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Mini review

Chemokine binding proteins: An immunomodulatory strategy going viral

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ABSTRACT

Chemokines are chemotactic cytokines whose main function is to direct cell migration. The chemokine network is highly complex and its deregulation is linked to several diseases including immunopathology, cancer and chronic pain. Chemokines also play essential roles in the antiviral immune response. Viruses have therefore developed several counter strategies to modulate chemokine activity. One of these is the expression of type I transmembrane or secreted proteins with the ability to bind chemokines and modulate their activity. These proteins, termed viral chemokine binding proteins (vCKBP), do not share sequence homology with host proteins and are immunomodulatory *in vivo*. In this review we describe the discovery and characterization of vCKBP, explain their role in the context of infection *in vivo* and discuss relevant novel findings.

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1. Introduction

1.1. Chemokines

Chemokines are small, basic cytokines that orchestrate the migration of leukocytes during development, homeostasis, tissue damage and infection [1]. Deregulation of chemokine function plays a key role in cancer development, immunopathologies and induction of pain [2,3]. Chemokines are secreted, with the exception of CXCL16 and CX3CL1, which are transmembrane proteins and can be shed following cleavage (reviewed in [4]). Interaction with glycosaminoglycans (GAGs) on the cell surface is required for chemokine retention on the endothelium, presentation to the chemokine receptor and thereby activity *in vivo* [5,6]. Binding of the chemokine to its receptor at the leukocyte plasma membrane triggers signalling cascades leading to a coordinated reorganization of the cytoskeleton, activation of adhesion molecules and leukocyte extravasation [7] (Fig. 1). Chemokines can interact with both GAGs and the chemokine receptor simultaneously through distinct domains, although for some chemokines these domains may overlap [8,9]. Chemokine receptors are 7 transmembrane G protein-coupled receptors (GPCR) that signal through heterotrimeric G proteins, normally of the Gαi-type. There are, however, atypical chemokine receptors that act as chemokine scavengers and do not induce G protein signalling [10]. For a detailed description of the nature and nomenclature of atypical chemokine receptors see the report by Bachelerie and colleagues [11].

Chemokines form the largest family of cytokines, with approximately 50 chemokines and 20 chemokine receptors discovered to date [10]. Most receptors interact with more than one chemokine and most chemokines use more than one receptor [10]. This peculiarity led to the assertion that there is a high level of redundancy in the chemokine network. This notion is partially supported by failure of therapeutic strategies aimed at blocking single chemokine activity and by the use of knock out and transgenic mouse models. However, several sets of data indicate that there is a certain degree of selectivity in the chemokine network. This selectivity seems to be achieved by (i) biased signalling; (ii) differential interaction with GAGs; (iii) effect of GAG binding on chemokine oligomerization and (iv) chemokine–chemokine interactions [5,12–15].

Chemokines are classified as homeostatic, inflammatory or dual function according to their main functional activity and as CXC, CC, C, and CX3C according to structural criteria based on the relative position of their N-terminal cysteines [13,16]. Inflammatory chemokines are essential in controlling the recruitment of leukocytes during inflammation whereas homeostatic chemokines are involved in directing the migration of leukocytes during development, adaptive immune response, and in peripheral healthy tissues [13]. Homeostatic chemokines seem to be less promiscuous and more conserved between species than

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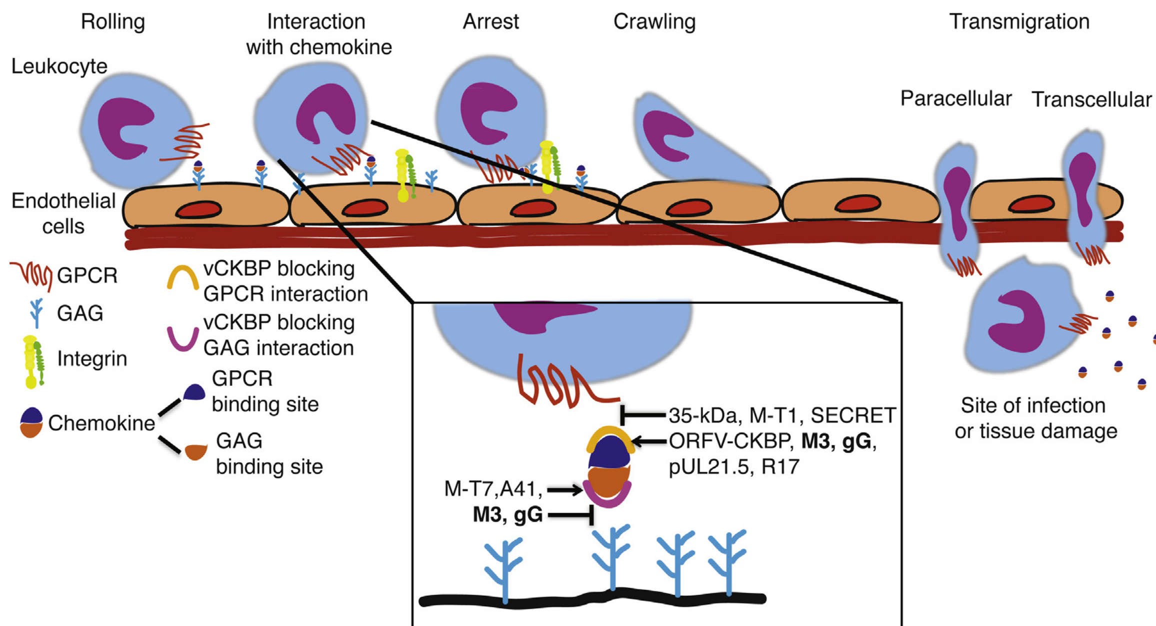

Fig. 1. Viral chemokine binding proteins (vCKBP) interfere with chemokine-mediated migration of leukocytes to the site of infection or tissue damage. Leukocytes rolling on the surface of endothelial cells detect GAG-bound chemokines. The interaction of the chemokine with the GPCR of the rolling leukocyte activates signalling cascades in the leukocyte. Arrest, crawling and transmigration depend also on adhesion molecules, mainly integrins and selectins. The process terminates with the para- or transcellular migration of the leukocyte to the site of injury or infection [7]. Inlet: vCKBP modulate chemokine activity through interacting with the GPCR-, GAG-binding domain of the chemokine or both (in bold). Examples for both types of vCKBP are indicated. Interaction with the GPCR-binding site results in inhibition of chemokine activity *in vitro* and *in vivo*. Binding to the GAG-binding site of the chemokine may affect chemokine retention at the cell surface and generation of a chemotactic gradient inhibiting chemokine activity *in vivo*. An exception to this rule is HSV gG, which interacts through the GAG-binding domain of the chemokine and enhances chemokine activity *in vitro* and *in vivo*.

inflammatory chemokines, probably reflecting the evolutionary pressure exerted on the latter by different types of pathogens [13]. Dual function chemokines include those that share functions of both inflammatory and homeostatic chemokines [16]. Chemokine receptors show a higher degree of conservation between mammals than their ligands and they are classified according to the chemokine group they interact with [17].

Despite differences in sequence, chemokines share some structural features: a long, flexible N-terminal loop followed by a three-stranded β sheet and a C-terminal α helix [18]. Recently, the structure of chemokines bound to their receptors was solved [19,20]. These studies reported the crystal structure of human CXCR4 complexed with the Kaposi's sarcoma-associated herpesvirus (KSHV) chemokine vMIP-II [20], and the structure of human cytomegalovirus (HCMV) chemokine receptor US28 bound to CX3CL1 [19]. In both cases the core of the chemokine interacts with the receptor N-terminus whereas the chemokine N-terminal residues bind to the transmembrane pocket of the receptor [19,20]. The predicted model suggests a two-step interaction of the chemokine with its receptor, triggering conformational changes in the latter leading to insertion of the N-terminal residues of the chemokine between the transmembrane helices [21].

1.2. Viral chemokine binding proteins (vCKBP)

Due to the essential role of chemokines in the antiviral response, some viruses express proteins that are able to interfere with the host chemokine network, modulating its activity and thereby interfering with leukocyte migration (Table 1). To do so they express viral chemokine receptors, viral chemokines and soluble receptors interfering with extracellular chemokines [22]. Viral chemokine receptors and viral chemokines share high degree of identity with host proteins suggesting that the virus has acquired them from the host and modified them in a process termed viral piracy (reviewed in Ref. [22]). However, in the case of the soluble receptors, also known as vCKBP, there is very little or no sequence identity with host proteins. Moreover, the amino acid homology between the distinct vCKBP in different viruses is very low or non-existent [23]. Despite this, the crystal structures of several vCKBP show common structural patterns [24,25], probably due to parallel evolution as suggested by Lubman and Fremont [24].

All known vCKBP have been so far discovered in members of the *Pox* and *Herpesviridae* families. Similar to chemokines, most vCKBP are secreted proteins but some are also structural proteins present at the viral envelope or at the plasma membrane of infected cells [26–29]. Also, like the chemokines, some vCKBP interact with GAGs and this seems to be relevant for their function [30–33]. While the majority of known vCKBP inhibits chemokine activity *in vitro* or *in vivo*, a vCKBP with the ability to potentiate chemokine function was found recently in herpes simplex virus type 1 and 2 (HSV-1 and HSV-2, respectively) [27]. In the following paragraphs we discuss the properties of the different vCKBP groups in more detail.

2. Discovery and characteristics of vCKBP

2.1. vCKBP that inhibit chemokine activity

All but one of the vCKBP described to date inhibit chemokine activity *in vitro* or *in vivo*. To inhibit chemokine activity, vCKBP bind to the chemokine through either its chemokine receptor- or its GAG-binding pocket or both, thereby impairing the interaction between the chemokine and its receptor or GAGs (Fig. 1). Impairment of GPCR binding can be functionally addressed *in vitro* by performing classical transwell assays. However, when the interaction takes place exclusively through the GAG-binding pocket of the chemokine, this type of assay may not provide information regarding the inhibitory properties of the vCKBP. Nevertheless, both types of binding impairment may result in inhibition of chemotaxis *in vivo*. The known vCKBP are described

Table 1
Interactions and effects of vCKBP.

	vCKBP	Virus	Interacting partners	Targeted chemokine domain	Effect of Δ-vCKBP virus *in vivo*	References
Inhibitory vCKBP	35-kDa	Orthopoxviruses: Camelpox, CPXV, Rabbitpox, Racoonpox, VACV Lister, VARV	CC chemokines, CXCL1 and CXCL8 (low affinity), GAGs (MYXV M-T1 (canonical motif)	GPCR binding domain	chemotaxis↑, immunopathology↑, viral replication at low MOI↑, Leukocyte infiltration↑,	[30,34–39,41–43,81–83]
	T1	Leporipoxviruses: MYXV, Shope fibroma virus; (homolog to 35-kDa)				
	M-T7	Leporipoxvisuses: MYXV	IFN-γ; CXC, CC, C chemokines	GAG binding domain	Viral pathogenicity↓, viral spread↓, leukocyte infiltration↑	[45–48]
	ORFV-CKBP	Parapoxviruses: ORFV	CC chemokines, CL1	GPCR binding domain	Not tested	[50–52]
	Crm SECRET domain	Orthopoxviruses: CrmB (VARV, CPXV), CrmD (CPXV, ECTV),	CC, CXC Chemokines, CrmB: CL1 (low affinity), CrmD: CX3CL1 (low affinity)	GPCR binding domain	Virulence↓	[53,55,84]
	SCP	Orthopoxviruses: CPXV (SCP-1, V128), ECTV(E12, E184), VACV (B7R)	CC, CXC chemokines	GPCR binding domain	Lesion size↓	[53,86]
	M-T2C-terminal domain (predicted)	Leporipoxvisuses: MYXV (CrmB structural homolog)	Chemokine binding predicted but not tested, except for CCL5 (no interaction)	GPCR binding domain (predicted)	Virus highly attenuated	[47,85]
	A41 E163	Orthopoxviruses: VACV (A41) ECTV (E163)	Some CC chemokines, CXCL9, CXCL10, CXCL11 (Weak interaction), GAGs (canonical motif) for E163	GAG binding domain	A41: Leukocyte infiltration↑, viral clearance↑, CD8^{+}T-cell response↑ E163: Not tested	[31,56–58]
	M3	*Gammaherpesvirinae*: MHV-68	C, CC, CXC, CX3C chemokines	GPCR binding domain and GAG binding domain	C57BL/6: Attenuation in CNS, altered lymphoid infiltrate BALB/c: viral load↓, B-cell activation↓ Wood mice: chemokine activity↓, lymphoid tissue (iBALT, splenic follicles)↓ Latency↓	[59–62,80,87–89]
	R17	*Gammaherpesvirinae*: RHVP	C, CC chemokines, GAGs (canonical BBXB motif)	GPCR binding domain	Not tested	[24,32]
	pUL21.5	*Betaherpesvirinae*: HCMV	CCL5, others not tested	GPCR binding domain	Not tested	[65]
	gG	*Alphaherpesvirinae*: EHV-1, BHV-1, BHV-5, FeHV-1, ILTV, PRV	C, CC, CXC chemokines (different set of chemokines depending on the virus)	GPCR binding domain and GAG binding domain	Leukocyte infiltration↑, Immuno-pathology↑	[26,27,68–73,91]
Enhancing vCKBP	gG	*Alphaherpesvirinae*: HSV-1 and HSV-1	CC, CXC chemokines, neurotrophic factors, GAGs (non-canonical motif)	GAG binding domain	HSV-1: viral replication↓ HSV-2: not tested	[27,33,78,92,93,95]

below, starting with those expressed by poxviruses followed by those found in herpesviruses. In both cases they appear in chronological order of discovery.

2.1.1. Ortho and leporipoxvirus 35-kDa/T1

Most poxviruses from the *Orthopoxvirus* or *Leporipoxvirus* genera express a protein with the ability to interact with CC chemokines [34–36]. This vCKBP expressed by orthopoxviruses is termed 35-kDa whereas in leporipoxviruses is termed T1. Collectively, these two proteins are called viral CC chemokine inhibitor (vCCI) because they target nearly exclusively CC chemokines. The binding activity was initially discovered by performing crosslinking experiments with radio-iodinated chemokines and supernatants from cells infected with several viruses or by the use of surface plasmon resonance (SPR) with purified protein. Binding activities were found in the orthopoxviruses camelpox, cowpox (CPXV), rabbitpox, raccoonpox, vaccinia virus (VACV) strain Lister, variola virus (VARV) and the leporipoxviruses myxoma virus (MYXV) and Shope fibroma virus [34–36]. Despite the low amino acid sequence identity (40% between T1 and 35-kDa), a study comparing 35-kDa from VACV Lister strain and T1 from MYXV showed that they share similar binding and functional properties [37]. Binding to CC chemokines is of high affinity and specific since members of other chemokine subfamilies are not targeted by 35-kDa, with the exception of low affinity binding to CXCL1 and CXCL8 [35–38]. This selectivity is probably due to the presence of conserved epitopes in the CC chemokines [39]. Interaction with CC chemokines results in inhibition of chemokine activity *in vitro* and *in vivo* through impairment of the chemokine-GPCR interaction [35–37,40]. The chemokine residues responsible for GPCR interaction are shared with the vCKBP as shown using CCL2 mutants [41,42]. Structural and site-directed mutagenesis studies have shown that VACV 35-kDa β-sheet II binds to the N-terminal region and residues within the 20's region and 40's loop of the chemokine [43]. Apart from binding chemokines, MYXV T1 (M-T1) also binds to cell surface GAGs through basic residues located in its C-terminal domain, an ability not present in VACV 35-kDa [30]. M-T1 contains two canonical GAG-binding motifs, XBBXBX and XBBBXXBX where B indicates a basic residue and X any amino acid, characteristic of heparin-binding proteins [44]. This permits

M-T1 to bind simultaneously GAGs and chemokines, probably inhibiting chemokine activity in the proximity of infected cells *in vivo* [30].

2.1.2. Myxoma virus M-T7

The discovery of the second vCKBP expressed by leporipoxviruses came following the finding that MYXV lacking expression of M-T7, a protein that binds and inhibits IFN-γ [45], had higher leukocyte infiltration in the site of virus replication *in vivo* [46]. Later, the secreted M-T7 was shown to act as a broad range binding protein of CXC, CC and C chemokines through the chemokine GAG-binding site [47]. M-T7 probably disrupts the GAG-chemokine interaction required for chemokine activity *in vivo* [47]. Binding to chemokines and IFN-γ seems to occur through overlapping or neighbouring sites in M-T7 [47], making it difficult to address each activity independently *in vivo*. However, since the IFN-γ activity is rabbit-specific whereas M-T7 binds chemokines of different species [47,48], the relevance of chemokine inhibition was proven in several *in vivo* models using rodents [40,49].

2.1.3. The orf virus CKBP

The only vCKBP discovered to date in a poxvirus belonging to a genus other than *Orthopoxvirus* or *Leporipoxvirus* was described in orf virus (ORFV), a member of the *Parapoxvirus* genus. This vCKBP was discovered due to sequence homology of ORFV with granulocyte–macrophage colony-stimulating factor/IL-2 inhibitory factor protein [50]. This sequence encodes a vCKBP that, despite low sequence identity to the 35-kDa/T1 family of vCKBP, binds CC chemokines with high affinity as shown by SPR [50]. However, its binding range is wider, being also able to interact with CL1 [50]. ORFV-CKBP inhibits chemokine-induced calcium mobilization *in vitro* [50] and chemotaxis *in vitro* and *in vivo* in an LPS-induced inflammation model [51,52]. The mechanism of action involves interaction with the receptor-binding site of the chemokine [50].

2.1.4. Orthopoxvirus SECRET domain

Most poxviruses express soluble receptors of TNF termed cytokine response modifiers (Crm) [53]. There are four Crms (CrmB-E) with the ability to bind to and inhibit TNF through a conserved N-terminal, cysteine-rich domain, which is similar to the cellular TNF receptor [54]. Interestingly, CrmB and CrmD have a C-terminal domain of unknown function and with no sequence similarity to host proteins. The work by Alejo and coworkers elegantly showed that this C-terminal domain was able to bind chemokines. They termed this novel domain SECRET for smallpox virus-encoded chemokine receptor [53]. Both VARV CrmB and ectromelia virus (ECTV) CrmD can bind simultaneously TNF and chemokines and inhibit their functional activities *in vitro* making these Crms excellent immunomodulatory tools. The SECRET domain is also present in other poxvirus proteins termed SECRET-containing proteins (SCP) that lack the TNF-binding domain. There are 3 SCP described in poxviruses, with some viruses expressing more than one. CPXV CrmB and SCP-1, VARV CrmB, and ECTV CrmD inhibit chemokine activity [53]. CrmB,

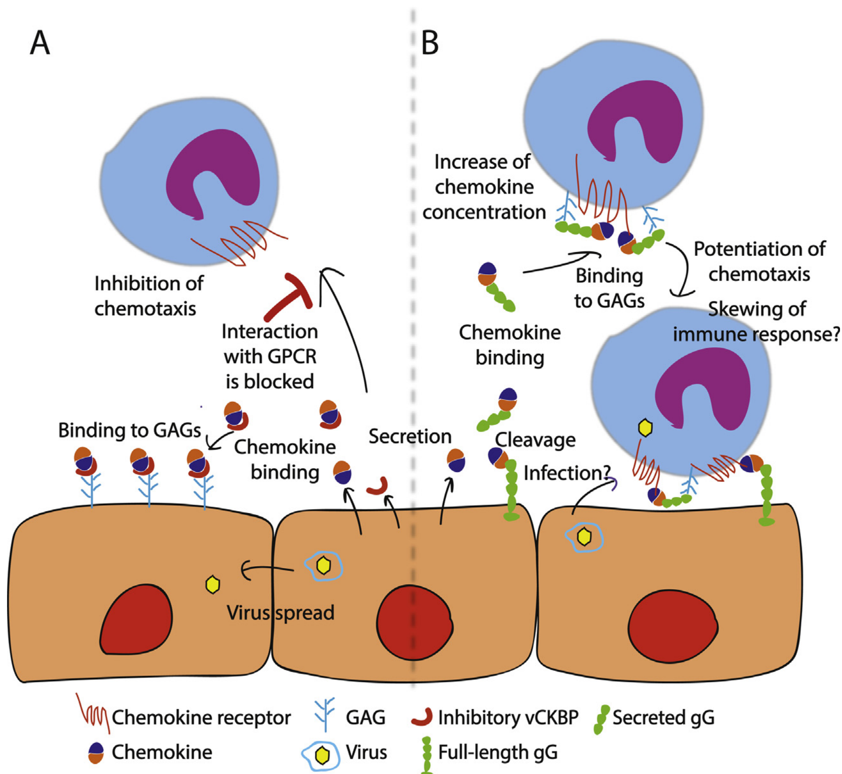

Fig. 2. Two possible outcomes of vCKBP-GAG interactions. (A, B) Certain viruses express vCKBP that modulate chemokine activity and have also the ability to bind GAGs. The functional relevance of vCKBP-GAG interaction is not well characterized. However, depending on the nature of the vCKBP, at least two scenarios are possible. (A) vCKBP that inhibit chemotaxis: Some vCKBP that interact with the chemokine through its receptor-binding site can bind to GAGs at the surface of the infected and neighbouring cells. The chemokine is thereby retained in the cell surface and cannot interact with the GPCR at the plasma membrane of the leukocyte, inhibiting chemotaxis and facilitating virus spread to the neighbouring cells. (B) vCKBP that enhances chemotaxis: HSV gG is expressed as a type I transmembrane protein (for HSV-1) that sheds the secreted N-terminal domain following proteolytic cleavage (for HSV-2). Both transmembrane and secreted gG interact with chemokines. Interaction with the chemokine takes place through the GAG-binding domain of the chemokine and results in potentiation of chemokine activity. HSV gG can simultaneously bind to the chemokine and GAGs, increasing the concentration of the chemokine in the proximity of the GPCR, modifying its activity and potentiating chemokine function. This could facilitate the infection of particular subsets of leukocytes or it could modify the nature of the infiltrating leukocytes, dampening the local immune response. None of these hypotheses have been tested yet.

CrmD and the analyzed SCP (CPXV V218, ECTV E12 and E184) bind a similar subset of chemokines from the CXC and CC subfamilies with nanomolar affinities [53]. CrmB can also interact with CL1 and the CrmD SECRET domain also binds CX3CL1 with low affinity, as shown in a study solving the crystal structure of the complex [55]. The fact that several poxviruses express more than one vCKBP targeting similar chemokines points to the relevance of these chemokines in their viral cycle. Further characterization of the different proteins *in vivo* is required to decipher the reasons of such an apparent redundancy.

2.1.5. Orthopoxvirus A41

A secreted protein of 30 kDa, A41, expressed by orthopoxviruses has the ability to bind chemokines. Although its immunomodulatory role was clear from previous studies [56,57], its role as a vCKBP was not discovered until 2008. An initial report showed that A41 from VACV Western Reserve (WR) was not required for *in vitro* replication but played a role in pathogenesis [56] (see below). In that report, a role for A41 in inhibiting leukocyte migration *in vivo* was postulated. However, despite these results, the use of recombinant protein could not demonstrate the interaction of A41 with a limited number of chemokines by SPR [56]. Weak interaction with CXCL9, CXCL10 and CXCL11, the ligands of CXCR3, was observed although chemotaxis induced by these ligands was not inhibited by A41 [56]. Later, a more extensive chemokine screening showed that VACV A41 interacts with a limited number of CC chemokines [58]. Similarly, the ECTV E163 protein, an ortholog of VACV A41, binds a limited number of CC and CXC chemokines with high affinity [31]. The interaction of the A41 family of vCKBP with chemokines takes place through the GAG-binding domain of the chemokine [31,58]. Therefore, as expected, A41 does not inhibit chemokine migration in transwell assays since the contribution of GAG-binding to chemokine activity cannot be measured with this type of assay. However, previous results [56,57] suggest that it does so *in vivo*, probably by disrupting chemokine retention and presentation by GAGs. ECTV E163 can also interact with GAGs providing the possibility of vCKBP retention, and disruption of chemokine activity in the proximity of the infected cells [31] (Fig. 2).

2.1.6. Murine gammaherpesvirus 68 M3

The first vCKBP described in herpesviruses was M3, a 44-kDa secreted glycoprotein, expressed by the gammaherpesvirus murine herpesvirus 68 (MHV-68) [59,60]. M3 is a broad-spectrum vCKBP, interacting with high affinity with members of all four chemokine families although the affinities for CXC chemokines are lower compared with those for others, suggesting still some kind of specificity [59,60]. M3 blocks receptor binding by interacting with the N-terminus of the chemokine [59,61] and inhibits chemokine-mediated calcium mobilization and chemotaxis *in vitro* [59–61]. Further characterization revealed that M3 is also able to interact with the GAG-binding site of the chemokine and displace chemokines already bound to heparin [62]. These results indicate that M3 could inhibit also the formation of GAG-dependent chemokine gradients *in vivo*. Due to its broad binding activity, M3 has been widely used as a tool to decipher the role of the chemokine network during homeostasis and in disease models [63,64].

2.1.7. Human cytomegalovirus pUL21.5

HCMV, a betaherpesvirus containing the largest double stranded DNA genome among human viruses, expresses pUL21.5, a small secreted protein with high affinity for human CCL5 [65]. HCMV is a nearly ubiquitous pathogen that establishes latency in cells of the myeloid lineage. HCMV does not cause serious illness in healthy individuals but is a serious threat for immunocompromised patients, such as transplant recipients, and for developing fetuses or newborns. The interaction between pUL21.5 and CCL5 inhibits binding of the chemokine to its receptor, probably inhibiting migration, although this was not formally proven [65]. As only a small number of chemokines were included in the binding experiment it is still not clear whether this protein can bind chemokines other than CCL5.

2.1.8. Rodent herpesvirus Peru R17

The protein expressed by open reading frame 17, R17, of the gammaherpesvirus rodent herpesvirus Peru (RHVP), is a novel vCKBP [32]. R17 interacts with both CC and C chemokines with nanomolar affinity, but not with CX3C and the 6 CXC chemokines tested by SPR. R17 blocks chemokine-induced chemotaxis and calcium influx *in vitro* probably through inhibition of chemokine interaction with its receptor [32]. Interestingly, R17 enhances chemokine binding to cell surface GAGs through a simultaneous interaction with both chemokines and GAGs. Interaction of R17 with GAGs takes place through two consensus BBXB motifs not involved in chemokine binding [32]. These two sets of results indicate that R17 acts similarly to M-T1, interacting with plasma membrane GAGs to inhibit chemokine activity in the proximity of infected cells [30] (Fig. 2).

2.1.9. Glycoprotein G of animal alphaherpesviruses

In alphaherpesviruses so far only one vCKBP has been identified, glycoprotein G (gG), encoded by the *US4* ORF [28]. The gene encoding gG is present in most human and animal alphaherpesviruses with the notable exceptions of varicella zoster virus (VZV) and Marek's disease virus [66,67]. Chemokine binding activity was detected in the supernatant of cells infected by several animal alphaherpesviruses including equine herpesvirus 1 (EHV-1), bovine herpesvirus 1 and 5, and shown to correspond to gG [28]. Further reports showed similar activity for the gG of Felid herpesvirus 1 (FeHV-1), infectious laryngotracheitis virus (ILTV) and pseudorabies virus (PRV) [26,68,69]. The interaction of gG with CC, CXC and C chemokines is of high affinity as shown by SPR [26,28,68,69]. However, there is selectivity in the interaction with chemokines since not all the gG proteins interact with the same chemokines or members of the same chemokine subfamily. gG expressed by animal alphaherpesviruses inhibits chemokine activity *in vitro* [26,28,68–70] and *in vivo* as shown for EHV-1, ILTV and PRV [27,70–72] by blocking the interaction of the chemokine with its receptor [26,28,69]. Similar to MHV-68 M3, gG can also interact with the GAG-binding domain of the chemokine probably inhibiting gradient formation and chemokine presentation to the chemokine receptor *in vivo* [28,69]. The fact that gG from EHV-1 but not EHV-4 binds chemokines permitted the identification of the gG residues involved in chemokine interaction [73]. The hypervariable region of EHV-1 gG is required for chemokine interaction and N-glycosylation is essential for inhibitory activity [73]. For other alphaherpesviruses no binding or functional domains in gG have been characterized so far.

An interesting property of alphaherpesvirus gG compared to other vCKBP is that it is a type I transmembrane protein that sheds a secreted domain following proteolytic cleavage. This does not apply to HSV-1 gG, which is not secreted [74]. Most experiments regarding the chemokine binding activity of gG have been performed with the secreted form. However, transmembrane gG also binds chemokines at the surface of the cell [26–28] and, at least for FeHV, also on the viral envelope [29]. The functional relevance of chemokine interaction in these settings is unknown. It has been proposed that transmembrane gG may act as a chemokine sink or may signal following chemokine interaction [26], although there are no results to prove either hypotheses.

Chemokine-gG interaction at the viral envelope of FeHV did not play a role in the infection of feline kidney cells [29].

2.2. vCKBP that enhance chemokine activity

The vCKBP described so far inhibit chemokine activity by preventing formation of the chemokine gradient or blocking receptor interaction. Interestingly, in contrast to this, the vCKBP gG encoded by the human alphaherpesviruses HSV-1 and HSV-2, has been shown to enhance chemokine activity *in vitro* and *in vivo* [27]. Both proteins bind a limited number of chemokines with high affinity through their GAG-binding pocket and enhance the activity of hCXCL12-β and hCXCL13 *in vitro* and hCXCL12-α and hCCL28 *in vivo*. Interaction with the chemokine is required for this activity since gG does not have chemotactic properties when used alone or together with a chemokine not bound by gG [27]. HSV gG is also able to interact with GAGs [33], despite gG lacking canonical GAG-binding motifs. Binding to GAGs results in higher level of chemokine localized in the proximity of the chemokine receptor, indicating that gG can interact with GAGs and chemokines simultaneously [33] (Fig. 2). The mechanism of action of HSV gG also involves modification of receptor localization at the plasma membrane, clustering, signalling and internalization [27,33]. The fact that HSV gG enhances chemotaxis is intriguing since one would expect that viral proteins would aim at inhibiting chemokines due to their antiviral role. However, enhancing leukocyte migration can also be pro-viral depending on the specific requirements in the viral life cycle as other viruses have been shown to express viral chemokines with agonist activity that enhance leukocyte migration. Examples include KSHV vCCL1 and vCCL2 [75,76]. Most of these viruses have the ability to infect leukocytes whereas this is not a property of HSV, with the exception of dendritic cells whose infection results in impairment of dendritic and T cell activity [77]. In this regard, it is important to recall that chemokine receptors are not limited to leukocytes. They are also expressed in epithelial cells and neurons, two cell types that are essential in HSV biology and pathogenesis. The modulation of chemokine activity through GPCR signalling could play a role in these cellular settings, facilitating infection of epithelial cells or neurons. In this context HSV gG is a structural protein present at the viral envelope. Whether it binds chemokines like FeHV-1 gG and enhances their activity in this setting is currently unknown. However, one could envisage that the interaction of the virion with chemokines could play a role during cell entry through enhancing the activation of GPCR-dependent signalling pathways, modulating them to benefit viral gene expression and replication. Furthermore, alphaherpesviruses establish latency in neurons and this is required for their *in vivo* persistence and pathogenesis. Interestingly, HSV-1 and HSV-2 gG binding activity is not limited to chemokines. They also bind neurotrophic factors including nerve growth factor (NGF). However, only HSV-2 gG enhances NGF activity [78]. Whether this activity has any relevance in the infection of particular subsets of neurons deserves further investigation.

2.3. vCKBP interaction with GAGs

As mentioned above some vCKBPs also show high affinity for GAGs, underscoring the relevance of these polysaccharides in chemokine activity. Interestingly, this has been shown both for inhibitory vCKBP that interfere with GPCR or GAG interaction such as RHVP R17, ECTV E163 and MYXV M-T1, and for a potentiating vCKBP, gG of HSV [30–33]. Consensus GAG-binding motifs are present in chemokines and also in some vCKBP such as RHVP R17, ECTV E163 and MYXV M-T1 [30–32]. Other vCKBP that interact with GAGs such as HSV gG, do not contain known canonical GAG-binding sites. Therefore, interaction with GAGs must take place through other motifs or through the generation of basic stretches in their quaternary structure. M-T1, R17 and HSV gG can interact simultaneously with chemokines and GAGs [30,32,33]. It is predicted that GAG-binding permits inhibitory vCKBP to act locally in the proximity of the infected cells, facilitating virus spread (Fig. 2). In case of potentiating vCKBP HSV gG, binding to GAGs may facilitate the interaction of the viral protein with the plasma membrane allowing it to increase the concentration of the chemokine in the proximity of the chemokine receptor. This, together with its ability to modulate the biology of the chemokine receptor could potentiate chemokine activity [33] (Fig. 2). The possible benefit of chemokine potentiation for HSV is discussed in Section 2.2.

2.4. Structural features of vCKBP

The crystal structure of several vCKBP, alone or complexed with chemokine, has been solved. A thorough analysis of these structures helps understanding the molecular mechanism of action of vCKBP and how such a divergence in amino acid sequence is able to result in proteins with similar activity. Due to space constraints only a few important considerations are included here. The reader is referred to relevant manuscripts and references therein for further insight [24,25]. The current knowledge so far leads to the conclusion that several poxviral immunomodulatory proteins share a common fold consisting of a globular β-sandwich formed from two nearly parallel β sheets connected by loops [25]. This fold has not been previously observed in prokaryotic or eukaryotic proteins [25]. Proteins containing this structure signature include 35-kDa [79], A41 [58], SECRET [55] and it is predicted to be present in a large number of poxviral proteins with possible or confirmed immunomodulatory activity [25]. The characteristics of this fold, tentatively termed poxvirus immune evasion (PIE) domain, and the structures of different vCKBP have been reviewed elsewhere [24,25]. Similarly, comparative analysis of the crystal structures of chemokine receptors and CKBP coupled to ligands showed that they all interact with a disulfide present in all chemokine subfamilies [24]. The structures of two herpesvirus vCKBP, M3 and R17, coupled to chemokine have been solved [24,80]. An important difference is that M3 binds CCL2 as a dimer whereas a monomer of R17 is present in the crystal bound to CCL3. Nevertheless, they both share a similar structural scaffold to interact with the chemokine, despite their very low amino acid identity [24].

3. Relevance of vCKBP in the context of infection *in vivo*

The role of several vCKBP during infection *in vivo* has been extensively studied with several animal viruses in their natural host or using mouse models as surrogate for other species. This is not always possible since some of these viruses are species specific (*i.e.*, HCMV). The outcome of vCKBP deletion is normally unpredictable due to the multiple interactions with chemokines. This may cause lower disease due to virus attenuation or higher disease due to immunopathology.

VACV was used in the vaccination campaign led by the world health organisation to eradicate smallpox. VACV strains are widely studied and used as vaccine vectors for infectious diseases. Different strains have diverse immunomodulatory activities since they express different immune modulators. This has facilitated the investigation of the functional role of some of these proteins. For instance, insertion of the gene encoding the 35-kDa vCKBP from VACV Lister strain into the 35-kDa negative strain WR attenuates the virus in an intranasal mouse model of infection [81]. Expression of 35-kDa results in lower migration of inflammatory

cells into the lungs of infected mice, accompanied by lower virus replication, spread and reduced mortality. Moreover, the bronchoalveolar lavage of mice infected with the 35-kDa-expressing virus shows lower chemotactic activity, probably due to the inhibitory function of the vCKBP [81]. Intranasal infection of BALB/c mice with a rabbitpox lacking the 35-kDa protein results in higher pathogenicity than wild type infection at sub-lethal but not at higher doses of infection [82]. Other parameters of infection such as size of primary lesions or virus spread from skin to the lungs are not affected, indicating that this vCKBP is not a virulent factor in this animal model [82]. However, rabbits infected with the deletion mutant suffer a profound inflammatory response [34] indicating that rabbitpox 35-kDa inhibits chemotaxis *in vivo* in its natural host.

MYXV causes myxomatosis in rabbits, a lethal, systemic disease associated with immunosuppression. The functional role of two MYXV vCKBP, M-T1 and M-T7, has been analysed *in vivo*. M-T1 inhibits leukocyte migration during MYXV infection in its natural host. Lack of M-T1 expression does not affect virulence but results in increased leukocyte infiltration [83]. The IFN-γ and chemokine inhibitor M-T7 has also been studied *in vivo* in European rabbits [46]. Disruption of M-T7 expression attenuates the virus, resulting in lower disease symptoms and reduced spread to secondary infection sites. Leukocyte infiltration to the site of infection is evident in the dermis of rabbits infected with the M-T7 defective mutant whereas it is blocked in the wild type virus [46]. Moreover, lymph node, spleen and other secondary lymphoid organs contain high numbers of activated lymphocytes in animals infected with the defective virus [46]. These phenotypes suggest that M-T7 interaction with chemokines plays a role in the inhibition of leukocyte infiltration to the site of infection and the mounting of appropriate adaptive immune responses [46]. However, since M-T7 inhibits both IFN-γ and chemokines, the sole contribution of chemokine inhibition in pathogenesis could not be clarified in this study.

Initial attempts to discover the ligands of A41 were unsuccessful [56]. However, deletion of *A41L* gene from VACV WR results in a virus causing larger skin lesions and being cleared faster from the infected mice [56]. Similar experiments in rabbits show that, despite similar lesion size, lack of A41 protein results in higher leukocyte infiltration indicating that, as 35-kDa, A41 inhibits chemotaxis *in vivo* and its deletion facilitates virus clearance [56]. Another report using also VACV WR devoid of *A41L* showed that the deletion results in a mild increase in virulence following intranasal infection of mice [57]. In this model, and also when using VACV modified virus Ankara strain lacking *A41L* to immunize mice, cytotoxic and VACV-specific memory $CD8^+$ T cells are increased in the spleen [57]. Since $CD8^+$ T cells play a protective role against poxviruses, vaccination with VACV lacking A41L provides better protection than the parental VACV to a challenge with VACV WR [57].

The SECRET domain is present in several poxviruses including VARV [53]. Research with VARV is strictly limited due to its high infection and mortality rate in humans. CPXV, ECTV and monkeypox virus represent good models to study lytic viral infections in general and VARV pathogenesis in particular. The only report addressing the function of CrmB *in vivo* showed that CrmB from CPXV strain Brighton Red is a virulence factor in an intracranial model of mouse infection [84]. However, the role of the chemokine binding activity of CrmB SECRET domain was not specifically addressed. Another poxviral TNF receptor, M-T2 expressed by MYXV, shares a similar domain structure to CrmB and CrmD containing the TNF-binding domain followed by a C-terminal domain with no sequence homology to host proteins. Disruption of M-T2 expression results in a highly attenuated MYXV unable to cause lethal disease in most rabbits [85]. Moreover, survivor rabbits are protected against subsequent challenges with wild type MYXV [85]. However, the potential role of M-T2 C-terminal domain as a chemokine inhibitor has only been addressed for CCL5 with negative results [47]. Deletion of VACV *B7R*, an SCP-3, does not affect virulence in an intranasal mouse model of infection. However, there is a decrease in lesion size in an intradermal infection model in the ear pinnae when compared with wild type virus [86]. Overall, the relevance of the SECRET domain in pathogenesis is still unknown.

MHV-68 is a natural pathogen of murine species belonging to the *Apodemus* genus such as the wood mice. MHV-68 intranasal infection of mice can result in pneumonia during acute infection, normally followed by establishment of latency mainly in B cells of the spleen. Moreover, MHV-68 is widely used as a model for the two human oncogenic gammaherpesviruses, KSHV and Epstein-Barr virus, which are host specific and do not infect mice. Experiments with recombinant virus lacking M3 expression have been performed in both inbred mice and in its natural host, the wood mice, with different results. Lytic replication in the respiratory tract and spread to lymphoid tissue are not affected by lack of M3 expression, but establishment of latency in B cells is impaired following intranasal infection of BALB/c mice [87]. Lack of M3 expression also affects total viral load and virus-driven B cell activation [87]. This phenotype is reverted when CD8 T cells are depleted suggesting that M3 ability to inhibit chemokine activity may reduce the recruitment of this cell population during expansion of latently infected B cells [87]. In another study using C57BL/6 mice M3 was not required for establishment of latency following intranasal infection but the virus lacking M3 was attenuated after intracerebral injection compared to wild type. This attenuation correlates with differences in the leukocyte infiltrate. Lack of M3 results in infiltration of lymphocytes and macrophages, whereas the infiltrate in the wild type-infected mice is composed predominantly of neutrophils, causing meningitis [88]. MHV-68 infection induces the expression of several chemokines and inhibition of their activity by M3 may explain the differences in leukocyte infiltration [88]. Interestingly, experiments performed using the natural host wood mice show that M3 has immunomodulatory properties during infection [89] and plays an important role in the establishment of latency in the lung and spleen [89]. The kinetics of M3 expression differ also between wood and BALB/c mice, especially at 14 days post infection [89] where M3 expression is detected within lymphocytes in inducible bronchus-associated lymphoid tissue (iBALT) and splenic follicles [89]. These iBALT are composed mainly of B cells and are not present in the mice infected with the M3 stop codon mutant [89]. In the spleen, lack of M3 expression results in no germinal centre formation and a reduction in latently infected cells [89]. Therefore, M3 is required for the formation of iBALT in the lungs and for the organization of splenic follicles following intranasal infection of the natural host of MHV-68 [89]. These results underscore the importance of choosing the appropriate animal model when studying immunomodulatory proteins *in vivo*.

Alphaherpesviruses are characterized by establishing latency in ganglia of the peripheral nervous system. They cause a variety of diseases: neurological disorders, encephalitis, meningitis, blindness, chickenpox, shingles, skin lesions, and abortion among others. Work with EHV-1 has been fundamental to understand the relevance of gG inhibition of chemotaxis *in vivo*. Deletion of gG results in increased EHV-1 pathogenicity in an intranasal mouse model of infection [90]. Mice infected with the deletion virus lose more weight and recover slower than those infected with a gG-expressing virus despite having similar virus titers in the lungs [70]. This is probably due of the presence of a higher inflammatory infiltrate in the lugs of mice infected with the gG-negative EHV-1 [70]. A report using CCL3 knockout mice showed the relevance of

gG inhibition of CCL3-mediated neutrophil migration and subsequent inflammation in the lungs [71]. Studies with ILTV, a virus that causes respiratory tract disorders in poultry, indicate that gG modulates leukocyte migration to the infection site. Deletion of gG results in higher levels of inflammatory infiltrate in the trachea of birds [68,72]. Deletion of PRV gG, on the contrary, does not seem to affect the virus during infection of pigs [91].

The role of HSV-1 gG has been addressed in the mouse model. Intracerebral infection of mice with HSV-1 lacking gG expression results in lower levels of virus replication in the central nervous system [92]. Similarly, a virus lacking gG and US3 expression is attenuated following intracranial infection of mice [93]. However, the contribution of gG could not be separated from that of US3, a kinase with multiple relevant modulatory functions [94]. Finally, lower titres were observed following infection of mice using the ear scarification model [95]. The role of HSV-2 gG *in vivo* has not been investigated.

4. Concluding remarks

Some viruses express type I transmembrane or secreted proteins with low or no sequence identity to host proteins that bind and modulate chemokine activity. Until now all vCKBP have been discovered in members of the *Poxviridae* and *Herpesviridae*, viruses that despite their differences in viral cycle and subcellular localization share one important characteristic: they contain a large double stranded DNA genome that permits them to include many genes involved in host interaction, including these encoding for vCKBP. This raises the question of whether other large DNA viruses such as members of the *Asfarviridae* also express this kind of proteins. It is noteworthy that such proteins have not yet been discovered in several human herpesviruses (VZV, KSHV, Epstein-Barr virus, human herpesvirus 6A, 6B and 7) or in the human poxvirus molluscum contagiosum virus. Whether these human viruses express vCKBP or whether they rely on other strategies to modulate the chemokine network is currently unknown. In this regard, we have recently found a vCKBP in VZV and are currently investigating its modulatory properties (V.G.-M. and A.V.-B., unpublished). Several studies have shown the presence of CKBP in more complex organisms such as the parasite *Schistosoma mansoni* and ticks [96–98]. Moreover, a similar activity has been recently discovered in a human protein, TNF- stimulated gene/protein-6 [99], showing that the expression of soluble proteins with the ability to bind and modulate chemokine function is more common than initially anticipated. Further research in this exciting field may uncover novel vCKBP in relevant pathogens with clinical implications.

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