (also referred to as herpes simplex virus (HSV) –1), HHV-2 (also referred to as HSV-2) and HHV-3 (also referred to as varicella zoster virus (VZV)). The α-herpesviruses are characterized by a relative short replication cycle and establishment of latent infection in sensory neurons. None of the human α-herpesviruses are known to encode chemokines or chemokine receptors. Only gallid herpesvirus 2, also called Marek's disease virus, encodes a protein, vIL-8, with similarities to human CXCL8 (IL-8).[1] The β- and γ1 herpesvirus genomes have acquired homologs of both chemokines and their receptors which may reflect the importance of host leukocytes in acute, persistent and latent phases of infection in the host. The β-herpesviruses HHV-5 (also referred to as human cytomegalovirus or HCMV), HHV-6 and HHV-7 have a relatively long replication cycle and interact with mononuclear as well as polymorphonuclear leukocytes. Mononuclear myeloid lineage cells are thought to be critical for both acute infection and latency of cytomegaloviruses. All members of the

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β-herpesvirus family, including HCMV, chimpanzee (CCMV), simian (SCMV), rhesus CMV (RhCMV), murine CMV (MCMV), rat CMV (RCMV), Guinea pig CMV (GpCMV) HHV-6, HHV-7 and tupaia herpesvirus (THV), encode chemokine and/or chemokine receptor homologs in their genomes. The γ-herpesviruses are divided into two biologically distinct classes, γ1-herpesviruses, which include HHV-4 (also referred to as Epstein-Barr virus (EBV)) and γ2-herpesviruses, which include HHV-8 (also referred to as Kaposi's sarcoma associated herpesvirus (KSHV)). Members of the γ-herpesvirus demonstrate specificity for lymphoid and epithelial cells, and have been implicated in transformation of infected cells.[2] Members of the γ1-herpesvirus family (e.g., Rhesus EBV (RhEBV), Callitricine herpesvirus 3 (CHV3), and porcine lymphotrophic herpesvirus 1 and 2 (PLHV1 and 2)) and the γ2-herpesviruses, (e.g., herpesvirus saimiri (HVS)), γ-herpesvirus 68 (γHV68), rhesus rhadinovirus (RhRV) and ateline herpesvirus 3 (AtHV3)) contain chemokine- and/or chemokine receptor- homologs in their genomes. Besides encoding chemokine ligands and receptors of similar structure to mammalian ligands and receptors, HCMV (Shenk T. personal communication) and γHV68[3] produce chemokine binding proteins that do not exhibit similarity to each other or to any known mammalian protein. Other, structurally distinct chemokine binding proteins are also encoded by several poxviruses (see Chapter 2).

Genes and Expression

β-Herpesvirus Encoded Receptors and Ligands

Sequence analysis of the 230 kb HCMV laboratory adapted strain AD169 genome revealed four open reading frames (ORFs), UL33, UL78, US27 and US28 with hallmarks of 7TM receptors.[4,5] US28 was originally identified as a chemokine receptor when the first CC chemokine receptor gene (CCR1) was cloned.[6] When genomic regions that are lost from strain AD169 but preserved in the limited passage Toledo strain genome were evaluated, two chemokine homologs, vCXCL1 and vCXCL2 were identified.[7] MCMV and RCMV, which have similar pathogenesis to HCMV in their rodent hosts, encode homologs of two of the 7TM receptors (M33/R33 and M78/R78)[8,9] as well as one chemokine homolog (MCK1/r131).[9,10]

UL33 is an orphan 7TM receptor, with sequence (and positional) homologs found broadly in the β-herpesviruses MCMV (M33) (47%),* RCMV (R33) (47%), THV (T33) (48%), guinea pig CMV (GpCMV) (Gp33) (41%), RhCMV (Rh33), CCMV (C33), HHV6 (U12) (24%) and HHV7 (U12) (24%) (Table 1 and Fig. 1). UL33 is approximately 25% identical to the closest related mammalian CC-chemokine receptors. UL33 is expressed from a spliced transcript both early and, most abundantly at late times during infection of human fibroblasts.[11,12] M33 is expressed starting at 3 hours post infection from a 4.5 kb transcript. At 24 hours post infection two M33 transcripts (4.5 and 5.5 kb respectively) are detected and both are downregulated when viral DNA synthesis is inhibited.[12] UL33, M33 and R33 transcripts all depend on viral protein synthesis and are down regulated by inhibitors of viral DNA replication.[12,13] The natural pUL33 has an N-terminal extension[5,12] compared to the originally annotated ORF[8] that results from mRNA splicing. The splicing pattern exhibited by UL33 is common to M33, HHV6-U12 and HHV7-U12 as well, but does not appear to be a characteristic of R33.[12,13] In HCMV infected fibroblasts UL33 is expressed as a heterogeneously glycosylated protein with a molecular weight ranging from approximately 58 to 100 kDa.[5]

UL78 is an orphan 7TM receptor, with sequence and/or positional homologs present in MCMV (M78), RCMV (R78), THV (T78) (30%) HHV6 (U51) and HHV7 (U51) (Table 1

*Numbers in brackets indicate % identity to homologous protein encoded by the homologous human pathogenic virus. % identity is based on BLAST search. (continue on page 34, footnote)

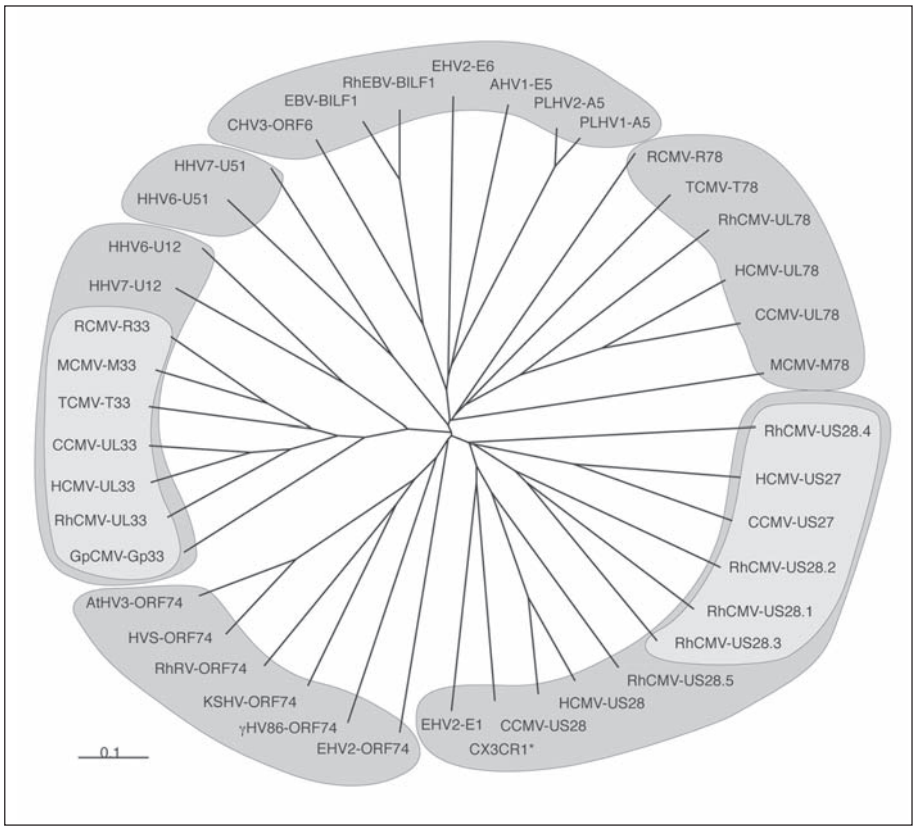

Figure 1. Phylogenetic dendrogram of herpesvirus encoded GPCRs and chemokine receptors. The phylogenetic tree are based on a Clustal X 1.81 alignments of the full amino acid sequence of all included receptors and the graphic presentation were generated using TreeView. GPCRs encoded by human and animal herpesviruses are included in the dendrograms together with human CX_3CR1 (marked with asterisk). The length of each branch predicts identity between the receptors. Shaded areas illustrate receptor clusters. Abbreviations are as in Table 1.

and Fig. 1). Besides showing hallmarks of a 7TM receptor, UL78 has minimal similarity to any known mammalian proteins, and the similarity between UL78 homologs (except between HHV6 U51 and HHV7 U51) is very limited (Fig. 1). U51 is expressed with early kinetics (not inhibited by viral DNA synthesis inhibitors) in HHV6 infected primary cord blood mononuclear cells.[14] Like HHV6-U51, R78 is transcribed as an early gene.[15]

US27 is an orphan 7TM receptor with approximately 30% identity to US28 and the greatest (27%) identity to mammalian CC-chemokine receptor, rat CCR4. The similarity and proximity to US28 suggests a gene duplication event rather than two independent acts of molecular piracy. US27 is transcribed as a late transcript during HCMV infection.[11,16,17]

US28 is approximately 38 % identical to human CX_3CR1 and is unique in the chemokine system due to the capability to bind a very broad spectrum of CC-chemokines as well as the CX_3C-chemokine fractalkine. US28 (and US27) homologous are not present in any other human β-herpesvirus or in any of the rodent CMVs analyzed to date. Nevertheless, US28 homologs have been identified in CCMV, SCMV and RhCMV (Table 1 and Fig. 1). CCMV

Table 1. GPCRs and chemokine receptors and chemokine ligands encoded by herpesviruses

	Virus Encoded		Host Target		Receptor / Ligand Function		
Virus	**Ligand**	**Receptor**	**Receptor**	**Ligand**	**In vitro Activities**	**In vivo Activities**	**Homologs in Other Herpesvirus**
β-herpesvirus							
HCMV (HHV5)		**UL33**		unknown	Constitutive signaling Intracellular localization (late endosomes) Envelope protein	Replication in the salivary gland or viral entry/dissemination to the salivary gland Virulence factor	MCMV (M33) RCMV (R33) THV (T33) CCMV (UL33) RhCMV (UL33) GpCMV Gp33) HHV6 (U12) HHV7 (U12)
		UL78		unknown (U51 (HHV6) binds CC-chemokines)	Viral replication Transcription regulation Intracellular localization Envelope protein	Viral replication Virulence factor	MCMV (M78) RCMV (R78) THV (T78) CCMV (UL78) RhCMV (UL78) HHV6 (U51) HHV7 (U51)
		US27		unknown	Intracellular localization (late endosomes) Envelope protein Constitutive recycling	unknown	CCMV (US27) RhCMV (US28.1) RhCMV (US28.2) RhCMV (US28.3) RhCMV (US28.4)

continued on next page

Table 1. Continued

	Virus Encoded		Host Target		Receptor / Ligand Function		
Virus	**Ligand**	**Receptor**	**Receptor**	**Ligand**	**In vitro Activities**	**In vivo Activities**	**Homologs in Other Herpesvirus**
		US28		CX_3CL1 CCL2,3,4,5,7 vMIP-II	Constitutive Signaling Ligand mediated signaling Chemokine scavanger Cell migration Intracellular localization (late endosomes) Constitutive recycling Attachment/membrane fusion	unknown (expressed in PBLs)	CCMV (US28) RhCMV (US28.5) EHV2 (E1) Simian CMV (like RhCMV) encodes 5 homologs of US27/US28 KSHV (vCCL2) functional?
	vCXCL1		CXCR2		Agonist Ca^{++} mobilization Neutrophil Chemotaxis	unknown	CCMV (UL146a and UL146b) MDV (vIL8)
	vCXCL2		unknown		unknown	unknown	unknown
MCMV	**MCK1/2**		unknown		Agonist Ca^{++} mobilization Monocyte adhesion	Pro-inflammatory (induce swelling) Induce a mononuclear infiltrate Promote virus dissemination	RCMV r131 GpCMV (MIP-1-like, GenBank AAN03823) HCMV (UL128, GenBank AY169800)
HHV6B	**U83**		unknown		Agonist Ca^{++} mobilization Monocyte chemotaxis	unknown	unknown

continued on next page

Table 1. Continued

Virus	Virus Encoded: Ligand	Virus Encoded: Receptor	Host Target: Receptor	Host Target: Ligand	Receptor / Ligand Function: In vitro Activities	Receptor / Ligand Function: In vivo Activities	Homologs in Other Herpesvirus
γ-herpesvirus							
KSHV (HHV8)		**ORF74**		CXCL1,2,3,5, 7,8,9,10 and 12 vMIP-II	Constitutive and ligand modulated signaling Initiate autocrine/ paracrine signals	unknown Possibly involved in KS lesion formation	γHV68 (ORF74) HVS (ORF74) EHV2 (ORF74) RhRV (ORF74) AtHV (ORF74)
	vCCL1		CCR8		Agonist Ca^{++} mobilization Angiogenic Chemotaxis	unknown	RhRV (R4)?
	vCCL2		CCR1,2,3, (4),5,(8) CXCR3,4 CX_3CR1 XCR1		Antagonist (Agonist on CCR3 and CCR8) Inhibit monocyte chemotaxis Angiogenic	Antagonize CCL4,5 and CX_3CL1 dependent leukocyte infiltration Reduce post traumatic inflammation Decrease graft infiltrating CTLs	RhRV (R4)? γHV68 (M3) functional? HCMV (US28) functional?
	vCCL3		CCR4		Agonist Th2 chemotaxis Angiogenic	unknown	RhRV (R4)?

continued on next page

Table 1. Continued

	Virus Encoded		Host Target		Receptor / Ligand Function		
Virus	**Ligand**	**Receptor**	**Receptor**	**Ligand**	**In vitro Activities**	**In vivo Activities**	**Homologs in Other Herpesvirus**
EBV (HHV4)		*BILF**		*unknown*	*unknown*	*unknown*	*RhEBV (BILF1)* *CHV3 (ORF6)* *EHV2 (E6)* *AHV1 (E5)* *PLHV1 (A5)* *PLHV2 (A5)*
α-herpesvirus **MDV**	**vIL8**		unknown		Agonist PBMC chemotaxis	Level of MDV in lytic infection	HCMV (vCXCL1)
Poxvirus Mollusc. cont.	**MC148R**		CCR8		Antagonist Inhibit I309 mediated chemotaxis	unknown	unknown

The in vitro and in vivo functions of human β- and γ-herpesvirus ancoded receptors and ligands are presented. See text for details. For completion the α-herpesvirus MDV and the poxvirus Molluscum contagiosum are also listed. In addition, homologous receptor and ligands in related animal herpesviruses are listed. Abbreviations used in this tables are CMV: cytomegalovirus (also known as HHV5, human herpesvirus 5), HCMV: Human CMV, CCMV: Chimpanzee CMV, RhCMV: Rhesus CMV, MCMV: Mouse CMV, RCMV: Rat CMV THV: Tupaia herpesvirus, GpCMV: Guinea pig CMV, EHV2: Equine herpesvirus 2, γHV68: γ- herpesvirus 68 (also known as murid herpesvirus 4), KSHV: Kaposi's sarcoma associated herpesvirus (also known as HHV8, human herpesvirus 8), RhRV: Rhesus rhadinovirus (also known as macaca mulatta rhadinovirus), HVS: herpesvirus saimirii, AtHV3:Ateline herpesvirus 3, HHV6: human herpesvirus 6, HHV7: Human herpesvirus 7, CHV3: Callitricine herpesvirus 3, EBV: Epstien-Barr virus (HHV4), RhEBV: Rhesus EBV, AHV1: Alcelaphine herpesvirus (also known as malignant catarrhal fever virus), PLHV1: Porcine lymphotropic herpesvirus 1, PLHV2: Porcine lymphotropic herpesvirus 2, MDV: Marek's disease virus. It should be noted that EBV-BILF1 and homologous receptors are not discussed in this review.

encodes two US28 homologs, US27 (NP_612799)* and US28 (NP_612800) (Davison A. personal communication), while SCMV[18] and RhCMV (Mark Penfold, personal communication) each encode five positional homologous receptors with similarity to HCMV US28 and US27 (RhCMV - US28.1 (AAN15200), US28.2 (AAN15199), US28.3 (AAN15198), US28.4 (AAN15197) and US28.5 (AAN15201) (Table 1 and Fig. 1). This distribution suggests that US27 and US28 are primate-CMV-specific genes. Furthermore, the duplication and amplification events that have occurred in this part of the viral genome suggests that some US28 characteristic properties such as the binding, signaling or recycling properties have been duplicated during evolution to serve the virus in additional ways. An early 1.3 kb US28-specific transcript can be detected two hours post infection in permissive cells[19] and throughout the progression of the infection.[16,17,20] An additional late 2.9 kb transcript containing both US27 and US28 is detected from 48 hours post infection.[11,16,17] US27 and US28 transcripts are present in infected undifferentiated THP-1 monocytes. Although the biological significance is unresolved, this has been taken as evidence that the receptors may be expressed during latency as well as during productive infection.[19,21] Furthermore, US28 transcripts have been detected in vivo in human peripheral blood mononuclear cells.[22]

vCXCL1 and vCXCL2 were discovered when cysteine spacing comparisons were performed on ORFs initially found to be encoded within a highly heterogeneous region of HCMV.[7,23] The limited passage Toledo strain and high passage Towne strain each had one ORF (UL146) encoding a protein with an ELRCXC motif and cysteine spacing similar to that of human CXC-chemokines such as CXCL8.[23] UL146 was initially called UL152 in strain Towne due to sequence divergence that was later found to characterize this ORF.[24] In strain Toledo, an additional adjacent chemokine like gene (UL147) that exhibited even less homology to human CXC chemokines was identified. Neither UL146 nor UL147 were present in the high passage AD169 strain whose genome had been entirely sequenced many years earlier.[25] UL146 varies by approximately 50% between clinical CMV isolates, whereas UL147 is more conserved among viral strains.[24] ORF UL146 encodes a 117 aa glycoprotein, vCXCL1, which is secreted into the culture medium of infected human fibroblasts late during infection.[7] Interestingly CCMV also encodes positional homologs of UL146 and UL147 with two copies of UL146, UL146 (AAM00768) and UL146A (AAM00769) and one copy of UL147 (AAM00761) (Davison A. personal communication). As with the 'US28 family', the 'UL146 family' seems to be a primate specific CMV gene family.

MCK1 is encoded from the fourth methionine of MCMV ORF m131, and would be predicted to encode an 81 aa precursor protein with similarities to mammalian chemokines[10] and to RCMV ORF r131.[9] MCK1 is not the major gene product of the *mck* gene but was found to constitute the N-terminus of a longer protein expressed from a spliced transcript. This in-frame fusion protein added 199 additional aa including the entire ORF m129 and was designated MCK2.[26,27] MCK2 is secreted into the medium of MCMV infected cells[27] and is expressed from a late transcript in infected fibroblasts.[26-28] The structure of MCK2 is unusual in comparison to other CC chemokines. The N-terminal, derived from ORF m131, is most similar to human CCL26, which binds to human CCR3, whereas the 199 aa C-terminal portion, derived from ORF m129, shares very limited sequence similarity with any proteins predicted from the public nucleic acid databases.**

U83 is encoded from the first methionine of HHV6B-ORFU83, as a 113 aa precursor protein with hallmarks of mammalian CC-chemokines.[29] U83 protein is expressed from a late

*BLAST search. *Number in brackets after gene name indicate GenBank accession number. **Recently a novel CC-chemokine has been identified in the genomes of both HCMV (GenBank AY169800) CCMV and SCMV, arising from a spliced UL128 transcript (Parvis Akter et al. Two novel spliced genes in human cytomegalovirus. J Gen Virol 2003; 84:1117-1122.

transcript in HHV6B infected MT4 cells and is, by immunofluorescence (IFA) determined to be localized to the plasma membrane and in the cytoplasm.[29] Besides a cysteine motif, which matches mammalian CC-chemokines, U83 does not have significant similarity to any known mammalian or other viral chemokines. Interestingly, U83 transcripts exists in different spliced forms.[30] U83 splicing abrogates production of the chemokine homolog by the introduction of a central stop codon. The spliced form of U83 is expressed with early kinetics, whereas the full-length product accumulates as a late gene product whose expression is dependent on viral DNA replication.[30]

γ-Herpesvirus Encoded Receptors and Ligands

Sequence analysis of the 145 kb KSHV genome revealed one ORF, ORF74, with the hallmark of a chemokine receptor and three ORFs, K6, K4 and K4.1, encoding the chemokines vCCL1, vCCL2 and vCCL3 respectively.[31] vCCL2 is unique in the chemokine system because it binds to and antagonizes receptors from all of the four chemokine receptor subfamilies (CC, CXC, XC and CX_3C).[32,33] Besides having specific pro- and/or anti- chemotactic properties, all the KSHV encoded chemokines display angiogenic activities.[34-36] The KSHV chemokine receptor ORF74 binds a broad spectrum of human CXC-chemokines as well as KSHV encoded vCCL2.[37,38]

vCCL1 or vMIP-I, encoded by ORF K6, is a CC-chemokine with 60% similarity to vCCL2 and with 44% similarity to human CCL3 (MIP-1α).[31] The high similarity to human MIP (Macrophage inflammatory peptide) 1α (CCL3) dictated the original name, 'viral MIP' (vMIP) of vCCL1 and vCCL2. vCCL1 is expressed as an early gene in 12-O-tetradecanoylphorbol-13-acetate (TPA) stimulated BC-1 cells (KSHV latently infected body cavity-based lymphoma (PEL) cell line). Expression begins at 13 hours post stimulation and maximum expression is seen 20-30 hours post stimulation.[39] Analysis of KS lesion biopsies showed, that vCCL1 is expressed in approximately 1-3% of KSHV infected spindle-shaped cells.[39] Microarray analysis of another PEL cell line, BC-3 cells activated with TPA revealed that vCCL1 is expressed early (0-10 hours) post activation.[40] A different expression pattern was observed in microarray analysis of yet another TPA activated PEL cell line, BCBL-1 cells,[41] where expression was shown to start at 12 hours post stimulation and peak at 48 hours post stimulation.

vCCL2 or vMIP-II, encoded by open reading frame K4, is 40% similar to human CCL3. vCCL1 and vCCL2 are 60 % identical to each other suggesting a gene duplication event rather than two independent acts of molecular piracy. vCCL2 is expressed as an early gene in TPA stimulated PEL BC-1 cells. Expression starts from 8 hours post stimulation and maximum expression is seen 13-20 hours post stimulation.[39] BC-3 cells activated with TPA confirmed the early expression pattern of vCCL2.[40]

Rhesus monkey rhadinovirus (RhRV) ORF R3 or R4 (no consensus in annotation, hereafter called R4) is the only γ-herpesvirus, besides KSHV known to encode a CC-chemokine.[42,43] R4 is approximately 40% identical to vCCL1 and vCCL2. It is still unknown whether R4 is a functional homolog of vCCL1 or of vCCL2.

vCCL3 or vMIP-III, encoded by ORF K4.1, is more distantly related to human chemokines, and shares approximately 35% identity to vCCL1 and vCCL2. vMIP-III protein has been shown to be expressed in KS lesions.[36]

ORF74 is a broad spectrum CXC-chemokine receptor with homologous sequences present in HVS (ORF74/ECRF3) (27%), γHV68 (ORF74) (21%), spider monkey herpes virus (AtHV-2) (ORF74) (27%), RhRV (ORF74) (35%) and EHV2 (ORF74) (Table 1 and Fig. 1). KSHV-ORF74 is approximately 24% identical to human CXCR2.[31,44] ORF74 is expressed in KS lesions[44,45] and in virus infected lymphoid cells primarily during the early phase of the lytic infection[46] with maximum expression at 20 hours post TPA stimulation.[39]

Agonistic and Antagonistic Activities of Viral Chemokine Ligands

Most herpesvirus encoded chemokines, including vCXCL1, MCK1, vCCL1 and vCCL3, are agonists and stimulate host chemokine receptors. However a few of the ligands characterized to date including KSHV vCCL2 and the poxvirus (molluscum contagiosum) encoded CC-chemokine MC148R act as antagonists on hosts receptors.

vCXCL1 secreted from the Toledo strain of HCMV infected HEL's is able to mediate neutrophil chemotaxis.[7] Recombinant vCXCL1 mediates neutrophil chemotaxis with a similar efficacy and potency as human CXCL8. Also, vCXCL1 was shown to cross desensitize calcium signaling in human neutrophils in response to human ELR CXC-chemokines (CXCR2 ligands), CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ), CXCL5 (ENA-78) and CXCL7 (NAP-2), but not CXCL8 and partially CXCL6 (GCP-2). Additionally, CXCL8 and partially CXCL6, but not CXCL7, cross-desensitized vCXCL1 activity on human neutrophils. The desensitization studies were performed on human neutrophils, which carry both CXCR1 and CXCR2. Since vCXCL1 can not desensitize CXCL8 which bind both CXCR1 and CXCR2, the desensitization data support that vCXCL1 interacts with CXCR2 and not CXCR1. Furthermore, vCXCL1 bound to human neutrophils with high affinity in competition against radiolabeled CXCL8, and vCXCL1 was shown to compete for CXCL8 binding to human CXCR2, but not to CXCR1 transfectants.[7]

MCK1 induced intracellular calcium mobilization in a small percentage (2-5%) of resident adherent peritoneal exudate cells (PECs) from uninfected mice.[47] Interestingly, a higher percentage of cells responded to MCK1 when PECs were isolated 48 hours after intraperitoneal inoculation of either wildtype or mck-mutant MCMV. Furthermore, rolling cells became adherent within seconds after calcium fluxing, and remained tightly anchored to the glass surface.[47] The host murine receptor(s) for MCK1 and MCK2 are still unknown. However, MCK1 was shown to bind to and desensitize human, but not murine CCR3.[47] MCK is the only virus encoded chemokine that has been studied using a reverse genetic approach in an infectious disease model in vivo (discussed later in 'Viral Immune regulation and dissemination').

vCCL1 has been shown to be a specific agonist on human CCR8.[48,49] vCCL1 specifically binds to CCR8 and competes for binding with the endogenous CCR8 ligand, CCL1 (I-309). vCCL1 stimulates a rapid calcium release from intracellular stores upon receptor binding and is able to mediate chemotaxis of CCR8 expressing Y3 cells. CCR8 is preferentially expressed on T_H2-type CD4 T cells. It has therefore been hypothesized, that vCCL1 selectively recruits CCR8 expressing cells to the site of infection, thereby influencing the balance of the immune response towards a T_H2 (less cytotoxic) phenotype. Also, vCCL1, as well as human CCL1, has been shown to mediate endothelial cell chemotaxis through interaction with CCR8.[50] Interestingly, the poxvirus (molluscum contagiosum) encoded CC- chemokine MC148R has been demonstrated to be a selective CCR8 antagonist.[33,49] MC148R competes for binding of CCL1 to CCR8, but in contrast to vCCL1, MC148R inhibits CCL1 induced calcium signaling and chemotaxis.

vCCL2 demonstrates a unique broad spectrum of receptor activities. vCCL2 is capable of displacing human chemokines from any of a number of chemokine receptors.[32] In competition binding assays, recombinant and synthetic vCCL2 was initially shown to bind with high affinity to human CCR1, CCR2, CCR3, CCR5 and CXCR4 and to HCMV encoded US28. The repertoire of receptors engaged by vCCL2 has now been expanded to also include CCR4, CCR8, CCR10, CXCR3, XCR1, CX_3CR1 and ORF74.[33,38,51,52] Hence, vCCL2 interacts with receptors from all four receptor subfamilies, XCR, CCR, CXCR and CX_3CR. vCCL2, however does not interact with CCR6, CCR7, CCR9, CXCR1 and CXCR2.[32,33] Also, vCCL2 was able to inhibit HIV-1 infection of US87/CD4 cells stably expressing CCR5, CCR3 or CXCR4[32,34] further underscoring the broad range of receptor interactions. In contrast to vCCL1

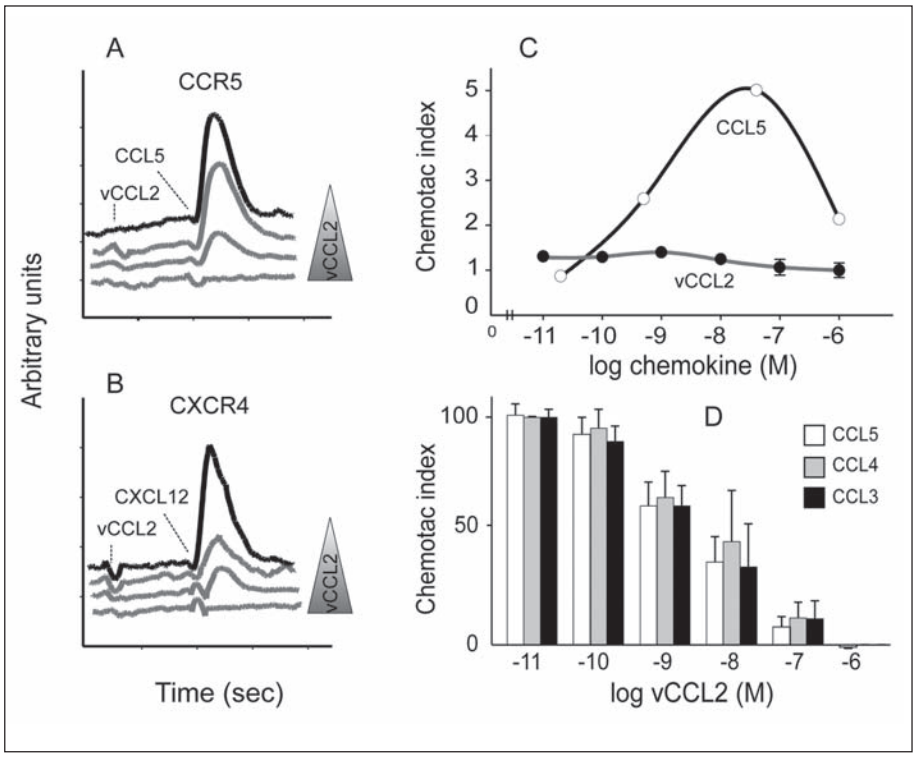

Figure 2. Antagonistic functions of vCCL2 on human chemokine receptor CCR5, CXCR4 transfectants and on human monocytes. A and B, effects of vCCL2 on intracellular [Ca^{2+}] in transfected cells. Upper traces (black lines) show [Ca^{2+}] response to the endogenous cognate ligands to CCR5 and CXCR4, CCL5 and CXCL12 respectively without prior treatment with vCCL2. Lower 3 traces (grey lines) show the response to endogenous ligands with prior treatment of increasing concentrations (-9, -8, -7 and -6 log [M]) of vCCL2 as indicated with the grey triangle. C, chemotaxis of monocytes to vCCL2 (grey line/closed circles) and to CCL5 (black line/open circles). D, dose response curve for inhibition by vCCL2 of CCL3 (black bars), CCL4 (gray bars) or CCL5 (white bars) induced monocyte chemotaxis (redrawn from ref. 32).

and vCCL3, vCCL2 is unable to induce calcium mobilization from intracellular stores through receptor binding (Fig. 2). Nevertheless, vCCL2 is an efficient and potent blocker of calcium mobilization induced by the relevant human chemokines through CCR1, CCR2, CCR5, CXCR3, CXCR4, XCR1 and CX_3CR1, and to a lesser extent on CCR3, CCR4 and CCR8[32,33] (Fig. 2). Thus, vCCL2 acts as an antagonist, inhibiting the action of the endogenous chemokines. Correspondingly, vCCL2 does not induce chemotaxis of human monocytes, but acts as an efficient and potent inhibitor of the chemotactic response to CCL3, CCL4 (MIP-1β) and CCL5 (RANTES)[32] (Fig. 2). Interestingly, vCCL2 has been reported to also possess agonistic properties. vCCL2 has been shown to induce calcium mobilization and migration of eosinophils through CCR3[34] and of CCR8 transfected Jurkat cells.[53] In addition, vCCL2 has been shown to trigger the arrest of eosinophils and T_H2 cells through CCR3 to IL-1β activated microvascular endothelial cells, while inhibiting arrest and transmigration of monocytes and T_H1 cells).[54] However, the study by Luttichau et al[33] did not reveal any agonistic activities of vCCL2 on any of the 16 human chemokine receptors tested. In this study, vCCL2 did not by itself induce chemotaxis of L1.2 cells stably expressing CCR8 but vCCL2 inhibited CCL1

induced chemotaxis. It is therefore still debatable whether vCCL2 by itself can signal through chemokine receptors or whether it is only an antagonist.

In vivo, vCCL2 seems to inhibit inflammation.[51,55,56] In a rat model of experimental glomerulonephritis induced by an antibody directed against a basement membrane protein, vCCL2 potently inhibited CCL2 (MCP-1), CCL4, CCL5 and CX_3CL1 induced chemotaxis of activated leukocytes isolated from nephritic glomeruli. vCCL2 significantly reduced macrophage and CD8+ T-cell infiltration to the glomeruli, and markedly attenuated proteinuria.[51] Using a rat model of spinal cord contusion injury, it was shown, that infusion of vCCL2 for up to 7 days, resulted in a reduction in the inflammatory response.[56] vCCL2 reduced macrophage infiltration to the site of injury, and concomitantly vCCL2 reduced neuronal loss and gliosis. Interestingly, vCCL2 infusion resulted in an increase in Bcl-2 expression and a reduction in the number of apoptotic cells compared to vehicle infused rats.[56] In mice, gene transfer by direct injection of vCCL2 plasmid DNA into cardiac allografts leading to expression of the viral chemokines within the graft resulted in a reduction in graft infiltrating CTL's and delay in IgM alloantibody production.[55] Importantly, the reduction in graft infiltrating CTL's correlated with a prolonged allograft survival, which was also reported for MC148R.[55] The receptors and downstream pathways through which the anti-inflammatory effects of vCCL2 and MC148R are mediated are still unknown, and it should be noted, that MC148R is unable to bind, and block response through murine CCR8.[57]

Whether the anti-inflammatory effects of vCCL2 observed in vivo, is due to pharmacological antagonistic activities on 'pro-inflammatory' chemokine receptors, or is due to pharmacological agonistic activities on 'anti-inflammatory' chemokine receptors, or both, is unknown. However the current literature suggests, that the KSHV encoded chemokine ligands, by acting alternatively as antagonists or agonists generate a specific pattern of the endogenous chemokine system that helps the virus to manipulate the host immune response to facilitate infection.

vCCL1, vCCL2 and MC148R all engage human CCR8, either as agonists or antagonists as measured by their ability to induce or inhibit a CCR8 dependent calcium response and chemotaxis. Besides being involved in cell migration, both the mouse and human encoded CCR8 ligands (murine- (TCA-3) and human- (I-309) CCL1) has been shown to protect cells from dexamethasone and Fas mediated apoptosis.[58,59] The virus-encoded chemokines may engage CCR8 to inhibit apoptosis. Interestingly, both vCCL1 and vCCL2 have been shown to inhibit dexamethasone induced apoptosis of KSHV infected lymphoma cell.[60] Liu et al[60] did not identify which receptor(s) mediated the anti-apoptotic effect of vCCL1 and vCCL2, however CCR8 seems to be a good candidate, and it will be interesting to see whether MC148R and other viral encoded chemokines also can protect infected cells from apoptosis.

vCCL3 has been shown to be an agonist on human CCR4.[36] Recombinant vCCL3 competes for binding to CCR4 with the endogenous CCR4 ligand, CCL22 (MDC). Interestingly, vCCL3 is a relatively low affinity (IC50 = 100nM) ligand for human CCR4 compared to the high affinity of CCL22 (IC50 = 150 pM) for this same receptor. This also contrasts the nM/sub-nM affinities reported for other KSHV chemokines (vCCL1 and vCCL2) (see above). Importantly, high concentrations of vCCL3 significantly stimulated chemotaxis of CCR4 expressing L1.2 cells, but not cells expressing CCR2, CCR3 and CCR5. The high efficacy of vCCL3 suggests that the low affinity (potency) interaction with CCR4 could be of biological relevance and play a role in both the pathogenesis of Kaposi's sarcoma and in KSHV immune evasion and/or dissemination. It is interesting to note, that vCCL3 was shown to selectively attract T_H2 cells over T_H1 cells. This could contribute to skewing the antiviral immune response towards a less cytotoxic phenotype, and vCCL3 could together with vCCL1 and vCCL2 contribute to the prominent leukocyte infiltrate observed in Kaposi's sarcoma lesions.

Viral Chemokine Receptor Activities and Receptor-Ligand Selectivity

Both US28 and ORF74 bind an unusually broad spectrum of ligands with high affinity, yet maintain a very high degree of ligand selectivity. Furthermore, both US28 and ORF74 maintain a high degree of constitutive activity. Interestingly, it has been shown that UL33 and the MCMV and RCMV homologs M33[61] and R33[62] all are constitutively active receptors.

UL33 and the homologous receptors from rodent CMVs are all considered orphan receptors. However, HCMV UL33, RCMV R33 and MCMV M33 signal without any known requirement for ligand engagement. UL33, M33 and R33 all stimulate phospholipase C (PLC) in transfected cells whereas only the rodent CMV receptors M33 and R33, but not HCMV receptor UL33 activates NF-κB driven transcription activation.[61,62] Interestingly, both UL33 and M33 activates cyclic AMP (cAMP) response element binding protein (CREB) through activation of $G\alpha_s$ and the mitogen activated protein (MAP) kinase, p38,[61] whereas R33 inhibits forskolin stimulated CREB through a $G\alpha_I$ mediated signaling.[62] It is of interest to note that the MCMV M33 signaling profile is more similar to the signaling profile of HCMV US28 then to HCMV UL33. It has therefore been suggested that M33 in some aspects could play a similar role to that of US28 during viral pathogenesis.[61] However, it should be noted that R33 whose primary structure is most identical to M33, signals very differently from US28.[62] Based on these studies, the rodent UL33 homologous (M33 and R33) receptors can not be considered functional homologs of US28.

US28 binds with sub-nanomolar affinities several CC-chemokines such as CCL2, CCL3, CCL4, CCL5 and CCL7 (MCP-3)[6,63,64] as well as CX_3CL1[65] and the KSHV encoded chemokine, vCCL2[32] (Fig. 3). Based on the unique ligand binding profile of US28, and the ability of US28 to sequester CC-chemokines in tissue culture, it has been suggested that US28 could function to remove chemoattractants from the surroundings of HCMV infected cells.[16,20,66] Therefore, US28 would disturb the chemokine gradient and possibly benefit the virus by obstructing the attraction of leukocytes to the site of infection.[16] The ability of US28 to bind CC-chemokines with high affinity has been determined in homologous competition binding experiments.* In heterologous competition experiments CX_3CL1 was able to displace, with high affinity, all the tested CC-chemokine tracers[65] (Fig. 3). However, when CX_3CL1 was used as the tracer all the CC-chemokines tested competed for binding to US28 with poor affinity (Fig. 3). The affinity of US28 for CX_3CL1 was approximately 1,000 fold higher than the affinity for CCL3, CCL4 and CCL5, and approximately 200 fold higher than the affinity for CCL2 in competition with radioactive CX_3CL1[65] (Fig. 3). This indicates that US28 is optimized to retain the membrane anchored CX_3C-chemokine once it is bound. The biological function of this ligand specificity is still unclear, but it has been hypothesized, that it could be involved in the cell to cell transfer of HCMV in humans.[65]

Interestingly, it has been shown that CX_3CL1 can mediate adhesion of both CX_3CR1 and US28 expressing cells, under both static conditions and under conditions resembling physiological flow.[67] Under flow conditions, CX_3CR1 and US28 expressing cells attached and remained arrested without further rolling. The ability of CX_3CL1 (but not of other chemokine-stalk chimeras) to capture US28 (or CX_3CR1) expressing cells correlated with a slow off rate of CX_3CL1 from the receptor. In contrast to CX_3CL1, a CCL5+stalk chimera, which was not capable of mediating adhesion to US28 under flow conditions, revealed a much faster off rate from US28.[67] It should be noted that US28 did not work as efficiently as CX_3CR1 in mediating adhesion. This could be due to both differences in on/off rates or differences in the receptor expression level, US28 showing a lower cell-surface expression level than CX_3CR1 and other chemokine receptors (discussed later in '(intra)-cellular receptor localization'). Both CX_3CL1

*In 'homologous competition binding experiments' the competitor ligand is identical to the tracer ligand. In 'heterologous competition experiments' the competitor is different from the tracer.

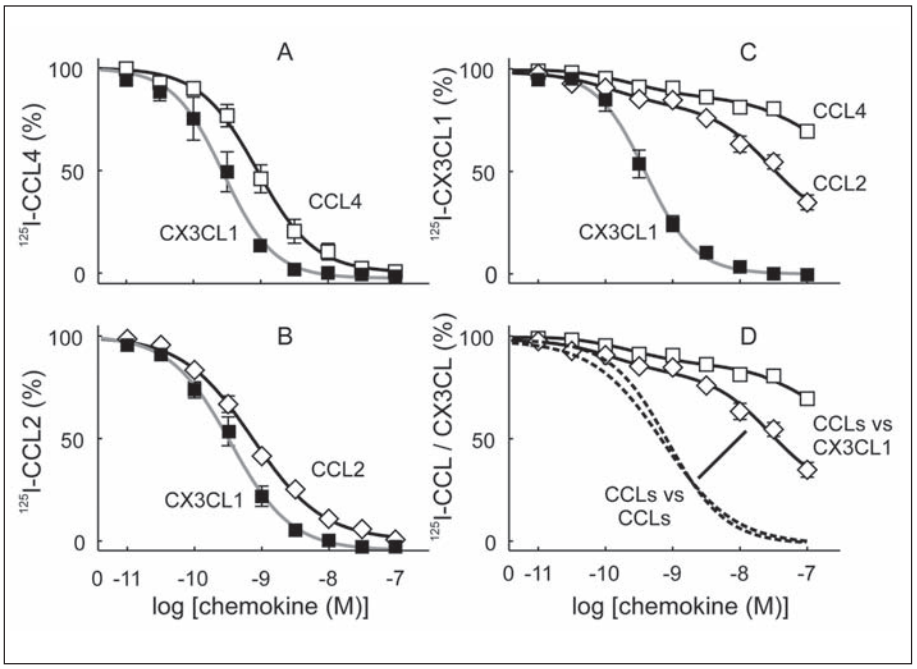

Figure 3. Competition binding experiments with US28 using radioactive chemokine tracers ^{125}I-CCL4 (A), ^{125}I-CCL2 (B) or ^{125}I-CX_3CL1 (C), competed with cold (unlabeled) CCL4 (A, C and D, black curves/white rectangles), CCL2 (B, C and D, black curves/white diamonds) or CX_3CL1 (A, B and C, grey curves/black rectangles). D illustrates the shift in affinity of CC-chemokines to US28 in homologous competition binding assays or in competition against ^{125}I labeled CX_3CL1 (redrawn from ref. 65).

and US28 are located appropriately for being involved in cell targeting and/or cell entry of HCMV. CX_3CL1 is expressed on endothelial and epithelial cells as well as on several other putative target-cells[68-70] and US28 is expressed, although at a low level, on the surface of HCMV infected cells.[16,17,20,22,71,72] Moreover, US28 coexpressed with CD4 has been shown to be able to mediate cell fusion with HIV-1 gp120.[73] Furthermore, US28 can function as a 'coreceptor' enhancing cell-fusion mediated by envelope proteins from vesicular stomatitis virus (VSV) and human T-cell lymphoma virus-1 (HTLV-1).[74] These results suggest that US28 can facilitate a cell to cell contact, which could aid in HCMV dissemination.

US28 has been observed to activate several different signal transduction pathways both in transfected cells and HCMV infected cells. Engagement of CC-chemokines, CCL2, CCL3, CCL4, CCL5 and CCL7 to US28, either expressed in K562 cells, 293 cells or during infection of fibroblasts or HUVECs, induced a transient increase in intracellular calcium.[17,63,71] However, US28 dependent calcium signaling was dependent on ligand concentrations substantially higher that the Kd values of the applied ligands. In addition, US28 expressed in 293 cells was shown to activate the MAP kinase pathway in response to CCL5.[71] The kinase activity as well as the calcium response was shown to be mediated through coupling to both pertussis toxin (PTX) sensitive Gαi and to PTX insensitive Gα16 G-proteins.[71] Besides ligand induced signaling, US28 signals constitutively, activating PLC and NF-κB signaling.[61,75] None of the CC-chemokines known to bind US28 with high affinity affected the constitutive signaling, whereas the chemokine domain of CX_3CL1 acted as a partial inverse agonist on US28 as it decreased signaling to approximately 70% of the basal activity level.[61,75] The constitutive PLC

activation was shown to be mediated via Gαq/11 subunits, whereas the constitutive activation of NF-κB depended on the βγ subunits with a preference for the β2γ1 dimer.[75]

ORF74 binds multiple CXC-chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL7, CXCL8, CXCL10 (IP-10) and CXCL12 (SDF-1)) and vCCL2 with high affinity.[37,38,76-78] Furthermore, ORF74 is both highly constitutively active and responds to ligand engagement.[37,38,77,79-87] Although CXCL8 was originally found to bind ORF74 with high affinity, CXCL8 did not affect the constitutive PLC activation mediated by the receptor.[37,38] Like US28, ORF74 seems to maintain a high degree of ligand selectivity. Since CXCL1 displaced ^{125}I-CXCL8 with higher affinity than unlabeled CXCL8 itself, ORF74 was probed with ^{125}I-CXCL1, resulting in a very distinct (and different) ligand binding profile. Consequently, the affinity of ORF74 for CXCL1, CXCL2 and CXCL3 (GRO-peptides) was approximately 10,000 times higher than the affinity for CXCL8 in competition with radioactive CXCL1.[38] Besides being optimized for binding the GRO-peptides, ORF74 was shown to be activated by the GRO-peptides resulting in an increase in the basal constitutive PLC activity.[38,76] CXCL8, CXCL7 and CXCL5 (IL-8-like peptides) were unable to stimulate ORF74 to above basic activity (except at micromolar concentrations), and CXCL10 and CXCL12 (non ELR CXC-chemokines) functioned as inverse agonist, inhibiting the constitutive activity.[38] Correspondingly, the ability of ORF74 to induce cell transformation was shown to be modulated by chemokines in accordance with the chemokines ability to function as either agonists (GRO-peptides), neutral ligands (IL-8-like peptides) or inverse agonists (non ELR CXC-chemokines).[38]

ORF74 has been shown to be able to activate and signal through different Gα proteins in addition to triggering βγ-subunit dependent signaling. ORF74 activation of Gαq activates PKC through PLC[37,38,87]whereas activation of Gαi (and pertussis toxin insensitive G-proteins) and βγ-subunits activates AKT/PKB through activation of PI3K.[87,88] Furthermore, ORF74 activates Rho (at least partially through activation of Gα13) leading to cytoskeleton rearrangements.[86] Also, ORF74 has been shown to activate several members of the MAPK superfamily (through both Gαi, Gαq and βγ dependent pathways) such as JNK and P38[79] and p44/p42 MAPK.[87] Furthermore, ORF74 has been shown to activate NF-κB and AP1 and cells expressing ORF74 has been shown to express various growth factors and angiogenic and proinflammatory cytokines (vascular endothelial growth factor (VEGF), granulocyte macrophage colony stimulating factor (GM-CSF), IL-2, IL-4, IL-6, CXCL8, CCL5, TNFα, VCAM, ICAM and E-selectin).[82,85,86,88]

ORF74 has been shown to stimulate the proliferation of transfected cells[37,79] and induce angiogenesis of human umbilical vein endothelial cells (HUVECs) in vitro through activation of VEGF secretion.[79,89] Additionally, ORF74 promotes cell survival of primary HUVECs, in a VEGF independent manner. This protective and anti-apoptotic effect of ORF74 was shown to be mediated by a constitutive induction of Akt/PKB. This signaling pathway required the function of PI3-kinase and was further shown to be dependent on βγ-subunit release from Gα-proteins.[88] Furthermore, ORF74 has been shown to induce spindle morphology in retroviral transduced primary HUVECs and dermal microvascular endothelial cells.[85] The constitutive proliferative, angiogenic and antiapoptotic properties of ORF74 indicates that ORF74 could be involved in the cell transforming properties of KSHV, and thereby be a part of the pathology of Kaposi's sarcoma and other KSHV lymphoproliferative disorders. Interestingly, transfection of 3T3 cells with ORF74 led to a gene dose dependent appearance of foci a few weeks after transfection, and foci derived transformed cells injected into the flank of nude mice were able to cause vascularized tumors.[79] Furthermore, it has been shown, that transgenic mice expressing ORF74 within hematopoietic cells do develop Kaposi's sarcoma like lesions in multiple organs,[90,91] characterized by intense angiogenic activity, the presence of spindle and inflammatory cells and the expression of ORF74, CD34 and VEGF.

(Intra-) Cellular Receptor Localization

Originally, UL33 was shown to be localized mainly in perinuclear cytoplasmic inclusions rather than in the plasma membrane, and UL33 was shown to be present in purified virus particles.[5] UL33 was present in virions, in noninfectious enveloped particles and in dense bodies derived from CMV infected human foreskin fibroblasts. The identification of UL33 in cellular inclusion and in purified virus particles suggests that UL33 could be targeted to this specific intracellular region to be incorporated into the envelope of the maturing virions.[5,92] This hypothesis is supported by a detailed study of the cellular localization of UL33 (and US27 (see below)) in transfected as well as in CMV infected cells.[93] The study by Fraile-Ramos et al[93] showed that the majority of UL33 was seen in intracellular organelles located in the perinuclear region of the cell. The intracellular pool of UL33 showed overlap with markers for endocytic organelles, CD63 and LAMP1. By immunogold labeling of cryosections and electron microscopy of transfected HeLa cells, UL33 was seen to localize to multivesicular bodies (MVBs or multivesicular endosomes). Furthermore, electron microscopy analysis of UL33 in CMV infected fibroblasts revealed the presence of UL33 in small membrane tubules and vesicles and in MVBs in which viral particles were also observed and presumably are membrane structures into which virus bud. In addition, the CMV glycoproteins gB and gH localizes to the same membrane structures within the infected cells as UL33, which is further evidence that UL33 is also incorporated in the viral envelope.[93]

In MCMV infected macrophages and fibroblast, M78 is mainly localized inside the cells in vacuole like structures which colocalizes with markers for medial and peripheral Golgi bodies.[94] Furthermore, M78 is present in virus preparations, and is as a possible virion envelope protein, presumably delivered to newly infected target cells. M78 has been proposed to be involved in modulating the intracellular milieu and regulating the transcription of selected immediate early viral mRNA's.[94]

U51 (from HHV6) transiently expressed in human monolayer cells (HEK293 or 143tk) could not be detected in the plasma membrane, but was found to accumulate in a reticular pattern partially overlapping with ER-staining.[14] However, the cellular localization U51 seems to be cell type dependent. In transfected T-cell lines (J-Jahn, Molt-3 and Jurkat cells) and in infected cord blood mononuclear cells, U51 seems to localize to the plasma membrane.[14]

US27 is closely related to US28, but until recently no activity has been ascribed to this receptor. However, a study of the cellular localization and trafficking of US27 (and UL33 (see above)) revealed that US27 is an active receptor.[93] In both transfected HeLa cells as well as in CMV infected cells, the majority of US27 (like UL33) was seen in intracellular organelles located in the perinuclear region of the cell.[93] The intracellular pool of US27 showed overlap with markers for endocytic organelles, CD63 and LAMP1. Importantly, US27 was shown to undergo constitutive endocytosis in transfected COS cells, indicating that US27 is an active receptor, which like US28 (see below) could be recycling between the plasma membrane and endosomal compartments. This hypothesis is further supported by the colocalization of US27 and US28 in cotransfected HeLa cells.[93] Also, electron microscopy analysis of US27 in CMV infected fibroblasts revealed the presence of US27 in small membrane tubules and vesicles and in MVBs in which viral particles were also observed.[93] Interestingly, the presence of US27 on viral membranes was evident.

US28 is mainly localized in intracellular compartments (like UL33, UL78 and US27, and in contrast to endogenous chemokine receptors) and not in the plasma membrane[72] (Fig. 4). This confirms earlier reports, describing US28 surface expression as 'less efficient' then that of endogenous receptors,[67,73] and that a majority of US28 seems to accumulate intracellularly[95] even though it can be detected on the cell surface.[65] Using tagged receptor constructs, US28 was found to accumulate in perinuclear endocytotic organelles including early and late

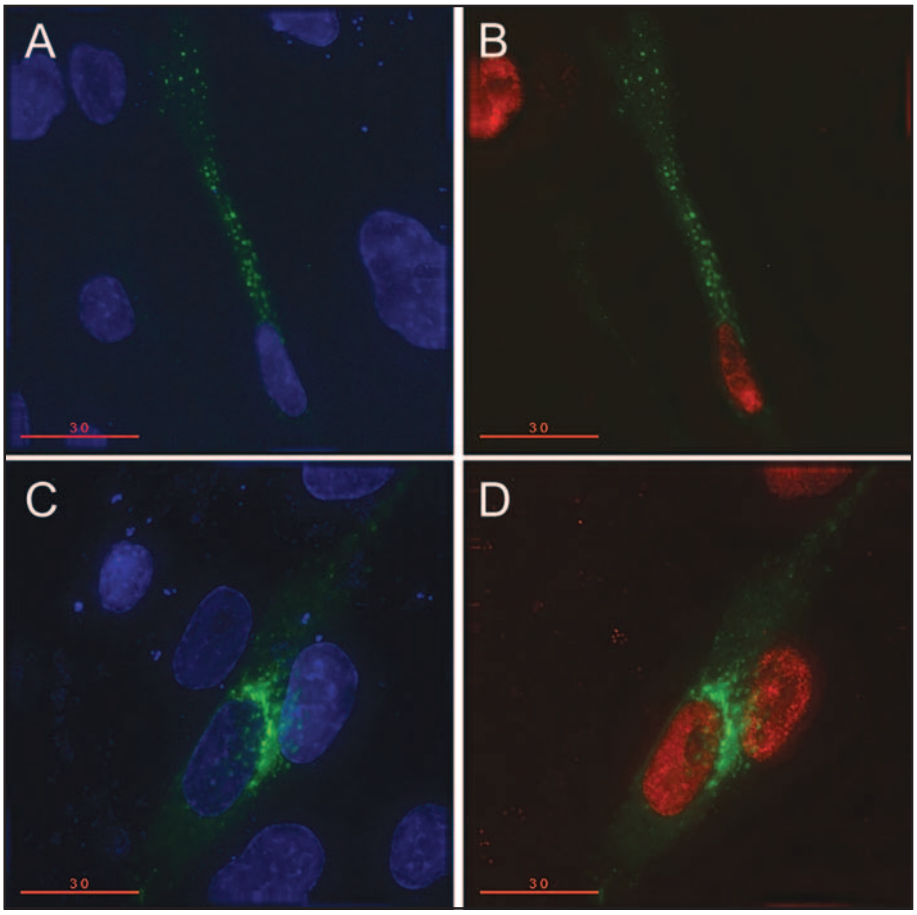

Figure 4. Localization of US28 (US28-YFP fusion protein) in transiently transfected human foreskin fibroblasts coinfected with the Towne strain of HCMV, 24 hours post infection (A and B) and 48 hours post infection (C and D). Antibody against HCMV IE1 (red staining) (D and F) is used to detect HCMV infected cells. Hoechst staining (blue) (C and E) show all nuclei. Scale bars are 30 μm. Transfections, infections and deconvolution images are prepared by TN Kledal as described in ref. 72.

endosomes[72] (Fig. 4). Biochemical analysis of US28 transfected cells with ^{125}I-labelled antibodies, before and after permeabilization, indicated that at most 20% of the tagged-US28 construct was present at the cell surface. Interestingly, antibody-feeding experiments showed that cell surface US28 undergoes constitutive, ligand independent endocytosis and recycling to the plasma membrane. Apparently, the high affinity ligand of US28, CX_3CL1, but not CCL5, reduced the steady state levels of US28 at the cell surface, presumably by inhibiting the recycling of the internalized receptor. It was further established that approximately 6-7% of the total cell surface pool underwent endocytosis per minute and that this rate was independent on the presence of either CCL5 or CX_3CL1. These constitutive internalization properties are very similar to those observed for activated endogenous chemokine receptors,[96] further establishing US28 as a constitutively active receptor. It has been suggested that the constitutive endocytosis and recycling of US28 could be a 'mechanism' for sequestering host CC-chemokines. In

addition, the distribution of US28 mainly on endosomal membranes may allow it to be incorporated into the viral envelope during HCMV assembly.[72] Interestingly, US28 may also influence the surface expression of some endogenous receptors. It has been shown that while the presence of US28, CCR5 or CCR1 has no apparent effect on the surface expression of CD4 and MHC-I, the surface expression of CXCR4 is downregulated in cells transfected with US28, but not in cells transfected with CCR1 or CCR5. Similarly, it has been shown that CCR5 surface expression is slightly lower in cells cotransfected with US28 than in cells cotransfected with CXCR4.[74]

It has been proposed, that the endocytosis of US28 provides a sink for clearing pro-inflammatory CC-chemokines from the tissue surrounding the CMV infected cell,[16] thereby antagonizing the recruitment of cells involved in the immune response to CMV. Whether the function of UL33 or US27 is similar to that of US28 remains unclear, as ligands for these proteins have yet to be identified. Nevertheless, the ability to undergo endocytosis and sort to endocytic compartments in the presence or absence of other viral proteins suggests, that US28 as well as US27 and UL33 contains intrinsic trafficking signals which may be important for their function and for their incorporation into viral membranes.[93]

Viral Immune Regulation and Dissemination

Herpesviruses have developed numerous mechanisms by which the virus can interact, inflect, exploit and subvert the host immune system. However, in recent years it has become evident that herpesviruses not only have developed immune-modulatory mechanisms to evade the host immune response but also to directly aid the virus growth and dissemination within the host.[97-101] All of the chemokine homologs vCXCL1, vCXCL2 and MCK1/2[7,23,47] and the 7TM receptors UL33, M33, R33, UL78, M78, R78, US27 and US28[5,12,13,15-17,94,102] are dispensable for viral growth in culture. However, several studies have revealed the importance of these genes for viral immune regulation, dissemination and/or replication in vivo.[12,13,15,47,94,103]

Both M33 and R33 have been investigated in vivo.[12,13] M33 deficient MCMV could not be detected in the salivary glands of inoculated mice, a phenotype in striking contrast to the behavior of wildtype MCMV.[12] R33 deficient RCMV is less virulent for immunocompromised rats than is wildtype virus.[13] Also, the R33 mutant RCMV does not replicate efficiently in the salivary glands, whereas the virus level did not differ significantly between most other organs tested from either wildtype RCMV or R33 knockout RCMV.[13] This phenotype was especially profound at late time points after infection (> 10 days) indicating that the decreased levels of virus in salivary glands results from a decreased ability to replicate in this organ, rather than to either a decreased dissemination capacity or a failure to enter salivary gland epithelial cells.

In vitro R78 deletion mutant RCMV replicated with 10-100 fold lower efficiency than wildtype virus, and fibroblast infected with the R78 deletion virus developed a syncytium like appearance which was not observed in wildtype RCMV infected fibroblasts.[15] In vivo the R78 deletion virus replicated and disseminated similar to or slightly less when compared to wildtype virus but showed a reduced virulence. This manifested itself in a lower mortality of R78 deletion mutant than wildtype virus infected immune compromised rats. In vitro, an M78 deletion mutant virus replicated with 50 fold lower efficiency than wildtype virus in IC21 macrophages infected under low multiplicity. However, the replication defect was not significant under high multiplicity or in infected fibroblasts. In vivo, in both immune competent and immunocompromised mice, M78 deletion mutant MCMV replicated and/or disseminated to a lower level in the spleen and liver, and persistently infected salivary glands revealed a 100 fold reduction in titer relative to the wildtype virus.[94]

In vitro MCK knockout virus has been shown to replicate as well as wildtype virus.[104,105] Also, following inoculation into BALB/c mice, MCK knockout virus grew as well as wildtype

virus in most organs tested, including spleen, liver, adrenal gland and lungs. However, replication in the salivary glands, was reduced by two to three orders of magnitude 14-21 days post infection.[26,47,104] The same phenotype was observed after inoculation of nude or SCID mice, indicating that the phenotype is independent of B or T lymphocytes.[104] Fleming et al[26] reported a different pattern where MCK knockout viruses were cleared more rapidly than wild type virus from spleen and liver. Depletion studies using anti-asialoGM1 or anti-CD4 plus anti-CD8 antibodies partially restored MCK knockout virus growth in the spleen and liver but did not rescue titers in the salivary glands at later times. These studies suggested an impact of MCK on the adaptive immune response; however, more recent experiments comparing MCK knockout viruses to control viruses could not confirm a more rapid clearance of mutant virus.[103] MCK mutant virus exhibited 20-50 fold lower viremia than wildtype virus.[47,103] The inability of the MCK mutant to reach levels of viremia as high as control virus correlated with a reduced salivary gland titer. When mice were coinfected with wildtype and MCK knockout virus together, both reached the high peak viremia levels of wild type, compatible with a role for MCK2 as a secreted factor/ligand capable of complementing MCK knockout virus in trans.[47] Coinfection also rescued the mutant phenotype in the salivary glands, subsequently supporting a link between levels of viremia and efficiency of seeding salivary glands.[103] Interestingly, it has been shown that expression of MCK lead to increased local inflammation two days after virus inoculation in mouse footpads. Feet inoculated with MCK knockout virus exhibited less than 50% of the swelling observed in mice inoculated with wildtype virus.[103] Levels of replication by these viruses at the site of inoculation and in the draining lymph nodes were equivalent, suggesting that the difference in inflammation did not result from varying amounts of virus. MCK knockout virus infection resulted in less cellular infiltration, reduced necrosis and a lower level of edema compared to wildtype virus. These effects seems to be consistent with MCK being a pro-inflammatory chemokine, and support the hypotheses, that MCK increases local inflammation in order to mobilize the mononuclear cells which subsequently contribute to peripheral blood leukocyte-associated viremia and dissemination of the virus to the salivary glands.

HCMV also encodesa pro-inflammatory chemokine, vCXCL1. vCXCL1 is a potent neutrophil chemoattractant secreted by HCMV infected cells. Neutrophils are associated with HCMV infection in several ways. Infiltrating neutrophils are prominent in CMV diseases such as retinitis[106,107] pneumonitis,[108] and central nervous system complications.[109] Furthermore, virus is carried in peripheral blood neutrophils (PBN) during acute infection.[110] Although the interaction of CMV with neutrophils does not result in productive infection,[111] cell culture experiments suggest that neutrophils may contribute directly to inflammation and to hematogenous dissemination in immunocompromised hosts.[112,113] Therefore, vCXC-1 (in analogy to MCK) may ensure an efficient dissemination during acute infection through an active recruitment of neutrophils to the site of infection.[7] By secreting chemokines and recruiting specific cells the virus is taking active control of the host chemokine system rather than just evading it by recruiting cells that the virus can infect and subsequently use as vehicle for dissemination to new target cells and tissues.

Most literature suggests that vCCL2 is anti-inflammatory[32,33,51,52,56,114] albeit also pro-inflammatory characteristics has been ascribed.[34,53,54] However, the finding that both vCCL1 and vCCL3 are pure and selective agonists[36,49] suggests that KSHV not only blocks one part of the immune system but, like CMV, also actively attracts a specific subset of immune cells. It has been suggested that the KSHV chemokines inhibit chemotaxis of monocytes/macrophages, NK cells, cytotoxic T cells and T_H1 cells, whereas the virus attracts T_H2 cells thereby polarizing the cellular infiltrate. It seems obvious why KSHV would inhibit the recruitment of immune cells involved in clearing intracellular pathogens whereas the significance of attracting T_H2 cells is unclear. The primary reservoir for persistent asymptomatic KSHV infection

appears to be CD19+ B cells.[115] The active recruitment of T_H2 cells to the site of KSHV infection, could be a mechanism by which KSHV disseminate from endothelial cells or epithelial cells via T_H2 to B-cells. Moreover, T_H2 cells could promote an environment, which is favorable for viral replication in B cells.

The structural and functional similarity of US28 to host chemokine receptors has led to speculation that US28 may be capable of mediating cell migration. Indeed, infection of vascular smooth muscle cells (SMC) with HCMV lead to an induction of SMC migration.[116] This cell type dependent migratory phenotype was shown to be dependent on US28, because US28 mutant virus did not induce migration whereas US28-expressing adenovirus did. It was shown that the migratory phenotype was partly due to random movements (chemokinesis) and partly due to directional movements (chemotaxis).[116] Whether US28 is directly involved in chemotaxis of infected cells is still at debate and many of the US28 functions described to date, binding, signaling and cellular localization are substantially different from the functions of homologous mammalian encoded chemokine receptors. Indeed, most US28 literature supports the hypothesis that US28 is anti-inflammatory by sequestering chemokines.[16,20,66] It is interesting to note that US28 by binding and sequestering CCL2, 3, 4, 5, 7 and CX_3CL1 conceivably antagonizes the receptors CCR1, 2, 3, 5 and CX_3CR1, which also are targeted and inhibited by vCCL2. Thereby, US28 and vCCL2 could play similar functions during viral pathogenesis, inhibiting chemotaxis of monocytes/macrophages, NK-cells and cytotoxic T-cells to the site of infection.

UL33, US27, US28, M78 and U51 all show a high degree of intracellular receptor localization.[5,14,72,93,94] Detailed analysis of US28 showed, that at most 20% of the total receptor pool was present at the cell surface and that US28 undergoes constitutive endocytosis and recycling to the plasma membrane.[72] It has been suggested that the constitutive endocytosis of US28 is the mechanism by which US28 mediates the clearance of pro-inflammatory CC-chemokines from the tissue surrounding the CMV infected cell.[72]

UL33, M78 and US27 have all been identified on viral particles.[5,93,94] GPCRs incorporated in the viral envelope would be delivered to the target cells upon membrane fusion, together with other envelope glycoproteins and tegument proteins and thereby contribute to the modulation of the target cell milieu immediately upon viral adhesion and entry. This hypothesis is supported by data, which show that M78 knockout MCMV fails to efficiently activate accumulation of the viral m123 immediate-early mRNA in infected macrophages.[94] Furthermore, it was shown that M78 facilitated the accumulation of the immediate-early mRNA in cycloheximide-treated cells, suggesting that it accomplished the transactivating effect in the absence of de novo protein synthesis.

HCMV binding and entry interferes with a number of signaling pathways[117-119] and infection alters the expression of a large number of cellular genes.[120] This behavior suggests that activation via cell surface receptors, delivery of tegument proteins or some other viral particle-mediated process plays a predominant role.[102] A number of these responses, including the activation of PLC as well as a rapid release of arachidonic acid metabolites and increases in cytosolic calcium and cAMP parallel the effects seen following G-protein activation through GPCRs.[118,119] Also, the early activation of NF-κB following CMV infection can be blocked by pertussis toxin suggesting the involvement of G-protein coupled receptor signaling.[121,122] The current literature on the signaling mediated by virus encoded GPCRs reviewed in 'Viral chemokine receptor activities and receptor-ligand selectivity' (see above) suggests that the receptors could play a role in the signaling events observed during viral infection. However, the literature is mainly based on expression studies on isolated receptors, out of the context of viral infection and it is therefore difficult to delineate the significance of the reported receptor activities for virus mediated signaling, for viral growth and dissemination or for viral pathogenesis.

The observed phenotypes of the receptor mutant viruses suggests either a cell type specific replication deficiency, a deficiency in the virus ability to disseminate to the salivary gland or a deficiency in the virus interaction with the host immune system resulting in increased clearance of the virus. However, it seem evident that signaling events induced by the virus encoded GPCRs are necessary for optimal viral growth in vivo. To further outline how the herpesvirus encoded GPCRs work to influence virus growth and dissemination and the virus ability to interact with the host immune system additional studies of receptor signaling and receptor ligand interaction during viral infection of the natural host are needed.

Over the last several years we have started to learn that chemokine mimicry by herpesvirus is common and important for the virus. What has come as a surprise is that herpesvirus encoded chemokines and chemokine receptors have positive effects and are not only used by the virus to block the chemokine system or evade the host immune response. Accordingly it seems inevitable that these pro-inflammatory chemokine elements play imporant roles in viral growth and dissemination in vivo.

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CHAPTER 4

Interferon-Inducible Chemokines in Poxvirus Infections

Surendran Mahalingam

Introduction

An important aspect that contribute towards the clearance of an infection is the migration of leukocytes to areas of viral replication and insults. It is now widely accepted that chemokines mediate chemotaxis, activation and the extravasation of immune cells from the blood to infectious sites. As described in Chapters 2 and 3 microbial pathogens use the chemokine system to their advantage through molecular piracy.[1-3] Interferons (IFNs) are involved in host-defense mechanisms and become activated within hours after infection. The importance of IFNs in the control of poxvirus infections are well established.[4,5] One mechanism by which IFNs control or inhibit the growth of poxvirus is via the upregulation of a variety of proteins as well as the activation of enzymatic pathways.[6] In this regard, two IFN-inducible chemokines namely CXCL9/Mig and CXCL10/Crg-2[7-9] could mediate some of the antiviral effects of IFNs. In vitro studies using macrophage cell lines RAW 264.7 established that CXCL10 could be induced by IFN-α/β, IFN-γ and lipopolysaccharide (LPS).[9] In contrast, CXCL9 expression was only triggered by IFN-γ.[7,8] Both chemokines share the same receptor CXCR3 on the surface of T cells and NK cells and mediate the chemotaxis of these cells.[10] This chapter provides an overview of the roles of CXCL9 and CXCL10 during poxvirus infection using vaccinia virus as an infectious model.

Expression Following Vaccinia Virus (VV) Infection in Vivo

The expression of the IFN-inducible chemokines in organs of infected mice were first determined. Using Northern blot analysis the expression of CXCL9 and CXCL10 were shown to be induced at high levels in various organs early after infection (Fig. 1). With the exception of liver the kinetics of CXCL9 and CXCL10 expression correlated with that of IFNs α and γ in all organs.[11,12] The high levels of CXCL9 expression seen in the liver may be due to circulating IFN-γ in the serum. Interestingly, this finding suggest that CXCL9's function is not restricted to cell recruitment locally at sites of inflammation, but may have roles at distant sites. When compared to control mice, CXCL9 expression were significantly diminished in mice lacking IFN-γ function (C57BL/6.IFN-$\gamma^{-/-}$). By contrast, CXCL10 expression was found to be only marginally reduced when compared to levels in control mice. However, both chemokines were not induced in mice lacking both functional IFN receptors. The findings from this study clearly demonstrate that the induction of CXCL9 and CXCL10 during VV infection in vivo is intimately dependent on IFNs.

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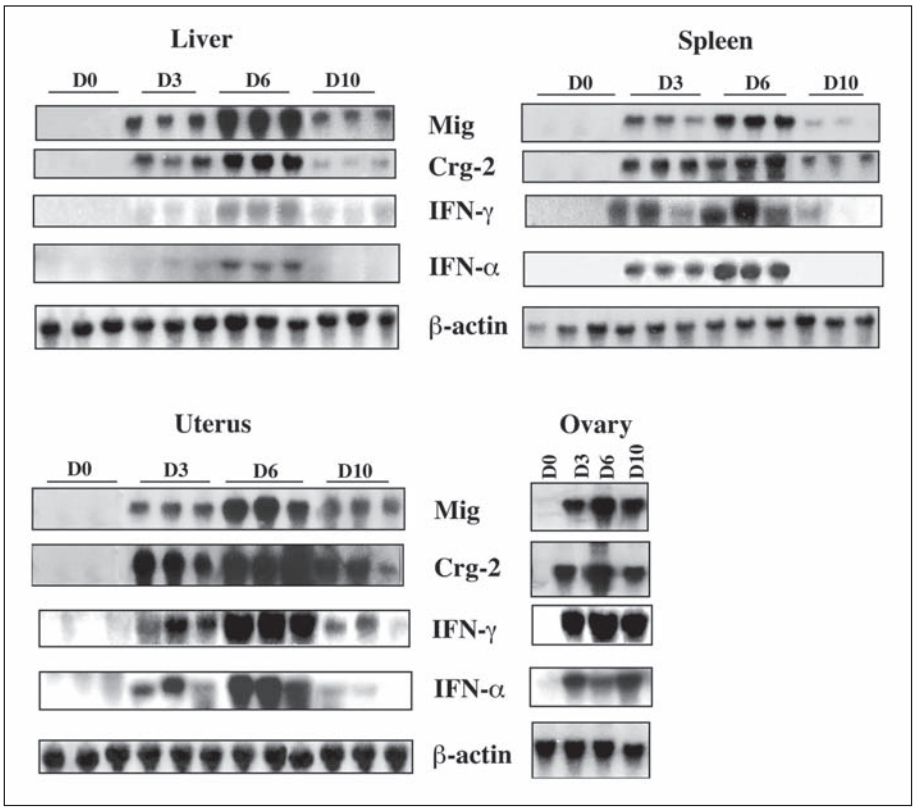

Figure 1. Expression of CXCL9/Mig, CXCL10/Crg-2, IFN-α and IFN-γ mRNAs in organs of VV infected C57BL/6 WT mice. RNA was extracted from organs harvested from mice that were uninfected (D0) or infected for 3 (D3), 6 (D6) and 10 (D10) days with VV. Twenty microgram samples of RNA were electrophoresed, transferred to nitrocellulose filters, and hybridized to CXCL9, CXCL10, IFN-α, IFN-γ or β-actin rRNA probes. Groups of 3 mice were used in each experiment. [Reproduced with permission from the journal Immunology and Cell Biology. Mahalingam S, Karupiah G. Expression of the interferon-inducible chemokines MuCXCL9 and CXCL10 following vaccinia virus infection in vivo. Immunol Cell Biol 2000; 78:156-160].

Recombinant VV Expressing CXCL9 or CXCL10

A number of cytokines expressed by rVV in vivo have been shown to influence the immune responses produced against the virus.[13] The expression of CXCL9 or CXCL10 by rVV, at foci of infection, has provided an ideal model to investigate the physiological effects of CXCL9 and CXCL10 in antiviral immunity.

Investigations in nude mice have shown that the recombinant vaccinia virus encoding chemokines are attenuated. Athymic, nude mice inoculated with 10^4 and 10^5 PFU of control virus (not expressing either of the chemokines), succumbed to infection and died (Table 1). By contrast, no mortality was observed when mice were given a similar dose of VV-CXCL9 or VV-CXCL10.[14] Infection with a higher dose (10^6 and 10^7 PFU) resulted in 100% mortality in all groups but the mean time to death was significantly delayed in mice infected with either of the chemokine-expressing viruses when compared to those infected with the control virus.

Table 1. Mean time to death after rVV infection in athymic Swiss nude mice

Virus Dose (PFU)[‡]	10^7	10^6	10^5	10^4
VV-X	5.5 ± 0.5	16.8 ± 2.0	22.7 ± 4.5	35.5 ± 8.3
VV-CXCL9	16.5 ± 2.3[¶]	35.2 ± 0.9[¶]	NM[†]	NM
VV-Crg2	10.5 ± 1.0[¶]	29.5 ± 1.0[¶]	NM	N/M

The effect of rVV infection on the survival of athymic Swiss nude mice. [Reproduced with permission from the Journal of Virology. Mahalingam S, Farber JM, Karupiah G. The interferon-inducible chemokines MuCXCL9 and CXCL10 exhibit antiviral activity in vivo. J Virol 1999; 73:1479-1491].
[*] Groups of 5 mice were inoculated with the indicated virus dose and observed for 60 days.
[‡] PFU, plaque forming unit
[§] MTD, Mean time to death in days ± SEM for 5 individual mice.
[†] NM, No mortality
[¶] Significant, $p < 0.001$ (Student's *t*-test) compared to MTD of mice inoculated with a comparable dose of VV-X

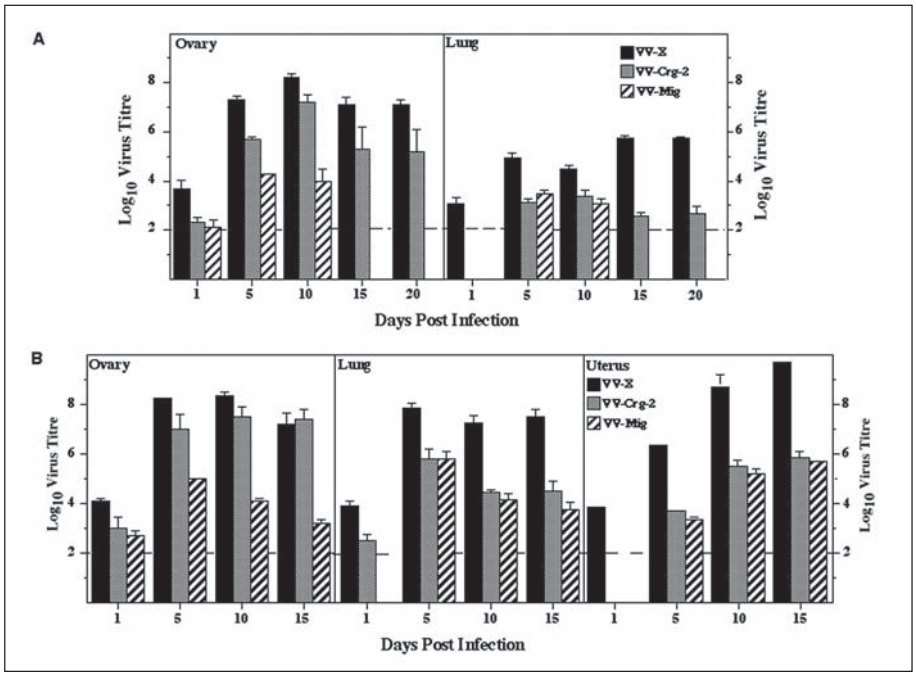

Figure 2. Kinetics of recombinant vaccinia virus replication in the ovaries, lungs and uterus of outbred nude mice. Female nude mice between the ages of 6 and 9 wks were infected intravenously with (A)10^5 or (B) 10^6 PFU of VV-X, VV-CXCL9 or VV-CXCL10 and on the days indicated organs were removed to determine virus titers. Data shown are the geometric means of four individual organs per day for each group ± SEM. [Reproduced with permission from the Journal of Virology. Mahalingam S, Farber JM, Karupiah G. The interferon-inducible chemokines MuCXCL9 and CXCL10 exhibit antiviral activity in vivo. J Virol 1999; 73:1479-1491].

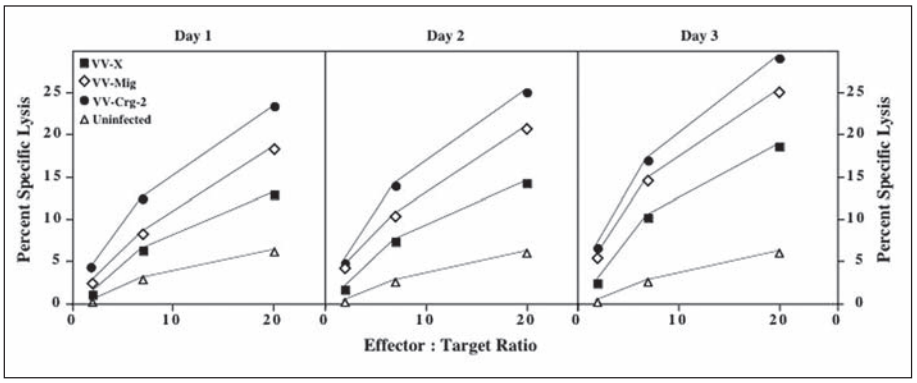

Figure 3. NK responses following infection with rVV. Female nude mice were infected i.v.with 10^6 PFU VV-control, VV-CXCL9 or VV-CXCL10. NK activity was measured using spleen cells from uninfected mice or from mice 1, 2 and 3 days following infection with rVV. Data are mean ± SEM (in most cases SEM is too low to be observed on the graph), n = 5 mice per group. [Reproduced with permission from the Journal of Virology. Mahalingam S, Farber JM, Karupiah G. The interferon-inducible chemokines MuCXCL9 and CXCL10 exhibit antiviral activity in vivo. J Virol 1999; 73:1479-1491].

Additionally, there were differences in organ virus titers. Organ virus titers were significantly lower in VV-CXCL9 and VV-CXCL10 infected mice than those of wild-type infected mice over the entire period investigated (Fig. 2). The viral kinetic studies demonstrate that the CXCL9- and CXCL10-induced antiviral activity is effective very early after infection (as early as day 1 post infection). This led to studies aimed at elucidating the mechanisms by which both CXCL9 and CXCL10 contributed to the accelerated clearance of the chemokine-encoding viruses.

Role for NK Cells

It is well established that IFNs can mediate the activation of NK cells.[15] NK cells express CXCR3 on their surface, which is the receptor for CXCL9 and CXCL10.[10] This implicates the possible role of CXCL9 and CXCL10 in the recruitment of NK cells as well as their activation. The latter may involve enhancement of cytolytic function and cytokine production. In this regard, it has been shown that recombinant CXCL10 enhances the cytolytic activity of NK cells in vitro.[16] Collectively, these findings reveal a role for NK cells in mediating the function of virus encoded CXCL9 or CXCL10. Interestingly, it was shown that the cytolytic activity of splenic NK cells obtained from the chemokine-encoding rVV-infected mice was at least 2-3-fold higher than that of the control virus-infected mice (Fig. 3).[14] This increase in killing activity is most likely related to the increased splenic cell numbers. There was also an increase in mononuclear cell infiltration in foci of infection in livers of VV-CXCL9- and VV-CXCL10-infected mice. NK cell depletion studies using anti-asiola-GM1 antibody, demonstrated an exacerbation of infection with the chemokine expressing viruses.

The mechanism by which CXCL9 and CXCL10 enhance the NK cell cytolytic activity is not clear, however, there are at least two possible mechanims. Firstly, an increase in chemokine expression as well as NK cell numbers at infectious foci may collectively contribute to enhanced NK cell adherence to target cells. Secondly, CXCL9- and CXCL10 may be involved in the upregulation of granule exocytosis which is one of the key factors in NK cell-mediated cell lysis.[17,18] Supporting this is the evidence that chemokines can induce NK cell degranulation.[16,19] It is possible that NK cells are induced to release granule contents upon conjugation with virally-infected cells as they enter sites of chemokine production.

Table 2. Importance of IFNs in the clearance of VV-CXCL9 and VV-CXCL10

rVV Infection +	Log_{10} VirusTtiters ± SEM	
Treatment	**Ovaries**	**Lungs**
VV-X		
+ GL113	8.0 ± 0.2	5.1 ± 0.3
+ α-IFN-α/β	8.2 ± 0.5	5.4 ± 0.3
+ α-IFN-γ	8.2 ± 0.1	5.4 ± 0.3
+ α-IFN-α/β + α-IFN-γ	8.3 ± 0.4	6.0 ± 0.2
VV-CXCL10		
+ GL113	6.4 ± 0.2	4.1 ± 0.1
+ α-IFN-α/β	7.4 ± 0.2	5.0 ± 0.1
+ α-IFN-γ	7.4 ± 0.2	5.1 ± 0.3
+ α-IFN-α/β + α-IFN-γ	8.1 ± 0.1	6.1 ± 0.4
VV-CXCL9		
+ GL113	5.0 ± 0.3	4.0 ± 0.1
+ α-IFN-α/β	5.5 ± 0.2	4.7 ± 0.3
+ α-IFN-γ	5.8 ± 0.1	4.9 ± 0.2
+ α-IFN-α/β + α-IFN-γ	7.5 ± 0.3	5.4 ± 0.2

*Groups of 6-9 week old female outbred nude mice were given mAb to IFN-γ, or 300 U of anti IFN-α/β or both antibodies. Mice treated with mAb GL113 were used as controls. Mice were infected i.v. with 10^6 PFU virus on day 0 and sacrificed 5 days later for determination of virus titers in organs. These experiments were repeated twice with reproducible results.
The effect of treatment with antibodies to IFN-γ or IFN α/β or both on rVV replication in nude mice. [Reproduced with permission from the Journal of Virology. Mahalingam S, Farber JM, Karupiah G. The interferon-inducible chemokines MuCXCL9 and Crg-2 exhibit antiviral activity In vivo. J Virol 1999; 73:1479-1491].

Role for Interferons

Recovery from VV infection is dependent on the involvement of IFNs. The combination of enhanced NK killing activity and the production of IFN by NK cells may have contributed to rapid viral clearance. To examine this possibility, the specific role of IFNs in the control of the chemokine-expressing viruses was investigated using specific neutralizing antibodies. It was found that VV-CXCL9 and VV-CXCL10 titres significantly increased and control virus titres remained unchanged, following treatment with anti-IFN-γ or anti-IFN-α/β antibodies (Table 2).[14] Neutralization of both IFN-γ and IFN-α/β simultaneously, did not increase virus titres and therefore completely abolished the chemokine-mediated antiviral effects of VV-CXCL9 and VV-CXCL10. In a similar study involving mice lacking both functional IFN receptors, similar results were obtained. Infection with the control virus was lethal for mutant mice lacking IFN-γ R (receptor), IFN-α/β R or IFN-α/β, γ R.[20] Infection of IFN-γ R GKO and IFN-α/β R GKO mice with chemokine-encoding rVV did not result in mortality, however virus clearance was delayed compared to wild-type mice. Interestingly, IFN-α/β, γ R GKO mice infected with VV-CXCL9 or VV-CXCL10 succumbed to vaccinia infection, however, mortality was significantly delayed compared to mutant mice given VV-control. From the results obtained, it can be deduced that the mechanisms of VV clearance in nude and normal mice is strictly dependent on the involvement of IFNs.

It appears that the mechanisms of CXCL9- and CXCL10-mediated viral clearance involve NK cells and IFNs. The increases in lymphoid cell numbers at foci of infection contribute to virus clearance through the action of cytolysis and IFN secretion. Through the induction of CXCL9 and CXCL10, IFNs facilitate the migration of NK cells to infectious sites so that infected cells can be eliminated more effectively.

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CHAPTER 5

CX_3C Chemokine Mimicry by Respiratory Syncytial Virus G Glycoprotein

Ralph A. Tripp

Introduction

Chemokines are small disulphide-linked polypeptides that act as potent chemoattractants for many cell types including lymphocytes, monocytes, macrophages, neutrophils and NK cells. As described in Chapter 1 chemokines are divided into subfamilies based upon cysteine signature motifs termed C, CC, CXC and CX_3C and have roles in immunity and inflammation affecting cell trafficking and activation. The only described CX_3C chemokine is fractalkine, an unusual chemokine in that it exists both as a membrane bound protein and as a cleaved soluble molecule. Several viruses have evolved strategies to alter immune responses by expressing proteins that mimic chemokines or their receptors. The G glycoprotein of respiratory syncytial virus (RSV) was recently shown to contain a CX_3C chemokine motif that competes with fractalkine for binding to its receptor, CX_3CR1, induces fractalkine-like leukocyte chemotaxis, and facilitates virus infection. The implications of this finding in the biology of RSV infection and disease pathogenesis are discussed.

Respiratory Syncytial Virus, Immune Modulatory Glycoproteins, and Disease Pathogenesis

Respiratory syncytial virus was first isolated in 1956 and has become recognized as the most important cause of serious lower respiratory illness in infants and young children worldwide.[1-4] Most children are infected with RSV by age 2, and RSV can cause repeat infections throughout life in older children and healthy adults.[4-6] RSV also causes substantial morbidity and mortality in younger and older children, adults, the elderly, and those with compromised cardiac, pulmonary or immune systems.[4,7-9] RSV, a member of the Paramyxoviridae family and type species of the *Pneumovirus* subfamily, has two major antigenic groups, A and B, and additional antigenic variability occurs within the groups.[4] It is an enveloped virus containing a negative sense, single-stranded RNA genome that encodes for 11 proteins including three transmembrane glycoproteins. These transmembrane glycoproteins assemble separately into homo-oligomers that include the attachment (G) glycoprotein, the fusion (F) glycoprotein, and the small hydrophobic protein (SH) of unknown function.

RSV primarily infects respiratory epithelium by interaction of heparin-binding domains (HBDs) on the G glycoprotein with glycosaminoglycans (GAGs) on the cell surface.[10,11] It is likely that the initial interaction of G glycoprotein with cell-associated GAGs is important to infection, however RSV infection may also be facilitated by interaction of the CX_3C chemokine motif on the G glycoprotein with the CX_3C chemokine receptor, CX_3CR1, expressed on some

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cell types.[11] Interestingly, G glycoprotein is not required for infection in vitro, as viruses that lack the G and SH gene can infect some cell lines probably through the F glycoprotein.[12] RSV infection of respiratory epithelial cells often results in destruction of the cells during virus replication, and severe infection in infants may result in bronchiolitis and pneumonia. The clinical pathology of RSV infection is obstructive airway disease and is associated with pulmonary leukocyte infiltration and mucous plugs.[4,13-17]

The mechanisms of obstructive airway disease and other manifestations of RSV disease are not completely understood, but probably include virus-mediated cytopathology and host responses to infection. Cytopathology mediated by RSV infection causes considerable damage to the respiratory epithelium and ciliary cells resulting in accumulation of cell debris and exudate in the bronchioles and alveoli.[18,19] In the young infant, the small diameter of the bronchioles probably predisposes to obstruction, a factor that likely contributes to the severity of RSV disease in this age group. The mechanism of RSV-mediated cell death is not known, but RSV proteins may affect cellular apoptotic mechanisms. RSV infection of epithelial cell lines has been shown to induce a variety of apoptosis regulators including caspase-12,[20] IEX-1L,[21] CD95,[22,23] caspase-3,[24] interferon regulatory factor-1 B,[24-27] and NFκB.[28-30] In addition, RSV infected epithelial cells have been shown to express a variety of pro-inflammatory mediators (e.g., IL-1, IL-6, CXCL8, TNFα) and undergo a self-defense strategy of programmed cell death to limit virus replication.[20,26,31-38]

The host response to RSV infection involves the innate, humoral and cellular immune responses. The innate immune response initiates the inflammatory response to infection, and involves the elaboration of cytokines and chemokines (e.g., IL-6, CXCL8, CCL5) and recruitment of various leukocytes including natural killer (NK) cells, polymorphonuclear (PMN) cells and granular cells (e.g., eosinophils) to the site of infection. The innate immune response also involves a variety of molecules that may alter infection that include members of the collectin family.[39,40] The RSV F and G glycoproteins have been shown to modulate the innate immune response to infection. The F glycoprotein has been shown to stimulate innate immunity through activation of the shared components of the innate immune activation pathway, CD14 and toll-like receptor (TLR)-4,[41] and TLR4 appears to be important in the innate immune response to RSV infection.[42] TLR-4-deficient mice challenged with RSV exhibit impaired NK cell and CD14+ cell pulmonary trafficking, deficient NK cell function, impaired interleukin (IL)-12 expression and impaired virus clearance.[42] In addition, the F glycoprotein has also been shown to alter immunity by delaying the transit from G_0/G_1 to S-phase in the cell cycle.[43] T cell contact with the F glycoprotein inhibited mitogen-induced T cell proliferation, an event that may be related to signaling events mediated by the F glycoprotein leading to unresponsiveness. In the mouse model, the G glycoprotein has also been shown to modify immunity, particularly the composition of the CD4 T cell compartment, by driving responses toward type-2 CD4+ T cells, and exaggerating Th2-type cytokine responses.[44-52] Studies comparing the immune response to infection with wild type RSV or a RSV mutant lacking G and SH genes showed that G and/or SH glycoprotein expression markedly decreased pulmonary trafficking of PMN and NK cells, and induced an exaggerated T helper (Th)-2 cytokine profile,[48] and reduced macrophage inflammatory proteins CCL3, CCL4, CXCL2 and CXCL10 mRNA expression by bronchoalveolar lavage (BAL) cells.[53] Since MIP chemokines interact with chemokine receptors CCR1 and CCR5, and CXCL10 with CXCR3,[54-56] preferentially expressed on Th1 cells,[57-59] these results may suggest that inhibition of G and/or SH glycoprotein expression may impair Th1 responses mediated by these chemokines. In BALB/c mice, immunization with a vaccinia virus expressing the G glycoprotein, or treatment with formalin-inactivated RSV, induces an exaggerated CD4+ T cell response with increased Th2-type (IL-4, IL-5) cytokine expression, and subsequent challenge with RSV results in enhanced

pulmonary eosinophilia and inflammation.[60-67] G glycoprotein sensitization for enhanced pulmonary disease has been shown to be associated with T cell epitopes on the G glycoprotein comprising amino acids 185-193,[68] 184-198[69] or 193-205,[70] however the response mediated by the G glycoprotein may not be epitope-specific, since the form and site of administration of G glycoprotein available for antigen processing and presentation has been shown to be an important factor sensitizing for enhanced pulmonary disease.[45,46,71] BALB/c mice immunized with vaccinia virus expressing the secreted form of the G glycoprotein had more severe illness following RSV challenge than did mice primed with membrane-anchored G glycoprotein.[45] Co-administration of purified G glycoprotein during priming with the vaccinia construct expressing the membrane-anchored G glycoprotein shifted the immune response following RSV challenge to a more Th2-like response characterized by increased interleukin-5 in lung supernatants, and an increase in G-specific immunoglobulin G1 antibodies.[46] In addition, RSV-specific CD8+ T cells have been shown to regulate the Th2 CD4+ T-cell response.[72,73] BALB/c mice primed for a RSV-specific CD8+ T cell response with a vaccinia virus expressing G glycoprotein and a matrix protein (M2) CD8+ T cell epitope inhibited pulmonary eosinophilia upon subsequent challenge with RSV.[73] Thus, the G glycoprotein can modify the host immune response at several levels and is likely important in disease pathogenesis.

G Glycoprotein CX_3C Chemokine Mimicry

G glycoprotein-associated changes in pulmonary cell trafficking and inflammation,[74] cytokine and chemokine expression,[74,75] and the evidence that the secreted form of G glycoprotein appears to be most potent in mediating these effects,[45,46] are suggestive of chemokine-like qualities of the G glycoprotein. Chemokines are crucial factors controlling the development and function of leukocytes, and are essential mediators of leukocyte trafficking, orchestrating the activation of immune cells and expression of cytokines through distinct chemokine receptors on the cells. There are four structural categories of chemokines, alpha (CXC), beta (CC), gamma (C) and delta (CX_3C) chemokines that are based upon cysteine signature motifs.[76,77] Chemokines mediate their activities through chemokine receptors that can be classified into three major categories: specific (i.e., a single ligand), shared (i.e., multiple ligand of the same chemokine family) and promiscuous (i.e., multiple ligands of different chemokine families).[78,79] The G glycoprotein was recently been shown to contain a CX_3C chemokine motif at amino acid positions 182-186 in the central conserved region of the G glycoprotein.[80] Comparison of G glycoprotein with fractalkine (FKN)/CX_3CL1, the only member of the delta or CX_3C subfamily of chemokines[81,82] revealed significant structural similarities between the proteins. For example, both FKN (397 amino acids) and G glycoprotein (289-299 amino acids) are large glycoproteins that have intracellular, transmembrane and extracellular domains, contain HBDs, and can be expressed as either membrane-bound or secreted forms.[4,80,82-85] FKN, unlike other chemokines, has a CX_3C chemokine domain at the top an extended mucin stalk, similar to the proposed structure of the G glycoprotein. These structural features afford FKN the properties of being membrane-bound and functioning as a tether protein for cell adhesion, or proteolytically released from the cell surface by tumor necrosis factor-alpha-converting enzyme (TACE,[86,87]) to function in a form that promotes chemotaxis of leukocytes expressing FKN receptors. The structural similarity between the G glycoprotein CX_3C motif and fractalkine CX_3C motif is shown in Figure 1.

The binding efficiency of the G glycoprotein to CX_3CR1 was investigated.[80] The CX_3C motif in the G glycoprotein was shown to compete with FKN for binding to CX_3CR1, and binding and binding inhibition studies showed that G glycoprotein specifically bound CX_3CR1-transfected human embryonic kidney cells (293-CX_3CR1) in the presence of heparin used to block HBD interaction with GAG (Table 1). In these studies, increased G

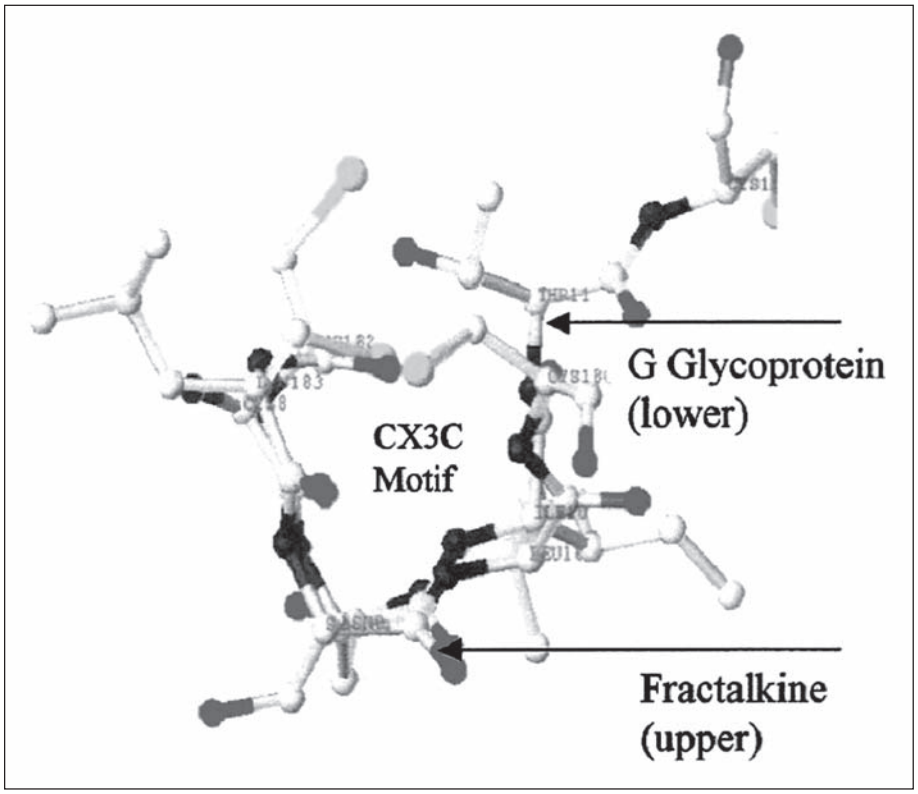

Figure 1. Comparison of the CX_3C motifs of RSV G glycoprotein and fractalkine. Superimposed images of the CX_3C motif of fractalkine and RSV G glycoprotein modeled using the molecular modeling program SYBYL. Forcefields were generated using SYBYL and charges calculated using the Gasteiger-Huckel and Kollman methods.

glycoprotein binding correlated with increased expression of CX_3CR1 on 293-CX_3CR1 cells, and was specifically inhibited using anti-G glycoprotein monoclonal antibodies. The specificity of G glycoprotein CX_3C-CX_3CR1 interaction was further supported by inhibition studies with reagents known to bind to CX_3CR1 including FKN, rabbit anti-CX_3CR1 serum and an anti-CX_3CR1 monoclonal antibody (Table 1). In addition, inhibition studies with 12-mer G glycoprotein peptides containing the CX_3C motif, or having an amino acid deletion (CX2C) or insertion (CX4C) in the motif indicated that G glycoprotein interaction with CX_3CR1 occurs through the CX_3C motif in the G glycoprotein (Table 1). Radioimmune and Scatchard analysis of the competition of unlabeled FKN or G glycoprotein with iodinated-FKN for binding to 293 or 293-CX_3CR1 cells showed similar binding parameters between FKN and G glycoprotein CX_3C binding to CX_3CR1 (e.g., FKN K_d = 5.8, G glycoprotein K_d = 2.1). These results showed that G glycoprotein could compete with FKN for binding to CX_3CR1, and suggested that G glycoprotein might alter FKN-mediated responses.

The possibility that G glycoprotein mediated FKN-like chemotactic responses was examined using modified Boyden chambers.[80] Leukocytes from naïve BALB/c mice, or leukocytes from normal human adults, were tested for chemotaxis toward FKN or G glycoprotein. Both FKN and G glycoprotein induced similar leukocyte chemotaxis that was inhibited with antibodies specific

Table 1. Percent inhibition of G glycoprotein and FKN binding to 293-CX_3CR1 cells by G glycoprotein, FKN, antibodies, and peptides in the presence and/or absence of heparin

Inhibitor	Range of Inhibition (%) G Glycoprotein[1]	Fractalkine[1]
G glycoprotein[1]	—-	50-58
FKN[1]	20-34	—-
Anti-G glycoprotein antibody[2]	86-92	0-4
Anti-CX_3CR1 antibody[2]	88-94	88-92
Heparin[3]	78-84	57-65
Heparin[3] + FKN[1]	85-98	—-
Heparin[3] + G glycoprotein[1]	—-	80-85
CX_3C peptide[4]	35-42	18-27
Heparin[3] + CX_3C peptide[4]	85-94	70-80
CX2C peptide[4]	8-12	0-4
Heparin[3] + CX2C peptide[4]	76-82	60-67
CX4C peptide[4]	16-22	0-8
Heparin[3] + CX4C peptide[4]	80-88	50-65
Normal rabbit sera[2]	6-10	5-14
Rabbit anti-CCR5 sera[2]	5-12	5-12

Inhibition of G glycoprotein and FKN binding to 293-CX_3CR1 cells by treatment with monoclonal antibodies to G glycoprotein or CX_3CR1, or treatment with G glycoprotein, FKN, or G glycoprotein peptides in the presence or absence of heparin.[1] 10nM of G glycoprotein or FKN; [2] 1 μg antibody; [3] 5 μg/ml heparin; [4] 1 mM peptide. The concentration of G glycoprotein was estimated using a molecular weight of 90kD.

for FKN or G glycoprotein and with anti-CX_3CR1 antibodies. Examination of CX_3CR1 expression on human leukocytes showed that cells that trafficked toward FKN or G glycoprotein expressed higher levels of CX_3CR1 compared to cells that did not migrate, a result consistent with chemokine-like responses. In addition, G glycoprotein was shown to be an antagonist of fractalkine-mediated chemotaxis, and fractalkine of G glycoprotein-mediated chemotaxis, suggesting similar mechanisms (i.e., CX_3C) induced chemotaxis (Fig. 2). Since cell trafficking to the site of infection or inflammation is mediated by chemotaxis, these results suggested that G glycoprotein expression might subvert leukocyte migration during infection by disrupting FKN-mediated chemokine gradients, or blocking FKN-CX_3CR1 interaction.

Since G glycoprotein is an attachment protein for RSV infection,[2,4,88] the possibility that G glycoprotein CX_3C-CX_3CR1 interaction facilitated virus infection was investigated using a plaque reduction assay[11] (Table 2). Vero cells are susceptible to RSV infection and express CX_3CR1 mRNA and detectable CX_3CR1 expression. G glycoprotein and FKN contain HBDs that interact with GAGs on the surface of Vero cells. To allow for identification of CX_3C-CX_3CR1 binding, and prevent HBD-GAG binding, Vero cells were treated with heparin prior to RSV infection. Plaque reduction assays were performed in the presence of FKN, G glycoprotein, 12-mer G glycoprotein peptides (with or without the CX_3C motif) or anti-CX_3CR1 antibody. The results showed that much of RSV binding to Vero cells occurred via HBD-GAG interaction and the remaining binding (~30%) occurred through G glycoprotein CX_3C-CX_3CR1 interac-

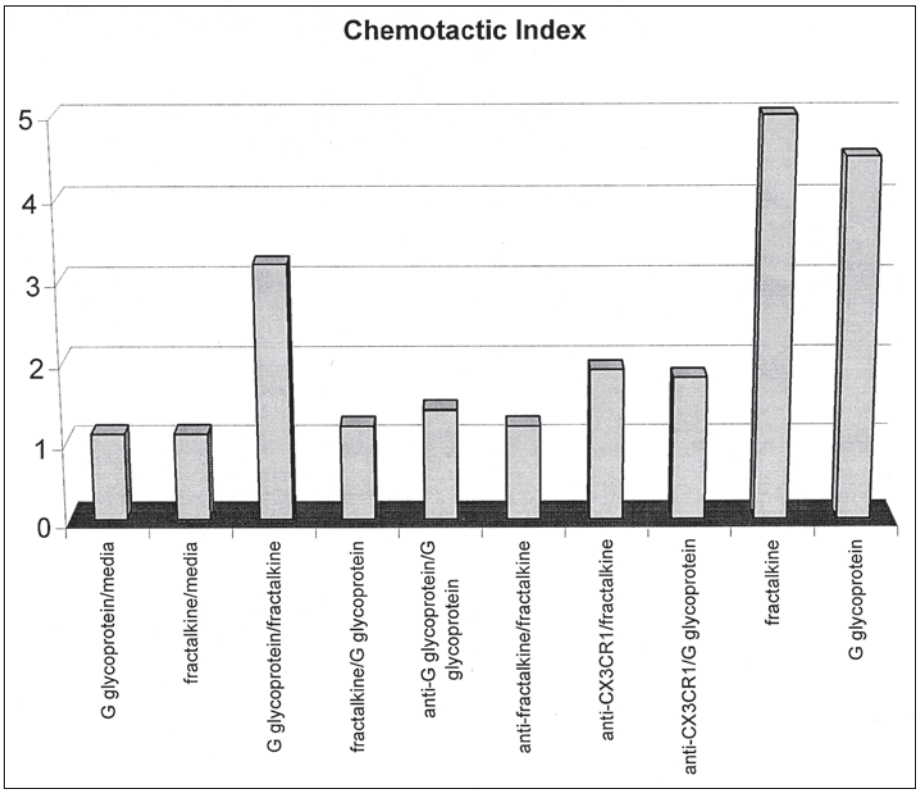

Figure 2. The chemotactic indices of murine leukocyte migration toward G glycoprotein or fractalkine in the presence and absence of inhibitors.The chemotactic index was determined from the fold increase of murine spleen leukocyte migration toward the chemoattractant (e.g., G glycoprotein or fractalkine) over the leukocyte migration toward media alone control using modified Boyden chambers. G glycoprotein, fractalkine, anti-CX_3CR1 antibody, anti-G glycoprotein or anti-fractalkine antibodies were added to the upper chamber to examine antagonism of cell migration toward the chemoattractant in the lower chamber (e.g. anti-CX_3CR1/G glycoprotein).

tion. Interestingly, FKN inhibited ~80% of RSV plaque formation, suggesting that FKN may have an important role in limiting RSV infection. Several cell types in the respiratory tract that have been shown to express FKN including dendritic cells,[89,90] T cells[91] and bronchial epithelial cells,[92,93] thus the degree of RSV infection may be affected by FKN expressed by these cell types.

Consequences of G Glycoprotein Mimicry

During RSV infection, G glycoprotein expression may affect a wide variety of immune components that regulate or participate in the response to infection. RSV infection of epithelial cells has been shown to induce expression of a variety of CC, CXC and CX_3C chemokines.[92,94-97] Many of these chemokines, including FKN, are upregulated by proinflammatory signals, such as IL-1, IFNγ and TNFα,[91,98,99] and G glycoprotein has been shown to alter expression of IFNγ and TNFα by BAL cells.[74] The exaggerated Th2-type cytokine response associated with G glycoprotein expression,[44-46,100] may also down regulate FKN

Table 2. RSV plaque reduction by G glycoprotein, FKN, G glycoprotein peptides, anti-CX_3CR1 antibody, and/or heparin on Vero cells

Treatment	Plaque Inhibition (%)
Heparin[1]	66
G glycoprotein[2]	61
Heparin[1] + G glycoprotein[2]	98
FKN[3]	80
Heparin[1] + FKN[3]	97
G glycoprotein[2] + FKN[3]	88
G glycoprotein[2] + FKN[3] + Heparin[1]	98
CX2C peptide[4]	0
Heparin[1] + CX2C peptide[4]	64
CX_3C peptide[4]	71
Heparin[1] + CX_3C peptide[4]	92
CX4C[4] peptide	30
Heparin[1] + CX4C peptide[4]	58

RSV plaque reduction following treatment with G glycoprotein, FKN, G glycoprotein peptides with the CX_3C motif, or having an amino acid deletion (CX2C) or insertion (CX4C) in the motif, and/or heparin. Percent inhibition was determined from the mean plaque forming units of treated vero cells over saline-treated vero cells using a dilution of RSV that would produce 40-80 pfu.[1] 5 μg/ml of heparin;[2] 100nM G glycoprotein;[3] 10 nM FKN;[4] 1 mM of peptide. The concentration of G glycoprotein was estimated using a molecular weight of 90kD.

expression, since IL-4 and IL-13 have been shown to inhibit IFNγ expression.[91,101-104] G glycoprotein modification of FKN-mediated responses may also affect the outcome of disease pathogenesis. NK cells and Th1-type cells express high levels of CX_3CR1,[91,105,106] respond to FKN, and are important components of anti-viral immunity promoting cell-mediated effector responses. In contrast, Th2-type cells express low levels of CX_3CR1, do not respond to FKN, and influence B cell development and augment IgE humoral responses.[91] Thus, CX_3C chemokine mimicry by the G glycoprotein may alter activation and trafficking of NK cells and Th1-type cells, resulting in reduced Th1-type cytokine expression and deregulated Th2-type T cell responses, leading to predominate Th2-type responses.

CX_3C chemokine mimicry may also affect the neuro-immunological synapse and affect inflammation and disease pathogenesis. Neurons express a wide variety of chemokine receptors, including CX_3CR1, that are important in signal transduction and linking the immune system with the nervous system.[107,108] FKN appears to be a neuron-to-microglia signal molecule whose expression is enhanced by proinflammatory cytokines including TNFα and IFNγ.[109-112] Neurons respond to FKN producing excitatory effects associated with substance P (SP) expression.[109,113] SP has diverse actions primarily mediated through specific NK-1R receptor[114,115] including induction of vascular extravasation of immune cells, increased adhesion of polymorphonuclear cells and eosinophils to endothelium, and potentiation of immune functions of lymphocytes, macrophages, mast cells, and eosinophils.[116-119] The functional relevance

of SP in pulmonary inflammation is indicated by studies of NK-1R knockout mice in which immune complexes, that induce vascular permeability and allow infiltration of inflammatory cells into the lungs of normal mice, had no effect in NK-1R knockout mice.[120] RSV infection of BALB/c mice has been shown to enhance pulmonary levels of SP, and induce pulmonary inflammation.[121] Mice infected with a RSV mutant lacking G and SH genes had lower levels of pulmonary SP and decreased pulmonary inflammation compared to wild type-infected mice, suggesting that G and/or SH glycoprotein expression affected the expression of SP and inflammation. Treatment of RSV-infected mice with anti-SP $F(ab)_2$ antibodies decreased inflammation associated enhanced Th1 and Th2 cytokine expression and leukocyte infiltration.[121] Thus, it is possible that during RSV infection, the CX_3C motif on the G glycoprotein interacts with CX_3CR1 expressed on neurons and other cells in the lung inducing SP in a fashion similar to the known activities of FKN, and that G glycoprotein and SP alter pulmonary physiology and inflammation.

Understanding CX_3C chemokine mimicry by the G glycoprotein may also benefit RSV vaccine development. There is currently no safe and effective RSV vaccine. The first candidate RSV vaccine was a formalin-inactivated RSV (FI-RSV) preparation evaluated in the 1960s. Unfortunately, many of the young children who received this vaccine experienced enhanced pulmonary disease when later infected with RSV.[122,123] Evidence from FI-RSV vaccine studies in mice suggest that G glycoprotein-mediated exaggerated Th2-type cytokine responses may have been associated with the enhanced pulmonary disease observed in the young vaccinees.[44-46,100] The experience with FI-RSV has precluded use of any non-live virus vaccine in infants and young children. Multiple candidate live attenuated RSV vaccines have been evaluated in adults and children,[124-129] all containing the G glycoprotein gene, however none has proven sufficiently safe to be used in the key target population, infants and young children. It is possible that the disappointing results observed with RSV vaccine candidates are related to G glycoprotein-mediated modification of the immune response, inflammation or disease pathogenesis.

Chemokine Mimicry

Several viruses have evolved mimicry mechanisms, such as viral chemokine homologs, chemokine receptor homologs or unique proteins that interfere with the normal host defense response. Viral chemokine homologs are common in herpesviruses and include CC and CXC viral chemokines. Gamma herpes viruses have been shown to encode several viral chemokine mimics including viral CC chemokine-like molecules MIP-1, MIP-2 and MIP-3,[130-134] and viral CXC chemokine receptor ORF 74.[135] Other examples of chemokine mimicry have been shown for beta herpes viruses, such as cytomegalovirus (CMV), which include viral CXC chemokines CXC-1,[136,137] viral CXC-2,[137] viral CC chemokine, MCK-1/-2,[138] and viral CC-CX_3C chemokine receptor US28.[139] In addition, other non-herpes viruses such as vaccinia virus express chemokine mimics, one being a soluble protein that binds to CC chemokines.[140,141] The expression of viral chemokine mimics are often used to reduce the efficacy of innate and acquired immune responses to infection, however viral chemokines may also act to recruit new targets for infection, or aid in infection or pathogenicity of the virus. For example, deletion of the CC chemokine homolog MCK-1 in CMV abrogates smooth muscle cell migration that has been linked to CMV-mediated vascular disease.[142] In addition, ablation of MCK-1 reduced the spread of CMV in vivo and resulted in more rapid viral clearance.[138] Perhaps the best-known example of chemokine mimicry is gp120 of the human immunodeficiency virus (HIV) envelope glycoprotein. Gp120 lacks chemokine sequence motifs, however gp120 can act at both CCR5 and CXC4 chemokine receptors to induce leukocyte signaling.[143-145] In addition, during the course of infection, HIV has been shown to exhibit chemokine

receptor promiscuity in which biological variants interchangeably interact with CXCR4 and CCR5 chemokine receptors as well as other chemokine receptors including CCR3 and CX_3CR1.[146] Thus, chemokine mimicry is important in the pathogenesis and immune control of HIV infection. Although viral chemokine mimicry is prevalent in large DNA viruses such as herpes and poxviruses, smaller RNA viruses such as HIV and RSV also exploit subversion of the immune response through chemokine mimicry. It is likely that other viruses also use this strategy to their benefit, and studies of these strategies should yield new understandings of the virus-host relationship.

Summary

RSV G glycoprotein CX_3C chemokine mimicry facilitates virus infection of cells and modifies the immune and inflammatory responses. Several immune and neural mechanisms induced by RSV infection are modified by G glycoprotein expression, possibly through CX_3C chemokine mimicry. It is also possible that G glycoprotein CX_3C chemokine mimicry may facilitate virus spread in the community by exacerbating pulmonary inflammation and aggravating coughing and production of infectious aerosols. A better understanding of the impact of CX_3C chemokine mimicry by RSV G glycoprotein should lead to a better understanding of RSV pathogenesis and may facilitate RSV vaccine development. For example, modifying the CX_3C region on the G glycoprotein, enhancing induction of antibodies, or designing inhibitors of CX_3C-CX_3CR1 interaction may prove useful for preventing or treating RSV disease.

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CHAPTER 6

Chemokine Expression and Granulocyte Recruitment in Response to Acute Pneumovirus Infection in Vivo

Helene F. Rosenberg and Joseph B. Domachowske

Introduction

The use of appropriate infectious agents in mice to mimic viral infection in man is essential to the understanding of human disease. In this Chapter, we focus on our recent findings on the inflammatory responses to respiratory virus infection using a novel model to study diseases caused by pneumoviruses. The group of pathogens collectively known as pneumoviruses are members of the family *Paramyxoviridae*, subfamily *pneumovirinae* (see Chapter 5). Briefly, they are enveloped viruses with negative sense, non-segmented single-stranded RNA genomes, each encoding ~10-12 open reading frames.[1] The best characterized of this group is respiratory syncytial virus (RSV), a human pneumovirus pathogen that is a common cause of bronchiolitis and pneumonia in pediatric populations and among the institutionalized elderly[2,3] (also described in Chapters 6 and 7). Despite advances in prophylaxis,[4,5] there are no specific therapies available to treat this infection. The limited efficacy of anti-viral approaches, such as ribavirin[6] together with the similar limited effectiveness of systemic anti-inflammatory therapies[7,8] suggest that RSV-mediated respiratory disease may include independent virus-mediated and proinflammatory pathophysiologic components.

Given these findings, there has been much interest in defining the inflammatory responses—both detrimental and beneficial—to acute pneumovirus infection. We have recently reviewed the literature on responses to infection with RSV, which are data that have been obtained primarily from studies performed in cell culture together with correlations from studies of bronchoalveolar lavage fluid from RSV-infected infants on ventilatory assistance.[9] A large link between these studies—namely, evaluation of responses of wild type and genetically-engineered mice—is severely hampered by the fact that RSV is not a rodent pathogen, and the conditions used to initiate even the minimal, abortive infection described in the literature lead to significant confusion in interpretation. This is considered in greater detail in the section to follow.

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Importance of Using Natural Rodent Pathogens for the Study of Inflammatory Responses to Virus Infection

There is no one rodent model of human disease that is absolutely perfect. For those of us engaged in inflammation research, there are two extremely important reasons why natural rodent pathogens present superior models for the study of acute responses to infection:

Rapid Evolution of Host Defense Proteins

As a group, proteins involved in inflammation and host defense are among the most highly divergent of all mammalian proteins. This was first examined systematically by Murphy[10] who evaluated 615 human-rodent coding sequence pairs, and found that proteins implicated in host defense (cytokines, chemokines, interleukins, interferons, etc.) were characterized by 35 +/- 1% amino acid sequence divergence, more than three-fold larger than the 1-12% divergence observed for most other human-rodent coding pairs. In our studies, we also identified host defense proteins as among the most rapidly evolving proteins known among primates, among them the anti-viral eosinophil secretory ribonucleases.[11,12] Although the constraints to which these proteins are responding may not always be evident, it is clear that one cannot always assume that responses of mice to human pathogens which are never experienced in nature have any relevance to physiology, not just on general principle, but based on the large degree of sequence divergence observed among the proteins designed to respond acutely to pathogen invasion.

Interpretational Issues

One often needs to go to great lengths to establish an infection with a human pathogen in a rodent host. This is certainly the case with RSV. While the RSV pre-sensitization studies in mice have been enormously successful in improving our understanding of adaptive immunity and responses to vaccine components,[13-15] this model does not translate effectively into the study of acute responses to virus infection. First, the aforementioned abortive infection is seen only in response to intranasal inoculation of a single mouse with 10 million or more plaque forming units (pfu). Second, from this inoculum, only ~1-10 thousand pfu per gram lung tissue (on the order of 10-100 pfu per set of mouse lungs) are recovered on days thereafter, with no significant clinical readouts (morbidity and/or mortality) to consider. Given these limitations, it is difficult to assess the extent to which a true infection has actually taken hold (i.e, significant replication of a pathogen in or around human tissues). Furthermore, the necessity of using huge inocula makes it impossible to discern whether the inflammatory responses measured are to an infection (however limited) or to a large bolus of foreign antigens that just happens to be viral in origin. This is not a trivial point, as the first scenario may have some relevance to physiology, but the second clearly does not. While it is certainly possible to measure chemokines produced and granulocytes recruited in response to foreign antigens, this is not at all the same as measuring responses to virus infection.

For these reasons, we have introduced pneumonia virus of mice (PVM) as a superior model system for the study of acute inflammatory responses of mice. PVM is also a virus of the family *Paramyxoviridae*, subfamily *Pneumovirinae*, and is the closest phylogenetic relative of RSV.[16] We can establish an acute infection with significant virus replication observed in lung tissue in response to intranasal inoculation with <10 pfu per mouse. From this inoculum, we typically recover 10 to 100 million pfu per gram lung tissue, accompanied by dose-dependent morbidity (fur ruffling and labored breathing) and significant (>60%) mortality.[17-19] This model is also not perfect—no one laboratory rodent can replicate the genetic diversity and thus varied susceptibility of the human population at large, nor can a single strain of virus mimic the variety and mutability of the strains of community-acquired infection, both factors which

Table 1. Comparison of disease models. Several characteristics of severe human RSV infection are compared to mouse models utilizing RSV and the natural rodent pathogen, PVM.

	Severe RSV Infection/Humans	PVM Mouse Model	RSV Mouse Model
Inoculum of virus	Very low	<10 pfu	>50,000,000 pfu
Initial pulmonary response	Granulocytic bronchiolitis	Granulocytic bronchiolitis	Few granulocytes
Eosinophil recruiment	Yes	Yes (10-30% at peak)	No
Progression to respiratory failure	10%	50-100%	0%
Peak virus titers	10^6 pfu/ml nasal secretion	10^6 – 10^8 pfu/gram lung tissue	Lower than titer in inoculum

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contribute to the variety and subtleties of the clinical response to this infection. Here, we are using genetically pure, inbred strains of mice and a highly infectious laboratory strain of PVM (J3666), which together have provided us with a model replicating the signs and symptoms of the most severe form of human RSV infection—that requiring hospitalization and ventilatory support. We compare the clinical and pathologic responses in severe RSV infection to both mouse models in Table 1, and conclude that PVM has a more significant chance of providing physiologically relevant and meaningful understanding of the inflammatory responses to acute pneumovirus infection.

Granulocytic Inflammation and Production of the CC Chemokine CCL3 Are Prominent Responses to Infection with PVM

We have summarized our published findings on the proinflammatory mediators produced in response to PVM infection in Table 2 . Among the most prominent sequelae of infection is granulocyte recruitment (Fig. 1A), with eosinophils peaking at 10-30% at the earliest time points (typically on day 3 in response to an inoculum of ~10^2 pfu) and disappearing rapidly, leaving the infiltrate with virtually 100% neutrophils by day 5. At the same time, the CC chemokine CCL3 is absent prior to infection but is detected in lung tissue at peak concentrations of ~100-300 pg/ml/mg protein in response to PVM infection. Interestingly, we and others have shown that CCL3 is synthesized in vitro in epithelial cells infected with RSV,[20,21] and can also be detected in BAL fluid from RSV-infected infants on ventilatory assistance.[20,22] Based on earlier studies implicating CCL3 as a central regulator of anti-viral inflammation,[23,24] we evaluated the responses of mice deficient in either CCL3 (CCL3 -/-[23]) or its major receptor on neutrophils and eosinophils, CCR1 (CCR1 -/-[25]) to infection with PVM. In our first set of experiments, we observed near complete ablation of the inflammatory response in CCL3-deficient mice, with only 10-60 neutrophils/ml, and no eosinophils detected in BAL fluid at any point in time after inoculation (down from counts on the order of 10^5 and 10^4 /ml, respectively), which, interestingly, was accompanied by a 6-fold increase in recovery of infectious virus from lung tissue. Similarly, when compared to CCR1-sufficient (+/+) mice,

Table 2. Production of proinflammatory mediators in lung tissue in response to infection with PVM

Mediator	pfu	Day 0	Day 4	Change	Ref.
CCL11	300	0.46 +/-0.07	0.41 +/- 0.05	0	17
Interleukin-5	200	0	0	0	27
CCL5	300	0.61 +/- 0.12	0.65 +/- 0.14	0	17
CCL3	300	0	0.35 +/- 0.04	↑	17
			Day 7		
CCL3	10	0	0.56 +/- 0.23	↑	19
CCL2	10	0	1.56 +/- 0.46	↑	19
			Day 3		
iNOS[a]	10	nd	↑	↑	19

Mediators detected in homogenized lung tissue by quantitative ELISA or [a]semi-quantitative Western blotting. Change in expression is scored as "0" for no change or "↑" for increase in response to PVM infection.

CCR1 -/- mice responded to PVM infection with minimal inflammatory response, accompanied again by a 6 to 8 fold increased recovery of infectious virus, as well as accelerated mortality. Taken together, these results suggest that the CCL3 / CCR1-mediated acute inflammatory response protects mice by limiting virus replication, and thereby attenuating the lethal sequelae of PVM infection

Double-Edged Sword of Inflammation

Can we use our model of acute PVM infection to aid in our understanding of issues relating to inflammation and its double-edged sword? This concept of the "double-edged sword" is often misunderstood. As it applies to the situation at hand, it is meant to imply that there are not unique and separable "beneficial" and "detrimental" inflammatory responses—it is simply that inflammatory cells participating in innate host defense are somewhat primitive in terms of specificity, and are not as skilled at definitive pathogen targeting as those participating in acquired immunity, and that responses that were designed to serve the host can "overshoot" resulting in tissue damage and functional pathology. In this case, inflammation alone is clearly not mediating all major pathology, as CCL3 -/- and CCR1 -/- mice with ablated cellular inflammatory responses are not at all protected from the ultimate sequela of this infection (i.e., death). In fact, as we have shown clearly with both CCL3 -/- and CCR1 -/- mice, elimination of the cellular inflammatory response entirely leaves virus replication at least partially unchecked. Yet, at the same time, mortality persists in wild type mice even when virus replication is blunted by administration of ribavirin (Domachowske et al, manuscript in review). We are exploring ways in which this model can be used to understand this double-edged nature of the inflammatory response to pneumovirus infection, with rational design of immunomodulatory strategies as an ultimate goal.

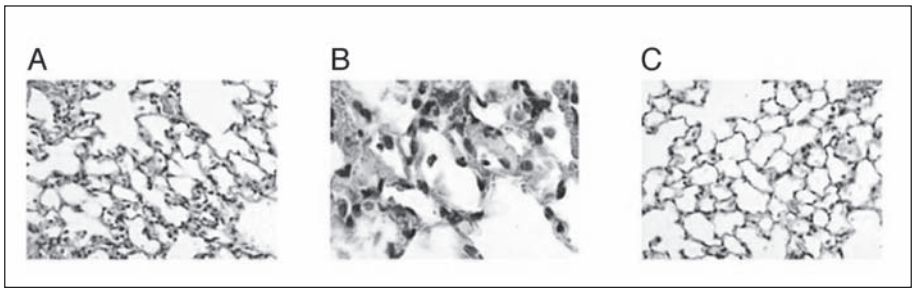

Figure 1. Microscopic pathology. Hematoxylin and eosin-stained lung sections from CCL3 +/+ (A, B) or CCL3 -/- (C) mice on day 5 post-inoculation with 60 pfu PVM. The prominent granulocytic infiltration observed in A and B is absent in C. Original magnifications, 10X (A, C) and 40X (B). Reprinted with permission from Domachowske and Rosenberg. Gene expression in epithelial cells in response to pneumovirus infection. Respiratory Research 2001; 2:225-233.

Systematic Evaluation of the Expression of Proinflammatory Response Genes

In order to provide a more comprehensive view of the differential expression of proinflammatory response genes, we harnessed the power of microarray technology via a comparison of responses of wild type mice to infection with two distinct strains of PVM—the pathogenic, continually mouse-passaged strain J3666, used in all of our previous studies described above and strain 15, a variant rendered non-pathogenic by repeated passage in tissue culture—to those of sham-infected controls.[26] In contrast to the severe symptomatology observed in response to infection with PVM strain J3666, infection with strain 15 resulted in few clinical symptoms, limited cellular inflammatory response, and no production of CCL3 or CCL2 despite ongoing virus replication. Microarray analysis of transcripts from lung tissue indicates that PVM J3666-infection promotes increased expression of specific pro-inflammatory gene transcripts, most notably interferon-β, interferon-response genes, and chemokines CCL2, CCL5, CCL7 and CCL11 (Table 3). Of these, only CCL5 expression also increased in response to infection with strain 15. These results suggest that pneumovirus replication alone is insufficient to promote antiviral inflammation, and that evaluation of the more divergent strain J3666 vs. strain 15-specific pneumovirus proteins may provide some intriguing leads toward defining the molecular basis of this differential response. Although the screen has demonstrated increased expression of mRNAs encoding both CCL5 and CCL11 in response to J3666 infection, we have shown previously (as noted above and in Table 2) that both proteins are present at relatively high concentration in lung tissue of uninfected mice with no significant change observed during the course of virus infection; this discordance between changes in mRNA and resulting protein expression, underscores the importance of protein-based immunologic and activity assays in the ultimate functional characterization of any of the responses detected via microarray analysis.

Conclusion

We have recently introduced PVM as a novel and physiologically relevant model for the study of inflammatory responses to acute pneumovirus infection in vivo in wild type and specific gene-deleted strains of mice. We have identified the chemokine CCL3 and its receptor CCR1 as central regulators of granulocyte recruitment observed in response to PVM infection, and found that deletion of either gene results in enhanced replication of virus in lung tissue.

Table 3. Specific mRNA levels in lung tissue observed in response to infection with pathogenic PVM strain J3666 and non-pathogenic PVM strain 15 as compared to uninfected controls

GeneBank #	Name	Fold Increase vs. Uninfected	
		J3666	15
Interferon and interferon-related mRNAs			
X56602	Interferon-induced 15 kDa protein	83	1[a]
M33266	CXCL10	29	3
U43085	Interferon-induced, GARG-39	25	1
U19118	Transcription factor, LRG-21	25	1
U43084	Interferon-induced, GARG-16	12	2
AJ007971	Interferon-induced GTPase, IIGP	7	1
U43086	Interferon-induced, GARG-49	7	1
K02236	Metallothionein	5	1
M31419	Interferon-activated gene 204	3	2
V00755	Interferon-β	3	1
U73037	Interferon regulatory factor, mirf7	3	1
**	Interferons-α (1, 4, 5, 6, 7, 8, 11)	1	1
K00083	Interferon-γ	1	1
Pro-inflammatory chemokine mRNAs			
M19681	ScyA2 (CCL2)[b]	30	1
AF065947	ScyA5 (CCL5)	15	13
X70058	ScyA7 (CCL7)	13	1
U77462	ScyA11 (CCL11)	3	1

[a]All values of 1 indicates no significant change when comparing expression to unifected mice. [b]Also identified as an interferon-regulated gene. **GeneBank accession numbers for interferons-α (1, 4, 5, 6, 7, 8, 11) in order: XO1974, XO1973, XO1971, XO1972, MI3710, D00460, M68944.[26]

Microarray analysis indicates that acute PVM infection is likewise accompanied by increased expression of mRNAs encoding interferon-beta and interferon response genes, as well as transcripts encoding proinflammatory chemokines CCL2, CCL5, CCL7 and CCL11. The contribution of these mediators to the cellular inflammatory response awaits formal immunologic and functional assessment.

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CHAPTER 7

Chemokines in HIV Infections

Anthony L. Cunningham and Katherine Kedzierska

Introduction

Viruses may interact with chemokines or chemokine receptors by mimicking key chemokines or their receptors or by producing molecules unrelated to either but able to bind to them, thus distorting their function. The chemokine and chemokine receptor mimics were probably acquired by transfer of genes from the host, as with oncogenes. The unrelated inhibitors which bind directly to chemokines and chemokine receptors are a feature of the poxvirus family. Overall these chemokine and chemokine receptor mimics and inhibitors have only been identified in the herpesviruses, poxvirus and rotavirus families, including HIV, human CMV, human herpesviruses 6, 7 and 8 and molluscum contagiosum[1,2,3] (Table 1).

The use of viral chemokine/receptor mimics and inhibitors identified in particular virus families such as herpesviruses, poxviruses and HIV indicates their importance in pathogensis of these viruses, either for evasion of the immune response as with poxviruses and herpesviruses or addtionally the use of recptors for viral entry as with HIV and poxviruses. Further studies will almost certainly identify a greater range of viruses producing such mimics.

Human Immunodeficiency Virus (HIV)

HIV mainly infects cells expressing the receptor CD4 and the coreceptors CCR5 or CXCR4 on their surface, mainly the CD4 subset of T lymphocytes in vivo, cells of myeloid lineage including monocytes, macrophages and dendritic cells (DCs). The co-receptors for HIV-1 entry, CCR5 and CXCR4 are G protein-coupled seven-transmembrane chemokine receptors, and are also important for the cellular tropism of HIV-1.[4] The β-chemokine receptor, CCR5, is the major co-receptor for macrophage (M)-tropic (R5) strains of HIV-1,[5,6] whereas the α-chemokine receptor, CXCR4, facilitates entry of T-tropic (X4) HIV-1 strains.[7] M-tropic or R5 strains of HIV-1 infect cells of myeloid lineage and primary CD4 T cells via CCR5 but not T cell lines in vitro.[8] R5 strains of HIV-1 do not usually induce syncytia (non-syncytium inducing NSI strains) and can be isolated at all stages of disease. In vivo the process of initial HIV transmission and initial infection usually selects CCR5 utilizing (R5) strains.[9] T-tropic strains of HIV-1 infect CD4 T cells and T cell lines in vitro, but by definition not in primary monocytes or macrophages.[8] However, recently productive infection of macrophages with primary isolates of HIV-1 utilizing CXCR4 (X4 strains) has been reported, demonstrating that the classification of X4 and SI strains may not always be congruent.[10-13] T-tropic or X4 strains of HIV-1 induce syncytia in T cells lines (SI phenotype) and are usually more cytopathic to T lymphocytes than M-tropic strains. They generally emerge several years after HIV-1 infection often at the peak of quasispecies diversity and at late stages of disease, predominate in approximately 40% of immuno-suppressed patients.[14,15] Some primary isolates of HIV-1 may be dual-tropic, exhibiting features of both M-tropic and T-tropic isolates and using either CXCR4 or CCR5 for infection of T cells (or macrophages).[16]

Chemokines in Viral Infections, edited by Surendran Mahalingam.

Table 1. Viral mimics of chemokines and chemokine receptors

Virus Family	Virus	Name (ORF)	Class of Chemokine or Receptor	Function
β Herpesviridae	Human CMV	US28	CCR3-CX_3CR	Chemokine sequestration, HIV entry. cell-cell fusion, constitutive NF-κB activation
		vCXC-1, UL146	CXC Chemokine	Neutrophil calcium flux; chemotaxis and degranulation (CXCR2-specific)
		vCXC-2, UL147	CXC Chemokine	NA
	Mouse CMV	M131/129	CC chemokine	Virulence factor; blocks NK and T cell response to MCMV in vivo; pro-inflammatory early in infection. Mutant virus→reduced viremia
	Rat CMV	R33	Putative CCR	Virulence factor (targeting and replication in salivary gland)
		R78	Putative CCR	Virulence factor
	HHV6	U12	CCR	Calcium flux in vitro
		U51	CCR	Downmodulation of CCL5 expression
		U83	CC chemokine	THP-1 cell chemotaxis
γHerpesviridae	Herpesvirus saimiri	ECRF3	ELR+ CXCR	Calcium flux in vitro
	HHV8 (KSHV)	ORF74	CC-CXCR	Pro-inflammatory
		KSHV GPCR	(constitutively active)	Angiogenic, oncogenic
		vMIP-I (K6)	CC chemokine	Angiogenic, CCR8 agonist
		vMIP-II, K6, K4	CC chemokine	Angiogenic, HIV-suppressive, eosinophil chemoattractant, antagonist at CCR1, CCR2,CCR3,CCR4, CCR5,CCR8,CXCR3, XCR1,CX_3CR1, CXCR4 and US28, inverse agonist at KSHV GPCR
		vMIP-III (K4)	CC chemokine	CCR4 agonist, angiogenic, T_H2 cell chemotaxis
	Mouse γHV68	M3	CC,C,CXC and CX_3C chemokine binding protein	Broad specificity chemokine scavenger

continued on next page

Table 1. Continued

Virus Family	Virus	Name (ORF)	Class of Chemokine or Receptor	Function
Poxviridae	Molluscum Contagiosum Virus	MC148R	CC chemokine	Blocks neutrophil, monocyte and T cell chemotaxis induced by multiple CC and CXC chemokines, antagonist at CCR2 and CCR8, blocks human hematopoietic progenitor cell proliferation
	Ortho and lepori-pox viruses	T1 (B29R [vaccinia]), 35 kD protein, vCCI vCKBP vCBP-1	CC chemokine-binding protein	Broad spectrum CC chemokine scavenger, anti-inflammatory in context of vaccinia infection and allergic airway inflammation in guinea pig
	Myxoma	M-T7, vCBP-2	CC,C and CXC chemokine-binding protein	Broad spectrum C, CXC and CC chemokine and IFN-γ scavenger, anti-inflammatory in context of myxoma infection
Lentiviridae	HIV	gp120	Chemokine mimic	HIV entry, leukocyte chemotaxis, apoptosis, calcium flux

ORF, open-reading frame; NA, not available; KS, Kaposi's sarcoma; KSHV, Kaposi's sarcoma-associated herpesvirus; GPCR, G protein-coupled receptor; CMV, cytomegalovirus; MCMV, mouse cytomegalovirus; MCK, murine cytomegalovirus chemokine; CCL5, CC chemokine ligand 5; IFN, interferon; IL, interleukin; NF-κB, nuclear factor κB; Tμ, T helper; NK, natural killer.
Modified with permission from Murphy P.M., Viral exploitation and subversion of the immune system through chemokine mimicry. Nature Immunology 2:115, 2001.

Expression of Chemokine Receptors on the Major Target Cells for HIV

The expression of chemokine co-receptors on T lymphocytes, monocytes, macrophages and DCs at various stages of their differentiation has been studied extensively in vitro and ex vivo.

T Lymphocytes

The major target for HIV infection is the CD4 lymphocyte subset in blood, lymph nodes and extra-nodal lymphoid tissue throughout the body. T lymphocytes express high levels of CD4, higher than those of monocytes and much higher than macrophages or dendritic cells, and high levels of CXCR4 on the plasma membrane. Resting T lymphocytes express very low levels of CCR5 which are rapidly upregulated after activation. In blood (and lymph nodes) there is a dynamic equilibrium between the turnover of plasma HIV, infected CD4 lymphocytes division of uninfected CD4 lymphocytes and de novo infection

of naïve CD4 lymphocyte. Naïve rather than memory T lymphocytes are preferentially targetted in the initial stages of HIV infection resulting in their depletion. The rapidly turning over HIV pool in blood, is provided by infection of activated T lymphocytes. Quiescent or latent infection of macrophages comprises a second group of cells turning over at a slower rate. Infection of resting T lymphocytes is associated with a very slow turnover with half lives exceeding four years.[16,17]

In lymph nodes and thymus, CD4 lymphocytes predominantly express CXCR4 (approximately 88% and 50% respectively) rather than CCR5 (10% and 2.5% respectively).[18] Despite the higher levels of CXCR4 relative to CCR5 on CD4 lymphocytes in blood and lymph nodes the R5 strains predominate in early infection, probably because of selection during transmission by infection of dendritic cells and/or macrophages, and remain dominant throughout the asymptomatic phase of infection.[13,19,20]

Both CCR5 and CXCR4 transduce intracellular signals and activate calcium fluxes and kinases in response to binding by chemokines or gp120 derived from either R5 or X4 primary isolates.[21,22]

Monocytes and Macrophages

The chemokine receptor CCR5 is the major coreceptor mediating HIV entry into adult monocytes and macrophages. Less commonly CXCR4 and very rarely CCR3 and CCR2b are used. Surface expression of CD4 and chemokine co-receptors on blood- and tissue-derived cells of this lineage differ significantly and may affect the efficiency of HIV-1 entry.[23-26] Threshold expression of both coreceptors are required for viral entry. Therefore if cells express abundant CCR5, even low densities of CD4 allow entry of R5 strains.[24] However, when CD4 levels are low (approximately 10^4 molecules), low CCR5 expression (1-2x10^4 molecules) inhibits HIV-1 infection.[27] Conversely, with high expression of CD4, minimal CCR5 levels (2x10^3 molecules) can support HIV-1 entry.

Monocytes

Only a very small proportion of blood monocytes (0.001 to 1%) are infected with HIV-1 at any time throughout the course of infection in vivo.[28] Both adult and neonatal monocytes express CD4. The expression of chemokine receptors on monocytes have been examined as mRNA levels, using semiquantitative RT-PCR, and as surface membrane expression by flow cytometry. The mRNA transcripts for CCR1, CCR2b, CCR5 and CXCR4 are present in freshly isolated blood monocytes, whereas CCR3 mRNA expression is undetectable. However, CCR5 surface expression is not detected on these cells.[25,26] CCR5 can be detected on monocytes from adult donors after 24 hours of adherence to plastic, but not on neonatal monocytes for 3-4 days.[29] CXCR4 and CCR2b are expressed at high levels as mRNA and membrane protein on freshly isolated monocytes. CCR3 receptor expression is not detected in or on adult monocytes.[25,30]

Macrophages

Monocyte-Derived Macrophages (MDMs)

Peripheral blood derived MDMs are susceptible to infection with clinical and laboratory adapted strains of HIV-1 in vitro. These cells can produce HIV-1 for weeks to months, without significant cytopathic effects. They are commonly used as a model to assess HIV-1 infection of tissue macrophages. The mRNA for CCR1, CCR2b, CCR5 and CXCR4, but not for CCR3, are easily detected in differentiated macrophages. Monocyte adherence to plastic and subsequent maturation into MDMs in vitro results in marked changes in the surface expression of CD4 and chemokine receptors.[25,31] Surface expression of CCR5 increases progressively during differentiation into MDM, peaks about 5 days after isolation, plateaus and then is followed by a decrease

over the next week of adherent culture. During differentiation of neonatal monocytes into macrophages membrane CCR5 appears more slowly. CXCR4 mRNA expression and membrane protein decline variably during differentiation of monocytes into MDM. CCR3 expression is not observed on the surface of adult MDM, as predicted by undetectable CCR3 mRNA.[25,31]

Laboratory-adapted R5 strains and primary isolates of HIV-1 differ in their co-receptor usage and the ability to infect monocytes and macrophages. Laboratory-adapted R5 strains of HIV-1 can infect freshly isolated monocytes (adult and neonatal sources) at earlier stages of maturation than primary isolates, as they can utilize lower levels of both CD4 and CCR5 than primary isolates. Neonatal monocytes are less permissive to infection with primary HIV-1 isolates than adult cells, and their susceptibility to infection correlates with maturation and increasing surface expression of CCR5.[27,29]

Genetic factors other than those affecting CCR5 may also influence the outcome of HIV-1 infection and replication in cells of macrophage lineage in vivo. The kinetics of HIV-1 replication in macrophages of identical twin pairs was very similar to each other more so than in those obtained from sex- and age-matched unrelated donors.[(32)] However, in these carefully matched studies, the level of surface expression of CCR5 did not exactly correlate with viral entry. HIV-1 entry and productive infection was restricted only at very low levels of CCR5, suggesting that the CCR5 co-receptor expression is one of many host factors determining the level of productive HIV-1 infection.

Tissue Macrophages

Tissue macrophages are major targets for HIV-1 (and SHIV) infection.[33] Resident tissue macrophages including alveolar macrophages, peritoneal macrophages, perivascular macrophages and microglia in brain are readily susceptible to HIV-1 infection in vitro.[23,34-36] The proportion of macrophages infected by HIV within tissues is relatively high, ranging from 1 to 50% depending on the site and stage of infection.[37,38] Infection of macrophages with R5 strains also results in transfer to adherent T cells within lymphoid tissue.

Microglia in brain express both CCR5 and CCR3. CCR5 is the most important receptor for HIV-1 entry into microglia, although several primary HIV-1 strains isolated from CNS appear to use CCR3 for entry. Microglia also express CXCR4 although T-cell tropic strains generally do not replicate in those cells.[39-41] Recently perivascular macrophages have also been identified as a target cell for HIV in brain. Similarly to microglia, alveolar macrophages express surface CCR5, CCR3 and CXCR4 receptors (and specific mRNA transcripts detected for all three receptors), with preferential infection being mediated via CCR5.[42,49] CCR5 or CXCR4 co-receptors are expressed at very low levels on the surface of placental macrophages, although transcripts for both CCR5 and CXCR4 are easily detectable. These low levels of CCR5 and CD4 surface expression on placental macrophages restricted HIV-1 infection with most primary isolates tested.[29,42] Placental and alveolar macrophage subsets have also been recently shown to express the C-Type Lectins, DC-SIGN, and mannose receptor respectively which may contribute to viral entry when CD4 and CCR5 are limiting.[44,45]

Dendritic Cells

Dendritic cells probably play major roles in HIV pathogenesis by transporting the virus from epithelia to lymph nodes and transferring it to T cells, resulting in explosive HIV replication.

Blood Dendritic Cells

Peripheral blood DCs are mostly immature DC precursors and comprise <1% of blood leucocytes.[51] Blood DCs express high levels of surface CCR5 and low CXCR4, and therefore are susceptible to infection with R5 not X4 strains.[47,48] In vitro maturation of DCs from blood

DCs is associated with an increase in the expression of both CCR5 and CXCR4 by 3- and 41-fold respectively.[47] Freshly isolated blood DCs do not express DC-SIGN or mannose receptor (MR), and show predominant binding of HIV gp120 to the CD4 receptor.[49,50]

Epithelial Dendritic Cells

Immature DCs in the epidermis (Langerhans cells, LCs) and dermis (dermal DCs) and their homologs in genital mucosa bind HIV mainly via interactions with C-type lectins (langerin, mannose receptor and DC-SIGN). Bound HIV is either endocytosed or transferred to CD4/CCR5 resulting in degradation or infection respectively. Immature LCs express moderate CCR5 and little CXCR4 probably partly explaining the selection of R5 HIV strains after sexual transmission.[45-50] Maturation of Langerhans cells results in upregulation of CXCR4 expression.

HIV-1 Entry via CCR5 and CXCR4 (Fig. 1)

HIV-1 tropism for T cells or macrophages and dendritic cells is defined by fusion and viral entry via CCR5 or CXCR4, and the determinants of cellular tropism are located in the env gene. The first step in the HIV-1 Env-mediated membrane fusion is the high affinity interaction between HIV-1 gp120 and CD4, which induces conformational changes in gp120 and subsequent interaction with CCR5 or CXCR4, which aggregate in the membrane within lipid rafts. The interaction between CD4 and gp120 exposes co-receptor binding sites located in the highly conserved V3 region of gp120 for CCR5, masked in unbound gp120 by the V1 and V2 loops.[51,52] The association of the gp120-CD4 complex with the extracellular amino-terminus of the co-receptor results in further conformational changes in HIV-1 gp41 fusion peptide. Initially, gp41 changes its configuration to the intermediate stage known as the pre-hairpin stage. This is followed by folding of gp41 into the hairpin structure and formation of a coiled-coil helix, which brings viral and cell membranes into close proximity, facilitating fusion and subsequent viral entry.[53] Interaction of gp120 with CXCR4 involves the V1 and V2 loops as well as V3.[54]

Which Are the Important Chemokine Receptors Used by HIV in Vivo?

More than a dozen chemokine receptors can be used as co-receptors by HIV strains in vitro.[4,54] The capacity to use multiple co-receptors increases during progression of disease. However, in the systemic circulation and lymphoid tissue in vivo, only CCR5 and CXCR4 are unequivocally used.

CCR5 is the main co-receptor for R5-strains of HIV-1 as shown by human genetic polymorphisms. A 32 base pair deletion in the CCR5 gene, named CCR5-Δ32, encodes a non-functional truncated CCR5 receptor which is not expressed in the cell membrane.[55] Individuals homozygous for CCR5-Δ32 are highly resistant to HIV-1 infection in vivo, whereas heterozygosity for CCR5-Δ32 delays the progression to disease by an average of 2 years.[56] The mechanism of this protection associated in heterozygotes is still unclear.

Variants in the promoter region of CCR5 can also influence CCR5 expression and the rate of disease progression. An allele of CCR2, CCR2-64I, is also associated with slower disease progression. This effect is probably due to interaction with CXCR4 (or CCR5) as CCR2 is unimportant biologically in HIV infection. Genetic variation in the CCL5 gene also modulate progression. A variant in the 3' untranslated region of the CXCL12 gene, the ligand for CXCR4, is also associated with delayed disease progression. The mechanism is also unclear.[57,58]

Some X4-utilizing HIV-1 isolates obtained from patients at late-stage disease can also infect MDM. Rare X4-utilizing HIV-1 strains can infect T lymphocytes and also MDMs obtained from individual homozygotes for the 32ΔCCR5 mutation.[59] Blocking with AMD 3100 prevented HIV-1 infection of MDM, showing that this primary isolate used CXCR4 but not CCR5 for infection of MDMs and proving the functional role of CXCR4 on macrophages.

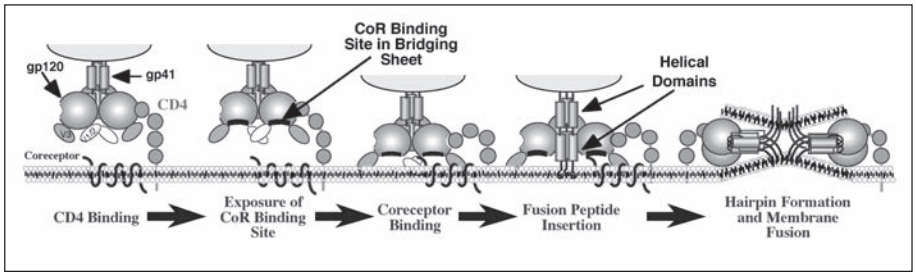

Figure 1. Scheme of HIV entry into target cells after binding of HIV envelope to CD4 and CCR5 or CXCR4 (coreceptor=CoR). Modified from Doms RW and Trono D. The plasma membrane as a combat zone in the HIV battlefield Genes Dev 14:2677-2688, 2000

Dual-Tropic Strains of HIV-1

Dual-tropic strains of HIV-1, such as HIV-1_{89}. and HIV-1_{DH12}, exhibit features of both M-tropic and T-tropic isolates and can use either CXCR4 or CCR5 for fusion with MDM.[11,16,60] Since the envelope of dual-tropic strains can bind to both CCR5 and CXCR4, those strains can infect both macrophages and T cell lines. CCR5-deficient MDM from individuals with CCR5Δ32 are susceptible to infection with HIV-$1_{89.6}$, as shown by blocking with CXCL12, and anti-CXCR4 antibody.[60] Since dual-tropic strains display characteristics of both M-tropic and T-tropic strains, they are thought to represent transitional isolates emerging during in vivo evolution from R5- to X4-utilizing isolates.[16]

Role of β-Chemokines in HIV Infection in Vitro and in Vivo

Three of the β-chemokines, CCL3, CCL4 and CCL5 are the natural ligands for CCR5.[61] CCL3, CCL4 and CCL5 are important inhibitors of R5 strains of HIV-1 in T lymphocyte cells of macrophage lineage including microglial cells.[61-64] Treatment of MDMs with β-chemokines inhibits CCR5 expression and HIV-1 entry.[62,63,65] However, the maturation state of monocyte/macrophages at the time of stimulation with β-chemokines may influence the outcome of HIV-1 replication in those cells. Exposure of MDM to the β-chemokines at the time of HIV-1 infection or after the infection significantly inhibits HIV-1 replication in MDMs. However, stimulation of freshly isolated monocytes with β-chemokines prior to HIV-1 infection renders them more susceptible to HIV-1 infection and increases HIV-1 replication in vitro.[63]

Inhibition of R5 strain replication in blood lymph nodes by β-chemokines may also contribute to the emergence and predominance of X4 strains in late disease.[66]

Blockade of Chemokine Receptors As a Strategy for Antiretroviral Therapy

Currently much effort is being devoted to designing β-chemokine analogues as inhibitors of R5 strain entry. For example the structural determinants of CCR5 recognition and HIV blockades in CCL5 have recently been reported and used to design a peptide analogue retaining HIV inhibitory properties. A modified CCL5 molecule (AOP-CCL5) has been shown to block HIV infection of macrophages, T cells and Langerhan's cells.[67-69]

Such strategies offer promise for a new generation of antivirals. The rarity of infection of CCR5 Δ32 homozygotes with X4 strains argues that concurrent administration of CXCR4 blockers will only occasionally be necessary. Further strategies of chemokine blockade in anti HIV therapy are discussed in Chapter 9.

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CHAPTER 8

A Role for Chemokine Activity in Alphavirus Pathogenesis:

Evidence from the Analysis of Polyarthritis and Myalgia Post Ross River Virus Infection

Brett A. Lidbury and Surendran Mahalingam

Introduction

Ross River virus (RRV) is an "Old World" alphavirus of the Semliki Forest group[1] and the etiological agent of the most common arthropod-borne viral disease in Australia. RRV has a positive strand RNA genome comprising 11,851 nucleotides in a single strand organized into regions encoding nonstructural proteins (nsP 1-4) and four structural genes (capsid, E3, E2, 6K, E1). The genetic organization of RRV also includes a 5' terminal cap and 3' poly(A) tract[1] resulting in the viral RNA acting in the host cell cytoplasm as a messenger RNA molecule. RRV is transmitted by both fresh and salt water mosquitoes.

"An unusual epidemic" reported by Nimmo in 1928[2] was the first reliable insight into what we now know of as RRV-associated disease, but it was not until 1963 that the viral agent of such unusual epidemics was isolated by Doherty and colleagues near the Ross River in North Queensland.[3] Over the past decade between 4000 to 7000 people per year have been identified as afflicted with RRV disease and there are concerns that the combination of changes to weather patterns and the encroachment of human populations in to new environments proximal to mosquito-breeding grounds may lead to the number of disease cases escalating to levels beyond those currently observed.[4-6] Human disease comprises a range of sequelae including myalgia, lethargy and rash, with polyarthritis very prominent amongst symptoms. Due to the viral nature of the disease, arthritis resulting from RRV infection is more widely recognized as "epidemic polyarthritis" (EPA).[7]

In this chapter we will investigate the putative role for chemokines in RRV disease, and will focus particularly on the polyarthritis often associated with RRV infection. Here we will address the precise role and activity of chemokines in EPA. This chapter will also draw on studies from other infectious and noninfectious arthritic syndromes in an attempt to understand the possible chemokine basis of RRV pathogenesis and EPA.

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Ross River Virus and Its Associated Disease: Human Studies and Animal Infection Models for Evidence of a Chemokine Connection

Early RRV studies showed that a number of cell types and tissues in mice, including muscle, brown fat and brain,[8,9] could be infected and support virus growth. Common to other alphaviruses, RRV showed similar infectivity for BHK-21 and Vero cell lines,[10] while more recent in vitro studies have demonstrated the ability of RRV to infect and replicate in a range of human and murine monocytes and macrophages, with infection not achieved in T-cell and B-cell lines.[11] In addition to the capacity of RRV to replicate in monocytic cells, the association of RRV with monocytes and macrophages will be shown to be a consistent feature of cellular responses to infection in both previous human studies of EPA and animal models of RRV disease, suggesting immediately a chemokine involvement post infection. By establishing this cellular framework from past RRV studies, we will have an ideal basis from which to investigate the associations between RRV infection, EPA and the putative role of chemokines.

Evidence for RRV-Induced Chemokine Activity: Human Disease Studies

Studies on humans with RRV disease (Table 1) have shown in both the virus-associated exanthum and in the synovium of EPA patients a predominant mononuclear cell infiltrate,[7,12-14] with functional NK cells also detected in one case.[14] In spite of the persistent nature of arthritis/arthralgia in a significant number of individuals with RRV infection, the virus has traditionally been very difficult to detect in synovial samples from EPA sufferers, with the initial successes of RRV isolation restricted to serum samples collected during the first seven days of disease.[15,16] RRV antigen was also successfully detected by immunofluorescence in synovial monocytes and macrophages during the early phase of illness,[17] and in basal epidermal and eccrine duct epithelia three days after the onset of RRV exanthem.[13] A recent report by Soden and colleagues has used molecular techniques, with partial success, to detect RRV RNA in synovial tissue from a cohort of EPA patients more than one month after the onset of symptoms, providing the first evidence of persistent infection in the inflamed synovium of an EPA sufferer.[18] It is feasible, therefore, that the long-term presence of RRV, or RRV antigens, is required in the synovium for the chronic arthritis/arthralgia symptoms seen in some EPA patients, although as pointed out by Soden and colleagues,[18] despite the many technical challenges inherent in detecting RNA in primary cell samples, the association of persistent virus with arthritis remains inconclusive as RRV was not detected in the majority of EPA patients who exhibited synovial inflammation.

What has been consistently observed by human EPA studies is the predominance of a synovial monocyte/macrophage infiltrate, unlike other arthritic conditions which feature neutrophils.[18] In fact, some of the early studies on synovial samples from EPA patients noted that the "…synovial fluid was devoid of neutrophil leukocytes…."[12] and there was a "… paucity of neutrophils…",[17] suggesting a different mechanism of pathogenesis for such an infectious arthritis as compared to, for example, rheumatoid arthritis.[18] Table 1 summarizes data on several human studies which have found RRV associated with EPA; in addition to this, Table 1 also lists the cells associated with RRV infection and polyarthritis, highlighting the dominance of mononuclear infiltrates as the cellular basis of disease, and therefore, the likely involvement of RRV-induced chemokine production at the site of inflammation and/or disease.

Evidence for RRV-Induced Chemokine Activity: Disease Studies in Animal Models

The first described studies using laboratory animals to model RRV disease were reported in consecutive articles from 1973,[8,9] although it is possible that preliminary work reported in 1975[10] on adapting RRV field isolates to laboratory mice and cell lines preceded these early

Table 1. Summary of attempts at Ross River virus detection in clinical samples from patients suffering epidemic polyarthritis (EPA) and the nature of the associated cellular response to infection

Study (Ref. #)	Year of Article	RRV Detected? (Time elapsed from onset of symptoms to detection of virus)	Predominant Cell in Infiltrate or Cell Infected (sample examined)	Other Laboratory, Clinical, Epidemiology Findings
Clarris et al (12)	1975	No (2 days)	Monocyte/ macrophage (synovial fluid)	Serum Abs low; monocytes/macrophages had enhanced phagocytic activity
Fraser et al (17)	1981	RRV Ag detected by IFA (1-7 days)	Monocyte/ macrophage (synovial fluid)	Neutrophils absent; macs vacuolated; RRV Ag in mono /macs
Tesh et al (15)	1981	RRV isolated from one EPA patient (weeks-months)	Not examined (serum)	Outbreak in American Samoa, 8/79-1/80; infection in 43.8% of samples; RRV detected in mosquitos
Aaskov et al (39)	1981	Yes, by i.c inoculation of suckling mice ("early infection")	Not examined; "small" effusions noted in ankles, knees etc…	Explosive RRV polyarthritis epidemic in Fiji, 4/79-6/79; up to 90% RRV antibody positive post epidemic for some communities
Rosen et al (40)	1981	RRV recovered from serum of 50/100 seropositive people; ID_{50} on C6/36 cells (1-7 days)	Not examined (serum)	Epidemic in the Cook Islands during early 1980; virus isolated from 6 pools of *Ae.polynesiensis*
Fraser et al (13)	1983	RRV Ag detected by IFA (3 days)	Ag in epidermal & eccrine duct epithelial cells; mainly T-cells + light mono. infiltrate (skin)	Rash with erythrocyte extravasation Also see Fraser & Becker, 1984 (Ref. 55)
Hazelton et al (14)	1985	No (synovial aspirate collected 23 days after the onset of symptoms)	Lymphocytes & mononuclear leukocytes (synovial exudate fluid)	Functional NK cells detected in synovial fluid; synovial NK activity similar to periphery

continued on next page

Table 1. Continued

Study (Ref. #)	Year of Article	RRV Detected? (Time elapsed from onset of symptoms to detection of virus)	Predominant Cell in Infiltrate or Cell Infected (sample examined)	Other Laboratory, Clinical, Epidemiology Findings
Aaskov et al (16)	1985	Yes, 2/4 patients by IFA (2 days)	Not examined (serum)	Virus isolates phenotypically diverse (plaque size variation on Vero cells)
Soden et al (18)	2000	Yes, 2/12 positive for RRV RNA by RT-PCR (5 weeks)	Monocyte / macrophage + lymphocytes (biopsy tissue from inflamed knee joints)	CD4+ were predominant T-cell; degenerate virus in joint tissue may not have been detected by RT-PCR

Abbreviations: RRV, Ross River virus; Ag, antigen; Ab, antibody; IFA, immunofluorescent antibody; RT-PCR, reverse transcriptase-polymerase chain reaction; NK, natural killer; EPA, epidemic polyarthritis; i.c, intra-cerebral.
A survey of the literature between 1990-1999 showed no attempts at recovering virus from human samples with clinical and epidemiological studies generally relying upon the detection of specific antibodies

pathogenesis studies. The studies on laboratory adaptation were performed with the relatively avirulent Nelson Bay strain (NB) and direct field isolates showed 86%-100% morbidity in day old outbred mice, but very low (5%-24%) mortality.[10] A feature of this infection model was that recovery in the surviving animals was complete by day 14 post infection, although it was noted that residual symptoms could persist for an additional 10 days, occasionally associated with muscle wasting. Mortality was increased significantly by serial mouse brain passage (in vivo) of virus stocks, whereas serial passage of RRV in cell lines (e.g., Vero cells) reduced mouse virulence. Another study by Taylor and Marshall showed that alternating passage through infant mice and mosquitoes did not result in a detectable change in virulence for field strains of RRV.[19]

The aforementioned seminal pathogenesis studies for RRV were described in two distinct parts, with a focus on RRV associated pathology in the brain of mice comprising the first section, and the pathogenic effects of infection on muscle, periosteum/perichondrium and brown fat comprising the focus of the second report.[8,9] The brain infection studies[8] reported that, in spite of persistent cortical and general neurologic lesions, mice survived and recovered from the symptoms of RRV disease. By day 13 post infection, by which time mice were recovering from symptoms, a generalized monocyte infiltration was noted in association with "cortical thinning", however this and other observations in the brain tissue suggested that such lesions were not responsible for the paralysis observed at between days 7-10 post RRV infection. Others have, therefore, posited that paralysis was more likely a result of severe necrosis in the muscle.[8,9] Specific studies on muscle from RRV-infected mice[9] showed that virus could be detected in muscle sections at day 3 post-infection by immunofluorescent antibody techniques, and RRV was observed by electron microscopy budding from myocytes at day 4 post-infection. By day 7 post-infection, severe "necrotic" changes in the muscle were noted, with only a small number of inflammatory cells reported in association with this tissue damage. "Confluent necrosis" was observed at day 10 post infection and this corresponded with the most severe clinical signs. Once the peak clinical signs were reached, "....Regeneration and repair...." were noted in the muscle tissue, returning the muscle to a completely normal appearance by day 34 post-infection with corresponding recovery of the mice from their symptoms.[9] Perichondrium and periosteum were also examined[9] and it was found that by day three post infection foci of infection were "prominent", with only occasional necrotic cells noted; only at very high doses of the mouse-virulent strain T48 were there signs of severe necrosis in periosteal and perichondrial cells at day three post infection. These observations led Murphy and colleagues to conclude that, "....there was no involvement of joints in the mice that paralleled the striking involvement of joints in the human disease". This suggested at the time that no correlation existed between the mouse model and human disease; however, they also noted the late appearance of monocytes in the brown fat (at day 13 pi), suggesting that monocyte involvement may vary depending on the tissue and day three could have been too early to observe significant inflammatory changes in perichondrium/periosteum. In spite of the doubts raised by these studies over the relevance of a mouse model to study human RRV disease (particularly joint pathology), these reports[8,9] were the first to note the association of RRV-induced pathological changes with monocytic infiltrates, providing early support to a role for chemokines in RRV pathology.

The early observations reported for muscle pathology[9] and the associations with mononuclear infiltrates post RRV infection were confirmed by additional studies in mice by Seay and colleagues.[20] Growth was detected in the serum and muscle of 7 day old mice by day 1, with virus detectable in muscle up until day 9 post infection, but only until day 4 post infection in serum, as assessed via plaque assay. In week old mice, foci of muscle necrosis were observed in association with local mononuclear infiltrates at day 5 post infection, which by day 7 post infection saw a prominent mononuclear infiltrate and significant muscle necrosis. In further agreement with previous observations,[9] "confluent necrosis" and inflammation almost

totally replaced muscle tissue at day 11 post infection; furthermore, Seay and colleagues also noted the reduction in inflammatory infiltrate by day 15 post infection, which by day 65 post infection manifested as the complete recovery of muscle structure and function. These studies in week old mice were also performed in 4 week old mice and as no clinical signs of infection were detected, confirmed the age-related resistance to RRV disease that had been previously observed.[8,9] No RRV antigen was detectable in muscle by immunofluorescent antibody (IFA) techniques for 4 week old mice, whereas antigen was detected in muscle from week old mice between days 3-7 post infection, but not after day 7 post infection. It appears, therefore, that while detectable infection was required for muscle pathology and disease to occur, peak disease symptoms reported for young mice were dependent upon the formation and persistence of a RRV-induced mononuclear infiltrate, and that recovery from muscle necrosis could only occur with the gradual disappearance of monocytes from the muscle. As humoral and cell-mediated responses to RRV were similar for the two age groups, and immunosuppressive therapies had no impact on age-related resistance to disease, Seay and colleagues concluded that RRV-induced myositis was a result of "viral lysis" of muscle fibres, rather than immune-mediated pathology.

Subsequent studies by Seay and Wolinsky on murine brain pathology post RRV infection further emphasized the earlier interpretation of viral replication as crucial to pathology rather than immunopathogenesis.[21,22] Again, while RRV could be detected in brain tissue during early infection, the early infiltrate was noted to consist primarily of neutrophils and monocytes, with monocytes eventually being the only infiltrating cell population present by day 8 (whereby virus was not detectable) post infection. Interestingly, Seay and Wolinsky presented evidence at the EM level showing "debris-filled" macrophages and "....cytoplasmic processes.....were inserted into myelin sheaths….", yet again concluded that immune responses were not responsible for pathology, once more based on negative results from studies with cyclophosphamide.[21,22] In this context, cells of the monocyte/macrophage lineage, which are insensitive to the action of cyclophosphamide, were not considered to be a component of the immune response, although they did report that the monocyte infiltrate was reduced in cyclophosphamide-treated animals (which was most likely due to impaired T-cell function).

The Identity of RRV-Associated Cellular Infiltrates

A recent study by Lidbury and colleagues has specifically identified the phenotype of the infiltrating cells into the muscle of outbred mice post RRV infection,[23] definitively confirming previous observations.[9,20] Immunohistochemistry (IHC) techniques revealed that the muscle infiltrating cells found at the height of clinical RRV disease were positive for the F4/80 surface marker which is diagnostic for monocytes and macrophages (Fig. 1). Like earlier studies, immunosuppressive agents were also used to unravel the nature of the immunology associated with disease; both cyclophosphamide and sub-lethal γ-irradiation resulted in the abolition of antibody production and prolonged in vivo virus growth, but only marginal changes in morbidity and mortality for infected mice were observed. In contrast to cyclophosphamide and irradiation, treatment of mice prior to infection with the macrophage toxic agent silica resulted in the significant amelioration of RRV disease, in contrast to other studies which have shown silica treatment to enhance virus morbidity/mortality in mice.[24] Repeat studies with another macrophage toxic agent, carrageenan, lead to the complete protection of mice from RRV-associated morbidity and mortality. These data firmly establish the macrophage as the cellular mediator of RRV disease in the mouse model and shows clearly that while cyclophosphamide and γ-irradiation sensitive progenitor T and B cells had only very minor roles (if any), contrary to previous opinion, the disease in the animal model can be considered to be immune-mediated. In agreement with previous studies cited above, the study by Lidbury and colleagues[23] also found that virus concentration was either falling or not detected at the peak of clinical disease.

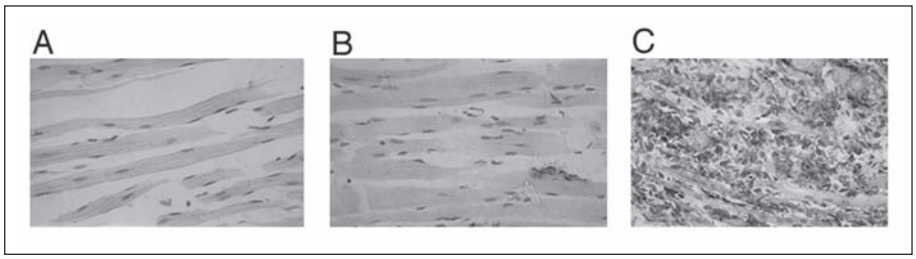

Figure 1. Infiltration of F4/80+ cells (monocyte/macrophage) into the muscle of Swiss outbred mice at day 8 post Ross River virus infection (which corresponded with the onset of clinical disease). Mice were subcutaneously inoculated at day 14-15 days of age with 10^3 plaque-forming units per mouse of the T48 strain of Ross River virus. A) Sham-inoculated; B) day 4 post Ross River virus infection; C) day 8 post Ross River virus infection (200x magnification). Reprinted with permission from: Lidbury BA et al. Macrophage-induced muscle pathology results in morbidity and mortality for Ross River virus-infected mice. J Infect Dis 2000; 181:27-34. © 2000 University of Chicago Press.

What is quite clear from the above-cited studies was that monocyte/macrophage infiltrates were a key correlate of muscle pathology, particularly as the kinetics of monocyte/macrophage infiltration mirrored the appearance of severe clinical disease and muscle necrosis in mice. Furthermore, a feature of recovery from RRV symptoms in mice was the clearance of monocytes /macrophages from the damaged muscle, which subsequently recovered its normal structure and function post disease. This poses fascinating questions for the role of chemokines in not only RRV-induced, macrophage-mediated muscle (and possibly joint) disease, but also for the function of chemokines in the recovery from RRV disease via the modulation of macrophage infiltration and activity at the site of pathology. A chemokine connection can be directly argued for disease, but are chemokine networks also necessary for the recovery and reconstitution of the damaged cells and tissues? With such issues in mind, it will be illustrative to examine clues from other arthritic conditions on the role for chemokines in disease and recovery.

The Role of Chemokines in the Pathogenesis of Rheumatoid Arthritis and Possible Connections with RRV and EPA

Research into the molecular immunopathology of rheumatoid arthritis has progressed solidly over the previous decade and with such a compelling basis for a role of chemoattractant proteins, has embraced the chemokine concept very rigorously, also impressively integrating knowledge of the cytokine network into these investigations.[25] A primary tool in this work has been the development of effective rodent models where arthritis can be either collagen- or adjuvant-induced,[26,27] as well as the availability of the MRL-*lpr* mouse strain which spontaneously develops a chronic arthritis with similarities to human rheumatoid arthritis.[28]

In reviewing these studies, it appears that this field has reached the consensus that chemokines are critical to arthritis pathogenesis. This does not suggest that chemokines are simply detectable during joint inflammation, but that there is also a sophisticated inter-relationship with cytokines and other chemokines which reveals the more subtle aspects of this inflammatory dysfunction.

Chemokines in Studies on Rheumatoid Arthritis

A survey of the literature over the last 10 years is summarized in Table 3. Early studies focussed on the chemoattractive cytokine CXCL8 and the associated neutrophil recruitment. An initial report by Endo and colleagues used radioimmunoassay (RIA) to measure CXCL8 in

the synovial fluids of patients suffering active rheumatoid arthritis (RA) and found increased levels of CXCL8 and an associated increase in infiltrating neutrophils correlating with disease.[29] Endo and colleagues confirmed their observation in humans by administering recombinant CXCL8 to the knee joint spaces of rabbits, noting again the infiltration of neutrophils into the joints post treatment. Another human study by Koch and colleagues found that synovial fluid samples from RA patients contained significantly more CXCL8 compared to identical samples from patients suffering osteoarthritis, as well as an increased CXCL8 concentration compared to "other arthritidies".[30]

Observations on the impact of CXCL8 on cellular recruitment into the joint and role in the corresponding disease were the precursor of future studies on chemokine involvement in RA, and as more chemokines became identified and characterized investigators in this field were quick to apply this knowledge to the aberrant inflammatory events which characterize arthritis.

The CC supergene family members CCL3 and CCL2 has been identified as the most prevalent chemokines detected using adjuvant- or collagen-induced arthritis models in rodents. In such a survey (Table 3) it was also instructive to see which cytokines were found by the various studies to promote the induction of these (and other) chemokines. One intensive study by Thornton and colleagues analysed the transcription of twenty-four cytokine and chemokine genes in a collagen-induced arthritis (CIA) model in mice.[27] These workers found IL-1β, IL-2 and IL-6 to be associated with the acute phase of disease, transforming growth factors beta 1-3 (TGF-β1, TGF-β2, TGF-β3) to be prominent for the chronic disease phase and IL-1Rα, IL-11, TNF-α and TNF-β to be significantly present throughout the acute and chronic phases. Another study by Szekanecz and colleagues has also considered the temporal nature of cytokine activity during adjuvant-induced arthritis in rats, concluding that TNF-α was more prevalent during early disease, whereas IL-6 was significant during the late disease,[26] disagreeing with the results of the study by Thorton and colleagues.[27] Furthermore, differences in the appearance of the chemokine CCL3 were also apparent when comparing these two studies.[26,27] Such observations may simply reflect differences between the mouse versus rat models, as well as differences in inducing arthritis by adjuvant or collagen. However, what is apparent from this work is that while chemokines have a role in arthritis, the cascade of events that leads to disease is complex and may be difficult to define absolutely. For EPA there are definite clues to be considered, particularly as the chemokine CCL2, which was noted above as prominent in arthritis studies, was most prominently expressed from cells of the monocyte/macrophage lineage.[31] With the massive infiltration of F4/80 positive cells into the muscle of RRV-infected mice (see Fig. 1), and the absence of neutrophils, it is clear that EPA is the result of chemokine/cytokine cascade which strongly favors the migration and activity of macrophages.

A Study of Chemokines Post RRV Infection

Compelling evidence has recently come to light from Mateo and colleagues for a role of chemokines in EPA, based on in vitro studies of RRV-infected primary human synovial fibroblasts and RAW 264.7 murine macrophages.[32] Infection of synovial fibroblasts was characterized by the pronounced upregulation of mRNA for CXCL8 and CCL2, as well as TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF). For RAW 264.7 macrophages, acute infection of naïve cells resulted in elevated mRNA for CXCL8 and CXCL2. This study also considered the response to acute RRV infection of RAW 264.7 cells which had both cleared and recovered from a primary RRV infection, and found that mRNA expression was enhanced for CXCL2, CXCL8, CXCL10 and CCL2. These results for acute infection of RAW 264.7 macrophages were found to be an interesting contrast with persistently infected RAW cells

which showed marginal increases in mRNA for CXCL2, CXCL8, CXCL10 and CCL28 and CXCL10, but no increase in CCL3 and no detectable mRNA for CCL2 and CXCL2. The contrast in chemokine gene activation between acute and chronically RRV-infected macrophages supports the general observation of EPA being episodic in some patients, with possible maintenance of persistent RRV in joint cells through periods of no disease as part of a cycle where persistent virus subsequently reinfects naïve macrophages, thus repeating the cycle of local chemokine-cytokine activity and overt disease.

The study by Mateo and colleagues[32] clearly showed for both synovial fibroblasts and macrophages that RRV can activate chemokine genes during early infection. While definitive in vivo evidence is limiting, the in vitro observations give some insight into the mechanism underpinning the previously observed infiltration of monocytes/macrophages into the muscle of RRV-infected outbred mice during clinical disease (Fig. 1).[23] Preliminary findings in our laboratory have identified several chemokines that are expressed at high levels in muscle tissues obtained from RRV-infected mice. Chemokines such as CXCL10, CCL2 and CCL5 were upregulated to high levels on days 5, 8 and 11 postinfection whereas CXCL9 and CCL3 expressions were extremely weak on all the days tested (Mahalingam and Lidbury, unpublished results). As discussed earlier, an interesting aspect to the early mouse studies with RRV were the observations that in spite of the massive mononuclear infiltrate and associated muscle damage, the infiltrate eventually cleared, muscle recovered its structural integrity and the mice made a full recovery from clinical disease. The investigations of chemokine activity modulation during recovery will also be of immense importance to our understanding of not only EPA pathogenesis, but possibly myalgia/arthritis pathogenesis generally; this model may identify chemokine/cytokine profiles associated with both the removal of inflammatory infiltrates from the muscle/joint and the restoration of local cells and tissues to predisease integrity and function.

Finally, this in vitro RRV-chemokine study[32] demonstrated a possibility of a considerable overlap with the chemokine basis of other arthritic diseases with CXCL2, CXCL8, CCL2 and CCL3 chemokines also being regularly observed in human and animal studies of rheumatoid arthritis (Table 3). A significant difference, however, is that generally TNF protein has not been detected in RRV studies[11,32] while it appears to be a feature of other arthritidies (Table 3), leaving open the question of the nature of the chemokine/cytokine collaboration in EPA pathogenesis. What is certain is that available knowledge on noninfectious arthritidies will be key to eventual understanding of arthritis resulting from RRV infection.

Caprine Arthritis Encephalitis Virus (CAEV) Offers Compelling Evidence for the Chemokine-Cytokine Basis of Infectious Arthritis

CAEV is a goat lentivirus which has been found to cause mononuclear infiltration and subsequent arthritis in the radiocarpel joints of infected animals. While CAEV infection of goats has obvious implications for agriculture, some researchers have suggested that there are also pathogenic parallels to rheumatoid arthritis in humans.[33] While this may be true, the viral nature of the disease etiology makes this infection of enormous benefit to our understanding of EPA, in spite of the difference in virus families. Like RRV in humans, CAEV has been shown to recruit mononuclear cells to the joints of goats, which as pointed out earlier for EPA appears to mediate disease at this anatomical site. CAEV, also similarly to RRV, is tropic for and replicates in macrophages which act as a major cellular host of the virus in vivo.[34,35] Similar to that discussed above for rheumatoid arthritis (RA), CAEV studies have also investigated the activity of cytokines in arthritis post infection, although the consideration of the role of chemokines has not been as thorough as for RA (but definitely more thorough than that for EPA).

Like RA, disease post CAEV infection has been associated with a dysregulation of cytokine expression, and with the monocyte/macrophage appearing to be pivotal in disease formation

these cells have been directly considered in this regard post infection. Lechner and colleagues[34] have reported that infected macrophages had downregulated TGF-β1 mRNA levels, whereas the constitutive expression of the chemokines CXCL8 and CCL2 was increased for CAEV-infected macrophages compared to noninfected controls, emphasising the ability of the virus to directly mediate chemokine activation. Furthermore, CAEV replication was found to influence the ability of macrophages to respond to stimulating agents such as LPS, with the expression of TNF, IL-1β, IL-6 and IL-12 p40 reduced for infected cells. Lechner and colleagues had also reported previously that while TNF expressing cells could be detected in the synovial membranes of arthritic joints, expression did not correlate with virus replication; this observation was confirmed in vitro by showing that infection did not prime goat macrophages for enhanced TNF expression post LPS treatment.[33] This group also found via in situ hybridization experiments that CCL2 expressing cells were more abundant than cells expressing IL-2 and IFN-γ;[36] the detection of CXCL8 and CCL2 agrees with evidence cited earlier for RRV infected macrophages.[32]

The above discussion of the correlation of disease phase with cytokine and chemokine activity for CAEV arthritis highlights some key features of relevance to EPA. From studies performed on RRV infection of macrophages in vitro, we know that RRV has the capacity to perturb the transcription and translation of the antiviral proteins TNF and iNOS,[11,38] while persistently infected macrophages showed increased expression of CXCL8 and CCL2.[32] RRV studies have not thoroughly considered the chronic stages of EPA and the profile of cytokines and chemokines during this phase, but the studies performed in the natural host of CAEV have suggested that immunopathological events are profoundly different during chronic arthritis compared to mild arthritis or asymptomatic infection.

Conclusion

RRV disease in humans, for example EPA, has been long associated with monocyte / macrophage infiltrates into the joint and other anatomical sites of pathology (Table 1), with strong supporting evidence from animal models which show associations between RRV infection, clinical disease and macrophage infiltration (Table 2). Recent in vitro evidence [32] has shown that chemokine genes (e.g., CXCL8 and CCL2) are induced in human synovial fibroblasts and macrophages post RRV infection, and hence may be considered a factor in EPA pathogenesis. While there is a current lack of other in vitro or in vivo studies to confirm chemokine induction post RRV infection, our confidence in this conclusion stems from the evidence of chemokine involvement, and associated cytokine activity, in the pathogenesis of rheumatoid arthritis (RA). RA, however, does not result from viral infection, making comparisons to EPA not wholly relevant. Inspiration for EPA insights can be found, however, in the animal infectious arthritis model of CAEV in goats. While the analysis of chemokines has not been as thorough for CAEV-arthritis as for RA, evidence from CAEV studies point strongly towards both an upregulation of chemokine expression and concomitant cytokine dysregulation during early infection.

Temporal studies have shown that particular cytokines and chemokines are associated with acute or chronic phases of RA, while RRV studies in animals have shown that after severe macrophage associated muscle necrosis post infection, tissue recovery could be observed with a return to normal function, which correlated with the disappearance of monocyte/macrophages from the site of pathology. Therefore, as well as elucidating the role for chemokines in the disease phase of EPA and other infectious arthritidies, future challenges will also center on the function of chemokines and collaborating cytokines in the recovery from arthritic/myalgic disease post infection.

Table 2. Review of experimental Ross River virus studies in animal models: Viral detection, pathology and identification of cellular infiltrates associated with infection and disease

Study (Ref #)	Year of Article (Species)	RRV Detected? (Method of detection)	Tissue Examined/ Pathology Produced	Nature of the Cell Infiltrate Associated with Infection/Pathology
Mims et al. (8)	1973 (mouse)	Yes (< day 1 pi by plaque assay; day 3 pi by IFA)	Brain tissue, serum, muscle/ neurologic lesions, cortical thinning, muscle necrosis	Monocyte infiltration associated with "cortical thinning" (day 13 pi)
Murphy et al (9)	1973 (mouse)	Yes (< day 1 pi by plaque assay; day 3 pi by IFA; day 4 pi by EM)	Muscle, brown fat, perichondrium, periosteum/ "confluent necrosis" of muscle by day 10 pi (fully recovered by day 34 pi)	Muscle only had a small number of "inflammatory cells" noted at day 7 pi; monocyte infiltrate into brown fat not observed until day 13 pi
Pearson et al (41)	1976 (sheep)	Yes, in lymph node at < 1.0 hour pi (plaque assay); detected to 36 hrs pi; clearance assoc. with ↑ antiviral Ab	Lymph node/no pathology noted	Increased "white blood cells"; particularly an increase in "large lymphocytes" noted post RRV infection in lymph node lavage
Seay et al (20)	1981 (1 week old mice)	Yes, in serum & muscle at day 1 pi (plaque assay); RRV persisted longer in muscle	Muscle/necrosis observed at day 5 pi; confluent muscle necrosis + inflammation by day 11 pi; recovery by day 65 pi	Local monocyte infiltrates noted at day 5 pi; prominent mononuclear infiltrate by day 7 pi + intrasarcoplasmic macrophages; inflammatory infiltrate decreasing by day 15pi
Seay & Wolinsky (21)	1982 (mouse)	Yes, 10 hours pi in the serum	Brain/demylination of brain tissue (recognized at day 8pi)	Monocytes & polymorphs observed in cerebellum & brainstem by 48 hours pi; at day 5 pi polymorphs decrease while monos/macs increase; macrophages present at sites of myelin loss and disruption

continued on next page

Table 2. Continued

Study (Ref #)	Year of Article (Species)	RRV Detected? (Method of detection)	Tissue Examined/ Pathology Produced	Nature of the Cell Infiltrate Associated with Infection/Pathology
Seay & Wolinsky (22)	1983 (mouse)	Yes, at day 2 & 3 pi (EM)	Brain/further studies on demylination post RRV infection. Cyclophosphamide did not reduce inflammation or damage in the CNS	+++ polymorphs & + macrophages at days 2-3 pi; polymorphs rare and macrophages numerous by day 5 pi; myelin debris noted in macrophages from days 5-14 pi
Lidbury et al (23)	2000 (mouse)	Yes, in serum, brain and muscle (day 1-6 pi; plaque assay)	Striated muscle of hind-leg/loss of muscle striations and structure; centralized muscle cell nuclei	Massive F4/80+ mononuclear cell infiltrate observed at day 8 pi (IHC on muscle sections) correlating with onset of clinical disease

Abbreviations: IFA, immunofluorescent antibody; pi, post infection; EM, electron microscopy; sc, subcutaneous; IHC, immunohistochemistry; CNS, central nervous system; RRV, Ross River virus

Table 3. Summary of chemokines detected in human and animal studies of rheumatoid arthritis and their association with cytokines which promote or inhibit their activity

Study or Review (Human/mouse) [Ref.#]	C-X-C Chemokines Detected??	C-C Chemokines Detected??	Chemokines Promoted by which Cytokines/Factors?	Chemokines Inhibited by which Cytokines/Factors?
Endo et al, 1991 Koch et al.,1991 (Human) [29,30]	CXCL8	Not examined	IL-1 LPS	Not examined
Kunkel et al, 1996 (Review) [25]	CXCL8 CXCL5	CCL2 CCL3	IL-1, IL-2, TNF IFN-γ, TGF-β	IL-10
Gong et al., 1997 (MRL-*lpr* mouse) [28]	Not examined	CCL2	Not examined	MCP-1(9-76)
Thorton et al, 1999 (CIA, mouse) [27]	Acute phase: CXCL2	None	IL-1β, IL-2, IL-6 (Acute)	
	Chronic phase: None Acute + Chronic: None	None CCL3, CCL5	TGF-β1-3 (Chronic) IL-1Ra, IL-11, TNF-α, TNF-β (Acute + Chronic)	Not suggested for any phase
Szenkenecz et al, 2000 (AIA, rats) [26]	Early: CXCL5 Late: None	CCL3 CCL2	TNF-α, IL-1β IL-6	Not suggested
Langdon et al, 2000 (Mouse) [42]	None	CCL2	OSM → IL-6 + metalloproteinase	Not suggested

Abbreviations: AIA, adjuvant-induced arthritis; CIA, collagen-induced arthritis; IL, interleukin; ENA, Epithelial-neutrophil activating protein; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IFN, interferon; TGF, transforming growth factor; IL-1Ra, interleukin-1 receptor antagonist; OSM, Oncostatin M.

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CHAPTER 9

Approaches to Viral Vaccine Development Involving Chemokine Receptors and Their Ligands, with Special Reference to Human Immunodeficiency Virus 1

Gordon Ada

Introduction

There are currently registered vaccines against 22 infectious agents pathogenic for humans and candidate vaccine preparations against 18 other infectious agents have undergone phase II clinical trials.[1] Some of these later preparations may become licensed for medical use within the next five years. The form of current viral or bacterial vaccines vary greatly, being either live, attenuated whole agent, inactivated whole agent, subunit preparations consisting usually of one or more surface antigens, bacterial toxoids, and polysaccharide preparations or more usually now, polysaccharide/protein conjugates. Most of these vaccines are against agents which show very little antigenic variation and mainly cause acute infections, i.e., a sublethal dose of the agent is cleared within a few weeks by the host's immune system.

There is now very well documented evidence that many of these vaccines, especially some of the childhood vaccines, are highly effective. Data from the Centers for Disease Control and Prevention in Atlanta show that, compared to the number of notified cases of disease during an epidemic prior to the availability of the specific vaccine, the number of cases in recent years (some years after the vaccine became available) has dropped by more than 99%.[1] Recently, the effect of a newly available vaccine on the reduction of cases of disease can be dramatic. The administration in 1999 of a new *N. meningiditis* type C vaccine in the UK reduced the incidence of disease by 92-95% in two different aged groups within one year.[2] These facts, together with the earlier global eradication of smallpox by vaccination, have encouraged the hope that it may be possible to control by vaccination at least some of the remaining infectious human pathogens.

There are still many infectious agents—viruses, bacteria and multicellular parasites which are major causes of morbidity and mortality in the world.[3] Many of these agents show considerable antigenic diversity which allows them to by-pass antibody responses following earlier infections or vaccinations. In one way or another, they can also evade or subvert cell-mediated immune responses so that the infection persists. For many years, diseases caused by agents such as malaria, tuberculosis and different viruses (including rotaviruses, hepatitis C and respiratory

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syncytial viruses) vied for the position as the world's leading cause of sickness and death. But very recently, HIV-1 has acquired that dubious honor and short of a new pandemic influenza virus outbreak in the next few years, is very likely to retain that position for some time to come. In some developed countries, HIV-1 infection can be avoided by safe sexual practices and/or kept under control by multidrug regimens. But in most developing countries, an effective vaccine offers the only hope of controlling in the future what has become an explosive pandemic.

Current Approaches to HIV-1 Vaccine Development

HIV-1 vaccine development has proceeded through several phases. Following the early, rapid elucidation of the structural properties of the virion and the sequencing of the viral RNA, there was optimism that an effective vaccine could be developed. Despite the growing evidence of the very great antigenic variation especially in the envelope protein, many pharmaceutical companies persisted with attempts to make the glycoprotein complex, gp160, or the main component, gp120, in one form or another, the basis of a subunit vaccine. An important event was the decision in 1995 of the Director of the National Institute of Allergy and Infectious Diseases, Washington, not to support the then leading candidate vaccine comprising monomeric rgp120, undergoing a phase III clinical trial. Many reasons were given but two important ones were: 1) That antibody produced by human volunteers immunized with this candidate vaccine did not neutralize the infectivity of freshly isolated field strains of virus; and 2) The preparation did not induce cytotoxic T cell (CTL) production in the volunteers. Although the statement is frequently made that the immune correlates of protection against HIV are unknown, most researchers by that time accepted that specific antibody was potentially the major mechanism for preventing infection, and that CD8+ CTLs were the major mechanism for controlling the infection for the first few years after the initial infection.

The finding that the envelope protein complex, gp160 composed of gp120 non-covalently bound to gp41, was present as a trimer on newly isolated field strains, raised the hope that immunizing with the polymer would induce antibodies which effectively neutralized the infectivity of newly-isolated field strains. To date, that has not turned out to be as promising as was hoped, and it would still suffer the disadvantage that the antibody formed, most likely, would be strain specific. There is a recent report that mucosal IgA isolated from an HIV and seronegative partner of an infected person neutralized the infectivity of an HIV field strain.[4]

The finding[5] that to be susceptible to infection by HIV, a target cell must bind to two different cell-surface receptors was a critical event. It was known that the CD4 molecule expressed especially on some T lymphocytes was a receptor for HIV. But when it was found that mouse cells transfected with DNA coding for human CD4 were still not infectible, a requirement for a second receptor was suspected. In 1995, R. C. Gallo and colleagues showed that the three β chemokines, CCL3, CCL4 and CCL5, suppressed the infection of otherwise susceptible cells.[5] Several groups rapidly confirmed[6-10] that different chemokine receptors could act as a second (fusion) co-receptor for HIV infection. This interpretation was supported by the findings[11-14] that infection did not occur if the target cells expressed certain mutant alleles of the chemokine receptors.

Opportunities Based on the Interaction of HIV-1 with Chemokine Cellular Coreceptors

CCR5 seems to be the main co-receptor for HIV on susceptible cells, especially macrophages. CD8+ CTLs are major producers of these infection-inhibiting factors as was first shown by Jay Levy and colleagues.[15] Cells of the innate immune system also secrete some chemokines, e.g., MDC by dendritic cells. In the absence of evidence for the presence of mutant alleles of CCR5, the high level of production of the chemokines by some individuals is associated with their natural resistance to infection by HIV-1.[16]

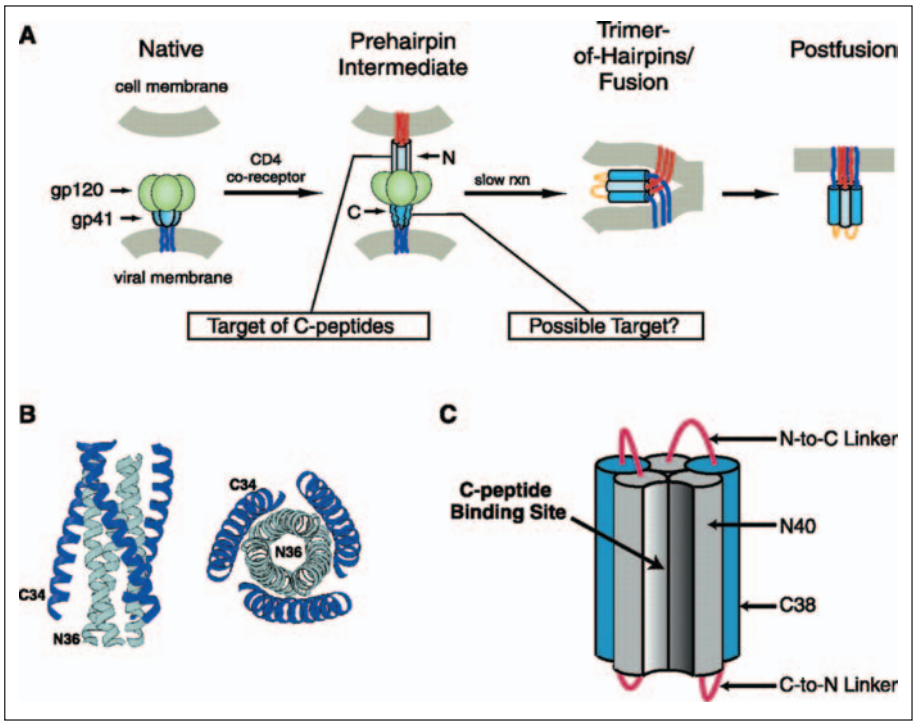

Figure 1. Targeting HIV-1 membrane fusion. (A) A schematic of HIV-1 membrane fusion depicting events that promote formation of the gp41 trimer-of-hairpins [adapted from ref. 1]. The NH2-terminal fusion peptide of gp41 (red), inaccessible in the native state, inserts into target cell membranes following gp120 interaction with CD4 and coreceptors. Formation of the prehairpin intermediate exposes the NH2-terminal coiled coil (gray), the target of C-peptide inhibition. This transient structure collapses into the trimer-of-hairpins state that brings the membranes into close apposition for fusion. (B) Lateral (left) and axial (right) views of a ribbon diagram representing the core of the gp41 trimer-of-hairpins. The ribbon diagram is derived from the crystal structure of a six-helix bundle formed by N36 (N-peptide, gray) and C34 (C-peptide, blue).[7] (C) A schematic model of the designed protein 5-Helix. Three N-peptide segments (N40, gray) and two C-peptide segments (C38, blue) are alternately linked (N-C-N-C-N) using short Gly/Ser peptide sequences (red loops).[21] The sequences of each segment in single-letter amino acid code are: N40, QLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILA; C38, HTTWMEWDREINNYTSLIHSLIEESQNQQEKNEQELLE; N-to-C linker, GGSGG; and C-to-N linker, GSSGG. Reprinted with permission from Root MJ, Kay MS and Kim PS. Protein design of an HIV-1 entry inhibitor. Science 2001; 291:884-888. Copyright (2001) American Association for the Advancement of Science.

A small percentage of frequently HIV-exposed individuals remain seronegative and apparently virus-negative. Examination has revealed that these individuals may demonstrate positive immune responses including

i. antibodies to cellular antigens including class I HLA and CD4;
ii. IgA-mediated, mucosally confined antibodies;
iii. HIV-specific systemic CMI responses including b-chemokine production;
iv. HIV-specific CTL-mediated cytotoxicity directed towards early-expressed HIV proteins; and
v. antibodies to CCR5 which down-modulate the surface expression of CCR5 in vivo (reviewed in ref. 17).

A decade ago, there was an (initially) surprising finding that monkeys immunized with SIV grown in human cells and then inactivated, were protected against a challenge with live SIV grown in human cells. However, they were not protected against a challenge with SIV grown in monkey cells. This initial success was due to the fact that when SIV budded from human cells, some cell-membrane-derived HLA antigen was included in the viral membrane and when used to immunize monkeys, this induced a strong anti-HLA response which neutralized the later challenge with human cell-grown virus. T. Lehner and colleagues[18] more recently reasoned that SIV grown in human CD4+T cells might also contain in the viral membrane, some cell membrane-derived human CCR5 transmembrane protein. This turned out to be the case. It was found that although there was only a slight difference in the composition of human and monkey CCR5, it was sufficient for antibodies against the human CCR5 to be formed by the immunized monkeys and these antibodies reacted with monkey CCR5. Such allo-immunization also results in a strong CMI response with a resulting high production of the three β chemokines. The net result is that monkey CCR5 is both down-regulated by the antibodies and access to the receptor blocked by the high level of β chemokines. Monkeys so immunized completely resisted a SIV challenge.

It was found initially with influenza virus and more recently with HIV-1 that when the virion attaches to the primary receptor on the target cell surface, a viral surface antigen 'shoots out a harpoon-like structure' that pierces the cell membrane. The 'extended' viral protein then 'collapses', thus bringing the viral and cell membranes close together and facilitating fusion, a pre-requisite to the virus entering the cell. In the case of HIV-1, a trimer-of-hairpins structure is formed that brings the amino- and carboxy-terminal regions of the gp41envelope glycoprotein ectoderm into close proximity.[19] This has made possible the synthesis of a new drug, a peptide called 5-helix, which binds to the carboxy terminal region and blocks the formation of the hairpins, so preventing fusion of the membranes. Binding of this small protein inhibits infection by a range of HIV-1 strains.[19] There is thus reason to expect that a vaccine could be developed by conjugating the 5-helix to an immunogenic protein carrier. The unknown is whether an IgG antibody would have sufficient access to the temporarily exposed region of gp41 to bind and remain bound.

The need to bind to two receptors, CD4 and CCR5/CXCR4, in order for infection of the host cell to occur, allows a still greater possibility of intervention. CCR5-dependent strains which preferentially infect macrophages and dendritic cells, predominate during the early clinical stages following infection (reviewed in ref. 20). On binding to both the CD4 and CCR5 receptors, the shape of the gp120 molecule is momentarily distorted, exposing, it is thought, a conserved sequence (Fig. 1). The complex of viral antigen and cellular receptors was 'fixed' with formalin and used to immunize mice. The antibody so formed was able to neutralize to different extents the infectivity of many different field isolates of HIV-1.[21] Currently, this appears to be the most promising of attempts to develop a vaccine with neutralizing- antibody activity against a range of freshly isolated HIV field strains.

Opportunities Based on Influencing Immune Responses by DNA Coding for Selected Chemokines

This is a field that has only recently become of widespread interest. In many respects, it has similarities to an earlier approach to influencing the type and extent of immune response following exposure to an infectious agent. In the case of a virus, e.g., vaccinia virus, acting as a vector of DNA coding for other viral antigens, DNA coding for one or more cytokines can also be inserted into the genome of the same virus particle. Some crucial findings using this approach are first reviewed. It should first be pointed out that there are at least two ways to set up such experiments. In the case of cytokine work, it is mostly done in the above fashion. This has

the advantage that the cytokine is produced precisely at the site of infection and it is therefore likely that the maximum effect will be shown. Alternatively, a preparation of DNA coding for the cytokine could be administered together with the virus being studied.

A Brief Summary of the Effects of Cytokines on Immune Responses to a Virus

Table 1 summarizes the effects of different cytokines on the immune response to a vaccinia virus infection in mice. These agents can be broadly classified as those favoring a T helper 2-type response (interleukins-4,-5,-6,-10 and –13) or a T helper type-1 response (interleukin-2, -12, tumor necrosis factor α and interferon γ). Their use in this way allows the immune response to the infection to be directed into giving a stronger humoral (systemic or mucosal) or a cell-mediated response. It is generally recognized that IL-4 and IL-12 are the two major cytokines which determine Th-2 and Th-1 responses respectively. Though no vaccine has yet been licensed which contains DNA coding for one or more cytokines inserted into the genome of the infectious agent, there are many reports of studies in experimental models (mice and monkeys) in the literature describing their effects during an infection. Clinical trials are about to start which involve constructs of this nature.

It should be born in mind that experiments of this nature can be a two-edged sword. Infection of mice genetically resistant to ectromelia virus (pathogenic for mice) with an ectromelia construct which expressed interleukin-4, resulted in high mortality rates. Even genetically-resistant mice which had been immunized to enhance their natural resistance, when infected with the interleukin-4-expressing ectromelia, showed a significant level of mortality.[22] NK cells and especially cytotoxic T lymphocytes (CTLs) are the major mechanism for controlling murine ectromelia infections. It was shown in these experiments that infecting mice with the IL-4-expressing ectromelia virus construct severely down-regulated CTL formation.

The Differential Effects of Some Inflammatory Chemokines on the Traffic of Different Cells of the Immune System, and Subsequently, the Types of Immune Responses Generated

Chemokines, like cytokines, are involved in the host response to infections, and are often divided into two groups—homing chemokines and inflammatory chemokines.[20, 23] The former group control the movement of cells within lymphoid tissue, and thus facilitate interaction between potential cellular partners. They are critical for the physiological development and homeostasis of the hematopoietic system. A deficiency of these agents can cause changes in the basic function of lymphoid tissues. For this reason, it is unlikely that vaccines against infectious agents would aim to disrupt or modify their basic function. In contrast, inflammatory chemokines affect the recruitment of different immune cells, varying from immature dendritic cells to memory T cells, to the sites of microbial invasion and inflammation. Vaccines against infectious agents might well aim at utilizing changes in the concentration and/or relative mounts of these chemokines. Certain possibilities can be proposed.

Dendritic Cells (DCs)

The role of Langerhan's cells, a form of DCs present in the epidermis, is to take up any foreign particles/antigens/peptides introduced by penetration of the skin. During their migration via afferent lymphatics to the draining lymph nodes, the cells, while maturing, process the foreign material and become highly effective antigen-presenting cells. They interact with immunocompetent T cells in the T-cell rich area of the node and so initiate an adaptive immune response. The roles of chemokine receptors and of Langerhan's cells to mature cells which can

process the antigen and interact with T cells in the nodes, is being actively investigated. Expression of the chemokine receptors, CCR-1, -3 and –5 increases during differentiation of monocytes to form immature CDs. This pattern is reversed as the DCs mature. CCR-7 expression is seen in mature DCs but not in monocytes or immature DCs.[24]

A study has been made of DCs generated and allowed to develop in vitro through the immature to the mature stage. When labeled with a dye and injected intradermally, the percentage of mature and immature cells which migrated to the local draining lymph node was about the same, but a low 1%. But the immature cells migrated more rapidly, indicating that after pre-priming DCs with antigen in vitro before injection, there seemed to be no benefit in inducing maturation with chemokines before injection.[25] In those cases where the antigen used is a T cell epitope, it may be beneficial to expose immature DCs to the epitopes in vitro and then to inject them intradermally. Whether in addition exposing the cells in vitro to ligands such as CCL-2, CCL3, CCL4 or CCL5, to improve the uptake of other antigens before injection, is not clear.

Preferential Induction of Humoral versus Cell-Mediated Immune Responses

Until quite recently, vaccines against infectious agents primarily aimed to induce a strong humoral response, especially antibody which would effectively neutralize the infectivity of the invading virus or bacteria. For this purpose, a T helper type 2 (Th-2) response was desirable. There are now many infectious agents which show such great antigenic variation in their surface antigens, HIV-1 being the prime example, that the alternative approach, that is, to induce a strong Th-1 and/or CTL response, would be advantageous. In this situation, a vaccine would not prevent infection, but within a few days after immunization, would aim to destroy virus-infected cells shortly after they became infected and before infectious progeny was produced.[26,27] CTLs are mainly responsible for clearing many acute primary viral infections. Th-1 responses may not only facilitate the generation of CTLs, but are important for the control of some intracellular bacterial infections, e.g., M. tuberculosis infections in mice.

Herpes simplex virus (HSV) infections are an important STD, and the mouse is often used as an experimental model, unlike the situation with HIV. Two recent reports have examined the influence of different chemokines on the type of CD4+T cell response in mice. In one,[28] DNA coding for HSV-2 gD protein together with plasmids encoding selected chemokines were injected i.m. Their immune modulatory effects were examined and the extent of any protective effects against an intra-vaginal HSV lethal challenge assessed. CXCL8 and CCL5 greatly enhanced the CD4+Th-1 type response and resulted in reduced morbidity and mortality following live virus challenge. T-cell subset deletions indicated that protection was due to CD4+T cells. In contrast, CCL2, CCL3 and CXCL10 increase mortality in the challenged mice.

In the second report, plasmid DNA encoding the gB protein of HSV together with the plasmid DNA encoding different chemokine ligands were administered three times i.n. to mice which were later challenged vaginally with HSV.[29] The use of CCL2 and CCL4 biased the responses to a Th-2 type, as judged by the pattern of immunoglobulin isotypes and levels of IL-4, whereas administering CXCL2 and CCL3 induced immune responses of the Th-1 type, as judged by enhanced IFNγ production. The latter rendered the mice more resistant to the HSV challenge, a finding consistent with other reports.

Other work has shown that XCL1[30] and CCL5,[31] when given i.n., can induce both mucosal and systemic immunity. Though both Th-1 and Th-2 pathways were induced by CCL5, type-1 responses dominated.

It seems too early to be completely sure that a particular chemokine ligand, when administered by any route with an antigen, will invariably favor a particular response. For example,

Table 1. Functions of cytokines and some effects in vivo

Cytokine	Major Known Functions
Type 1 factors	
IL-2	T cell growth factor; activates B cells, monocytes,NK cells;
TNFα	Anti-viral activity, activates CTLs;
IFNγ	Antiviral activity, up-regulates MHC expression on APCs;
IL-12	Promotes IFNγ production;
IL-18	Helps suppress IgE production
Type 2 factors	
IL-4	Enhances antibody responses, esp. IgE. Down-regulates Type 1 responses;
IL-5	Enhances antibody production, stimulates mucosal IgA Production.
IL-6	Involved in the maturation of B cells to ASCs;
IL-10	Promotes the production of IgG and IgM.
IL13.	Induces IgE production.
Other factors	
IL-1α	Activates T cells
IL-7	Early activator of T and B cells1

ASCs, antibody-secreting cells; CTLs, cytotoxic T lymphocytes; IFN, interferon; Ig, immunoglobulin; IL, interleukin; MHC, major histocompatibili ty complex; TNF, tumor necrosis factor; NK cells, natural killer cells.

two reports (from the same group) showed that CCL3 appeared not to favor a type-1 T cell response,[28] or directly to favor a Th type-2 response.[32] In contrast, two other reports clearly indicated the induction of a type-1 response by CCL3.[33, 34] It was also surprising that CXCL10 which induces IFNγ production and would be expected to favor a Th-1 response, caused increased mortality after the HV challenge.[28] CCL5 could also induce a mixed CD4+ T cell response, although a Th-1 response predominated.[31] It may be that a variety of factors, such as dose, form, and route of administration and the chemokine receptors involved on different cells, influences the outcome of this type of experiment.

Discussion

Though the study of chemokines is not exactly a new topic, the recent surge of interest was initiated by the finding[5] of R. C. Gallo and colleagues in 1995 that CD8+T cells secreted three soluble factors, identified as the chemokines CCL3, CCL4 and CCL5, which suppressed the infection of of susceptible T cells by HIV-1. Until that time, CD4 was the only known cellular receptor for HIV, though it had been suspected that there was a second co-receptor. By coincidence, 1995 was the year when it was finally accepted that antibody produced by volunteers immunized with the monomeric env protein produced antibodies which, though they neutralized HIV grown in CD4+ T cell lines, failed to neutralize newly isolated field strains of the same clade which preferentially infected macrophages. In addition, the very great antigenic variation of the env antigen had essentially overcome the many attempts to make a subunit vaccine similar to the hepatitis B vaccine. The 'holy grail'—to make a vaccine which induces broadly cross-reactive antibody so that field strains of different clades are neutralized—remains a major goal. There is still a possibility that this is achievable.[20]

In the meantime, there remains the possibility that vaccines to infectious agents, especially viruses, which show great variation in their surface antigens, might instead induce a strong type-1 response, generating especially strong CTL activity.[1] Theoretically, all proteins of an infectious agent may contain T cell epitopes and most internal proteins usually show less variation than a surface antigen. So the emphasis is now—what is the most effective way to induce the strongest immune response, antibody or cell-mediated? Can the addition of natural immune modulators—cytokines and chemokines—to a vaccine preparation generally or selectively facilitate reaching this goal? Activation of an adaptive response occurs in lymphoid tissues. So the first point is—can the uptake and transport of foreign antigen to the lymphoid tissue be facilitated? The major mechanism is by dendritic cells, especially Langerhan's cells. Though the uptake of antigen, especially individual T cell epitopes, can be improved by exposing immature DCs to antigen in vitro, the travel from the dermis to the draining lymphoid tissue via afferent lymphatics, during which antigen-processing and cell-maturation occurs, seems to work efficiently. However, the proportion of cells reaching the target tissue has been estimated at about 1%; the reason for this low figure is not yet clear.

The decision of the antigen-presenting cell, usually a dendritic cell, to induce a Th1type (predominantly cell-mediated) or a Th-2 type (predominantly humoral) response can be influenced by involving selected selected cytokines or chemokines in the antigenic mixture. Clearly, IL-4 on the one hand, and IL-12 or IFNγ on the other hand, can have very marked effects, especially if DNA coding for the interleukin is included in an infectious vector containing also DNA coding for foreign antigens. By this means, the interleukin is produced at the site of infection by the vector, ensuring a major effect on a subsequent immune response. Different chemokines have now been shown to have the potential to facilitate similar effects. But the distribution of different chemokine receptors does not follow a simple Th1/Th2 paradigm (discussed in ref. 35). For example, both Th1 and Th2 T cells may express the same chemokine receptors but at different levels. The next few years should see a considerable clarification of how the expression of different receptors controls the activity and traffic of the different subsets of effector and memory T cells. In the same way that insertion of DNA coding for IL-12 or IL-4 into a DNA viral vector now can greatly influence the type of immune response to the vector, it may be that one day, insertion of DNA coding for one or more particular chemokines may help to achieve a desired immune response to an antigen.

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APPENDIX

Abbreviations

Acquired immune deficiency syndrome	AIDS
Alpha interferon	IFN-α
Base pair	bp
Beta interferon	IFN-β
Bronchoalveolar lavage	BAL
Caprine Arthritis Encephalitis Virus	CAEV
CC chemokine receptor	CCR
CC ligand	CCL
Cell mediated immunity	CMI
Cowpox virus	CPV
CXC chemokine receptor	CXCR
CXC ligand	CXCL
Cytokine responsive gene 2	Crg-2
Cytotoxic T lymphocyte	CTL
Dendritic cell	DC
Epidemic polyarthritis	EPA
Formalin-inactivated RSV	FI-RSV
Fractalkine	FKN
Gamma interferon	IFN-γ
Glycosaminoglycans	GAGs
Heparin-binding domains	HBDs
Herpes simplex virus	HSV
Human cytomegalovirus	HCMV
Human herpesvirus	HHV
Human immunodeficiency virus	HIV
Human leukocyte antigen	HLA
Interferon	IFN
Interferon-inducible protein 10	IP-10
Interleukin	IL
Kaposi's sarcoma associated herpesvirus	KSHV
Lipopolysaccharides	LPS
Macrophage inflammatory protein	MIP

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Macrophage tropic	M-tropic
Molluscum contagiosum virus	MCV
Monocyte chemoattractant protein	MCP
Monocyte-derived macrophage	MDM
Monokine induced by interferon gamma	Mig
Murine cytomegalovirus	MCMV
Natural killer cells	NK cells
Open reading frame	ORF
Pneumonia virus of mice	PVM
Recombinant vaccinia virus	rVV
Regulated upon activation, normal T-cell expressed and secreted	RANTES
Respiratory syncytial virus	RSV
Ross river virus	RRV
Simian immunodeficiency virus	SIV
Stromal cell-derived factor 1 alpha	SDF-1α
Substance P	SP
T helper 1	Th1
T helper 2	Th2
Tumor necrosis factor	TNF
Vaccinia virus	VV
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Viral seven-transmembrane-domain chemokine receptor	vCKR
Virus encoded chemokine homolog	vCK
Virus encoded secreted chemokine binding protein	vCKBP

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