Mini-Reviews in Medicinal Chemistry, **2005**, *5*, 833-848

Virally Encoded Chemokine Binding Proteins

Louise M.C. Webb[1] and Antonio Alcami*[,1,2]

[1]*Department of Medicine and Division of Virology, Department of Pathology, University of Cambridge, Addenbrooke's Hospital, Cambridge CB2 2QQ, UK and* [2]*Department of Molecular and Cell Biology, Centro Nacional de Biotecnologia (CSIC), Campus Universidad Autonoma, 28049 Madrid, Spain.*

Abstract: Virus-encoded immune evasion mechanisms provide information on viral pathogenesis and offer a unique opportunity to identify new strategies of immune modulation. Secreted proteins that bind a broad range of chemokines have been identified in recent years in poxviruses and herpesviruses. We discuss the properties of these viral chemokine inhibitors and their potential as new therapeutics to treat human inflammatory diseases.

INTRODUCTION

The ability of viruses to manipulate our immune systems for their own survival and propagation has provided us with a unique opportunity to study key effector arms of the immune response from a different view point – that of the pathogen. Since viruses have co-evolved with our immune systems over a period spanning millions of years, they have studied the immune system in great detail and also shaped it. Indeed, some viruses have been able to establish life-long infections in the face of vigorous immune responses. Since the discovery of a poxvirus-encoded regulator of complement activation and then a soluble tumour necrosis factor receptor, a myriad of viral immunomodulators have been found [1-3]. Such proteins can be classified into those that are homologues of cellular genes and those that are not. The latter probably represent a paradigm for co-evolution. They often have novel structures, and they have the potential to show us new ways of manipulating immune responses that are not found in mammals. We can also learn a great deal from viral homologues of cellular genes. Although these are probably acquired from the host, they are often manipulated by the virus to increase efficiency and alter their biology.

The poxviruses and herpesviruses encode the largest number of immunomodulatory proteins. The large genome of these DNA viruses allows them to carry many genes which are devoted solely to immune manipulation. In contrast, small RNA viruses do not have this extra coding capacity and so many of their proteins have to be multifunctional. Most work in the area of viral immunomodulation has focused on poxvirus and herpesvirus families. Studies in this area have uncovered proteins that manipulate both the innate and acquired arms of the immune response. The complement system, CD8 and NK cell activity, antigen processing and presentation, immune cell apoptosis, and many cytokine networks have all been targeted by these viruses [1-3]. The fact that viruses from different families will target the same immune effectors underscores the importance of these molecules in host defence. A key example of this is the chemokine system. Chemokines have been attacked by poxviruses, herpesviruses, retroviruses and paramyxoviruses [4]. HIV utilizes chemokine receptors for entry into susceptible cells, HIV Tat protein encodes chemokine activity, and the respiratory syncitial virus glycoprotein gG has a domain with amino acid sequence similarity to CX3CL1 and induces cell migration by interacting with the chemokine receptor CX3CR1. Poxviruses and herpesviruses modulate the chemokine system through various mechanisms described below. It is likely that in the future, other virus families may be found that also manipulate this system.

Manipulation of the Chemokine System by Viruses

There are four ways in which viruses can interfere with chemokines as summarised in Fig. (**1**). They can encode (a) chemokine agonists, (b) chemokine antagonists, (c) chemokine receptor homologues, and (d) chemokine binding proteins [4-7]. Studies of all these different proteins have provided us with new insights into chemokine biology and shown us how the system can be manipulated to modulate immune cell trafficking, resulting in an altered immune response. Chemokine homologues can act as either agonists or antagonists by either binding the chemokine receptor and eliciting a signal, or by occupying the receptor and thereby preventing other chemokines from binding and transmitting their signal. They often have a broader range than the chemokine they mimic, and may also have altered receptor activation. By eliciting a signal through the chemokine receptor, an agonist can redirect the outcome of the immune response. An example of this is vMIP-II (encoded by human herpes virus-8 [HHV-8]), which triggers the arrest of eosinophils and Th2-like cells by engaging CCR3, whilst blocking the transmigration of monocytes or Th1-like cells to CCL5 by antagonising CCR1 and CCR5 [8]. In agreement with this, immunohistochemical analysis of HHV-8-associated Kaposis sarcoma lesions revealed a predominance of Th2-like cells over Th1-like cells [9]. Many viruses encode chemokine receptor homologues which are thought to subvert the immune system in a variety of ways [4,7,10]. Some virally-encoded chemokine receptor homologues are constitutively active and may induce proliferation or migration of the infected cell. They could also act as a sink, thereby reducing the local concentration of chemokines [7]. Virally encoded chemokine binding proteins (vCKBPs) also neutralise chemokine activity by binding the chemokine itself. This review will focus on these proteins.

*Address correspondence to this author at the Department of Molecular and Cell Biology, Centro Nacional de Biotecnologia (CSIC), Campus Universidad Autonoma, 28049 Madrid, Spain; Tel: +34 91 5854837; E-mail: aalcami@cnb.uam.es

1389-5575/05 $50.00+.00 © 2005 Bentham Science Publishers Ltd.

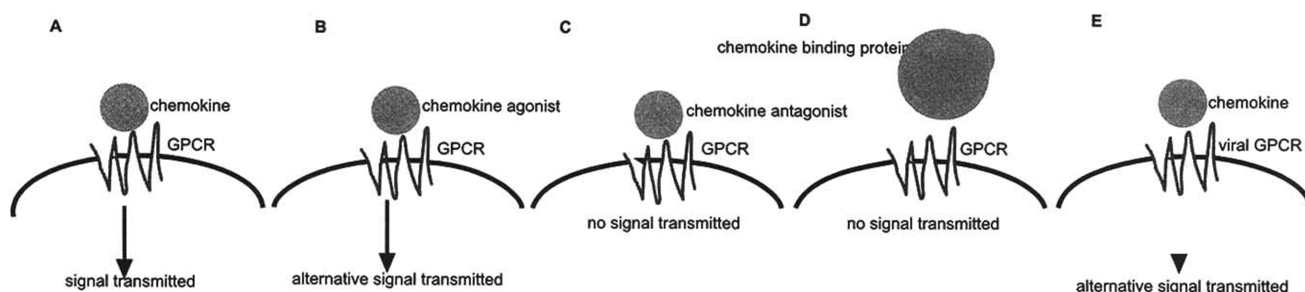

Fig. (1). Different strategies employed by viruses to alter chemokine responses. (A) activation of GPCR by a chemokine in the absence of virally encoded chemokine inhibitor, (B) chemokine agonist which will transmit a signal to a GPCR, (C) chemokine antagonist which binds to the GPCR and prevents its native ligand from binding and transmitting a signal, (D) chemokine binding protein which binds directly to the chemokine preventing its interaction with the GPCR, and (E) a virally-encoded GPCR that can transmit signals or sequester chemokines in the vicinity of the infected cell.

They differ from the chemokine and chemokine receptor homologues encoded by viruses, because they do not have any known cellular homologues and their evolutionary source remains obscure. By studying these proteins, we can uncover their secrets to design more effective chemokine inhibitors that supersede manmade efforts based on pharmaceutical small molecular weight antagonists, antagonistic chemokines and antibodies directed against chemokines or their receptors.

Classification of vCKBPs

vCKBPs can be divided into four subfamilies (vCKBP-1 to vCKBP-4) on the basis of their source and sequence homologies (see Table **1**). vCKBP-1 consists of two members, M-T7 from myxoma virus (MV), and S-T7 from Shope fibroma virus (SFV). The vCKBP-2 subfamily is composed of the 35kDa protein (also called vCCI) encoded by vaccinia virus (VV), cowpox virus (CPV), ectromelia virus (EV), camelpox virus (CaPV), rabbitpox virus (RPV), SFV (also called S-T1), racoonpox virus (RcPV), variola virus (VaV), and MV (also called M-T1). M3 is produced by murine gammaherpesvirus-68 (MHV-68) and is the only member of the vCKBP-3 subfamily. Glycoprotein G (gG) is the most recently published vCKBP (vCKBP-4) and is found in alphaherpesviruses including equine herpesvirus-1 (EHV-1), EHV-3, bovine herpesvirus-1 (BHV-1), BHV-5, rangiferine herpesvirus–1 (RanHV-1), caprice herpesvirus–1 (CapHV-1), and cervine herpesvirus-1 (CeHV-1). Bioinformatic studies showed that VV encodes another protein related to S-T1, A41L. Deletion of this gene from VV strain Western Reserve (VVWR) showed that it could

Table 1. Binding Specificity of vCKBPs

vCKBP family	Virus	vCKBP name	Binds to					Prevents binding to	References
			CXC	CC	C	CX3C	GAG	GPCR	
vCKBP-1	MV	M-T7	+	+	+				15
vCKBP-2	MV	M-T1	-	+	-	-	+	+	17, 62
	RPV	35kDa	-	+	-	-		+	16, 17
	CPV	P35, vCCI	-	+	-	-		+	16, 17, 18
	VV	35 kDa, B29R, vCCI	-	+	-	-	-	+	16, 17, 57, 62
	VaV	35 kDa, G5R	-	+	-	-			18
	EV	35 kDa	-	+	-	-			20
vCKBP-3	MHV-68	M3	+	+	+	+	-	+	21, 22, 67
vCKBP-4	EHV-1	gG	+	+	+	-		+	25
	EHV-3	gG	+	-	-	-			25
	BHV-1	gG	+	+	+	-		+	25
	BHV-5	gG	+	+	+	-		+	25
	RanHV-1	gG	+	+	+	-			25
	CaHV-1	gG	+	+	+	-			25
	CerHV-1	gG	+	+	+	-			25

reduce the infiltration of inflammatory cells into the infected site. However, no chemokine binding activity has been found for A41L, in either crosslinking assays using supernatants from virus infected cells, or BIAcore analysis using recombinant protein [11]. Thus, it appears that A41L inhibits leukocyte migration by a mechanism that does not involve it directly binding to chemokines and so cannot be classified as a vCKBP.

DISCOVERY OF vCKBPs

vCKBP-1 (M-T7, S-T7)

MV is a leporipoxvirus of rabbits that causes only benign lesions in its natural host, the South American tapeti rabbit, but it causes a lethal infection (known as myxomatosis) in European rabbits [12]. MV carries many immune evasion genes, including M-T7, which has significant sequence similarity to rabbit interferon (IFN) receptor and is able to bind and inhibit rabbit IFN [13]. The chemokine binding properties of M-T7 were first suspected when susceptible rabbits were infected with MV, which had been engineered to have no M-T7 [14]. The apparent block in inflammatory cell invasion into the dermal sites of virus replication was relieved in the absence of M-T7. Since IFN had not been shown to influence leukocyte migration, M-T7 was assessed for its ability to bind to other cytokines and shown to bind both IFN and CXCL8, but not IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, or IFN [15]. Further analysis revealed that M-T7 could bind to a broad range of chemokine from the CC, CXC, and C subfamilies including, CCL2, CCL5, CCL7, CXCL1, CXCL2, CXCL4, CXCL10, and XCL1 [15]. The IFN receptor from VV does not bind chemokines, in spite of the sequence similarity to M-T7 [16]. Interestingly, unfractionated proteins obtained from cells infected with a MV construct in which the M-T7 gene had been disrupted, still exhibited a chemokine binding species indicating that MV produced an additional chemokine binding activity – namely M-T1, a member of the vCKBP-2 subfamily.

vCKBP-2 (35kDa, M-T1, vCCl)

vCKBP-2 members also come from poxviruses. They were shown to bind to CC chemokines with high affinity by several different groups in the late 1990's. Graham *et al.* discovered it by screening supernatants from cells infected with a series of different poxviruses for CCL5 and CXCL8 binding [17]. They found that MV, SPV, RacPV, SPV, CPV, RPV and VV strain Lister (VVLis), but not VVWR, were able to bind both CCL5 and CXCL8. The VVWR and VVLis sequences are very similar and exhibit few genetic differences, enabling them to identify a 35kDa protein that was present in VVLis, but truncated to a 7.5kDa protein in VVWR. To examine the role of this protein during infection, two recombinant viruses were used to examine the role of this protein in chemokine binding. In the first, the M-T1 open reading frame (ORF) from MV (encoding a 35kDa protein) was inserted into VVWR, and in the second an RPV mutant in which the gene encoding the 35kDa protein had been deleted was used. Supernatants from cells infected with these different recombinant viruses were used to show that the 35kDa protein was responsible for CCL5 and CXCL8 binding.

Smith *et al.* expressed the CPV and VaV genes as recombinant proteins and used BIAcore analysis to show that an interactive partner was present in conditioned media from different cell lines [18]. The 35kDa protein was then shown to specifically bind to an 8-10kDa protein, which was identified as CCL2. A total of 29 proteins were screened for 35kDa binding. Virtually all 16 CC chemokines bound to 35kDa, whilst none of the 13 CXC chemokines or the XCL1 were able to bind. Included in their screening of CXC chemokines was CXCL8. CXCL8 could be crosslinked to the 35kDa protein as reported by Graham *et al.*[17].

The ability of the 35kDa protein to bind to chemokines was also reported by Alcami *et al.*[16]. They looked at VVLis and 11 other orthopoxviruses (representing 3 species of CPV, VV, and CaPV) and used crosslinking assays to show the presence of vCKBP that could bind CCL5, CCL3 (MIP-1) but was negative for CXCL8 and CXCL1. They used a VVLis mutant which lacked the 35kDa gene to show that this gene was responsible for chemokine binding. Expression of the 35kDa protein confirmed it as a vCKBP and showed that it bound CCL11, CCL7, CCL1, but was unable to bind to the CXC chemokines CXCL8, CXCL1, CXCL5, CXCL10, CXCL7, CXCXL12, CXCL3 or XCL1. Alcami *et al.* also showed that the 35kDa protein from VV inhibits the biological activity of CC chemokines, but not that of several CXC chemokines including CXCL8, suggesting that the binding affinity for CXCL8 is very low and biologically irrelevant. This was later confirmed by Lalani *et al*. [19]. Smith and Alcami also confirmed this and showed that the 35kDa protein was unable to bind to CX3CL, the only member of the CX3C subfamily of chemokines [20].

vCKBP-3 (M3)

M3 is a vCKBP produced by the which is a natural pathogen of wild murid rodents related to the primate gammaherpesviruses, herpes saimiri virus, HHV8 and Epstein-Barr virus [21].

The chemokine binding properties of M3 were first seen when MHV-68-infected cell supernatants were used in crosslinking assays with CXCL8, CCL5, CCL3, and CX3CL [22]. Complexes of chemokines with a soluble protein were detected with all chemokines tested, but not with the control supernatant from mock-infected cultures. Using a mutant virus where the M3 ORF had been inactivated by insertion of a LacZ expression cassette, it was shown that that chemokine binding activity was encoded by the M3 protein. Recombinant protein was then used to show that M3 is a broad spectrum vCKBP, able to bind chemokines from the CC, CXC, C, and CX3C chemokine subfamilies.

Van Berkel *et al.* also demonstrated that M3 was a vCKBP, using a crosslinking assay to show the presence of chemokine binding activity in supernatants of MHV-68-infected cells [23]. They suggested that M3 was primarily a CC chemokine binding protein, since although it was able to bind human CXCL8, it was unable to bind to many murine CXC chemokines. To date, M3 has been shown to bind to the majority of CC chemokines and many CXC chemokines – particularly human.

vCKBP-4 (gG)

Members of the alphaherpesvirus subfamily include pathogens such as herpes simplex virus 1 (HSV-1) and HSV-2, and varicella zoster virus (VZV), the causative agent of chickenpox and herpeszoster [24]. In domestic horses, the alphaherpesviruses equine herpes 1, 3, and 4 (EHV-1, EHV-3, and EHV-4) are important pathogens that cause immunosuppression, rhinitis, bronchiolitis, abortions and neurological disorders. Bovine herpesvirus 1 (BHV-1) is a major pathogen of cattle associated with abortions, respiratory and genital infections. It is related to alphaherpesviruses that cause disorders in ruminant (BHV-5, caprice herpesvirus (CaHV-1), cervine herpesvirus 1 (CeHV-1) and rangiferine herpesvirus (RanHV-1). Recently, Bryant *et al*. showed that the glycoprotein G (gG) from many of these viruses had chemokine binding activity [25]. Using supernatants from infected cells in crosslinking assays with chemokines, they showed that EHV-1, EHV-3, BHV-1, BHV-5, RanHV-1, CaHV-1 and CerHV-1 could bind a broad range of CC and CXC chemokines. Each virus had its own signature of chemokine binding specificities. This probably reflects their host cell and tissue tropism requirements to block different subsets of chemokines *in vivo*, or possibly the similarities between different species of chemokines. Expression of recombinant gG demonstrated that it encodes the chemokine binding activity. A unique feature of gG is that it is a membrane protein and is released into the medium after proteolytic cleavage. To date, no chemokine binding activity has been associated with HSV-1 or HSV-2. It is possible that the chemokine binding activity has been lost from HSV gG during evolution. HSV-1 gG is significantly shorter than that of other alphaherpesvirus gGs and is not secreted into the medium of infected cell cultures. Moreover, the gene encoding gG is not found in VZV or Marek's disease virus, which suggests that it is not an essential gene for all alphaherpesviruses.

ROLE OF vCKBPs IN VIRUS INFECTION AND PATHOGENESIS

If vCKBPs are to have any therapeutic value, they must be able to neutralise or alter chemokine activity *in vivo*. A first step in this evaluation is to establish the role of these proteins in the context of virus infection by constructing virus mutants in which the gene of interest has been deleted. Many studies have used wt virus as a control or comparison. However, the use of revertant viruses (where the active gene is restored to its wild type form) is the most appropriate control, since genetic manipulation of viruses may alter the expression/function of other non-target genes. Thus, caution should be used in the interpretation of studies where no such revertant viruses have been constructed and compared in the experiments.

vCKBP-1

As discussed earlier, it was only when the effect of deleting the M-T7 gene from MV on infection was evaluated that it became clear that M-T7 was able to influence leukocyte migration [14]. In the absence of M-T7, the virus was severely attenuated compared to parental virus. The majority of the animals infected with M-T7 deleted virus exhibited none of the most severe symptoms of myxomatosis that are associated with the wt virus, but instead recovered completely. The loss of M-T7 expression allowed the development of a more effective cellular immune response. Dramatic differences were seen in the size and progression of skin lesions, the onset and severity of Gram-negative bacterial infections and the ability of the animal to clear the viral infection. The primary site of infection revealed extensive necrosis of the skin down to the deeper dermis, with an intense cellular inflammatory reaction localised in the deeper dermis. In wt virus infection, the cellular infiltration and accumulation of large numbers of cells occupied about one-half of the depth of the deep dermal layer, suggesting a block to the free cellular migration into the primary site of viral replication. This block was relieved in M-T7-deficient virus. A common feature of myxomatosis is the rapid and efficient dissemination of virus *via* lymphatic channels from primary inoculation sites to secondary sites. The viral load at secondary sites following dissemination was significantly reduced in rabbits inoculated with M-T7 deficient virus. M-T7 deficient virus showed a dramatic level of lymph node cellular reactivity, characterised by edema of both the subcapsular and medullary sinusoids, and an increase in the number of cells within the sinusoidal areas and a reduced population in the central areas of the lymphoid germinal centers. It appears that M-T7 establishes a partial blockade on the effective migration of reactive inflammatory leukocytes into the infected sites, and substantially reduces the extent of communication between sentinel immune cells at the primary site, and resting lymphocytes in the secondary immune organs. IFN has yet to be shown to influence leukocyte migration, so these results are most likely due to inhibition of chemokine activity.

vCKBP-2

To examine the role of the 35kDa protein *in vivo*, rabbits were infected with either low or high doses of a mutant of a VV strain, RPV, that does not express the secreted 35kDa protein [17]. This revealed an alteration in the influx of extravasating leukocytes into virus-infected rabbit tissues. At the high viral dose, differences were seen only between days 3 and 7 postinfection. At the lower viral dose, such differences were less evident. While the deep dermal layer of lesions infected with wt virus still exhibited only a few scattered infiltrating cells at 3 days postinfection, the lesions infected with deleted virus were characterised by a significant leukocyte influx and an accompanying edema typical of an acute inflammatory reaction. The cells appeared to be predominantly neutrophils and about 30% lymphocytes and monocytes. This suggests that the expression of the 35kDa protein functions during the early stages of RPV infection to reduce the initial influx of extravasating leukocytes into the site of infection.

In a separate study, European rabbits were infected with either recombinant M-T1 deleted mutant MV or M-T1 revertant MV and assessed for differences in pathological profiles [26]. This work showed that M-T1 markedly influenced the chemotaxis of inflammatory cells, particularly macrophages into infected tissues sites during the initial phases of virus infection. However, the increased numbers of phagocytes seen following infection with M-T1 deficient

virus were relatively ineffective at clearing the virus suggesting that other viral proteins are able to neutralise the antiviral activities of the infiltrating macrophages. Rabbits infected with either virus developed the classic symptoms of myxomatosis, including the development of fulminating lesions at the primary site of inoculation, multiple secondary lesions, blepharaconjunctivitis and supervening bacterial infections. In the absence of M-T1, infected rabbits experienced more edema, conjunctivitis and a slightly accelerated development of bacterial infections. There was also augmented inflammation at the primary lesions from the M-T1 deficient virus-infected rabbits during the early stages of the disease. M-T1 modulates inhibition of infiltration significantly earlier that the inhibition of infiltration observed previously with M-T7, suggesting that these proteins are functionally nonredundant during MV infection. However, the lack of M-T1 had no major significant effects in attenuating the progression of disease or on the mortality rate of infected European rabbits [27].

More recently, the *in vivo* role of the 35kDa protein has been examined by constructing a recombinant of VVWR expressing the 35kDa protein from VVLis [28]. Expression of the 35kDa protein in VVWR led to a marked attenuation compared to wt and revertant virus following intranasal infection of mice. This attenuated phenotype correlated with a reduced cellular inflammatory response in the lungs of infected mice. The expression of the 35kDa protein blocked the ability of bronchiolar lavage (BAL) fluid to direct leukocyte chemotaxis *in vitro*, and *in vivo* fewer leukocytes were recovered from the lungs of infected mice compared to control viruses. Expression of the 35kDa protein by VVWR resulted in reduced mortality and weight loss, and decreased virus replication and spread. This could be attributed to the ability of the 35kDa protein to reduce cellular infiltration into the lungs. Excessive accumulation of inflammatory cells may be harmful and contribute to the clinical symptoms associated with virus-induced pneumonia, and may aid virus spread throughout the body. In agreement with this, intranasal infection of mice with RPV in which the 35kDa protein had been inactivated by insertion of LacZ resulted in an earlier onset and a more severe illness than observed with wt RPV. However, such differences were not seen following intradermal infection [27].

vCKBP-3

There have been two studies examining the role of M3 in MHV-68 infection. In the first, Bridgeman *et al.* used an M3-deficient virus, M3LacZ, in which a LacZ cassette was inserted into the M3 gene and compared it to a revertant virus [29]. No significant deficit in lytic phase replication was observed after intranasal infection of mice with either M3-deficient or revertant virus, and there was no evidence that lack of M3 led to an enhanced infiltration of NK, CD4, or CD8 cells after infection. Thus, M3 does not appear to play an essential role in protecting MHV-68 against the immune response during acute, lytic phase replication in the lung. Following epithelial infection, MHV-68 establishes a latent infection in lymphoid tissue, colonising germinal centers and driving antigen-non-specific B cell activation. This is associated with an amplification of virus, and marks the onset of an infectious mononucleosis-like illness that lasts several weeks. Although M3-deficient virus replicated normally in the lung, subsequent amplification of M3-deficient latent virus was grossly impaired compared to revertant virus. Expansion of the latently infected population in germinal centers was lacking in the absence of M3. CD8-depleted mice infected with wt MHV-68 showed a 35-fold increase in recoverable splenic virus relative to the undepleted infected controls. In contrast, animals infected with M3 deficient virus showed more than 2000-fold increase in virus reactivation. This study suggests that M3 chemokine blockade protects wt MHV-68 infected splenocytes against elimination by CD8+ T cells.

In a separate study, van Berkel *et al.* constructed an M3-deficient virus by inserting a stop codon and a frameshift mutation early in the ORF of the M3 gene [30]. They used this mutant to show that M3 plays a critical role in acute viral meningitis, but does not have a role in chronic vasculitis or in the establishment of, or reactivation from latency, by comparing it to a M3-rescue virus. Since MHV-68 is capable of infecting numerous cell types within the brain, they tested the role of M3 during intracerebral infection of mice. They found that at the peak of viral replication in the brain, there was approximately a tenfold decrease in viral titre in the brain that was specific to the M3 deficit. Virus was present at the sites of inflammation in similar patterns for both wt and M3-deficient virus. However, whereas wt infection was associated with a marked preponderance of neutrophils in the meninges, the proportion of lymphocytes and macrophages was increased in the meninges of mice infected with M3-deficient virus. The capacity of M3 to inhibit lymphocyte-dependent inflammation raises the possibility that M3 may alter inflammatory processes that are induced by chemokines, without compromising all chemokine-based host responses and may be useful clinically to combat diseases in which lymphocytes and macrophages cause tissue destruction. However, the brain is not the natural site of virus infection and so the role of M3 in viral pathogenesis remains unclear.

vCKBP-4

The role of gG in alphaherpesvirus infection and pathogenesis still remains obscure. It has been proposed to play a role in virus cell-to-cell attachment due to its localisation at cell junctions during *in vitro* infection, and the effect of deleting gG from different viruses [31-34]. When cells are infected with wt BHV-1 adherence between the cells and substratum becomes loose, this is not seen when cells are infected with gG deleted BHV-1 [31]. Thus, it has been proposed that gG acts as a glue-like substance to maintain cell-to-cell junctions. However, although insertions into the gG locus of pseudorabies virus (PRV) reduce cell-to-cell spread, a mutant with a nonsense mutation in the gG signal sequence had no spread defect, and it was proposed that this phenotype is due to indirect effects on an upstream gene [32]. In addition, PRV strains with mutations in the gG locus have no discernible phenotypes in most model systems. Since the role of gG as a vCKBP has only recently been established, its potential role in regulating cell trafficking during infection has not been analysed. Deletion of gG from BHV-1 causes viral attenuation in calves and increases immunogenicity [35]. For HSV-1, a gG mutant showed no

phenotype *in vitro* and only a marginal attenuation in the mouse ear model [33]. A further study has implicated HSV-1 gG in virus entry through apical surfaces of polarised epithelial cells [34].

MECHANISM OF vCKBPs ACTION

Close scrutiny of each vCKBP has revealed that they all do not all use the same mechanism to neutralise chemokine activity. Their mode of action is dependent upon the way in which they bind to chemokines – which sites they bind to and how this affects other regions important for chemokine activity (Fig. **2A**).

To understand how vCKBPs inhibit chemokines, we must first look at how chemokines work. Chemokines play a key role in directing the migration of leukocytes from the blood to sites of infection and to guide the migration of leukocytes through lymphoid compartments during leukocyte development, differentiation and response to infection [36]. In addition, non-migratory activities of

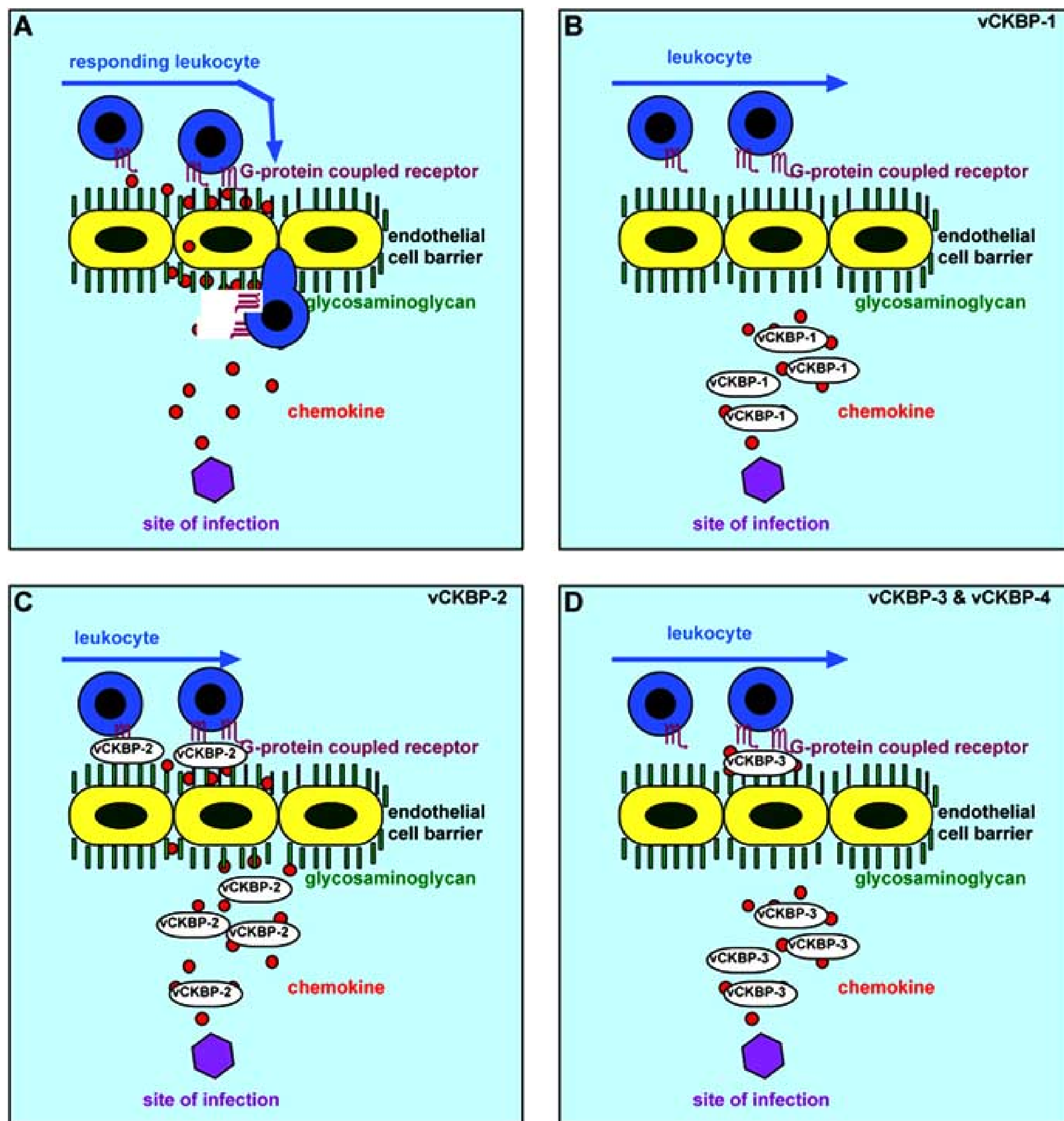

Fig. (2). Schematic diagram showing mode of action employed by different vCKBP subfamily members to disrupt chemokine-induced chemotaxis. (A) In the absence of vCKBP chemokines are produced at the site of infection and a chemokine gradient is established with chemokine presented on GAG molecules on the surface of endothelial cells. Leukoytes carrying the appropriate GPCR respond to the chemokine and transcytose through the endothelial cell barrier in order to migrate to the site of infection. (B) In the presence of vCKBP-1, chemokine is prevented from binding to GAG molecules and so a gradient is not established. (C) In the presence of vCKBP-2, the GPCR binding region of the chemokine is masked, thus preventing the chemokine from interacting with its receptor resulting in a block in GPCR induced chemotaxis. **(D**) In the presence of either vCKBP-3 or vCKBP-4, the chemokine is prevented from interacting with both GAG and GPCR so chemokine gradients do not form, chemokines do not interact with responding leukocytes, and any chemokine gradients that are already established are disrupted.

chemokines are beginning to be appreciated [37]. They can participate in leukocyte activation, cytokine production, proliferation and apoptosis. All of these activities are thought to be mediated though GPCRs to which specific chemokines will bind. In addition to GPCR-binding, the majority of chemokines also bind to glycosaminoglycans (GAGs).

The interaction of chemokines with GAGs is thought to be crucial for effective chemokine-mediated cell migration [38]. GAG-mediated tethering of chemokines permits chemokine retention on the luminal surface of endothelial cells surface under flow condition, and also allows the formation of chemokine gradients within the extracellular matrix [39-42] (See Fig. **2A**). GAG binding can also protect chemokines from proteolytic degradation and increase chemokine receptor sensitivity, probably by aggregating chemokines, and thereby increasing their local concentration [43]. GAGs can also affect chemokine receptor binding by interacting with receptor binding motifs within the chemokine [44,45]. However, this latter phenomenon is chemokine-specific and depends on the location of the GAG binding site within the chemokine, and whether it overlaps with the site for receptor binding. Until recently, the *in vivo* relevance of the chemokine-GAG interaction was obscure. However, Proudfoot *et al*. recently showed that mutant forms of CCL2, CCL4, and CCL5 that could no longer bind to GAGs, but could induce *in vitro* chemotaxis, were unable to induce recruitment of cells into the peritoneal cavity [38]. This data suggests that GAG binding is a prerequisite for *in vivo* chemotaxis.

So vCKBPs have potentially two activities of chemokines which they can target to neutralise their activity – namely their GPCR or GAG binding. By preventing chemokines from binding to GPCRs, vCKBPs will block the signal transmitted to the target cell. In addition, by blocking the chemokine-GAG interaction, they can prevent *in vivo* chemotaxis and the establishment of chemokine gradients.

Mapping studies have defined the key domains involved in GPCR and GAG binding by chemokines. All chemokines show the same overall tertiary structure [36] (Fig. **3A**). This consists of an elongated N-terminus that precedes the first cysteine. This N-terminus appears to be mobile and in most cases is unobservable in high-resolution structural studies. After the two cysteines, there is the N-loop which is about 10 residues long and is succeeded by one strand of a 3^{10} helix. The 3^{10} helix is succeeded by three strands and a C-terminal helix. Each secondary structural unit is connected by turns referred to as the 30s, 40s, and 50s loops (Fig. **3A**). The N-terminus and N-loop are critical for GPCR binding and activation. GAG binding can occur at different positions within the chemokine. It has been proposed that there are four different heparin binding modes that are created by the folding and oligomerisation of chemokines [46].

vCKBP-1

To date, M-T7 has been shown to bind to CCL5, CCL2, CCL7, CXCL8, CXCL4, CXCL10, CXCL7, CXCL1 and XCL1 [15]. It shows a broad specificity, but its affinity is relatively weak. This suggests that it binds to a site which is common to all chemokines. In order to determine the vCKBP-1 binding site in CXCL8, the interaction of M-T7 with chemically synthesised analogues

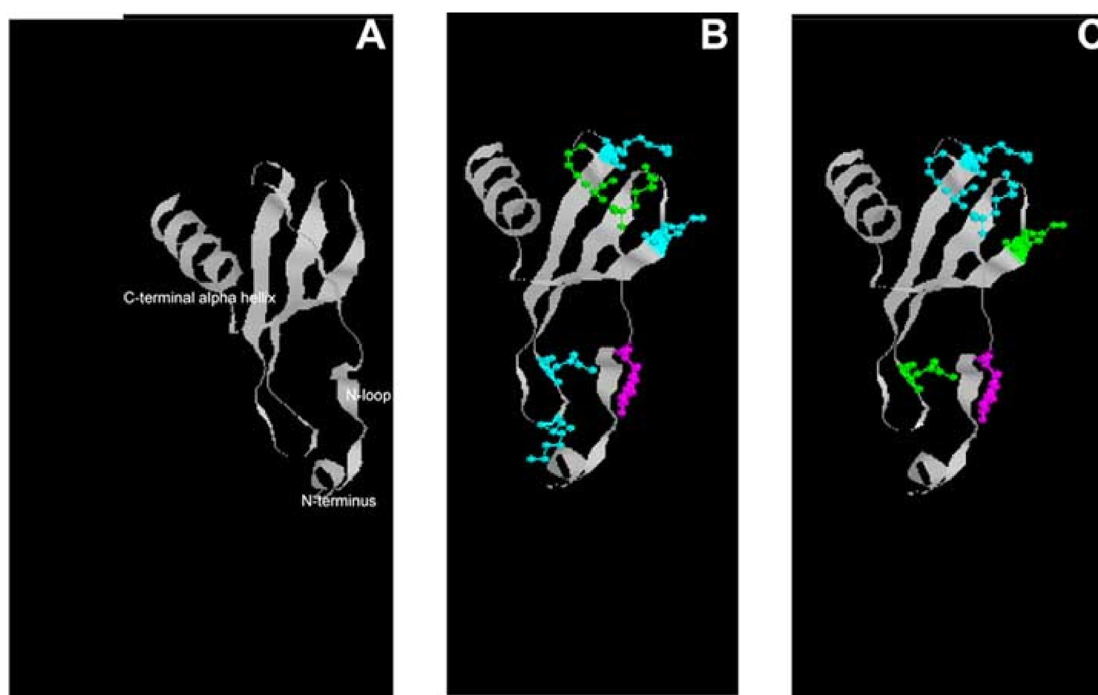

Fig. (3). Comparison of the binding determinants of CCL2 for CCR2b and vCKBP-2. Tertiary structure of CCL2 (**A**) is depicted with the key residues involved in (**B**) CCR2b, and (**C**) vCKBP-2 binding highlighted. Residues shown in magenta represent aromatic residues that affect binding, residues shown in cyan represent basic residues that affect binding greater than 5-fold, and green represent residues basic residues that affected binding by a factor of 5 or less. Figure generated using Rasmac, adapted from Seet *et al.* PNAS **2001**, 98, 9008-9013.

of CXCL8 with different truncations was assessed [15]. Removal of the first seven N-terminal amino acid residues of CXCL8 (containing the receptor binding domain) had no effect on binding to vCKBP-1. In contrast, removal of just the last 6 residues abrogated vCKBP-1 binding. This particular analogue had been used to show that the GAG binding site of CXCL8 resides in the C-terminus, since truncation of the last 6 residues resulted in a markedly lower heparin affinity [40]. At the time of this work, heparin binding sites of chemokines were all thought to lie within the C-terminus, and it was proposed that M-T7 will interfere with the ability of many chemokines to interact with GAGs. However, it is now clear that the GAG binding site can reside within areas of the chemokine molecule other than the C-terminus [47]. Whether M-T7 is able to inhibit GAG binding independently of the type of chemokine-GAG structure formed is not clear. To date, only the binding of CXCL8 to M-T7 has been shown to be inhibited by exogenous heparin [15]. M-T7 has a submicromolar affinity for CCL5, a value reported similarly for chemokine-GAG interactions. This implies that M-T7 may mimic GAGs in its interaction with chemokines. If this were the case, it would be expected that M-T7 would interact with other GAG binding cytokines. Many cytokines have been shown to bind to heparin (e.g. interleukin –2 [IL-2], IL-3, IL-4, IL-5, IL-6, IL-7, IL-12, transforming growth factor- , fibroblast growth factor, IL-10, and granulocyte-macrophage colony-stimulating factor [47-56]. Yet, when tested for its ability to bind to IL-2, IL-3, IL-4, IL-6, or IL-7, no binding was seen. This shows that the interaction between M-T7 and chemokines is more complex than merely mimicking the chemokine-GAG interaction.

The ability to bind to chemokines is not a property shared by all poxviral IFN R homologues. MV appears to have evolved differently from its related viruses. IFN specifically competes with CCL5 binding to M-T7, implying that the sites for these two cytokines overlap or that upon binding to IFN , a conformational change is induced in M-T7 that prevents its binding to chemokines or vice versa. It has been suggested that M-T7 will disrupt established chemokine gradients, since it has a higher affinity for chemokines than heparin does [15]. *In vivo*, it should at least be able to prevent chemokines from binding to heparin and thereby act as a chemokine sink, akin to the duffy antigen receptor for chemokines found on erythrocytes (Fig. **2B**).

vCKBP-2

Members of the vCKBP-2 subfamily are able to inhibit *in vitro* chemokine-mediated calcium flux and chemotaxis [16-18]. Their binding to chemokines is unaffected by exogenous heparin and heparan sulfate [16]. Thus, the mechanism by which vCKBP-2 can inhibit chemokines is distinct from that of vCKBP-1. vCKBP-2 binds with high affinity to CC chemokines. The dissociation constant of vCKBP-2 is in the subnanomolar range, and the affinity for CC chemokines is often higher than that for their native chemokine receptors [16-18]. Alcami *et al*. also showed the 35kDa protein was an effective inhibitor of CC chemokines only [16]. They demonstrated that it could prevent CCL3 and CCL5 from binding to their receptors, but had no effect on the binding of CXCL1 to its receptor. In addition, they also found that the 35kDa protein prevented calcium mobilization by CCL11, CCL13 and chemotaxis induced by CCL3 *in vitro*, and CCL11 *in vivo* [16]. Together these data reaffirm that the 35kDa protein is an inhibitor of CC, but not CXC chemokines. Lalani *et al*. confirmed this chemokine specificity [19].

Recently, the chemokine binding profile of the 35kDa protein has been assessed [57]. Burns *et al*. tested 80 chemokines for their ability to displace radiolabelled signature chemokines from 35kDa protein. Only 26 of these chemokines were shown to be high affinity ligands. Interestingly, they found that two herpesvirus-encoded chemokines, HHV-8 vMIP-1 and HHV-8 vMIP-II were very effective in displacing CCL3 from the 35kDa protein. Although most CC-chemokines exhibited vCKBP-2 binding, CCL22, CCL17 and CCL25 did not. They also found that different chemokines which had the same affinity for the 35kDa protein showed different degrees of cooperativity e.g. CCL11 seemed to associate with simple one-site kinetics, whereas CCL3 displayed marked positive cooperativity. Thus, the 35kDa protein shows distinct co-operation profiles for different chemokines.

The sequences of vCKBP-2 from different poxviruses have between 40 and 95% amino acid sequence identity, all

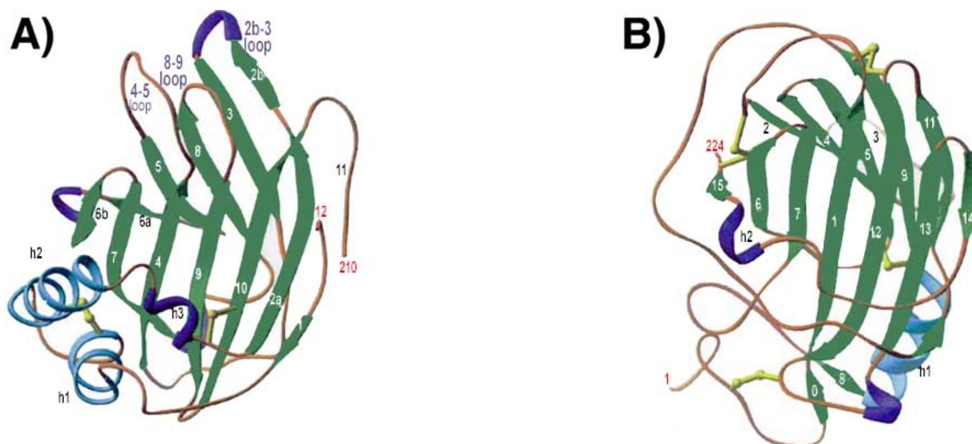

Fig. (4). Three dimensional structure of (**A**) vCKBP-2 and (**B**) N-terminal domain of vCKBP-3. Adapted from Alexander *et al.* Cell **2002**, 111, 343-356.

having the same number and pattern of conserved cysteines [19]. Despite the significant heterogeneity in sequence identity between the 35kDa proteins from leporipox and orthopox viruses, their functional activities remain equivalent. This suggests that their CC-chemokine binding or inhibitory properties may occur through conserved motifs or moieties within these viral proteins, or that members of the vCKBP-2 family adopt a uniform conformation that allows their functional inhibitory activities. The structure of vCKBP-2 from CPV has been solved (See Fig. **4A**) [58]. It is a compact globular protein composed primarily of two parallel -sheets, two short -helices, and a few large loops connecting these secondary structure elements. The -sandwich topology of CPV 35kDa protein is unique and has not been observed in other protein structures. This fold is reminiscent of the collagen-binding domain from Staphylococcus aureus adhesin, although the number of strands forming the -sheets and their order is different between the two molecules [58].

It is well established that the functional binding sites of protein-protein interactions are dominated by only a few residues located in the interface. Given the chemokine binding profiles and activities of vCKBP-2, it is predicted that they recognise common structural features shared by most CC-chemokines and are able to occlude receptor binding. To address this, Seet *et al*. used a panel of deletion and site-directed CCL2 mutants to define the vCKBP-2 binding site [59]. Using the CCL2 mutant P8A (which is unable to dimerise), they first showed that VV 35kDa protein binds to monomeric CCL2. They found that the first seven N-terminal amino acids of CCL2 (which are critical for receptor binding) were dispensable for VV 35kDa protein binding. This region is highly disparate in chemokines. Although mutations of most residues in CCL2 had little or no effect on the binding affinity for vCKBP-2, significant effects were observed for three residues within the "N-loop" (Y13, R18, and K19) and one residue (R24) within the 3^{10} helix. Independently, Beck *et al*. also showed that VV 35kDa inhibited CCL2 by masking its CCR2b binding site [60]. Probing of all surface-exposed residues of CCL2 for their importance in the interaction with vCKBP-2 revealed a functional binding site dominated by the three residues Y13, R18 and R24. CCL1, which is unable to bind vCKBP-2, carries a glutamine instead of R18, therefore an arginine (or its equivalent) at position 18 seems to be a precondition for binding to vCKBP-2. Some residues when mutated to alanine caused an increase in vCKBP-2 affinity, e.g. K49 which is adjacent to R24. Interestingly, removal of the first eight amino acid residues resulted in a 7-fold increase in vCKBP-2 binding. Analogous to the binding surface mapped by mutagenesis for CCR2b, Y13, R18, K19 and R24 define two discontinuous largely basic, regions of the chemokine surface separated by a hydrophobic groove. Because vCKBP-2 is large in comparison to CCL2 and makes contact with residues that flank the groove, it is possible that the viral protein also contacts the intervening hydrophobic groove where CCR2b's N-terminus purportedly binds, thereby forming a continuous interaction surface. So, vCKBP-2 not only binds to the same molecular face on CCL2 that is used for CCR2b, but it also interacts with the same residues that constitute hotspots for the host receptor (Fig. **2B**&**C**). This interaction explains how vCKBP-2 is able to interact with many CC-chemokines, and how vCKBP-2 can obstruct CCL2 and other CC chemokines from binding to their receptors. CCL5 requires R17 (CCL2's R18 equivalent) to bind CCR1, F12 (CCL2's Y13 equivalent) to bind CCR3, and both F12 and I15 to bind CCR5. Likewise CCL4 requires F13 (CCL2's Y13 equivalent) to bind CCR5, and it is predicted that the corresponding residue in CCL3 will also be necessary.

How does vCKBP-2 manage to bind to so many CC chemokines given their large sequence diversity? It is possible that there are physiochemical features on the surface of chemokines that are conserved in the absence of any strict amino acid identity and that vCKBP-2 evolved to retain a certain flexibility to accommodate slightly different arrangements of key determinants in different ligands. In an analogous case, the hinge region of the Fc fragment of human immunoglobulin G has been shown to interact with completely unrelated proteins with high affinity using a common binding site [61]. This site is highly accessible, adaptive and hydrophobic. CXC chemokines are also basic proteins and fold similarly to CC chemokines, yet they do not bind vCKBP-2 with appreciable affinity. The residues identified as important for CCL2 binding are found in many CXC chemokines. It is possible that the CXC motif itself may prevent binding to vCKBP-2.

Together, these data clearly showed that vCKBP-2 interacted with sites of the chemokine involved in GPCR, but not GAG binding. This correlates with the inability of exogenous heparin or heparan sulfate to inhibit the chemokine-vCKBP-2 interaction. Curiously, M-T1 from MV has evolved an additional function which allows it to indirectly prevent chemokine-GAG interactions [62]. Seet *et al*. showed that in addition to binding CC chemokines, M-T1 can specifically interact with cell surface GAGs and can bind both CC chemokines and GAGs simultaneously, suggesting that the chemokine-binding domain of M-T1 is distinct from its heparin-binding domain [62]. There are several examples of heparin-binding proteins encoded by poxviruses. Several virion-associated proteins of VV, certain secreted viral proteins, and a complement-binding protein have been shown to bind to GAGs [63-65]. No other vCKBPs have a GAG binding ability. M-T1 elutes from heparin at a salt concentration of 0.4M, comparable to that required to elute many chemokines [62]. This suggests that M-T1 could displace GAG-bound chemokines.

The GAG-binding site of M-T1 is found in its C-terminus [62]. This region has a high occurrence of basic residues, the bulk of which make up the surface region of -sheet 1 and contains two clusters that closely resemble canonical Cardin and Weintraub heparin-binding motifs. A model of M-T1 constructed on the basis of the structure reported for CPV 35kDa protein shows a region on the opposite side to the proposed chemokine binding site that is likely to be the heparin binding region. This region also forms a potential heparin-binding structure found in human lactoferrin and fibronectin known as a cationic cradle. Comparison of the models of M-T1 and the VV-35kDa protein revealed differences in surface electrostatics [62]. Consistent with its inability to bind heparin, the VV 35kDa protein notably lacks the region of positive charge, and the putative heparin-binding consensus sequence seen in M-T1.

In contrast, the two proteins share a negatively charged surface within the exposed face of -sheet II consistent with their shared ability to bind positively charged chemokines.

The ability of M-T1, but not other vCKBP-2s to bind GAGs, probably reflects the distinct evolutionary history of the two genera of poxviruses that these viruses represent. It also suggests that M-T1 will exhibit distinctive tissue distribution and clearance profiles to that of other vCKBP-2s. The dual ability of M-T1 to simultaneously bind GAGs and chemokine may impart several distinct functions *in vivo*, not shared by the VV 35kDa protein. GAG binding by M-T1 within the extracellular matrix may prevent diffusion effects *in vivo* and enable the protein to persist in the tissue microenvironment, thereby increasing its local concentration at sites of infection. M-T1 may share the ability of other heparin-associated proteins that are protected from protease degradation when bound to GAGs. Also, M-T1 may displace other GAG-bound proteins present with in the extracellular matrix, thereby altering their biology by removing them from heparin. Finally, M-T1 may be able to capture and neutralise host chemokine gradients established at sites of infections. However, this is likely to be a very inefficient mechanism of chemokine inhibition, since it would have to saturate all local GAGs in order to do this.

vCKBP-3

M3 is a broad spectrum vCKBP that is able to bind both CC, CXC, CX3C and C chemokines [22,23]. However, it does not utilise the common GAG-binding motif of chemokines as M-T7, since exogenous heparin or heparan sulfate have no effect on its interaction with chemokines [22]. M3 blocks the interaction of chemokines with their cell-surface receptors and inhibits chemokine-mediated signal transduction [22,23]. Like all vCKBPs, vCKBP-3 has no homology to any described mammalian protein.

The structure of M3 has been determined. It forms an asymmetric dimer, with two M3 chains pairing in an anti-parallel fashion to create a flat, rectangular complex. The core of the N-terminal domain displays remote structural similarity to vCKBP-2 (Fig. 4**A**&**B**). In general, both structures are composed of a core sandwich, each uniquely decorated with loops and helices [58,66]. While the majority of the strands in the core of M3 have analogous strands in vCKBP-2, the connecting topology of the two structures appears completely distinct. The C-terminal domain adopts a sandwich fold similar to a V-type Ig fold. M3 has been co-crystallised with a monomeric variant of CCL2 [66]. In the complex, M3 forms a symmetric homodimer, similar to that observed for the unliganded protein, with two chemokines bound into niches at distal ends of the dimer. CCL2 binds to M3 as a monomer in a 2:2 stoichiometry, with no interactions observed between the two chemokine molecules (Fig. **5**). The chemokine binding sites of M3 are deep clefts formed between the N and C terminal domain sandwiches. There are 26 residues from CCL2 and 29 residues from M3 at each ligand-receptor interface. The

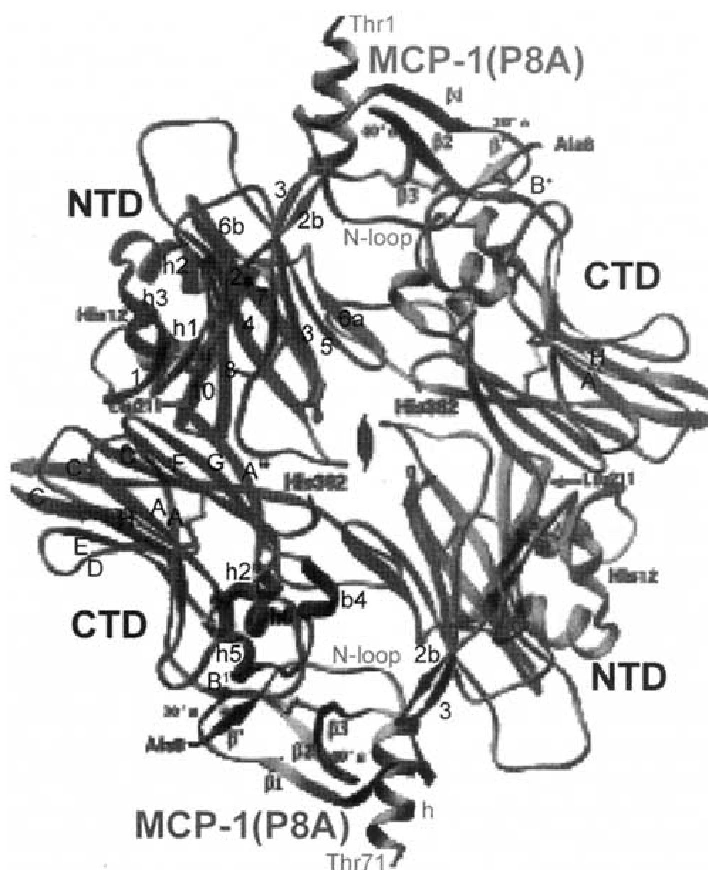

Fig. (5). Structure of vCKBP-3 complexed to CCL2. Dimeric vCKBP-3 binds two monomers of CCL2. The location of disulphide bonds in vCKBP-3 are indicated by sequence numbers and dashed lines; CTD, C-terminal domain; NTD, N-terminal domain.

CCL2 binding interface includes the N-terminus, the entire N-loop and to a lesser extent the 30's loop, 40's loop, and the C-terminal helix. To validate these interactions, alanine mutants of CCL2 of residues Y13, K19, R29, I46 and E50 of CCL2 were tested for their ability to competitively bind M3 [66]. Y13 and K19 of CCL2 directly contact M3 in the complex, E50 loses only a small amount of solvent-accessible surface area, whilst R29 and I46 are outside the binding interface. Only substitutions of Y13, and to a lesser extent K19, had a measurable effect on binding affinity. The CCL2 surface engaged by M3 contains the same residues that were previously identified by mutational analysis as important endogenous receptor CCR2b contacts. CCL2 N-terminal residues 1-7, essential for endogenous receptor signalling were not visible in the structure and are presumptively not in contact with M3. M3 engages CXC family members with a slightly lower affinity and higher selectivity than CC family members [22,23]. The center of the N-loop of non-binding CXC chemokines appears to pack closer to the C-terminal helical region as a result of a deletion at the position corresponding to CCL2 R18. In contrast, the N-loops of CXC chemokines which bind to M3 bulge outwards. In agreement with this, we have found that it is the N-loop of CXCL8 that binds to M3 and residues that affect the bulge of the N-loop directly affect M3 binding [67]. Using CXCL8 analogues with truncations and point mutations, and hybrid molecules containing the N-loop of CXCL8 on a non-M3 binding chemokine (e.g. CXCL4, or CXCL12) or vice versa, we showed that the N-loop of CXCL8 is critical for binding to M3, with some modest contribution from the first seven N-terminal amino acids. In addition, mutation of L49 to Alanine caused a marked drop in M3 affinity. L49, packs into the N-loop causing it to bulge out. The contribution of the N-terminus of CXCL8 for M3 binding is in contrast to that seen with vCKBP-2, which uses only the N-loop of CC chemokines (removal of the N-terminus of CCL2 actually increases vCKBP-2 affinity). This may explain the broader specificity of vCKBP-3. We found that most contribution to M3 binding came from I10 within the CXCL8 molecule, which is the equivalent molecule to Y13 of CCL2.

The N-loop of chemokines is used to bind to the GPCR and confer specificity. Thus, by binding to this site, M3 should be able to mask the receptor binding motifs of the chemokine, and thereby prevent GPCR activation. Parry *et al*. first showed that M3 was able to prevent CCL3 and CXCL8 from binding to their receptor and also prevented CCL5-induced calcium mobilization. Van Berkel *et al*. were also able to demonstrate that M3 could prevent calcium mobilization induced by CCL2, CCL3, CCL5, CX3CL1 and CXCL8 [23].

Analysis of M3/CCL2 contact surface has revealed several general features associated with promiscuous chemokine binding. Broad chemokine binding by M3 may be attributed in part to the oligomeric construction of the combining sites and the use of flexible loops as primary contact regions. This design is highly reminiscent of adaptive immunoreceptors that likewise use loops projecting from two sandwich folds to create ligand binding niches [61].

Jensen *et al*. have demonstrated that the CCL19/CCL21-M3 binding exhibits positive cooperativity. The functional implications *in vivo* are unclear, but it suggests that its chemokine antagonism could occur within a very narrow dose range. These findings should be taken into consideration when evaluating the therapeutic potential of M3 [68].

We have also found that M3 prevents chemokines from binding to GAGs (Webb, L.M., Smith, V.P., and Alcami, A. submitted). The ability of vCKBP-3 to prevent chemokine binding to heparin depended upon whether the individual chemokine could bind to vCKBP-3. CXCL8, CCL2 and CCL1 have been shown to bind vCKBP-3, CXCL10 binds vCKBP-3 but with a lower affinity, and CXCL12 is unable to bind vCKBP-3. In agreement with this, we found that vCKBP-3 could inhibit heparin binding of CXCL8, CCL2 and CXCL1 but not CXCL12, and only partially inhibited CXCL10. This implies that M3 is able to inhibit the chemokine-GAG interaction by binding to the chemokine and not the GAG. In agreement with this, we have been unable to detect direct binding of M3 to heparin. It has been previously shown that preincubating either CCL3 or CXCL8 with $3x10^6$ molar excess of heparin was unable to prevent either chemokine from binding to vCKBP-3 [22]. This indicates that heparin-bound CCL3 or CXCL8, both have similar affinities for vCKBP-3 as unbound chemokine. Interestingly, we were able to show that vCKBP-3 could completely displace both CXCL8 and CCL5 from heparin. Differences in the kinetics of this displacement suggest that M3 abrogates the chemokine-GAG interaction by different mechanisms. CCL5 is displaced within 1 hour, and CXCL8 within 5 hours after vCKBP-3 addition. This is unlikely to be due to the different affinities of CXCL8 and CCL5 for vCKBP-3, since CXCL8 has a higher affinity than CCL5 for vCKBP-3. It is more likely due to the different regions used by CCL5 and CXCL8 to bind to heparin. Previous work has shown that residues K20, R60 and R67 in CXCL8 and residues R44, K45 and R47 in CCL5 are responsible for heparin binding [38,44,69]. However, there is evidence that other residues in CCL5 also participate in GAG binding. CCL5 binds to the GPCRs CCR1 and CCR5. It has been postulated that there is some overlap in the CCR1 and GAG binding sites of CCL5. It is possible that the GAG-binding residues within CCL5 may also participate in vCKBP-3 binding. We would predict from the mapping studies done with CCL2 and CXCL8 that CCL5 also binds to vCKBP-3 *via* its N-loop. We would also predict from the displacement kinetics that for CCL5, there is likely to be an overlap in the GAG and vCKBP-3 binding domains (See Fig. **6A**&**B**). The residues of CXCL8, important for GAG binding, are not though to participate in M3 binding. M3 appears to mimic receptor binding to CXCL8, thereby masking the sites that bind to the receptor within the N-terminus. It is conceivable that upon receptor binding, conformational changes are induced within the chemokine molecule that abrogate GAG-binding, thereby allowing the chemokine to be removed from the cell surface and internalised. Hence, by mimicking receptor binding, vCKBP-3 is able to simultaneously inhibit GPCR and GAG binding.

vCKBP-3 appears to act at two distinct levels to inhibit chemokine activity. By binding to the N-loop of CCL2, CXCL8, and presumably other chemokines, it prevents the chemokine engaging its GPCR, but it also prevents the chemokine binding to GAGs. vCKBP-1 also prevents chemokines interacting with GAGs, but unlike vCKBP-3, it

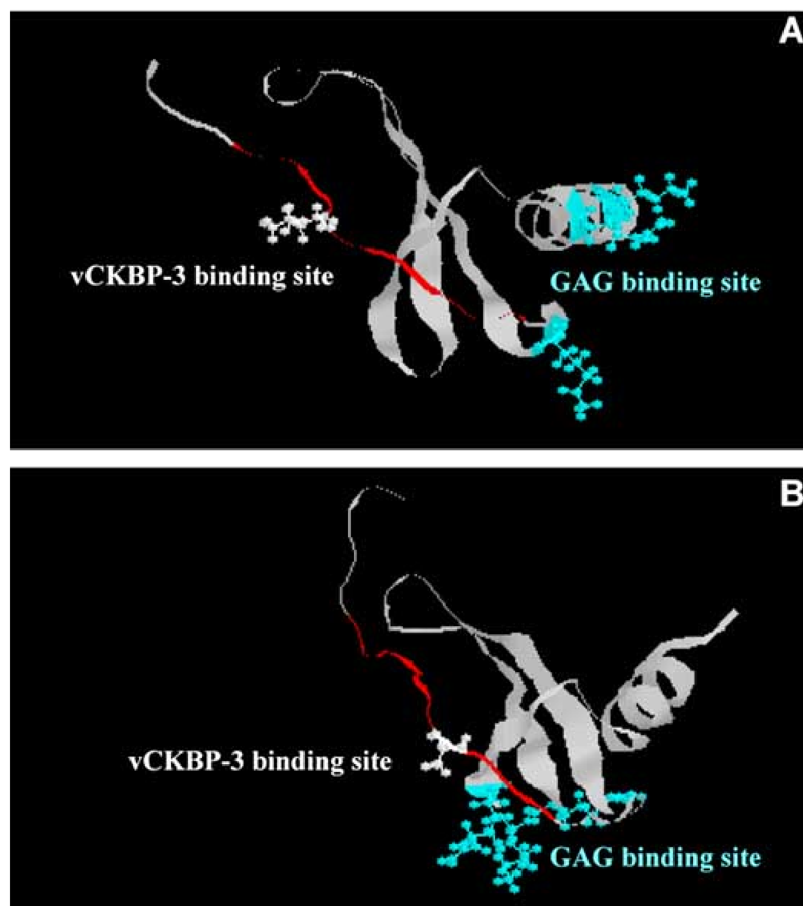

Fig. (6). Structure of (**A**) CXCL8 and (**B**) CCL5 showing areas of the molecule involved in GAG binding, vCKBP-3 binding and the N-loop.

has no effect on GPCR binding. Since GAG binding is now thought to be a prerequisite for the *in vivo* activity of chemokines, it would appear that inhibition of only GAG or GPCR binding would be sufficient for the *in vivo* neutralisation of chemokine activity by vCKBP-3. However, by preventing the chemokine-GAG interaction, vCKBP-3 is able to disrupt established chemokine gradients making it a more effective inhibitor of chemokine activity. To date, the only other vCKBP known to affect both GPCR and GAG binding by chemokines is vCKBP-4.

vCKBP-4

The structural features of chemokines required to bind to gG remain undetermined. Like M3, gG is a broad spectrum vCKBP that inhibits both GPCR and GAG binding. Like M3, it is also able to displace GAG-bound chemokines. Based on the similar mechanisms adopted by vCKBP-2 and vCKBP-3 to occlude receptor binding, we would predict that gG is also likely to interact with the N-loop of chemokines, and probably utilises this site to induce conformational changes to abrogate GAG binding.

A unique feature of gG is that it is a membrane protein, anchored by a C terminal transmembrane domain, which may be secreted after proteolytic cleavage. Secretion of gG has been demonstrated during infections with EHV-1, EHV-4, BHV-1, BHV-5 and HSV-2 [70-74]. Bryant *et al.* were able to show chemokine binding to recombinant EHV-1 gG expressed at the surface of insect cells [25]. It is possible that cell bound gG acts as a decoy receptor preventing the interaction of chemokines with cellular chemokine receptors and subsequent signal transduction. It has also been speculated that the chemokine binding activity of gG, incorporated into the EHV-1 virion envelope may mediate initial virus attachment to cell surfaces presenting chemokines, and thereby play a role in determining tissue tropism *in vivo* [25].

POTENTIAL THERAPEUTIC USES

The broad and high affinity chemokine binding patterns of these different vCKBPs suggests that they may be particularly useful in two settings. Firstly, they can be used to dissect the biological roles of chemokines in inflammatory diseases and secondly, they could be used as a clinical anti-inflammatory agents. There are currently few studies assessing the effect of vCKBPs *in vivo*. However, those that have been published suggest that vCKBPs do have potential for the treatment of inflammatory disorders.

vCKBP-1

M-T7 has been shown to inhibit plaque development *in vivo* using both rabbit and rat models of angioplasty-mediated vascular injury [75]. Since its effects are not species

specific, it is likely that they are due to its ability to inhibit chemokine activity rather than its rabbit IFN inhibitory activity. The rat model of vascular balloon injury is characterised by a predominant smooth muscle cellular response with less pronounced fatty plaque areas or macrophage/foam cell invasion at sites of damage. The cholesterol-fed rabbit model of balloon injury has a more pronounced inflammatory cell component with areas of lipid-filled macrophage invasion. In both cases, significant reductions in plaque area were detected with M-T7 infusion [75]. The data suggests that M-T7 is acting at an early initiating step in atherogenesis. These findings also support chemokine activity as a central mediator in the initiation of plaque growth after angioplasty.

vCKBP-2

The potential of vCKBP-2 as a therapeutic agent *in vivo* was first illustrated in a guinea pig skin model by the blockade of eotaxin-induced eosinophil infiltration, a feature of allergic inflammatory reactions [16]. Guinea pigs were first pretreated with IL-5 to induce blood eosinophilia and then given eotaxin, C5a, or LTB4 with or without the 35kDa protein (all injected intradermally). The VV 35kDa protein inhibited local eosinophil infiltration induced by intradermal injection of eotaxin, but not other non-chemokine reagents. The inhibitory effect of various doses of 35kDa on eosinophil accumulation in the skin, in response to eotaxin was determined in guinea pigs injected i.v. with 111In-labelled eosinophils. A complete blockade of eotaxin activity was seen with a threefold molar excess of 35kDa protein and 50% inhibition by an equal molar concentration.

The effect of the 35kDa protein in a murine model of allergen-induced asthma has also been examined [76]. For this, ovalbumin (OVA) was administered twice intraperitoneally (i.p.), and three times intra-nasally (i.n.) into mice, resulting in a disease that mimics human allergen-induced asthma. A dimeric form of the 35kDa protein fused to the human IgG1 Fc domain, or an equivalent amount of purified human IgG1 was administered i.n. two hours before each of the three i.n. does of OVA on days 14, 24 and 25. 35kDa protein treatment significantly reduced the number of total leukocytes and eosinophils in BAL, and reduced inflammation in the lung. A single dose of the 35kDa protein blocked the recruitment of inflammatory cells into the lungs, but had no effect on the recruitment of cells into the peritoneal cavity of mice subsequently challenged with OVA i.p. Local intrapulmonary administration of the 35kDa protein was effective in preventing the increased airway hyperreactivity,characteristic of the late phase response. The circulating levels of total and OVA-specific IgE at day 26 showed that the 35kDa protein had no effect on the increase in total IgE levels induced by OVA administered i.p., implying that it was not affecting any potential role of CC chemokines in augmenting lymphocyte function (e.g. production of IgE by B cells). CD4+ T cell activation or function did not appear to be affected since the levels of KLH-specific IgG1 were no different in control versus 35kDa protein treated mice. Thus, the 35kDa protein appears to be highly effective in blocking both the inflammatory and airway physiological consequences of allergen-induced asthma *in vivo*, without significantly altering systemic antigen-specific immunity. So 35kDa protein treatment for asthma might have advantages over conventional local or systemic glucocorticoid therapy, such as avoiding inhibition of neutrophil migration to the lung and airways, and the systemic negative effects on bone homeostasis.

vCKBP-3

Jensen *et al*. showed that M3 can inhibit chemokine activity *in vivo* using transgenic mice, where M3 was expressed in the pancreas by placing the gene downstream of the RIP promoter (which targets transgenic expression predominantly to the pancreatic islets and the kidney) [68]. These mice were then crossed with mice expressing CCL21 under the same promoter. Transgenic expression of CCL21 in pancreatic islets to the development of lymphoid aggregates (primarily composed of T and B lymphocytes) that resemble lymph nodes. In contrast to CCL21, transgenic mice where 18% of the islets had infiltrates, double transgenic mice showed only 2% of the islets having infiltrates. Such infiltrates were composed of only a few scattered mononuclear cells, in contrast to the large infiltrates found in the CCL21 transgenic mice. Thus, expression of M3 in pancreatic islets can reduce the accumulation of mononuclear cells induced by the ectopic expression of CCL21, demonstrating the *in vivo* efficacy of M3 as a chemokine inhibitor.

Rice *et al*. recently showed that M3 can interfere with epitope-specific CD8+ T cell attack on a lymphoid tumour in a subcutaneous site [77]. EL4-FrC cells were transduced with M3 expressing retrovirus and secreted active M3. Cytotoxic T-cell lines (CTLs) derived from EL4-FrC vaccinated mice were able to kill parental EL4-FrC cells. Expression of M3 by ELF-FrC cells led to a slight decrease in the level of killing – possibly reflecting some requirement for chemokine-dependent migration. The effect of M3 expressing tumour cells was examined *in vivo* in naive or vaccinated mice. Following vaccination, protective immunity was induced against parental EL4-FrC cells. This protection is completely dependent on CD8+ T cells. Strikingly, these CTLs were unable to control ELF-FrC-M3 cell, even though the tumour could be lysed by CTL *in vitro*. Thus, M3 protein secreted by the tumour cells was capable of *in vivo* inhibition of anti-tumour CD8+ T cell immunity.

FUTURE PERSPECTIVES

Viruses have co-existed with the host immune system and have evolved a number of strategies to modulate the immune response. The secretion of soluble protein that can bind chemokines is clearly an anti-chemokine mechanism chosen by several viruses. How can we exploit vCKBPs for our own benefit? At the very least, vCKBPs can be used to establish the effect of blocking chemokine activity in different diseases. Their potential as therapeutics is only beginning to be appreciated. vCKBPs offer many advantages over other inhibitors of chemokines, such as small molecule antagonists, antibodies directed against chemokines and antagonistic chemokines themselves. Other technologies being developed include gene therapy, antisense inhibitors and ribozymes. Most of these non-viral inhibitors are directed against only one chemokine receptor or chemokine

[78,79]. However, there are over 40 known human chemokines with many overlapping activities, ligands and receptors, and there is little evidence that a single chemokine or chemokine receptor is solely responsible for the pathogenesis seen in inflammatory disease. The redundancy within the chemokine system suggests that it may not be possible to only target a single chemokine receptor to produce a pharmacological response. Another important problem associated with non-viral chemokine receptor antagonists is species specificity. Evaluation in rodent disease models of chemokine receptor antagonists generated against human chemokine receptors is very difficult, because the affinity of a small molecule for a human chemokine receptor may be several fold higher than that for its murine counterpart. vCKBPs often bind to chemokines from several different species with similar affinities. Also, synthetic inhibitors developed against one chemokine receptor may cross-react with other members of the GPCR family with unknown side effects [78]. vCKBPs have many advantages over manmade efforts, since they not only target multiple chemokines, but they also show less species specificity making their evaluation in murine models of disease more relevant for development of therapeutics for human disease.

However, vCKBPs have some disadvantages. Because of their broad-spectrum nature, they may disrupt some of the homeostatic functions of chemokines. Chemokines are not only important in inflammatory reactions, but they can also play a crucial role in homeostatic functions [37]. Some are continuously produced and expressed, and are important for maintaining an active immune response and lymphoid architecture. However, this might be a desirable strategy in some cases of chronic inflammation. For example, disruption of lymphogenesis induced by homeostatic chemokines during chronic inflammatory conditions might break the perpetual state of inflammation [80]. Yet, neutralisation of CXCL12 could have very damaging effects, since mice deficient in CXCL12 suffer from impaired feetal development of the cerebellum, the cardiac septum, gastric vasculature and B cell lymphopoiesis [81-83]. Neutralisation of CXCL12 in developed mice may have equally devastating consequences. Interestingly, neither vCKBP-2 nor vCKBP-3 bind to CXCL12, so it is possible that some vCKBPs have already evolved to avoid neutralising chemokines that are critical for host survival. It is also important to remember that some virally-encoded immunomodulatory proteins have multiple activities (e.g. M-T7 inhibits binds to both IFN and chemokines through distinct domains). However, since most virally-encoded immunomodulatory proteins usually reduce inflammation, this may offer a significant advantage in the clinical setting. It may also be difficult to administer therapeutic levels of vCKBPs. Timing of administration, pharmacokinetics and immunogenicity may pose problems in the treatment of chronic inflammatory diseases. However, initial studies suggest that vCKBPs can be effective when administered at low doses [75]. It is thought that viruses have engineered and selected their immunomodulatory proteins not only to maximise their effectiveness, but also to minimise their immunogenicity. Potential immunogenicity problems could be overcome by strategies such as polyethylene glycol (PEG)ylation, which is a well established means of decreasing the immunogenicity of biopharmaceuticals. A more attractive way to overcome this problem would be the use of mammalian counterparts of vCKBPs. To date, no such proteins have been identified, and vCKBPs remain some of the few examples of virally-encoded immunomodulatory protein where there is no parallel in mice/humans [2,7]. We may simply have not yet identified the vCKBP that has a human homologue. Alternatively, vCKBPs may have evolved from a viral gene with an unrelated activity. To date, the evolutionary source of vCKBPs remains obscure.

So far, only vCKBP-2 and vCKBP-3 have been crystallised [58,66]. What this work has shown is that vCKBPs not only have unique amino acid sequences, but their tertiary structures also show novel folds. Thus, viruses appear to have evolved their very own method of inhibiting chemokines that is unique to them. These structures could be used as a scaffold to develop specific chemokine inhibitors by mutating specific positions in vCKBPs, and changing their chemokine specificity from narrow to broad or vice versa, depending upon the therapeutic requirement. Mapping studies and site directed mutagenesis may enable the rational design of chemokine inhibitors for specific disease types, where the key chemokines involved in the pathogenesis have been defined.

There are already clear examples of effective use of vCKBPs in different models of disease. They have been shown to be effective in models of allergic inflammation, airway inflammation, allergy induced perivascular and peribronchial inflammation, and hyperplasia following balloon angioplasty [16,75,76]. In addition, other viral proteins have been shown to have anti-inflammatory properties when administered systematically at very low doses [4,5]. Virally-encoded chemokine agonists and antagonists have been successfully used in many diseases models. An example of this is vMIP-II, which has been successfully used to attenuate inflammatory symptoms in rat models of glomerulonephritis, spinal cord contusion, heart plant rejection and brain ischemia [84-87]. vCKBPs may be useful for the treatment of inflammatory diseases in which excessive, local elaboration of chemokines and tissue-specific influx of leukocytes are hallmarks, e.g. chronic autoimmune disorders and allograft rejection. These broad spectrum, high affinity binding proteins that disrupt specific chemokine/receptor or chemokine/GAG interactions have general promise for the treatment of immunologically mediated diseases. The fact that many different viruses have evolved these and not other strategies of chemokine modulation suggests that the efficacy of this strategy is worth exploring, and valuable lessons can be learnt by studying how these vCKBPs work.

ACKNOWLEDGEMENTS

The work in the authors laboratory was supported by the Wellcome Trust.

REFERENCES

[1] Alcami, A.; Koszinowski, U. H. *Immunol. Today* **2000**, *21*, 447.
[2] Seet, B. T.; Johnston, J. B.; Brunetti, C. R.; Barrett, J. W.; Everett, H.; Cameron, C.; Sypula, J.; Nazarian, S. H.; Lucas, A.; McFadden, G. *Annu. Rev. Immunol.* **2003**, *21*, 377.
[3] Tortorella, D.; Gewurz, B. E.; Furman, M. H.; Schust, D. J.; Ploegh, H. L. *Annu. Rev. Immunol.* **2000**, *18*, 861.

[4] Murphy, P. M. *Nat. Immunol.* **2001**, *2*, 116.
[5] Murphy, P. M. *J. Clin. Invest.* **2000**, *105*, 1515.
[6] Smith, V. P.; Bryant, N. A.; Alcami, A. In *Chemokines in Viral Infection*; Mahalingham, S., Ed.; Landes Bioscience, **2003**.
[7] Alcami, A. *Nat. Rev. Immunol.* **2003**, *3*, 36.
[8] Weber, K. S.; Grone, H. J.; Rocken, M.; Klier, C.; Gu, S.; Wank, R.; Proudfoot, A. E.; Nelson, P. J.; Weber, C. *Eur. J. Immunol.* **2001**, *31*, 2458.
[9] Sozzani, S.; Luini, W.; Bianchi, G.; Allavena, P.; Wells, T. N.; Napolitano, M.; Bernardini, G.; Vecchi, A.; D'Ambrosio, D.; Mazzeo, D.; Sinigaglia, F.; Santoni, A.; Maggi, E.; Romagnani, S.; Mantovani, A. *Blood* **1998**, *92*, 4036.
[10] Lalani, A. S.; Barrett, J. W.; McFadden, G. *Immunol. Today* **2000**, *21*, 100.
[11] Ng, A.; Tscharke, D. C.; Reading, P. C.; Smith, G. L. *J. Gen. Virol.* **2001**, *82*, 2095.
[12] Kerr, P.; McFadden, G. *Viral. Immunol.* **2002**, *15*, 229.
[13] Upton, C.; Mossman, K.; McFadden, G. *Science* **1992**, *258*, 1369.
[14] Mossman, K.; Nation, P.; Macen, J.; Garbutt, M.; Lucas, A.; McFadden, G. *Virology* **1996**, *215*, 17.
[15] Lalani, A. S.; Graham, K.; Mossman, K.; Rajarathnam, K.; Clark-Lewis, I.; Kelvin, D.; McFadden, G. *J. Virol.* **1997**, *71*, 4356.
[16] Alcami, A.; Symons, J. A.; Collins, P. D.; Williams, T. J.; Smith, G. L. *J. Immunol.* **1998**, *160*, 624.
[17] Graham, K. A.; Lalani, A. S.; Macen, J. L.; Ness, T. L.; Barry, M.; Liu, L. Y.; Lucas, A.; Clark-Lewis, I.; Moyer, R. W.; McFadden, G. *Virology* **1997**, *229*, 12.
[18] Smith, C. A.; Smith, T. D.; Smolak, P. J.; Friend, D.; Hagen, H.; Gerhart, M.; Park, L.; Pickup, D. J.; Torrance, D.; Mohler, K.; Schooley, K.; Goodwin, R. G. *Virology* **1997**, *236*, 316.
[19] Lalani, A. S.; Ness, T. L.; Singh, R.; Harrison, J. K.; Seet, B. T.; Kelvin, D. J.; McFadden, G.; Moyer, R. W. *Virology* **1998**, *250*, 173.
[20] Smith, V. P.; Alcami, A. *J. Virol.* **2000**, *74*, 8460.
[21] Simas, J. P.; Efstathiou, S. *Trends Microbiol.* **1998**, *6*, 276.
[22] Parry, C. M.; Simas, J. P.; Smith, V. P.; Stewart, C. A.; Minson, A. C.; Efstathiou, S.; Alcami, A. *J. Exp. Med.* **2000**, *191*, 573.
[23] van Berkel, V.; Barrett, J.; Tiffany, H. L.; Fremont, D. H.; Murphy, P. M.; McFadden, G.; Speck, S. H.; Virgin, H. I. *J. Virol.* **2000**, *74*, 6741.
[24] Roizman, B.; Pellet, P. E. In *D. M. Knipe and P. M. Howley (eds.) Firelds Virology,*; Lippincott-Williams and Wilkins, Philadelphia, PA, **2001**; Vol. *2*, pp. 2381-2397.
[25] Bryant, N. A.; Davis-Poynter, N.; Vanderplasschen, A.; Alcami, A. *EMBO J.* **2003**, *22*, 833.
[26] Lalani, A. S.; Masters, J.; Graham, K.; Liu, L.; Lucas, A.; McFadden, G. *Virology* **1999**, *256*, 233.
[27] Martinez-Pomares, L.; Thompson, J. P.; Moyer, R. W. *Virology* **1995**, *206*, 591.
[28] Reading, P. C.; Symons, J. A.; Smith, G. L. *J. Immunol.* **2003**, *170*, 1435.
[29] Bridgeman, A.; Stevenson, P. G.; Simas, J. P.; Efstathiou, S. *J. Exp. Med.* **2001**, *194*, 301.
[30] van Berkel, V.; Levine, B.; Kapadia, S. B.; Goldman, J. E.; Speck, S. H.; Virgin, H. W. t. *J. Clin. Invest.* **2002**, *109*, 905.
[31] Nakamichi, K.; Matsumoto, Y.; Otsuka, H. *Virology* **2002**, *294*, 22.
[32] Demmin, G. L.; Clase, A. C.; Randall, J. A.; Enquist, L. W.; Banfield, B. W. *J. Virol.* **2001**, *75*, 10856.
[33] Balan, P.; Davis-Poynter, N.; Bell, S.; Atkinson, H.; Browne, H.; Minson, T. *J. Gen. Virol.* **1994**, *75* (Pt. 6), 1245.
[34] Tran, L. C.; Kissner, J. M.; Westerman, L. E.; Sears, A. E. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1818.
[35] Kaashoek, M. J.; Rijsewijk, F. A.; Ruuls, R. C.; Keil, G. M.; Thiry, E.; Pastoret, P. P.; Van Oirschot, J. T. *Vaccine* **1998**, *16*, 802.
[36] Baggiolini, M. *Nature* **1998**, *392*, 565.
[37] Yoshie, O.; Imai, T.; Nomiyama, H. *Adv. Immunol.* **2001**, *78*, 57.
[38] Proudfoot, A. E.; Fritchley, S.; Borlat, F.; Shaw, J. P.; Vilbois, F.; Zwahlen, C.; Trkola, A.; Marchant, D.; Clapham, P. R.; Wells, T. N. *J. Biol. Chem.* **2001**, *276*, 10620.
[39] Rot, A. *Immunol. Today* **1992**, *13*, 291.
[40] Webb, L. M.; Ehrengruber, M. U.; Clark-Lewis, I.; Baggiolini, M.; Rot, A. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 7158.
[41] Tanaka, Y.; Adams, D. H.; Hubscher, S.; Hirano, H.; Siebenlist, U.; Shaw, S. *Nature* **1993**, *361*, 79.
[42] Tanaka, Y.; Adams, D. H.; Shaw, S. *Immunol. Today* **1993**, *14*, 111.
[43] Hoogewerf, A. J.; Kuschert, G. S.; Proudfoot, A. E.; Borlat, F.; Clark-Lewis, I.; Power, C. A.; Wells, T. N. *Biochemistry* **1997**, *36*, 13570.
[44] Martin, L.; Blanpain, C.; Garnier, P.; Wittamer, V.; Parmentier, M.; Vita, C. *Biochemistry* **2001**, *40*, 6303.
[45] Ali, S.; Palmer, A. C.; Banerjee, B.; Fritchley, S. J.; Kirby, J. A. *J. Biol. Chem.* **2000**, *275*, 11721.
[46] Lortat-Jacob, H.; Grosdidier, A.; Imberty, A. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 1229.
[47] Lortat-Jacob, H.; Garrone, P.; Banchereau, J.; Grimaud, J. A. *Cytokine* **1997**, *9*, 101.
[48] Hasan, M.; Najjam, S.; Gordon, M. Y.; Gibbs, R. V.; Rider, C. C. *J. Immunol.* **1999**, *162*, 1064.
[49] Najjam, S.; Mulloy, B.; Theze, J.; Gordon, M.; Gibbs, R.; Rider, C. C. *Glycobiology* **1998**, *8*, 509.
[50] Alvarez-Silva, M.; Borojevic, R. *J. Leukoc. Biol.* **1996**, *59*, 435.
[51] Lipscombe, R. J.; Nakhoul, A. M.; Sanderson, C. J.; Coombe, D. R. *J. Leukoc. Biol.* **1998**, *63*, 342.
[52] Mummery, R. S.; Rider, C. C. *J. Immunol.* **2000**, *165*, 5671.
[53] Salek-Ardakani, S.; Arrand, J. R.; Shaw, D.; Mackett, M. *Blood* **2000**, *96*, 1879.
[54] Clarke, D.; Katoh, O.; Gibbs, R. V.; Griffiths, S. D.; Gordon, M. Y. *Cytokine* **1995**, *7*, 325.
[55] McCaffrey, T. A.; Falcone, D. J.; Du, B. *J. Cell Physiol.* **1992**, *152*, 430.
[56] Schlessinger, J.; Plotnikov, A. N.; Ibrahimi, O. A.; Eliseenkova, A. V.; Yeh, B. K.; Yayon, A.; Linhardt, R. J.; Mohammadi, M. *Mol. Cell* **2000**, *6*, 743.
[57] Burns, J. M.; Dairaghi, D. J.; Deitz, M.; Tsang, M.; Schall, T. J. *J. Biol. Chem.* **2002**, *277*, 2785.
[58] Carfi, A.; Smith, C. A.; Smolak, P. J.; McGrew, J.; Wiley, D. C. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 12379.
[59] Seet, B. T.; Singh, R.; Paavola, C.; Lau, E. K.; Handel, T. M.; McFadden, G. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 9008.
[60] Beck, C. G.; Studer, C.; Zuber, J. F.; Demange, B. J.; Manning, U.; Urfer, R. *J. Biol. Chem.* **2001**, *276*, 43270.
[61] DeLano, W. L.; Ultsch, M. H.; de Vos, A. M.; Wells, J. A. *Science* **2000**, *287*, 1279.
[62] Seet, B. T.; Barrett, J.; Robichaud, J.; Shilton, B.; Singh, R.; McFadden, G. *J. Biol. Chem.* **2001**, *276*, 30504.
[63] Lin, C. L.; Chung, C. S.; Heine, H. G.; Chang, W. *J. Virol.* **2000**, *74*, 3353.
[64] Hsiao, J. C.; Chung, C. S.; Chang, W. *J. Virol.* **1998**, *72*, 8374.
[65] Hsiao, J. C.; Chung, C. S.; Chang, W. *J. Virol.* **1999**, *73*, 8750.
[66] Alexander, J. M.; Nelson, C. A.; van Berkel, V.; Lau, E. K.; Studts, J. M.; Brett, T. J.; Speck, S. H.; Handel, T. M.; Virgin, H. W.; Fremont, D. H. *Cell* **2002**, *111*, 343.
[67] Webb, L. M.; Clark-Lewis, I.; Alcami, A. *J. Virol.* **2003**, *In Press*.
[68] Jensen, K. K.; Chen, S. C.; Hipkin, R. W.; Wiekowski, M. T.; Schwarz, M. A.; Chou, C. C.; Simas, J. P.; Alcami, A.; Lira, S. A. *J. Virol.* **2003**, *77*, 624.
[69] Kuschert, G. S.; Hoogewerf, A. J.; Proudfoot, A. E.; Chung, C. W.; Cooke, R. M.; Hubbard, R. E.; Wells, T. N.; Sanderson, P. N. *Biochemistry* **1998**, *37*, 11193.
[70] Marsden, H. S.; Buckmaster, A.; Palfreyman, J. W.; Hope, R. G.; Minson, A. C. *J. Virol.* **1984**, *50*, 547.
[71] Su, H. K.; Eberle, R.; Courtney, R. J. *J. Virol.* **1987**, *61*, 1735.
[72] Crabb, B. S.; Nagesha, H. S.; Studdert, M. J. *Virology* **1992**, *190*, 143.
[73] Engelhardt, T.; Keil, G. M. *Virology* **1996**, *225*, 126.
[74] Drummer, H. E.; Studdert, M. J.; Crabb, B. S. *J. Gen. Virol.* **1998**, *79* (Pt. 5), 1205.
[75] Liu, L.; Lalani, A.; Dai, E.; Seet, B.; Macauley, C.; Singh, R.; Fan, L.; McFadden, G.; Lucas, A. *J. Clin. Invest.* **2000**, *105*, 1613.
[76] Dabbagh, K.; Xiao, Y.; Smith, C.; Stepick-Biek, P.; Kim, S. G.; Lamm, W. J.; Liggitt, D. H.; Lewis, D. B. *J. Immunol.* **2000**, *165*, 3418.
[77] Rice, J.; de Lima, B.; Stevenson, F. K.; Stevenson, P. G. *Eur. J. Immunol.* **2002**, *32*, 3481.
[78] Horuk, R. *Methods* **2003**, *29*, 369.
[79] Schwarz, M. K.; Wells, T. N. *Nat. Rev. Drug Discov.* **2002**, *1*, 347.
[80] Weyand, C. M.; Kang, Y. M.; Kurtin, P. J.; Goronzy, J. J. *Curr. Opin. Rheumatol.* **2003**, *15*, 259.
[81] Tachibana, K.; Hirota, S.; Iizasa, H.; Yoshida, H.; Kawabata, K.; Kataoka, Y.; Kitamura, Y.; Matsushima, K.; Yoshida, N.;

Nishikawa, S.; Kishimoto, T.; Nagasawa, T. *Nature* **1998**, *393*, 591.

[82] Zou, Y. R.; Kottmann, A. H.; Kuroda, M.; Taniuchi, I.; Littman, D. R. *Nature* **1998**, *393*, 595.

[83] Nagasawa, T.; Hirota, S.; Tachibana, K.; Takakura, N.; Nishikawa, S.; Kitamura, Y.; Yoshida, N.; Kikutani, H.; Kishimoto, T. *Nature* **1996**, *382*, 635.

[84] Chen, S.; Bacon, K. B.; Li, L.; Garcia, G. E.; Xia, Y.; Lo, D.; Thompson, D. A.; Siani, M. A.; Yamamoto, T.; Harrison, J. K.; Feng, L. *J. Exp. Med.* **1998**, *188*, 193.

[85] Ghirnikar, R. S.; Lee, Y. L.; Eng, L. F. *J. Neurosci. Res.* **2000**, *59*, 63.

[86] Holzknecht, Z. E.; Platt, J. L. *Nat. Med.* **2000**, *6*, 497.

[87] Takami, S.; Minami, M.; Nagata, I.; Namura, S.; Satoh, M. *J. Cereb. Blood Flow Metab.* **2001**, *21*, 1430.

Copyright of Mini Reviews in Medicinal Chemistry is the property of Bentham Science Publishers Ltd.. The copyright in an individual article may be maintained by the author in certain cases. Content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.