Herpes simplex virus glycoprotein G enhances chemotaxis through modification of plasma membrane microdomains and receptor trafficking

Ph.D. Thesis

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Herpes simplex virus glycoprotein G enhances chemotaxis through modification of plasma membrane microdomains and receptor trafficking

Memoria presentada para optar al grado de Doctora en Biología Molecular

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Abbreviations
ACP: acyl-carrier protein
BHV: bovine herpes virus
BRET: bioluminescence resonance energy transfer
BSA: bovine serum albumin
C-tail: C-terminal tail
CapHV: caprine herpesvirus
CCPs: clathrin-coated pits
CCV: clathrin-coated Vehicle
CerHV: cervine herpesvirus
CNS: central nervous system
CoA: Co-enzyme A
CPXV: cowpox virus
DAG: diacylglycerol
DARC: Duffy antigen receptor for chemokines
DBC: DeepBlueC
EBV: Epstein-Barr virus
ECTV: ectromelia virus
EE: early endosome
EEA-1: early endosomal antigen-1
EGFR: epidermal growth factor factor
EHV: equine herpesvirus
EHV: equine herpesvirus
EM: electron microscopy
ERK: extracellular signal-regulated kinase
FACS: flow cytometry
FAK: focal adhesion kinase
FBS: foetal bovine serum
FeHV: felid herpesvirus
FF: freeze fracture
FIP-2: family interacting protein-2
FMI: forward migration index
GAGs: Glycosaminoglycan
GalNAc: N-acetylgalactosamine
gB: glycoprotein B
gC: glycoprotein C
gG: glycoprotein G
gG1: HSV-1 gG
gG2: HSV-2 gG
GlcNAc: N-acetylgalactosamine
GPCR: G-protein coupled receptor
GRK: GPCR-kinases
HCMV: human cytomegalovirus
HCMV: human cytomegalovirus
HHV-6: human herpesviruses 6
HHV-7: human herpesviruses 7
HIV: human immuno deficiency virus
HSE: sporadic herpetic encephalitis
HSV: herpes simplex virus
ICA: intensity correlation analysis
ICQ: intensity correlation quotient
IE: immediate-early
IF: immunofluorescence
IFN: type 1 interferon
ILTV: infectious laryngotracheitis virus
IP3: inositol 1,4,5 triphosphate
IRL: long component internal repeat
JAK: Janus kinase
JNK: Jun N-terminal kinase
KSHV: Kaposi’s sarcoma-associated herpesvirus
LAMP-1: lysosomal-associated membrane protein 1
LATs: latency-associated transcripts
LE: late endosome
MAPK: mitogen-activated protein kinase
mgG2: mature membrane-anchored form
MHV-68: murine γ-herpesvirus
MM-1: MonoMac-1
MYXV: myxoma virus
NFκB: nuclear factor κ B
NGF: Nerve growth factor
NK: natural killer
PBMC: peripheral blood mononuclear cells
PC: Pearson’s Coefficient
pDCs: plasmacytoid dendritic
PI3K: phosphoinositide-3 kinase
PIP3: phosphoinositol triphosphate
PKC: protein kinase C
PLC: phospholipase C
PRV: pseudorabies virus
QDots: Quantum Dots
RanHV: rangiferine herpesvirus
RE: recycling endosome
ROI: regions of interest
RRP: rapid recycling pathway
RU: response unit
SA: streptavidin
SCG: superior cervical ganglion
SDF-1: stromal derived factor
SgG1: secreted gG1
SgG2: secreted gG2
SPR: surface plasmon resonance
SRP: slow recycling pathway
STAT: Signal Transducer and Activator of Transcription
Tfr: transferrin
TLR: Toll-like receptor
TM: transmembrane
TNF: tumor necrosis factor
TRL: long component terminal repeat
TRS: short component terminal repeat
VACV: vaccinia virus
VARV: variola virus
VZV: varicella-zoster virus
WB: Western blot
WHIM: warts, hypogammaglobulinemia, infections and myelokathexis syndrome
PFA: paraformaldehyde
β2AR: β2Adrenergic receptor
Abstract
Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2) are two highly prevalent, neurotrophic human pathogens. The interplay between HSV and the immune system is important to determine the onset and the outcome of the viral infection, but yet, the precise modulation of the immune system by HSV remains poorly understood. We have previously identified secreted HSV glycoprotein G (SgG) as the first viral chemokine binding protein (vCKBP) that, in sharp contrast to all vCKBPs described to date, enhances chemokine function both in vitro and in vivo. Furthermore, we have recently shown the ability of SgG to interact with and modulate the function of neutrophins such as nerve growth factor (NGF), essential elements of the biology of the nervous system.

HSV gG interaction with chemokines induces an enhanced chemotaxis mediated by an augmented receptor signalling and directionality of cell movement. Surface plasmon resonance (SPR) and cell binding assays show that SgG binds to the cell surface through the interaction with glycosaminoglycans. We demonstrate that SgG modulates membrane trafficking, delays chemokine-mediated internalization, and leads to an increase in the level of chemokine receptors at the cell surface. Moreover, SgG-induced impact on the architecture of the plasma membrane microdomains promotes the incorporation of CXCR4 into lipid rafts and differentially alters the fate of other receptors. The link between the aggregation state of chemokine receptors and their functionality has not been characterized. Here, we provide evidences showing that the enhancement of chemokine function is associated with an SgG-induced increase in the presence of CXCR4 small oligomers on the surface, accompanied by conformational rearrangements in CXCR4 homodimers.

SPR-based assays were set up in order to characterize the ability of HSV viral particles to interact with chemokines. Our results demonstrate that HSV virions bind chemokines. The functionality of the virus-chemokine interaction was analyzed in vitro. We show that HSV particles are chemotactic in transwell assays and that they synergize with chemokines to enhance migration. Importantly, we found that envelope-anchored gG has an important contribution to these activities, suggesting the relevance of the HSV gG-chemokine interaction during early stages of the viral infection.
Los virus herpes simple 1 y 2 (HSV-1 y HSV-2) son patógenos humanos altamente prevalentes. La interacción entre HSV y el sistema immune es muy importante para determinar la evolución de la enfermedad; sin embargo, no ha sido bien caracterizada. Trabajos anteriores de nuestro laboratorio identificaron por primera vez la capacidad de unión a quimiocinas de la glicoproteína G secretada de HSV-1 y de HSV-2 (SgG1 y SgG2, respectivamente). De forma opuesta al resto de las proteínas virales de unión a quimiocinas descritas, la gG de HSV potencia la función de las quimiocinas, tanto in vitro como in vivo. Recientemente, hemos identificado la capacidad de SgG para interactuar con neurotrofinas como el factor de crecimiento nervioso (NGF), elementos esenciales para el funcionamiento y regulación del sistema nervioso.

Hemos descrito que la potenciación de la función de las quimiocinas está asociada a una mayor activación del receptor, así como con un aumento en la direccionalidad del movimiento celular. Ensayos de resonancia de plasmón superficial (SPR) y de unión a células muestran que la SgG interacciona con la membrana plasmática a través de la unión a glicosaminoglicanos. Hemos demostrado que la SgG modifica el tráfico de receptores en la membrana y disminuye la internalización de estos en respuesta a la quimiocina, aumentando la cantidad de receptores disponibles en la superficie. La redistribución de los microdominios de la membrana plasmática mediada por SgG2 promueve la incorporación de CXCR4 en balsas lipídicas, y afecta diferencialmente a otros tipos de receptores. Se desconoce la relación entre el estado oligomérico y la funcionalidad de los receptores de quimiocinas. Durante este trabajo, mostramos que la potenciación de la función de las quimiocinas mediada por SgG2 está asociada a la presencia de un mayor número de oligómeros de CXCR4 en la superficie celular. Esta reorganización en superficie está acompañada por cambios conformacionales en los dímeros de receptor.

Mediante la adaptación de ensayos basados en SPR, hemos demostrado que los viriones de HSV interaccionan con quimiocinas. La funcionalidad de esta interacción fue analizada mediante ensayos in vitro. Hemos mostrado que las partículas de HSV son quimioatractivantes y sinergizan con las quimiocinas aumentando la migración celular. El hecho de que la gG presente en la envuelta viral sea importante para esta actividad sugiere la relevancia de la interacción entre la gG del virión y las quimiocinas durante estados tempranos de la infección viral.
Introduction
1. HERPESVIRUSES

Diseases caused by human herpesviruses were recognized by the earliest practitioners of Medicine, who described cutaneous lesions that crept (from the Greek term herpein) over the patient’s skin. It was 1930 when Burnet and Williams stated the notion that herpes simplex virus (HSV) persists for life: “it remains for the most part latent; but under the stimulus of trauma, fever, and so forth it may at any time be called into activity and provoke a visible herpetic lesion”. To date, the Herpesvirales order is divided into three families, Alloherpes-, Malacoherpes- and Herpesviridae family, the latter composed by more than 130 members, eight of which infect humans: HSV type 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), Epstein-Barr virus (EBV), human herpesviruses 6, 7 (HHV-6, HHV-7) and Kaposi’s sarcoma-associated herpesvirus (KSHV) 1.

Based on molecular phylogeny and biological properties, herpesviruses belonging to Herpesviridae are divided into three subfamilies: Alpha, Beta and Gammaherpesvirinae. The Alphaherpesvirinae subfamily is characterized by viruses with a wide host range, an efficient and rapid replicative cycle, and a strong neurotropism that determines the capacity to establish a life-long latent infection in sensory ganglia of their host2. The simplex genus within this subfamily contains two of the most prevalent human pathogens worldwide, HSV-1 and HSV-2, whose study represents the main scope of this Thesis. The third human member, the prototype of the varicello genus, is VZV. The Alphaherpesvirinae also contains viruses that infect animals causing serious diseases and huge economic losses, such as the equine herpesviruses (EHV-1 and EHV-4), infectious laryngotracheitis virus (ILT) or pseudorabies virus (PRV)1.

2. THE BIOLOGY OF HSV

HSVs are among the most successful human pathogens, with more than 90% people worldwide being infected with one or both viruses. HSV-1 seroprevalence is higher than 60% in Europe and in the US, and is almost universal in developing countries. HSV-2 is less frequent, although 15-80% of people are seropositive, depending on the geographical region3-5. The first global estimate on HSV-2 infection by the World Health Organization concluded that 536 million individuals were infected prior to 2003 with more than 23 millions becoming newly infected during that year, numbers that might be underestimated given the often assymptomatic nature of genital herpes infection6.

HSV is a highly complex virus7, 8. The diameter of the mature HSV virion is approximately of 200 nm and consists, from the surface inward, of a lipid envelope, an amorphous proteinaceous region termed the tegument composed by more than 30 viral proteins, an icosahedral capsid organized in 162 capsomers and a double-stranded DNA genome (Fig. I-1A). The 152 kb genome allows the virus to encode for more than 80 polypeptides, including the proteins responsible for initiating viral gene expression, shutting off host cell protein synthesis, virion maturation and egress, and proteins involved in diverse immune modulatory roles2, 9, 10. The HSV genome is
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...forming protruding spikes that are involved in many aspects of the virus life cycle including attachment and entry, cell-to-cell spread and immune evasion\(^2\),\(^{15}\),\(^{16}\). Moreover, HSV envelope glycoproteins elicit strong cell immune responses and therefore they are candidates for vaccine development\(^17\). Interestingly, all glycoproteins elicit cross-reactive T and B cell responses, with the exception of HSV gG, which induces type-specific human antibody responses and therefore is used as a serological marker to distinguish HSV-1 and 2 infections\(^18\),\(^{19}\). The lack of cross-reactivity has been associated to significant structural differences between gG1 and gG2\(^20\). The functional implications ...

Figure I-1. HSV morphology and genome organization. (A, top) Electron microscopy image of an HSV-2 virion negatively stained with uranyl acetate. Structural elements of the virus are indicated. Scale bar, 100 nm. (A, bottom) Cryo-electron tomography of a single virion. Glycoprotein spikes, shown in yellow, protrude from the viral envelope, shown in blue (left). Cutaway view of the virion showing the capsid (light blue), the tegument (orange) and the envelope harboring the viral glycoproteins (right). (B, top) Genome organization and nucleotide sequence comparison between HSV-1 and HSV-2. The position of the segments and repeats within the HSV genome is schematized, showing the unique long (U\(_L\)) and the unique short (U\(_S\)) segment each bound by inverted repeats (IR) elements. The location of the essential and nonessential genes regarding their requirement for \textit{in vitro} replication are indicated. Viral glycoproteins and genes that play a relevant role in pathogenesis are indicated. (B, bottom) Both genomes show a high sequence similarity at the nucleotide level. Note that the greatest disparity is found is the U\(_S\) gene, encoding for gG (indicated with a yellow ellipse). Image A (top) was obtained during the current work; Image A (bottom) was taken from Grünewald et al.\(^7\). Image B (top) was taken from Frampton et al.\(^20\); plot in B (bottom) is from Baines et al.\(^21\). Abbreviations: L, long component; S, short component; TRL, long component terminal repeat; IRL, long component internal repeat, TRS, short component terminal repeat.
of such differences for the biology of HSV-1 and 2 remain unknown. The following two sections describe different important aspects of HSV biology.

2.1 HSV entry into the host cells and replication

HSV entry into the host cell is a complex multi-step process involving several envelope glycoproteins that is initiated by virus attachment to the cell surface via the interaction of glycoprotein B (gB) and C (gC) with surface GAGs. Interaction with GAGs is not essential, but it enhances viral infectivity. Entry of HSV into the host cell occurs either through fusion of the viral envelope with the plasma membrane or by endocytosis, which contrary to the former is pH-dependent (Fig. I-2). Although HSV enters most cells via the pH-independent pathway, it has been hypothesized that the route of entry may depend on the cell type infected. Moreover, different results are obtained when using polarized rather than non-polarized cells. HSV gG has been proposed to play a role in the infection of polarized cells through the apical surface. Initial binding to GAGs allows a close association of glycoprotein D (gD) with specific entry receptors triggering viral fusion with the cell plasma membrane upon recruitment of three additional

Figure I-2. HSV entry into a susceptible host cell. HSV entry into most cells occurs by fusion with the plasma membrane, although it can also take place through a pH-dependent endocytic process (schematized on the left). (A) Initial attachment to the cell through binding of gB and gC to GAG moieties on the surface. (B) Interaction of gD with specific entry receptors. The use of entry receptors varies for HSV-1 and HSV-2. Some receptors, such as nectin-1 that is found in both skin cells and neurons, is used with equal affinity by both viruses. (C) Fusion of the viral envelope and the cell membrane through the action of gB, gD, and gH/gL complex. (D) After fusion with the plasma membrane, the nucleocapsid and the tegument proteins are released into the cytoplasm and capsids travel along microtubules to the nucleus, where the DNA is released. For simplicity, only the viral glycoproteins involved in the entry process have been represented.
progeny exits the cell by exocytosis. 

2.2 HSV life cycle and disease

HSV initial replication in the skin or mucosa results in progeny virus released to the skin surface, allowing transmission to other hosts, and to the epidermis, allowing the virus to access the free nerve endings of the sensory neurons that innervate the infected tissue (Fig. I-3). Within neurons, HSV nucleocapsid and tegument components are transported in a retrograde manner to the neuronal soma, where the viral genome is released into the nucleus. The speed of the viral transport along the axon indicates an active transport process to which viral and host components are thought to contribute. Once the virus reaches the trigeminal ganglion or the dorsal root ganglia (as is normally the case for HSV-1 and HSV-2), the virus establishes latency, persisting as an episome in the neuronal nucleus for the lifetime of the host. HSV latency has been commonly associated with the lack of virus gene expression but the presence of specific LATs (latency-associated transcripts) in the infected nervous tissue, which represses lytic infection promoting the maintenance of the latent state. Nonetheless, since low level virus production is detected in some infected ganglia, HSV infection has been described by some authors as a persistent rather than a latent infection.

Several stimuli induce reactivation within the ganglia followed by anterograde transport of the virus to the initial site of infection, where it is released giving rise to a recurrent infection at the site of primary inoculation. Although the mechanisms of
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Figure I-3. HSV life cycle and pathogenesis. (A) HSV primary infection typically proceeds through the oro-labial mucosa and the genital tract in the case of HSV-1 and HSV-2, respectively. However, both viruses can infect either mucosa, often as a consequence of oral-genital sex. Following virus entry into host epithelial cells, capsids are transported to the nucleus, where viral replication takes place. After a regulated cascade of lytic gene expression, the newly synthesized particles assembly within the nucleus, and virions egrees from the mucosal cell. HSV primary infection usually shows mild-symptoms, such as stomatitis, although it can be a fatal disseminated disease in the neonate. (B). The progeny virus access the nervous system through the axonal termini that innervate the mucocutaneous zone and nucleocapsids are retrogradely transported to the sensory ganglia. (C) Once in the soma and after a short replication phase with limited neuronal death, latency is established. The virus persists as an episome within the neuron wherein viral lytic gene expression is silenced and LAT genes are produced. (D) Following stimulation by a variety of physiological and environmental stimuli, HSV can reactivate from latency and start the lytic cascade, resulting in the production of viral particles that travel to the site of primary infection by anterograde axonal transport. (E) Upon encountering of a mucosal cell a productive recurrent infection occurs again resulting in the development of lesions at the site of the primary infection. The pathological outcome of viral recurrences varies from asymptomatic to fatal if the virus spreads to the CNS, leading to the development of encephalitis or keratitis. The causes for the differential outcomes are not well understood but depend on the interplay between the virus and the host immune system. Modified from Frampton et al.18
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HSV transport along axons have yet to be elucidated; a great amount of data suggest that nucleocapsids and the rest of viral proteins travel independently along axons, in an active process mediated by cellular and viral factors, similar to retrograde movement. About 30% of population serologically positive for HSV-1 suffer more than one recurrence per year; HSV-2, in turn, is the most common etiologic agent of genital ulcers worldwide, with one third of the infected individuals showing more than 6 recurrences per year. HSV reactivation is usually associated with mild symptoms or is even asymptomatic, complicating the detection of the virus and then enhancing its transmission. Nevertheless, reactivation can lead to severe disease in immunocompromised individuals and in neonates, who can develop a disseminated “sepsis-like” infection with fatal consequences. Furthermore, HSV is the most common cause of sporadic herpetic encephalitis (HSE) and the major cause of ocular scarring and visual loss, both illnesses causing high morbidity and mortality rates in healthy individuals. Besides causing painful lesions, genital herpes is often associated with social distress, psychosexual problems, depression and other behavioural alterations during recurrences. Moreover, HSV infection is associated with an increase in human immunodeficiency virus (HIV) acquisition and transmission. HIV viral loads are higher in HSV-2 patients, and virus spread is facilitated due to the increase in HIV target cells in the genital mucosa. The causes beneath the variability in the pathological outcome following HSV infection are not well understood, but the interaction between the virus and the host immune system appears to be highly relevant.

3. BIOLOGY OF CHEMOKINES AND CHEMOKINE RECEPTORS

Chemokines are essential guidance cues that orchestrate leukocyte activation and migration to sites of infection or injury, and, as such, chemokines are pivotal elements of the antiviral immune response. The coordination of cell trafficking by chemokines is also of major importance during homeostasis and in several pathological conditions such as metastasis or angiogenesis. Moreover, the role of chemokines is not restricted to the immune system, since they display crucial functions in different aspects of the mature and developing nervous system (reviewed in section 4). Disregulation of the chemokine function is beneath several immunopathologies and therefore it may impact dramatically on the development of HSV-induced pathologies, including HSE.

The human chemokine network comprises more than 53 chemokines and around 23 receptors, which belong to the G-protein coupled receptor (GPCR) family. All chemokines are secreted, while CXCL16 and CX3CL1 are also present as membrane-anchored forms. According to the position of conserved cystein residues, chemokines are classified as C-, CC-, CXC-, and CX3C-chemokines. The spatial structure of many chemokines has been solved, showing a high degree of conservation throughout the 4 chemokine classes. The general structure consists of a disorderly N-terminal domain of 6-10 residues followed by a long loop, a $\alpha_{10}$ helix and a triple-stranded antiparallel...
3.1 GAG binding shapes the interaction of chemokines with their receptors

The chemokine network is complex and promiscuous, with some receptors interacting with more than one chemokine and some chemokines binding to more than one receptor. Besides, chemokines bind to GAGs, found ubiquitously at the cell surface and in the extracellular matrix. GAGs are linear polysaccharides that consist of a succession of disaccharide units in which one of the
sugars is either N-acetylgalactosamine or N-acetylglucosamine or derivatives, both of which could be sulphated, making the polysaccharide acidic. GAGs are divided into four subgroups, namely heparan sulfate and heparin, hyaluronic acid (non sulphated), chondroitin sulfate and dermatan sulphate. Binding to GAGs normally occurs through consensus sequences for heparin binding (BBXB, B for basic and X for any amino acid) and involves other chemokine residues that cluster creating a positively charged surface on the folded protein\(^{56, 59}\). The interaction with GAGS goes beyond cell surface retention, since it is believed to contribute to chemokine function in vivo by protecting chemokines from proteolytic degradation and by enhancing chemokine presentation to its receptor facilitating the formation of chemotactic gradients, either by modifying the local concentration of the chemokines or their oligomerization\(^{60-64}\). The modulation of chemokine function by GAG binding is particularly notable for some isoforms of CXCL12, such as CXCL12\(\gamma\), whose longer C terminus allows this chemokine to bind heparan sulphate with a 100-fold higher affinity compared to its \(\alpha\) counterpart promoting cell migration in vivo with much higher efficiency than CXCL12\(\alpha\)\(^{64-66}\).

GAG interaction with chemokines is seen as a mechanism to tune the function of chemokines and to generate functional diversity without disrupting the chemokine fold itself\(^{61, 64, 66-68}\). In addition, GAGs contribute to the specificity of the cellular response mediated by chemokines. The induction of the expression of particular chemokines together with the differential expression of a subset of chemokine receptors determine which immune cells migrate in each particular situation.

GAGs expression is not random either, it is temporally and spatially regulated and thus different cells and tissues express a specific GAG repertoire, and chemokines in turn, bind different GAGs with variable affinities. Moreover, it has been suggested that free or GAG-bound chemokines have different GPCR selectivity\(^{69}\). Altogether, it can be concluded that the GAG environment and binding shapes the function of chemokines, modulating the interaction with its specific GPCR and adding specificity to the promiscuous chemokine system.

The interaction of the chemokine with its specific receptor initiates a series of signalling pathways that result in a variety of responses such as chemotaxis, cell survival or proliferation, gene transcription and immune cell activation\(^{60, 70}\) (Fig. I-5). Chemokine binding to the GPCR induces conformational changes in the extracellular receptor domains that are then transmitted to the intracellular loops, activating coupled heterotrimeric G-proteins. The heterotrimer is composed of \(G_{\alpha}\), \(G_{\beta}\) and \(G_{\gamma}\) subunits, which associate with the GPCR through the conserved DRYLAIV motif in the second intracellular loop. Upon activation by ligand binding, the GDP bound to \(G_{\alpha}\) subunit is exchanged by GTP, leading to the dissociation of \(G_{\beta\gamma}\) from the \(G_{\alpha}\) monomer. The latter hydrolysis of GTP to GDP results in the reassociation of \(G_{\beta\gamma}\) with the \(G_{\alpha}\) monomer. The latter hydrolysis of GTP to GDP results in the reassociation of \(G_{\beta\gamma}\) with the \(G_{\alpha}\) monomer. On the basis of sequence similarity \(G_{\alpha}\) subunits have been divided into four families: \(G_{\alpha_1}\), \(G_{\alpha_2}\), \(G_{\alpha_3}\) and \(G_{\alpha_4}\). Chemokine signalling pathways are mediated by pertussis toxin-sensitive \(G_{\alpha_1}\) and \(G_{\alpha_2}\) components. The \(G_{\beta\gamma}\) subunit stimulates phospholipase C (PLC) generating the second messengers diacylglycerol (DAG) and inositol 1,4,5
receptors also induces several mitogen-activated protein kinases (MAPKs) such as the extracellular signal-regulated kinases (ERK), Jun N-terminal kinase (JNK) and p38\(^\text{\textsuperscript{70}}\). MAPKs activation has a role in many processes, including cell proliferation, cytokine production, migration or activation of nuclear transcription of transcription factors through ERK translocation to the nucleus. In addition, chemokines such as CXCL12 activate the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway in a pertusis toxin-independent manner, a process that has been associated to the ligand-induced homodimerization of receptors\(^\text{\textsuperscript{71}}\). Binding of chemokines to their receptors also activates phosphoinositide-3 kinase (PI3K) that in turn activates AKT and nuclear factor \(\kappa\) B (NF\(\kappa\)B)-mediated pathways. Chemotaxis highly relies on PI3K-dependent activation of focal adhesion kinases (FAK) among other components they play a role in the leading edge of migrating cells\(^\text{\textsuperscript{72, 73}}\). PI3K also contributes to the activation
of the small GTPases Cdc42, Rac and Rho, involved in the maintenance of cell polarity, directionality of cell movement and adhesion to the matrix. In addition, the activation of chemokine receptors such as CXCR4 involves Ras-mediated pathways and the Src-related kinases Src, Lyn, Fyn and Lck.

### 3.2 Conventional and unconventional routes of chemokine receptor trafficking

The complexity of the chemokine system raises the question of how the information is integrated and translated into a physiological output. Membrane trafficking is a fundamental means to regulate the sensitivity to a chemokine as well as to provide spatio-temporal control of the downstream signals. The rate of internalization of chemokine receptors regulates the amount of receptor exposed to the ligand, and, as such, it is a highly controlled process with direct implications in the responses elicited by chemokines. Nevertheless, internalization is not required for signaling, but it serves as a regulatory mechanism to control the strength and duration of cellular responses.

Clathrin-dependent endocytosis is the major pathway directing the internalization of most chemokine receptors, including CXCR4 (Fig. I-6). Chemokine receptors undergo a constitutive level of endocytosis that is greatly enhanced upon chemokine binding. Following chemokine exposure, desensitization mechanisms act in order to control the magnitude and duration of the signal. The cytosolic C-terminal tail (C-tail) of the activated GPCR is rapidly phosphorylated at Ser and Thr residues by GPCR-kinases (GRKs), resulting in the interaction of the GPCR with β-arrestins and its subsequent uncoupling from G-proteins, a process termed desensitization. β-arrestins are key regulators of GPCR functionality, since they mediate signal quenching, targeting the desensitized receptor to clathrin-coated pits (CCPs) for endocytosis. Next, the clathrin-coated vesicles bud from the membrane and the receptor traffics within the endosomal and then lysosomal compartment where the GPCR is finally degraded (downmodulation), although a small percentage can recycle back to the membrane being available to interact again with the chemokine (resensitization).

Despite the predominant role of clathrin-dependent endocytosis, several chemokine receptors internalize through clathrin-independent mechanisms. In the last few years, non CCP-dependent endocytosis through lipid rafts and caveolar pathways has emerged as a second crucial trafficking route (Fig. I-6). The plasma membrane of eukaryotic cells consists of a complex assembly of lipids and proteins in regions on lateral homogeneity known as microdomains. Among these, the best studied are lipid rafts and caveolae (a subtype of lipid rafts that forms membrane invaginations rich in caveolin), membrane domains enriched in cholesterol, glycosphingolipids, glycosylphosphatidylinositol-anchored proteins and acylated signalling molecules. Lipid rafts are heterogeneous and highly dynamic entities, forming microclusters (50-100 nm) that aggregate into larger rafts, such as the ones found at the leading edge of migrating cells or during the formation
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whether clathrin-independent mechanisms use the same compartments in intracellular trafficking as the clathrin-dependent ones or whether any lipid raft component mediate the intracellular signal transduction events triggered upon raft-dependent endocytosis, requires further study.

The internalization of CCR2, CCR4 and CCR5 has been reported to rely on both, clathrin and caveolar/lipid raft-mediated
mechanisms\textsuperscript{96-99}. Moreover, several receptors, including CCR5, CXCR1, CXCR2 or CXCR4, have been found to some degree in raft membranes\textsuperscript{90-92}. The internalization of CCR2 and CCR4 depends on both CCPs and lipid raft integrity, whereas CCR5, in turn, partially colocalizes with endogenous caveolin positive vesicles. Although it is well established that CXCR4 is preferentially located in the non-raft fraction, a number of reports have indicated that CXCR4 can associate with rafts under specific circumstances, an event linked to changes in receptor functionality\textsuperscript{90, 93, 94}. The cell type, the relative abundance of specific adaptor proteins, the endocytic machinery components, and the lipid raft composition of the membrane in the proximity of the receptor may, in part, determine the likelihood of utilization of one endocytic pathway over the other one.

3.3 Modulation of chemokine receptor function

The link between the steady-state oligomerization status of chemokine receptors and its functional significance is insufficiently understood\textsuperscript{95}. The most straightforward model of chemokine and GPCR interaction involves the binding of a monomeric chemokine to its cognate monomeric receptor. In support of that hypothesis, in vitro studies have shown that obligate monomers are able to induce efficient leukocyte responses\textsuperscript{96, 97}. Chemokine receptors belong to the large subgroup of rhodopsin-like class A GPCRs, where evidence demonstrating that a monomeric ligand can activate G proteins exists\textsuperscript{98}. Despite these considerations, the in vivo situation is more complex, and many reports indicate that the formation of chemokine oligomers enhances leukocyte recruitment\textsuperscript{54, 61, 99, 100}. Not only chemokines oligomerize, but also their receptors are found as dimers or higher order oligomers at the cell surface\textsuperscript{101-103}. A growing body of biochemical and biophysical evidences indicate that chemokine receptors, such as CXCR4 or CCR2, form homodimers in the absence of the cognate chemokine. Although some initial controversy arised\textsuperscript{71, 104}, many studies have shown that binding of the chemokine induces conformational rearrangements within these pre-formed homodimers without promoting receptor dimer formation or disassembly\textsuperscript{105-107}. Moreover, the existance of chemokine receptor heterodimers has been reported. Many groups have shown that chemokine receptors such as CXCR4 and CXCR7 or CCR2, CCR2 and CCR5, or CXCR1 and CXCR2 constitutively heterodimerize\textsuperscript{108-112}. The functional consequences of such heterodimerization have been poorly characterized, although the formation of heterodimers might act as a regulatory mechanism to coordinate the responses to the cognate chemokines.

Emerging data indicate that ligand-free chemokine receptors, including CXCR4, exist in an equilibrium between transient inactive and active states, and that chemokine binding shifts the equilibrium towards the active state. Thus, instead of one single active and inactive conformation, CXCR4 presents conformational flexibility, showing an array of different conformations all capable of activating G protein\textsuperscript{95, 103, 113}. Moreover, different active conformations of a given receptor can be preferentially stabilized by different chemokines\textsuperscript{87}, or even
when the same chemokine is presented in a distinct cellular environment\textsuperscript{114}.

In addition to the regulation of chemokine receptor function through changes in the oligomeric status of chemokine, in the aggregation state of the receptor itself, and in the cellular context or the membrane microdomains where the receptor is found, it is also conceivable that stable homodimer pairs regulate each other allosterically. Once an active conformation of the receptor is stabilized, such conformational changes could have allosteric influences on the conformation of proximal dimers, extending that change throughout neighboring receptors in a sort of “domino” effect\textsuperscript{103, 115, 116}.

An additional level of regulation is achieved by post-translational modifications of chemokine receptors, which modify the interactions with their signalling partners and hence the biological functionality of the GPCRs. Such modifications are located in the extracellular face of the receptor, in the intracellular loops or in the C-tail, and include sulfation and glycosylation, or phosphorylation, acylation and ubiquitination, respectively. The former mainly affects ligand/receptor interactions, while the latter modifications tune membrane location, trafficking, turnover and signalling pathways associated to the chemokine receptor\textsuperscript{70, 76}.

Altogether, many different factors and mechanisms act coordinately to shape the fate of a chemokine receptor, which eventually determines the length, strength and type of cellular responses elicited by chemokines. Dysregulation or alterations of any of such regulatory mechanisms may lead to immunopathology or influence the immune response to pathogens.

4. CXCL12 AND ITS RECEPTORS, CXCR4 AND CXCR7

CXCL12 (also termed SDF-1, for stromal derived factor) is expressed in many different tissues and organs, including skin, lymph nodes, liver, lung, heart, kidney, bone marrow and brain. So far, six different isoforms ($\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$ and $\phi$) that differ in the length of their C-terminus have been described in human tissues\textsuperscript{117}. CXCL12 secretion is also associated with tissue damage (ischemia, liver damage, excessive bleeding) or chemotherapy\textsuperscript{118}. CXCL12 binds to CXCR4 which is expressed in a broad number of immune and non-immune cells and tissues, including hematopoietic cells such as T and B lymphocytes, monocytes, neutrophils and eosinophils, as well as microglia, astrocytes, neurons, and progenitor endothelial and smooth muscle cells. Mice deficient in CXCR4 or CXCL12 are embryonic lethal and show similar phenotypes, with defects in hematopoiesis, recruitment of hematopoietic stem cells from the foetal liver to the embryonic bone marrow and deficits in vascularization and heart and brain development\textsuperscript{119, 120}.

The CXCL12-CXCR4 axis has pleiotropic functions in the immune system. CXCL12 is an efficient chemoattractant for lymphocytes and monocytes, regulates the maturation and effector functions of T and B lymphocytes, and contributes to survival and generation of memory T cells. The CXCL12/CXCR4 axis is not only essential during homeostasis, but is also involved in many pathological conditions, such as ischemia, hypoxia, viral infections, and in the development of tumours and metastasis\textsuperscript{118, 121, 122}.

CXCL12 was believed to interact only with...
CXCR4, until the role of CXCR7 as a second cognate GPCR for CXCL12 was described a few years ago\textsuperscript{123, 124}, CXCR7 belongs to the group of “atypical chemokine receptors” that also includes the human decoy receptors D6, the Duffy antigen receptor for chemokines (DARC) and CCX-CKR1. Atypical receptors show different chemokine specificities and expression patterns and, although they are characterized by the lack of G-protein activation, most decoy receptors are competent for signalling\textsuperscript{125, 126}. Disruption of the CXCR7 gene leads to a phenotype similar to that of CXCR4 knockouts animals, suggesting that CXCR7 compromises CXCR4-dependent signalling\textsuperscript{127, 128}. CXCR7, whose affinity for CXCL12 is almost 10-fold higher than that of CXCR4, presents different trafficking kinetics. CXCR7 rapidly cycles between the plasma membrane and endosomal compartments, removing extracellular CXCL12 that is degraded within lysosomes\textsuperscript{124, 129}. Thus, CXCR7 reduces the amount of CXCL12 available to interact with CXCR4, shaping the chemotactic gradient. The scavenging function of CXCR7 is essential for the proper migration of primordial germ cells during zebrafish development, and it may have a role in tumour survival and progression, where CXCR7 is highly expressed\textsuperscript{130-134}. There are emerging data indicating that the CXCR7 function goes beyond scavenging, since the receptor signals in a ligand-dependent manner biased through the alternative arrestin-mediated signal pathway\textsuperscript{109, 135, 136}. Moreover, CXCR7 heterodimerizes with CXCR4, resulting in different responses to the chemokine in comparison with CXCR4 alone\textsuperscript{108, 109}. Although the precise role of CXCR7 interaction with CXCL12 is still a matter of debate, CXCR7 has emerged as a key regulator of CXCR4 function, adding complexity to the intricated chemokine network.

4.1 CXCL12 in the nervous system

The first indication of the important role of CXCL12 in the nervous system comes from reports showing CXCR4 immunoreactivity in almost all central nervous system (CNS) cells: neurons, astrocytes, microglia, oligodendrocytes and endothelial cells. In the last few years, many groups have clearly denoted that interaction of chemokines and their receptors goes far beyond their original functions in the immune system, playing also an important role in the establishment and maintenance of homeostasis of the nervous system\textsuperscript{50, 137}. CXCR4/CXCL12 are key for the correct development of the nervous system, where they coordinate migration of neuronal precursors, axon guidance/pathfinding and maintenance of neuronal progenitor cells\textsuperscript{119, 133}. In the mature CNS, where CXCR4 is constitutively expressed, CXCL12 modulates neurotransmission, neurotoxicity, neuroinflammation and neuroglial interactions\textsuperscript{137, 138}. Moreover, the prominent expression of CXCR7 in ganglia and in the CNS suggests a relevant function of this receptor in these tissues\textsuperscript{136, 139}. Indeed, recent studies show that both CXCR7 and CXCR4 differentially regulate the migration of interneurons\textsuperscript{140, 141}. The implication of chemokines during the demyelination/remyelination and blood brain barrier breakdown processes has been also proposed\textsuperscript{142}. An unorganized CXCR4/CXCL12 response is behind the pathogenesis of several neurodegenerative and neuroinflammatory disorders, such
as HIV-associated encephalopathy, brain tumour or multiple sclerosis53.

5. NEUROTROPHINS ARE SECRETED FACTORS THAT REGULATE THE FUNCTION ON THE NERVOUS SYSTEM

The ability of HSV to colonize, persist and reactivate from the nervous system, usually without severe collateral damage for the host, relies on a complex and finely tuned interaction between the virus and different aspects of the host’s response. Commonly, HSV-1 establishes latency in the trigeminal ganglia whereas HSV-2 usually persists in the sacral ganglia. The viral determinants for such differential neurotropism, the neurevmodulatory strategies developed by HSV as well as the mechanisms through which the virus initially gains access to the axon termini are not well understood.

Free nerve endings in the skin are dynamic structures, capable to degenerate and regenerate, and to respond to nerve ending navigational cues. Nerve growth factor (NGF) is the founding member of the neurotrophins, a family of secreted proteins essential for the proper development, patterning, maintenance and functions of the mammalian nervous system143-145. Neurotrophins are also important guidance cues that modulate nerve ending navigation146. NGF engages two structurally distinct receptors in neurons; the tyrosine kinase receptor TrkA, and p75, which belong to the tumor necrosis factor (TNF) receptor superfamily147, 148. The biological output of NGF/TrkA axis depends on multiple factors, including the interaction with p75, which exerts multiple regulatory roles ranging from promotion of axonal outgrowth to cell death146, 149. Peripheral neurons innervating skin show a strong dependency on neurotrophins, which are required for the survival and regeneration of mature neurons146, 150.

6. IMMUNE RESPONSE AGAINST HSV

The hallmark of human herpesviruses infection is that these viruses have reached a state of equilibrium with their host that allows them to establish a persistent infection for the life span of the host. To enable such lifestyle, herpesviruses employ a great proportion of their genome to the expression of a plethora of genes to modulate or evade the host immune system. The close relationship established between herpesviruses and their hosts during millions of years of co-evolution has shaped not only the appearance of viral immunomodulatory strategies, but also the development of specific host immune pathways to counteract the viral challenges. Therefore, the characterization of herpesviruses immune regulatory strategies offers the opportunity to gain insights into key aspects of the immunology field from the viral perspective, as viruses are repositories of knowledge acquired during their constant struggle with the immune system.

Innate immunity against HSV is marked by the production of type I interferon (IFN), and represents the first line of the host defense, being key to determine the pathogenesis of HSV infection51, 152. The innate immune response includes virus recognition by Toll-like receptors (TLRs) and the complement
system, early cytokine production, and cellular effector responses, where natural killer (NK) cells and plasmacytoid dendritic cells play a prominent role\textsuperscript{153-155}. Innate and adaptative immunity are closely integrated, with the latter allowing lifelong immunological memory and affording the control of persistent HSV infection and protection against recurrences. During primary infection, the virus rapidly travels to the neuronal body of sensory neurons, facilitating the escape from the adaptative immune responses, making the innate immune response the primary protection mechanism against the HSV. Nevertheless, the cellular effectors of the adaptative arm of the immune response also play a crucial role. Specific CD8\textsuperscript{+} T cells are key contributors to limit viral spread and to maintain the latent state in the infected ganglia. In addition, virus-specific CD4\textsuperscript{+} T-lymphocytes coordinate the adaptative immune response by directing the antiviral activity of CD8\textsuperscript{+} T-lymphocytes and NK cells, and further stimulating the activity of phagocytic and B cells\textsuperscript{153,156}.

\textbf{6.1 Chemokines are important for immunity against HSV}

Accumulating data underscore the importance of the chemokine network for HSV pathogenesis. Although the temporal and spatial expression of chemokines during an HSV infection requires further study, a number of reports have provided evidence of chemokine production in HSV-infected tissues, including the CNS\textsuperscript{51,52}. The expression of some chemokines is upregulated upon HSV infection causing strong leukocyte infiltration that may lead immunopathological damage\textsuperscript{157}. In fact, chemokine expression increases during HSE development in humans\textsuperscript{158}. Whether this is a cause or a consequence of HSE is unknown at present.

Consistent with infections by other neurotropic viruses, the levels of CCL2, CCL3, CCL5 and CXCL1 are also increased in the spinal cord of HSV-2 infected animals\textsuperscript{53,159}. CCL2 is highly produced by microglia, and it has been associated with HSV-2 neuropathogenesis of the CNS\textsuperscript{51,160}. CCL3, produced by astrocytes among other cell types, is elevated during HSV-2 infection in mice, leading to IFN-\gamma production\textsuperscript{159}. CCL5, which has a detrimental impact on hepatitis virus infection in mice by increasing neuroinflammation, is significantly upregulated in the vaginal tissue, spinal cord and brain stem of HSV-2 infected mice\textsuperscript{52,159}. However, CCR5, the receptor for both CCL5 and CCL3, appears to be necessary to control HSV-2 vaginal infection through directing natural killer cell trafficking\textsuperscript{159,161}. Deficiency in CXCR3, the receptor for CXCL9, CXCL10 and CXCL11, also increases susceptibility to genital HSV-2 infection\textsuperscript{162-164}. Mice lacking CXCR3 show higher viral titers in the vaginal tissue and in the spinal cord, as well as a reduced cytotoxic T lymphocyte effector function and an impaired expression of plasmacytoid dendritic (pDCs) cells within the lymph node\textsuperscript{164}. The IFN-inducible chemokines CXCL9 and CXCL10 have specific roles in recruiting NK and virus-specific T cells into the primary site of infection and to the CNS, and they also facilitate the generation of effector T cells. In fact, these chemokines are highly upregulated upon HSV-2 infection, and animals deficient in either CXCL9 or CXCL10 deficient present a higher mortality.
after HSV-2 infection than wt animals\textsuperscript{159, 165}.

Other chemokines are expressed in tissues where HSV replicates and spreads. CCL25 is expressed by mucosal cells and found upregulated during oral wound healing\textsuperscript{166}; CCL28 is secreted by airway epithelial cells\textsuperscript{167} whereas CXCL14 is present at high levels in the human saliva\textsuperscript{168}. CXCL13, in turn, controls leukocyte recruitment to and proper function of nasal-associate lymphoid tissue\textsuperscript{169}. The tissue pattern-expression as well as the functions coordinated by CXCL12 suggests an implication of this chemokine in distinct aspects of HSV infection. Altogether, these and other findings support the involvement of chemokines in HSV biology.

7. VIRAL CHEMOKINE BINDING PROTEINS (vCKBPs)

Cytokines, including chemokines, play a variety of essential roles in the antiviral defence and therefore constitute main targets for viral immune modulation. Large DNA viruses, such as poxviruses and herpesviruses, encode proteins that mimic chemokines, cytokines and their receptors meant to counteract or evade the immune response of the host. Compared to poxviruses, herpesviruses are specialized in the expression of molecules that target the chemokine system, including homologues of chemokines and chemokine receptors, a fact that reflects the need to block different aspects of the immune system by each viral family\textsuperscript{170, 171}. The production of secreted versions of receptors for cytokines, such as TNF and IL-1 receptors, is a mechanism employed by both pathogens and hosts to limit the activity of cytokines in order to avoid immune pathology and undesired cell activation. A great number of such receptors have been identified in poxvirus, including four vTNFRs\textsuperscript{172-175}. The structure of GPCRs on the other hand, makes the expression of secreted versions of these receptors not feasible, and the immune system has evolved alternative mechanisms to control chemokine activity. One of such is the expression of decoy receptors that transport, internalize or degrade a specific set of chemokines creating functional chemokine patterns in tissues. Moreover, rather than activating conventional signalling routes, these receptors trigger G\alpha protein-independent signalling events that modulate the function of “classical” chemokine receptors\textsuperscript{125, 126}. Poxviruses and herpesviruses, however, express vCKBPs, secreted proteins that bind chemokines in solution (Table I-1). vCKBPs do not share any sequence similarity between themselves or with any host molecule, making difficult their identification by sequencing approaches\textsuperscript{170}. An interesting property of vCKBPs is that they inhibit the function of chemokines by means of either interfering with the interaction with their specific receptors, with GAGs or disrupting the binding to both, leading to a blockade of chemokine-induced signalling and cellular response\textsuperscript{176-179}. The redundancy of the chemokine network makes specific chemokine inhibitors not as effective as broad-spectrum chemokine antagonists. Thus, due to their broad and high affinity binding to chemokines, vCKBPs have been proposed as promising therapeutic agents for the treatment of chemokine-induced immunopathologies\textsuperscript{180-183}. Nearly all vCKBPs have been shown to effectively inhibit
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<td>smCKBP</td>
<td>Schistosoma mansoni</td>
<td>Binds some CC, CXC and CX3C chemokines, and blocks migration. Expressed by schistosome eggs</td>
<td>190</td>
</tr>
<tr>
<td>Ticks</td>
<td>Evasins</td>
<td><em>Rhipicephalus appendiculatus</em></td>
<td>Family of proteins (Evasins 1, 2 and 4) of restricted chemokine binding specificity, block binding of chemokines to cellular receptors and cell migration</td>
<td>189</td>
</tr>
</tbody>
</table>

Table I-1. CKBPs encoded by pathogens. Abbreviations: MHV-68, murine γ-herpesvirus; EHV, equine herpesvirus; BHV, bovine herpes virus; PRV, pseudorabies virus; ILTV, infectious laryngotracheitis virus; FeHV, felid herpesvirus; RanHV, rangiferine herpesvirus; CapHV, caprine herpesvirus; CerHV, cervine herpesvirus; HSV, herpes simplex virus; HCMV, human cytomegalovirus; MYXV, myxoma virus; VACV, vaccinia virus; CPXV, cowpox virus; ECTV, ectromelia virus; VARV, variola virus.
chemotaxis either \textit{in vitro} or \textit{in vivo}\textsuperscript{179, 184-188}. The expression of vCKBPs is restricted to the complex poxvirus and herpesvirus families, with the exception of a CKBP codified by the parasite \textit{Schistosoma mansoni} and a family of CKBPs codified by ticks\textsuperscript{189-191} (Table I-1).

\section*{7.1 vCKBPs encoded by herpesviruses}

Despite the key relevance of chemokines for the host response against herpesviruses, only three vCKBPs have been identified in \textit{Herpesviridae} so far: M3 from murine-\textgamma- herpesvirus 68, pUL21.5 encoded by the \textbeta-herpesvirus human cytomegalovirus (HCMV) and gG from \textalpha-herpesviruses. In addition, interaction of HSV gB with a reduced number of chemokine has been reported, although this interaction showed 1000-fold less affinity compared to the rest of vCKBPs (micromolar vs. nanomolar range), and did not have any effect on chemotaxis\textsuperscript{203}.

\textbf{The M3 protein from MHV-68.} M3 was the first example of a vCKBP identified in a herpesvirus\textsuperscript{192, 193}. M3 presents a broad binding-specificity, interacting with chemokines from all subfamilies. The crystal structure of M3 has been solved, alone and in combination with the chemokine CCL2\textsuperscript{204}. Despite the lack of sequence similarity to chemokine receptors, the chemokine-M3 interaction structurally mimics that of the chemokine with its receptor thereby impairing chemokine binding to the GPCR. Moreover, M3 prevents the binding of chemokines to GAGs, and disrupts pre-established chemokine-GAG interactions, probably by inducing conformational alterations in the chemokine\textsuperscript{178, 205}. \textit{In vivo}, M3 limits chemokine-mediated responses of particular cell types, reducing the immune response against the virus, and it is involved in the establishment of latency in lung and spleen\textsuperscript{186, 206-208}. Moreover, expression of M3 in transgenic mice has been used to analyse the role of chemokines during homeostasis or inflammation\textsuperscript{183, 208, 209}.

\textbf{HCMV pUL21.5.} In contrast to other vCKBPs, pUL21.5 was suggested to selectively bind CCL5 blocking its interaction with the GPCR. Nevertheless, since a very limited number of chemokines was tested in that study, the possibility that pUL21.5 binds other chemokines remains open. pUL21.5 mRNA is packaged into HCMV virions, suggesting the implication of pUL21.5 at very early stages of the virus infection\textsuperscript{197}.

\textbf{gG from non-human \textalpha-herpesviruses.} Previous results from our lab and others have reported the chemokine-binding ability of gG from several non-human \textalpha-herpesviruses, and shown that gG is able to efficiently inhibit chemokine function by blocking chemokine interaction with both receptor and GAGs\textsuperscript{179, 207}. Some of the animal \textalpha-herpesviruses that share such activity are EHV, PRV, ILTV and bovine herpesvirus-1 and 5\textsuperscript{179, 185, 194, 210}. Although gG is dispensable for viral replication \textit{in vitro}\textsuperscript{211, 212}, the high affinity interaction of gG with a broad spectrum of chemokines suggests a role for gG in immunoevasion during a viral infection. In fact, deletion of gG from ILTV results in attenuation of the virus in its natural host and causes an increase in tracheal mucosal thickness, reflecting an exacerbated inflammatory infiltrate at the site of infection when gG is absent\textsuperscript{213}. Moreover, a gG-deficient EHV-1 virus caused enhanced weight loss, higher virus titres in lungs and severe signs of respiratory disease due to massive
infiltration of immune cells when compared to the wt virus\textsuperscript{210}. These studies suggest that gG from non-human \textalpha{}-herpesviruses has a function in viral pathogenesis that is related to its chemokine-binding potential.

\section*{8. HSV gG}

In initial studies, no chemokine binding activity was detected when supernatants of cells infected with VZV, HSV-1 and HSV-2 were tested using different radio-iodinated chemokines\textsuperscript{179}. The gene encoding for gG is not present within the genome of VZV\textsuperscript{214}. On the contrary, both HSV-1 and HSV-2 encode gG, termed gG1 and gG2, respectively. HSV-1 and HSV-2 are closely related viruses with a high degree of similarity at the protein level, with the exception of gG that has an overall amino acid identity <30%. In fact, the detection of anti-gG antibodies is used as a serological marker to distinguish HSV-1 and HSV-2 infection in clinical settings\textsuperscript{18, 19}. Both gG1 and gG2 are present in the virion and at the plasma membrane of infected cells. gG2 presents a unique property, since it is further processed by cellular proteases, giving rise to a 40-kDa product that is secreted to the extracellular medium (SgG2) and to a mature membrane-anchored form (mgG2)\textsuperscript{13, 14}. The processing site has not been precisely determined, but it has been proposed to occur between Arg 321 and Ala 322 or Arg 342 and Leu 343 residues\textsuperscript{215}. A scheme of gG2 processing is shown in Fig. I-7. Contrary to gG2, and similarly to the rest of HSV glycoproteins, gG1 is present at the membrane of the infected cell and in the viral envelope, but it is not secreted\textsuperscript{11}. Whether these differences determine a distinct functionality of gG1 and gG2 is currently unknown.

gG is the less well characterized HSV glycoprotein, and its role in the viral life cycle and pathogenesis has not been clearly elucidated. However, several reports support the involvement of gG in the pathogenesis of the virus. HSV gG1 seems to be implicated in entry into, but not initial binding to, polarized epithelial cells through the apical surface\textsuperscript{27}. In vivo, three reports indicate that the absence of gG leads to different degrees of HSV-1 attenuation\textsuperscript{211, 216, 217}. Lower virus titres were found in sensory ganglia, spinal cord and brain of mice infected through scarification of the ear with an HSV-1 lacking gG1. A
double us3/us4 insertional mutant (U3 double gG1 and gG2 enhance chemokine-mediated cell migration. gG was not chemotactic on its own, and the increase in chemotaxis required the interaction of the viral protein with the chemokine, since no effect was observed when migration was assayed towards CCL2, a chemokine not bound by gG. The enhancement of chemokine-directed recruitment of immune cells by SgG2 was also demonstrated in vivo, by using the air-pouch model of inflammation. Furthermore, we have shown that signalling pathways triggered by chemokines, including activation of G proteins and phosphorylation of MAPKs, are also upregulated upon SgG interaction with chemokines. Most of our previous work was performed with recombinant soluble gG. Whether gG present in the virion binds chemokines and the functional relevance of such interaction is unknown at present.

We have reported the first vCKBP encoded by a human pathogen that modulates chemokine-induced signalling leading to an enhanced migration in vitro and in vivo (Fig. 1-8). Furthermore, recent unpublished data from our laboratory shows that HSV SgGs are able to bind neurotrophins, including NGF, with high affinity (Viejo-Borbolla A., Martinez-Martin N., Wandosell F., Alcami A. and Cabrera JR., manuscript in preparation). Similarly to chemokines, SgG binds secreted neurotrophins and modifies their signalling properties. We hypothesize that gG could behave as a local regulator of axonal navigation providing an advantage in facilitating infection of sensory ganglia.

Our work supports the notion that the differences in pathogenicity and viral colonization of the nervous system observed in HSV gG-deficient mutants
may be associated with the dysregulation of chemokine and neurotrophin function exerted by gG. Besides, these observations point towards a previously undescribed strategy of immune modulation, and may uncover a novel neuroregulatory mechanism developed by HSV.
Introduction

Figure I-8. Potential mechanisms mediating HSV gG enhancement of chemokine function. During an HSV-1 or an HSV-2 infection, infected cells express gG1 in their plasma membrane and secrete SgG2 to the extracellular medium, respectively. gG binds chemokines impairing their interaction with surface GAGs. Contrary to what is expected, such binding results in an improved interaction with the chemokine receptor that leads to an increase in chemokine-mediated signalling and to a higher recruitment of immune cells to the site of HSV infection. The molecular basis for such increase in chemokine-triggered activation and migration of cells had not been determined.

The upper panel shows the recruitment of immune cells to HSV-infected tissues in response to chemokines. The lower panel illustrates potential effects of gG1 and gG2 on chemokine activity, causing changes in the chemokine structure, altering the conformational status of chemokine receptors, influencing chemokine receptors location and trafficking or differential recruitment of signalling components. These effects cause enhanced chemokine-mediated signalling and a more active migration of cells in response to a lower concentration of chemokines.
AIMS
Previous studies on the role of gG during HSV infection have revealed that gG is a virulence factor. Notwithstanding such important role, the mechanisms beneath such phenotype are unknown. We have previously identified HSV gG as the first vCKBP that enhances chemokine function both in vitro and in vivo, but the molecular mechanism causing this unique effect on chemokines is unknown. Our results point towards a novel strategy of viral immunomodulation mediated by HSV gG that could be involved in the different outcomes observed in pathogenesis following HSV infection. Given the essential role played by chemokines in both the immune and the nervous system, the dysregulation of chemokine function induced by HSV gG may be important for viral-related immunopathogenesis and neuropathology. Recently, we have also shown that HSV SgG interacts with neurotrophins, such as NGF, and modulates their function. We have just started to uncover the functional meaning of such interactions. A deeper understanding of SgG regulation of NGF-dependent responses will provide new insights into the interplay between HSV and the nervous system.

During this thesis work we have focused our efforts on three main objectives:

1. Characterization of the molecular mechanism(s) beneath gG-induced enhancement of chemokine function and the cellular partners involved.

   1.1 To further evaluate cell chemotaxis in the presence of SgG2 by time-lapse videomicroscopy.
   
   1.2 To determine the ability of SgG to bind to the cell surface and to identify the ligand involved in such interaction.
   
   1.3 To study the impact of SgG on chemokine receptor trafficking, focusing on the CXCR4/CXCL12 axis.
   
   1.4 To analyze the internalization dynamics and surface localization of other chemokine receptors, non-related GPCRs and other types of receptors.
   
   1.5 To characterize the regulatory effects exerted by SgG2 on several aspects of CXCR4 functionality, including microdomain partitioning in the plasma membrane, post-translational modifications and conformational rearrangements of the receptor.
   
   1.6 To address the aggregation state of CXCR4 upon SgG2-stimulation of the cells by electron microscopy analysis of cell surface replicas.
2. Evaluation of the chemokine-binding potential of HSV particles.

2.1 To assess the ability of HSV virions to bind chemokines and the contribution of gG present in the viral envelope to that interaction by means of SPR.

2.2 To analyze the function of virion-chemokine interaction in vitro.
Materials and methods
Table MM-1. Antibodies used during this work.

<table>
<thead>
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<th>Antibody</th>
<th>Clone/number</th>
<th>Description</th>
<th>Use/dilution</th>
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<td>WB 1/1000, EM 1/50</td>
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<td>SgG2</td>
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<td>Everett et al.²²²</td>
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<td>GM1</td>
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Table MM-1 (continuation)

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<th>Technique</th>
<th>Source</th>
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Abbreviations: WB, Western blot; EM, electron microscopy; FACS, flow cytometry; IF, immunofluorescence.

Table MM-2. Reagents and other materials.

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<td>Invitrogen</td>
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<td>Ficoll</td>
<td>17-1440-02</td>
<td>Gradient separation</td>
<td>PBMC purification</td>
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Materials and methods

Heparin: H8537, Porcine intestinal mucosa, SPR, pulldown, Sigma
Chondroitin sulfate A: C9819, Bovine trachea, SPR, Sigma
Chondroitin sulfate B: C3788, Porcine intestinal mucosa, SPR, Sigma
Heparan sulfate: H7640, Bovine kidney, SPR, Sigma
Heparin-agarose beads: H3025, Heparin-agarose, Cyanogen-activated, Pulldown-assay, Sigma
Collagen: Rat collagen, Coating, BD Biosciences
VCAM-1: 809-VR, Recombinant human VCAM-1, Coating, 3 μg/ml, R&D Systems
Fibronectin: F2006, Human fibronectin, Coating, 20 μg/ml, Sigma
Poly-D-lysine: P0899, Not applicable, Coating, 100 μg/ml, Sigma
Poly-L-ornithine hydrom bromide: P3655, Not applicable, Sigma
BSA fatty acid free: A8806, Time-lapse buffer, Time-lapse, Sigma
Prolong gold antifade: 36930, Mounting media, IF, Invitrogen
Transwell M96 plates: ChemoTx101-3, Chemotaxis plates, migration, NeuroProbe
Glass-bottom Culture dishes: 80826, 15 μl slide 8 well, Time lapse, Ibidi
Glass-bottom Culture dishes: P35G-1.5-10, 35mm Petri dish 10mm microwell, Time lapse, Mat-tek

Abbreviations: IF, immunofluorescence; FACS, flow cytometry; PBMC, peripheral blood mononuclear cells; SPR, surface plasmon resonance.

Table MM-3. Plasmids used during this work.

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<th>Description</th>
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<td>M. Thelen</td>
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<td>M. Thelen</td>
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<td>pcDNA Flag-CXCR4</td>
<td>N-terminal Flag-tagged CXCR4</td>
<td>IF, internalization assays</td>
<td>J. Benovic</td>
</tr>
<tr>
<td>pcDNA GFP-CXCR4</td>
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<td>Time-lapse</td>
<td>M. Mellado</td>
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<tr>
<td>Flag-β&lt;sub&gt;2&lt;/sub&gt;AR</td>
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<td>IF, internalization assays</td>
<td>J. Benovic</td>
</tr>
<tr>
<td>β-arrestin2-GFP</td>
<td>Wt β-arrestin2 GFP tagged</td>
<td>Time-lapse, IF</td>
<td>Braun et al.</td>
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**Materials and methods**

**Table MM-3 (continuation)**

<table>
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<tr>
<th>β-AR-Rluc</th>
<th>C-terminal tagged β-AR</th>
<th>BRET&lt;sub&gt;2&lt;/sub&gt;</th>
<th>JL. Benovic&lt;sup&gt;238&lt;/sup&gt;</th>
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<tr>
<td>β-arrestin&lt;sub&gt;2&lt;/sub&gt; (1-382)-GFP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Constitutively active β-arrestin&lt;sub&gt;2&lt;/sub&gt; fused to GFP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>BRET&lt;sub&gt;2&lt;/sub&gt;</td>
<td>JL. Benovic&lt;sup&gt;227&lt;/sup&gt;</td>
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<tr>
<td>CXCR4-Rluc</td>
<td>Rluc C-terminal tagged CXCR4</td>
<td>BRET&lt;sub&gt;2&lt;/sub&gt;</td>
<td>JL. Benovic&lt;sup&gt;196&lt;/sup&gt;</td>
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<tr>
<td>CXCR4-GFP&lt;sub&gt;2&lt;/sub&gt;</td>
<td>GFP&lt;sub&gt;2&lt;/sub&gt; C-terminal tagged CXCR4</td>
<td>BRET&lt;sub&gt;2&lt;/sub&gt;</td>
<td>JL. Benovic&lt;sup&gt;196&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Abbreviations: IF, immunofluorescence; BRET, bioluminescence resonance energy transfer.

**Chemokines.**

Recombinant chemokines used in the Biacore X biosensor (hCCL1, hCCL2, hCCL3, hCCL3L1, hCCL4, hCCL4L1, hCCL5, hCCL7, hCCL8, hCCL11, hCCL13, hCCL14, hCCL15, hCCL16, hCCL17, hCCL18, hCCL19, hCCL20, hCCL21, hCCL22, hCCL23, hCCL24, hCCL25, hCCL26, hCCL27, hCCL28, hCXCL1, hCXCL2, hCXCL3, hCXCL4, hCXCL5, hCXCL6, hCXCL7, hCXCL8, hCXCL9, hCXCL10, hCXCL11, hCXCL12α, hCXCL12β, hCXCL13, hCXCL14, hCXCL16, hXCL1, hX3CL1) and the cell migration assays were obtained from PeproTech (London, UK), with the exception of hCCL25 and hCXCL13, which were from R&D Systems (Minneapolis, MN). Wild-type CXCL12, CXCL12<sub>3/6</sub> (K24S/H25S/K27S), CXCL12<sub>4-67</sub>, and CXCL12<sub>P2G</sub> were kindly provided by F. Arenzana-Seisdedos<sup>58</sup>. CXCL12<sub>-biotin</sub> conjugated was a gift from M. Thelen lab (IRB, Switzerland). CXCL12<sub>-biotin</sub> was conjugated with Quantum Dots (QDots)-streptavidin (Invitrogen) at a ratio 4:1 (CXCL12-biotin/QD605, mol/mol) for 15 min at RT immediately before the experiments. Recombinant neurotrophins NGF and Artemin were purchased from Alomone labs (Jerusalem, Israel) and Peprotech, respectively.

**Cells.**

Jurkat (human, peripheral blood, leukemia, T cell) and MonoMac-1 (MM-1, human, monocyte-like) cells were grown in RPMI 1640 (Sigma) containing 10% foetal bovine serum (FBS) (R-10 medium). m300-19 cells (mouse B cells) and m300-19-hCXCR5 (stably transfected with hCXCR5) were a gift of Dr. Bernhard Moser (Cardiff University) and were grown in R-10 medium supplemented with β-mercaptoethanol (5 x 10<sup>-5</sup> M) and sodium pyruvate (1%). m300-19-hCXCR5 were also supplemented with puromycin (1.5 μg/ml). HEK-293T, MDCK, Hela and Vero cells were grown in DMEM containing 10% FBS. CHO-K1 and CHO-618 cells were grown in DMEM-F12 1:1 medium containing 10% FBS. All mammalian cell lines were cultured at 37°C, 5% CO<sub>2</sub> in a humidified incubator. ACP-CXCR4 and Lck<sub>10</sub>mCherry HEK-293T expressing cells were a gift of Dr. Marcus Thelen (IRB, Switzerland) whereas Flag-CXCR4 and Flag-β<sub>2</sub>AR HEK-293T cells were kindly provided by Dr. JL. Benovic (Thomas Jefferson University, USA). HEK-293T cells expressing the construct Lck<sub>10</sub>mCherry were generated during this work by lentiviral transduction as previously described<sup>225</sup>, using the plasmids provided by Dr. Marcus Thelen (IRB, Switzerland). SF9 and Hi-5 insect cells were cultured in TC-100 medium (Invitrogen) containing 10% foetal bovine serum. When necessary, adherent Hi-5 insect cells were grown in EX-Cell 405 medium (SAFC Biosciences) to avoid the presence of FBS. Conditioned Hi-5 cells
were grown as suspension cultures in serum-free Five Express medium (Invitrogen). All insect cells were grown at 28°C.

**Viruses and purification procedure.**

HSV-1 wild type (wt), strain SC-16, HSV-1ΔgG and HSV-2 wt strain 333 were a gift from Dr Helena Browne (Cambridge, UK). HSV-1ΔgG is a virus mutant that does not express gG and has been described previously\(^{211}\) gE, gl or the putative gJ. HSV viruses were amplified by infecting subconfluent Vero cells at a low multiplicity of infection (moi) (0.1). Viral absorption was performed in the presence of 2% FBS and replaced by DMEM medium containing 10% FBS following 2h incubation at 37°C. wt HSV-1, ΔgG HSV-1 and wt HSV-2 were purified by Ficoll gradient, as previously described\(^{229}\). Typically, around 600x10^6 cells grown in P150-cm² culture dishes were infected. In brief, supernatants from infected cells were collected and clarified by centrifugation at 2,000 rpm during 10 min to remove cell debris. Subsequently, viral particles were concentrated by ultracentrifugation at 18,000 rpm during 2 h, using a SW28 Beckman Coulter rotor. The pellet was resuspended in sterile PBS, sonicated and layered onto a 15-30% Ficoll 400-DL gradient (Sigma). Following ultracentrifugation the samples at 12,500 rpm in a SW28 rotor during 90 min 1ml fractions were collected from top to bottom of the gradient. Aliquots of each fraction were analyzed by SDS-PAGE and western blot using a polyclonal anti gB/gD antibody provided by Enrique Tabarés. Fractions enriched in viral particles were diluted in PBS and ultracentrifuged at 21,000 rpm during 90 min. The resultant pellet formed by purified virions was resuspended in 400μl of PBS and kept at -80°C. All the procedure was performed at 4°C using pre-cooled instrumentation. For comparative reasons, HSV particles were purified by a 15-30% sucrose cushion. Prior to any further manipulation, viruses were inactivated by trioxsalen treatment and UV-light irradiation, as previously described\(^{230}\). The protein concentration in the viral stocks prepared was measured by using a BCA protein assay kit (Pierce) following manufacturer’s instructions, and the purity of the preparation was further assessed by direct visualization by electron microscopy.

**Generation of recombinant baculoviruses and purification of recombinant proteins.**

Recombinant proteins were generated using the recombinant baculovirus expression system followed by protein purification by affinity chromatography, as previously described\(^{196}\). The SgG1 and SgG2 constructs used in this study have been previously described\(^{196}\). The honeybee melittin secretion signal peptide present in pMelBac (Invitrogen) was cloned into the pFastBac plasmid (Invitrogen) to produce pFastBacMel. Truncated forms of SgG2 were amplified by PCR using DNA from HSV-2-infected cells as templates using the primers listed in Table MM-3. The amplified DNA products were cloned into pFastBacHTb (Invitrogen) without the putative gG signal peptide sequence. A PCR reaction was performed to amplify truncated His-tagged-SgG2 versions that were subsequently cloned into the pFastBacMel vector. All constructs were sequenced to confirm the absence of undesired mutations. The pFastBacMel-HisG2s recombinant plasmids were transformed into competent DH₅Bac bacteria where the corresponding recombinant bacmids were generated by a transposition event. Purified
bacmids were transfected into Hi-5 insect cells using Cellfectin (Invitrogen) in order to obtain recombinant baculoviruses. The supernatant from the transfected cells was collected 72 h post-transfection and the viruses were amplified in Hi-5 or Sf9 cells. In order to express high amounts of recombinant proteins, Hi-5 cells were infected at a high multiplicity of infection (moi) and the supernatant was collected 72 h post-infection, clarified by centrifugation and concentrated using Stirred Ultrafiltration Cell 8200 (Amicon) and then desalted and buffer exchanged against phosphate buffer containing 10 mM imidazole (PD-10 desalting columns; Amersham Biosciences). The recombinant protein (expressed fused to a N-terminal six-His tag) was purified by metal chelate affinity chromatography (Ni-nitrilotriacetic acid resin; Qiagen). Purified recombinant protein-containing fractions were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining and then were pooled, concentrated, and dialyzed against HEPES buffer pH 7.4 containing 100mM NaCl, 20% glycerol by using a Vivaspin500 device (VivaScience).

**Table MM-4. Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’-3’ nucleotide sequence</th>
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<td>pFBHT-F</td>
<td>TAAGAATTCCATCACCATCACCATACGATTACC</td>
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<td>delCter gG2-1</td>
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<td>delCter gG2-2</td>
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<td>delCter gG2-3</td>
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<td>Nter3 sphl</td>
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<tr>
<td>delNter gG2-3</td>
<td>TAAGAATTCCATCACCATCACCATACGATTACC</td>
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</tbody>
</table>

**Determination of the chemokine binding specificity of SgG2 truncated versions by SPR.**

Binding specificity and affinity constants calculated during this work were determined by SPR technology using a Biacore X biosensor (GE Healthcare) available in the laboratory. Proteins were dialyzed against acetate buffer pH 5.5 prior to amine-coupling of the recombinant proteins in CM5 chips following manufacturer’s instructions. Chemokines were injected at a concentration of 100nM diluted in HBS-EP buffer (10 mM HEPES, 150 mM, NaCl, 3 mM EDTA, 0.005% (vol/vol) surfactant P20, pH 7.4) at a flow rate of 10 µl/min, and association and dissociation phases were monitored. All Biacore sensorgrams were analyzed with the software Biaevaluation 3.2. Bulk refractive index changes were removed by subtracting the reference flow cell responses, and the average response of a blank injection was subtracted from all
Materials and methods

**SgG-Heparin binding analysis by SPR.**

To measure the kinetic and affinity binding parameters between SgGs and heparin, we used a streptavidin (SA) sensor chip (BIACore, Inc.) where biotynilated heparin had been immobilized. The sensor chip was conditioned with three consecutive injections of 1 M NaCl in 50 mM NaOH according to the manufacturer’s instructions. For kinetic studies, SgG protein was serially diluted into HBS-EP buffer, and 60 µl of each protein concentration was injected over both flow cells at a rate of 30 µl/min. Following the association phase, the dissociation phase was monitored during 5 min. The surface was regenerated by eluting bound protein with a 30 µl injection of 2 M NaCl. Bulk refractive index changes were removed and curves were globally fitted using a 1:1 mass transport binding model.

**GAG competition assays using SPR.**

To examine the oligosaccharide structures that SgG2 was capable of binding, we used the heparin-SA sensor chip in an SPR competition assay that involved the preincubation of 100 nM of the viral protein with several concentrations (0, 1, 10, 100, and 1,000 µg/ml) of soluble GAG competitors (Sigma), including heparin, heparan sulfate, chondroitin sulfate A, and chondroitin sulfate B, for 10 min prior to SPR analysis. The protein preincubated with the corresponding GAG was injected over the chip at a 10 µl/min flow rate, and the response at equilibrium was recorded. gG binding to the heparin surface in the absence of any competitor GAG was considered 100%.

**Heparin-agarose based pull-down assay.**

Different concentrations of SgG1 and SgG2 or their truncated versions were incubated with 10 µl of heparin-agarose beads (50%, vol/vol) in 400 µl of binding buffer (PBS with 0.2% bovine serum albumin (BSA)) on a rotating wheel for 1 h at room temperature. The beads were then recovered by centrifugation in a microcentrifuge for 1 min at 14,000 rpm. The supernatant was discarded, and the beads were washed three times with 400 µl of PBS. Bound viral proteins were eluted from the beads by the addition of 25 µl of SDS-PAGE loading buffer, boiled for 1 min, and then subjected to SDS-PAGE and detected by immunoblotting.

**Negative staining of viruses and immunogold labelling of viruses.**

The purity and integrity of virion preparations was assessed by uranyl acetate negative staining and analysis by electron microscopy. Purified viruses were fixed with 2% glutaraldehyde and attached to copper grids during 3min at RT. Following washing with tridistilled water, grids were stained with 2% uranyl acetate during 40 sec. The copper grids were examined on a JEM1010 electron microscope (Jeol, Japan) operating at 80 kV. Images were taken with a slow scan CCD camera (Bioscan, Gatan, Pleasanton, CA).

For immunogold staining, Ficoll-puriﬁed viruses were fixed with 0.1% glutaraldehyde and subsequently attached to nickel grids. After washing with tridistilled water and TBS buffer (50mM Tris-HCl pH 7.5 150mM NaCl) the samples were incubated with TBS containing 3% BSA to block unespeciﬁc binding and incubated with the corresponding primary antibody...
during 1 h at RT. Following the washing step, samples were incubated with 10 nm gold-conjugated protein A (Cell Microscopy Center) or with a 10 nm gold-conjugated anti-mouse immunoglobulin (British Biocell) when the primary antibody used was produced in mouse. The grids were negatively stained and visualized on a JEM1010 electron microscopy (Jeol, Japan). Images were acquired with a slow scan CCD camera (Bioscan, Gatan, Pleasanton, CA).

**Coupling of viral particles to a BIAcore sensor chip.**

Purified virions were diluted in acetate buffer pH 4 (1:1) prior to their covalent immobilization to the short carboxy-dextran matrix of F1 (CM3) sensor chips (BIAcore AB, Sweden) via amino groups. This mild acification of viruses was found to be non-disruptive, as confirmed by subsequent cell infection assays (not shown). The immobilization was performed following the standard amino-coupling chemistry technique according to manufacturer’s instructions. Briefly, the carboxyl groups of the chip surface were activated by injecting a 1:1 mixture of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1 M) and N-hydroxysuccinimide (0.25 M) at 5 μl/min. One or two injections of HSV virions were performed, until the desired level of response units (RU) was reached, normally between 2000-3000 RU. Following attachment in the Fc-2 flow cell, the remaining surface carboxyl groups were quenched with 35 μl of 1 M ethanolamine pH 8.5 at 5 μl/min. The Fc-1 flow was left uncoupled as a control for non-specific chemokine binding to the dextran surface.

A similar procedure was followed to immobilize CXCR4-lentiviral particles to F1 (CM3) chips. Lentiviruses expressing ACP-CXCR4 or an irrelevant construct (the PH domain of PKB fused to mCherry) were produced in HEK-293T cells. Supernatants containing the lentiviral particles were collected 48 and 72 h post transfection, pelleted by centrifugation and filtered through 0.45 μm filters (Millipore). Lentiviral particles were purified by sucrose cushion and stored at -80°C. The purity and integrity of the viral preparations were visualized by electron microscopy. Around 3000 RUs of CXCR4-lentiviruses were covalently coupled to the Fc-2 flow cell of the sensor chip while control lentiviral particles were attached to the Fc-1 flow cell as a reference surface.

**Determination of HSV binding specificity to chemokines using SPR.**

For screening purposes, chemokines were injected at a 100nM concentration over the surface of a F1 (CM3) sensor chip containing viral particles in HBS-N buffer (10 mM HEPES, 150 mM, NaCl, pH 7.4) at a flow rate of 5 μl/min and association and dissociation were monitored at 20°C. HBS-N was also used as a running buffer. The chip surface was regenerated after each chemokine injection by 2 or 3 30 μl pulses of HBS-N buffer pH 4.5 to 5.5. Regeneration conditions were optimized empirically by testing various reagents in order to remove bound protein while maintaining surface activity. For kinetics analysis, different concentrations of the chemokine were injected at a flow rate of 30 μl/min over a 2 min period and allowed to dissociate for 5 min. Chemokines that did not bind under screening conditions were considered negative and were not taken into further consideration for the study. Bulk refractive index changes were removed by subtracting the background corresponding to the reference flow cell, and the average response of a blank
injection was subtracted from all analyte sensorgrams to remove systematic artifacts. BLACore sensorgrams were analyzed using BIAevaluation 3.2 software, and kinetic data were globally fitted to a 1:1 Langmuir model. A similar procedure was used for CXCR4-lentiviral chips.

Chemotaxis assays.
Chemokines were placed in the lower compartment of 96-well ChemoTx System plates (Neuro Probe Inc., MD, USA) with or without recombinant SgG2 or HSV virions in RPMI 1640 containing 1% FBS. Between 5x10^9 and 5x10^10 virions alone or in combination with chemokines were used in each well. Primary monocytes or MM-1 cells were placed on the upper compartment (typically 2.5x10^5 cells were used). Both chambers were separated by a 3 µm pore size filter. The plates were incubated at 37ºC during 1–2 h and the number of MM-1 in the lower chamber was determined by staining them with CellTiter 96 aqueous one solution cell proliferation assay (Promega, USA) during 2 h at 37ºC and measuring absorbance at 492 nm. In the case of monocytes, the total number of migrated cells present in the lower compartment was counted with a light microscope. When chemical inhibitors were used, the cells were incubated with the compounds for 30min at 37ºC prior to the assay. Drugs were maintained during the migration assay. The inhibitors were used at the following concentrations: MβCD 7mM (Sigma), filipin 50 µg/ml (Sigma), nystatin 1.5 µg/ml (Sigma) chlorpromazine 2 µM (Sigma) and the Src kinase inhibitor PP2 (Sigma) and the PI3K inhibitor LY294002 (Calbiochem) were used at 10 and 35 µM respectively. For the preparation of the virion stocks used in chemotaxis assays, Vero cells were infected at a low moi and grown in Optimem serum-free medium (Gibco). Supernatants were collected after 72 h, clarified by centrifugation, and inactivated by trioxsalen and UV-light exposure230. The virus-containing supernatants were concentrated 100x using 100K cut-off centrifugal filter units (Millipore). The number of viral particles in the concentrated stocks was quantified by electron microscopy. Viruses were incubated with a solution of 100nm latex particles used as an standard of known concentration. The combination of virions and beads was attached to copper grids and visualized by electron microscopy following negative staining. Images from at least 20 different fields were taken at 15,000x magnification and viral particle counts were obtained using Image J software. Independent preparations of viruses were used during the transwell assays shown. The stocks obtained typically contained between 1x10^9 and 1x10^10 viral particles/ml.

Activation of MAPKs.
Different concentrations of CXCL12 alone or in combination with viruses (10^10–10^11 particles) were added to 5x10^5 MM-1 cells and incubated during 1 min at 37ºC. The reaction was stopped with cold-PBS on ice and cells were lysed in lysis buffer (20 mM triethanolamine pH 8.0, 300 mM NaCl, 2 mM EDTA, 20% glycerol, 1% digitonin and proteinase inhibitors). The lysate was analyzed by western blotting using anti-phospho-ERK, anti-phospho-P38 (Cell Signaling), anti-phospho-JNK1/2 polyclonal (Abcam) or anti-phospho AKT (Cell Signalling) antibodies. Blots were scanned and the densities of the bands were analyzed and compared with the Image J 1.43 software normalizing the densities obtained from each band from the MAPK blots to their respective loading controls.
Materials and methods

Isolation of human monocytes.
Monocytes were isolated from blood of healthy donors by negative selection using Monocyte Isolation kit II (MACS Miltenyi Biotec), following manufacturer’s instructions. In brief, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll (Lymphoprep) gradient centrifugation. Cells were resuspended in MACS buffer and incubated with FcR blocking reagent at 4ºC. Non-monocytes were indirectly magnetically labelled using a cocktail of biotin-conjugated antibodies followed by anti-biotin microBeads. Highly enriched unlabelled monocytes were obtained by depletion of the magnetically labelled cells.

Time-lapse analysis of chemotaxis.
Time-lapse video microscopy analysis of chemotaxis was performed immediately after monocyte isolation with a Leica DIL6000 microscope stand connected to a SP5 scan head equipped with a temperature controlled chamber (Cube, LIS, Basel). Freshly isolated monocytes were placed in a humidified and CO₂-controlled incubator, which was mounted on the microscope stage (Brick, LIS, Basel). Cells were resuspended in D-PBS containing calcium and magnesium (Invitrogen) supplemented with 1% FBS, Pen/Strep, 0.04 mM sodium pyruvate, 1 mg/ml fatty acid free BSA (Sigma) and 1 mg/ml glucose. Cells were plated on glass bottom petri-dishes (MatTek culture-ware) which were coated previously with poly-D-lysine (5 mg/ml) and subsequently overlaid with 3 mg/ml VCAM-1 (BD Biosciences) O/N at 4º. Before plating the cells, coated-dishes were treated with PBS containing FBS and BSA to block non-specific binding. Chemokine was dispensed with a micropipette (Femtotip II, Eppendorf) controlled by a micromanipulator (Eppendorf) at a constant backpressure of 30 hPa (Femtojet, Eppendorf).

Analysis of primary monocyte polarization.
Freshly isolated monocytes were adhered to poly-D-lysine-coated coverslips, and the cells were mock-treated or stimulated with 8n M CXCL12, 800 nM SgG1, SgG2 or PRV-SgG or with the vCKBPs:CXCL12 at a 50:1 molar ratio during 15 or 30 min at 37ºC. After stimulation the cells were PFA-fixed and stained using a polyclonal antibody against CXCR4 (Abcam) and an anti-GM3 antibody (Dr. Illa) or FITC-conjugated cholera toxin β-subunit (Sigma) to detect GM3 and GM1 lipid rafts, respectively. The incorporation of CXCR4 into GM3, as leading edge markers, and GM1 rafts, as uropod markers, was quantified. Images were subjected to background correction and threshold analysis in order to quantify the area corresponding to lipid rafts in the stimulated leukocytes. In additional experiments, stimulated monocytes were simultaneously stained against CXCR4, GM3 and GM1, obtaining the same results (not shown).

Analysis of protein binding to cells by flow cytometry.
Cells in suspension were washed and resuspended in 1% FBS-containing RPMI 1640 medium to perform the experiment. Adherent cells were harvested with 4 mM EDTA in PBS, washed and resuspended in 1% FBS DMEM-F12 or 1% DMEM medium. 10⁵ cells were incubated on ice with different amounts of purified proteins, supernatants from HSV-2 infected-cells or supernatants containing equivalent amounts of VACV B18 and ECTV sema proteins during 30 min at 4ºC.
Materials and methods

VAVC B18 and ECTV sema proteins have been previously described\(^{231}\) during 30 min at 4°C. Following incubation, cells were washed with PBS 1% BSA, 1% FBS, and protein binding was assessed by flow cytometry using an anti-gG2 polyclonal antibody (Austral Biologics) or an anti-His tag antibody (QIAGEN) to detect B18 and sema, followed by Alexa Fluor 488 donkey anti-mouse IgG secondary antibody (Invitrogen). As a control, cells were incubated with primary and secondary antibodies in the absence of the viral proteins. Data were collected on a FACSCalibur (Becton Dickinson) and analyzed using FlowJo 7.2.2 software (Treestar, OR, USA).

Analysis of protein binding to cells by immunofluorescence.

CHO cells or primary monocytes were attached to untreated or to poly-D-lysine-coated coverslips, respectively. CHO cells were incubated with DMEM 1% FBS containing 400ng of SgG2, supernatants from HSV-2 infected cells or supernatants containing equivalent amounts of VAVC B18 and ECTV sema proteins during 30 min at 4°C. VAVC B18 and ECTV sema proteins have been previously described\(^{231}\). Primary monocytes were incubated with 400 ng of SgG2 during 30 min at 37°C. Afterwards, the cells were washed and fixed with 4% PFA, and subsequently stained using a polyclonal antibody raised against SgG2 (Liljeqvist et al.\(^{215}\)), an anti-His tag antibody (QIAGEN) to detect B18 and sema (in the case of CHO cells), and a polyclonal antibody to detect CXCR4 (Abcam) in the case of monocytes. Primary antibodies were detected by incubating the cells with specific fluorophore conjugated-secondary antibodies (Alexa antibodies, Invitrogen) during 45 min at 37°C and nuclei were stained using DAPI (Invitrogen).

Primary mouse neurons from the superior cervical ganglion (SCG), isolated as described in the section “Isolation of primary mouse neurons and culture” below, were grown on collagen-coated coverslips, and incubated with DMEM 1% FBS in the presence of 800nM SgG2, 8nM CXCL12 or the complex SgG2:CXCL12 at a 100:1 molar ratio. The stimulation was performed for different time points and the cells were fixed with 4% PFA and stained using an anti-mouse CXCR4 antibody (eBioscience) followed by Alexa 555-conjugated streptavidin. For SgG2 binding studies, the neurons were incubated with 400ng of SgG2, washed and fixed with PFA. The presence of SgG2 at the surface was detected using a polyclonal antibody raised against SgG2 (Austral Biologics).

Receptor internalization assays.

CXCR4 internalization was analyzed by flow cytometry in MM-1, Jurkat cells, primary monocytes and HEK-293T cells. Cells (10\(^5\)) were mock treated or incubated with 1.5 μM SgG or PRV-SgG, 15nM CXCL12 15nM or the complex vCKBP:CXCL12 at a 100:1 molar ratio for different time points in 1% FBS-containing RPMI medium, washed with ice-cold PBS, and incubated with PBS containing 2% FBS, 2% BSA to block unspecific binding. A 50:1 molar ratio between the vCKBPs and CXCL12 was used for primary monocyte stimulation. PE-conjugated anti-CXCR4 (R&D Systems) was added to the cells for 1h at 4°C. Finally, cells were washed and analyzed by flow cytometry to quantify the receptor present on the surface. The same procedure was used to measure surface CCR2. In this case, MM-1 cells were stimulated with 5nM CCL2, 500nM SgG2.
or SgG2:CCL2 at 100:1 molar ratio, and incubated with APC-conjugated CCR2 (R&D Systems). For CD71 internalization studies, MM-1 cells were serum starved for 1 h prior to the assay, and then stimulated with transferrin (Sigma), 1.5 μM SgG2 or both. The cells were washed and incubated with anti-CD71 (BD Pharmingen) for 1 h at 4ºC, washed again, and incubated for 45 min at 4ºC with anti mouse Alexa488-conjugated antibody (Invitrogen). The excess of secondary antibody was removed by washing steps, and receptor levels at the surface were analyzed by flow cytometry. Data were collected on a FACSCalibur (Becton Dickinson) and analyzed using FlowJo 7.2.2 software (Treestar, OR, USA).

For HEK-293T cells stably expressing Flag-CXCR4, an enzyme-linked immunosorbent assay that measures the level of epitope-tagged cell surface receptors was performed, as described previously. Briefly, cells were stimulated with 8nM CXCL12, 800 nM SgG2 or the complex SgG2:CXCL12 at a 100:1 molar ratio for different time points at 37ºC, washed and fixed. The levels of CXCR4 remaining at the surface were detected using a monoclonal antibody directed against the Flag-tag (Sigma) followed by incubation with a goat anti-mouse alkaline phosphatase-conjugated antibody (Sigma). Cells were washed three times, and antibody binding was detected by adding 0.25 ml of alkaline phosphatase substrate (Bio-Rad). Development was stopped by removing 0.1 ml of the substrate to a 96-well microtiter plate containing 0.1 ml of 0.4 M NaOH. Plates were read at 405 nm in a microplate reader (Bio-Rad) using Microplate Manager software. HEK-293T cells stably expressing Flag-β2AR were stimulated with isoproterenol (1 μM) or 800nM SgG2 during different time points at 37ºC and the surface receptor levels were analyzed following the same procedure used for Flag-CXCR4 HEK-293T cells.

For immunofluorescence analysis of CXCR4 internalization in Flag-CXCR4 HEK-293T expressing cells, cells grown on poly-D-lysine-coated coverslips were stimulated with 8nM CXCL12, 800nM SgG2 or SgG2:CXCL12 at a 100:1 molar ratio during 30 and 60 min at 37ºC. The cells were fixed with PFA 4%, permeabilized with PBS containing 0.1% Triton X-100 and stained using an anti-Flag antibody (Sigma) followed by a fluorophore-conjugated Alexa secondary antibody. The same procedure was used to analyze β2AR internalization by immunofluorescence of Flag-β2AR HEK-293T cells after 30 min stimulation with SgG2 (800nM) or isoproterenol (1 μM).

For the analysis of CD44 surface levels, Jurkat cells were attached to fibronectin-coated coverslips, and then stimulated with 8nM CXCL12, 800nM SgG1 or SgG2, or with the vCBKP combined with the chemokine at a 100:1 molar ratio. Cells were PFA-fixed after 30 min and then stained for endogenous CXCR4, GM3 lipid rafts and CD44 followed by specific fluorophore-conjugated Alexa secondary antibodies.

Analysis of the phosphorylation status and degradation of CXCR4 by immunoblotting.
HEK-293T cells stably expressing Flag-CXCR4 were serum starved for 1 h at 37ºC, and then incubated in DMEM 1% FBS, containing 8nM CXCL12, 800nM SgG2 or SgG2:CXCL12 at a 100:1 molar ratio for 2 or 4 h. Cells were lysed in SDS loading buffer during 30 min at 4ºC and subsequently centrifuged at 12,000 rpm during 15 min at RT. Samples were not boiled to avoid CXCR4 aggregation. Proteins were resolved in 10% SDS-PAGE gels, and blots were incubated with an anti-CXCR4 polyclonal antibody (Abcam). For phosphorylation studies, Flag-CXCR4 or
ACP-CXCR4 expressing cells were serum starved for 1 h and then stimulated with 8nM CXCL12, 800nM SgG2 or the complex SgG2:CXCL12 at a 100:1 molar ratio during different time points (5 to 60 min). After stimulation cells were lysed using 2X sample buffer during 30 min at 4°C and centrifuged at 12,000 rpm during 15 min. Cell lysates were separated for ~1h 45 min at ~135V, subsequently blocked with TBS-Tween buffer containing 0.25% gelatin and incubated O/N with the phosphospecific antibodies, pSer330 and pSer324/325 (Dr. JL. Benovic). Blots were stripped and probed with anti-tubulin as a loading control (Sigma).

BRET assays.
BRET assays were performed as previously described with minor modifications. Briefly, HEK-293T cells were transfected with the donor construct Rluc-CXCR4 (0.01 μg) and acceptor construct GFP\textsubscript{2}-CXCR4 (1 μg) using FugeneHD reagent (Promega) in 6-well plates. The total amount of DNA was adjusted to 2 μg with empty vector. For titration acceptor/donor analysis curves, cells were co-transfected with 0.1 μg of Rluc-CXCR4 and increasing concentrations of GFP\textsubscript{2}-CXCR4 (0.1-2 μg). The cells were detached 24 h later and seeded at 5x10^5 cells per well in 96-well white plates (Corning) pretreated with poly-D-lysine and poly-L-ornithine and cultured for an additional 24 h. The growing medium was replaced for PBS containing 0.1% (w/v) glucose for 1 h prior to the assay. Cells were treated with 1.5 μM SgG2, 15nM CXCL12 or both at a 100:1 molar ratio prior to the addition of freshly prepared DeepBlueC (DBC, 5 μM). BRET readings were collected immediately after DBC addition, using a multidetector plate reader (Tecan Infinite F500). BRET signal was determined by calculating the ratio of the light intensity emitted by the acceptor protein CXCR4-GFP\textsubscript{2} (measured at 515±20nm) over the light intensity emitted by the donor protein CXCR4-Rluc (400±70nm). All measurements were corrected by subtracting the background BRET signal detected when Rluc-pcDNA vector was expressed alone. As a negative control for non protein-protein interactions, cells were co-transfected with the pair β\textsubscript{2}Adrenergic receptor (β\textsubscript{2}AR) fused to luciferase (β\textsubscript{2}AR-Rluc) and CXCR4-GFP\textsubscript{2}. As a positive control for the assays the interaction between CXCR4-Rluc and a constitutively active mutant form of β- arrestin2 (1-382) able to bind GPCRs independently of their phosphorylation state was measured.

Lentiviral transduction, transfection and electroporation of cell lines.
Supernatants used in transduction experiments were produced from transfected packaging HEK-293T cells using pALPS-CXCR4 or pALPS-Lck\textsubscript{10}mCherry, psPAX2 and pMD2-G plasmids, as described. Briefly, 70% confluent HEK-293T cells in 6-well-plates were cotransfected with the indicated plasmids at a ratio of 4:3:1 to a total amount of 3.3 μg of DNA, using JetPEI (Polyplus transfection) transfection reagent and serum-free Opti-MEM medium (Invitrogen). 48 h post-transfection, the supernatant containing lentiviral particles was collected, filtered through a 0.45μm filter (Millipore), and used to infect Jurkat cells during 96 h in the presence of 4 μg/ml polybrene (Sigma). Transfection of mammalian cells was carried out using Fugene HD reagent (Promega), according to manufacturer’s protocol. To electroporate Jurkat cells, the cells were washed, resuspended in 500 μl of Opti-MEM medium (Gibco) and mixed with DNA. Cells were then electroporated in 0.4 cm cuvettes at 0.26V and 900 μF using an EasyjlecT
apparatus (Eurogentec, Belgium), and grown O/N in R-10 medium. Typically, 20-30x10^6 cells were electroporated with 10-30 μg of the target DNA.

**Analysis of CXCR4 and β-arrestin2 localization.**

Jurkat cells were studied by time-lapse videomicroscopy. The cells were lentiviral transduced with ACP-CXCR4 for 72 h and then 30 x 10^6 cells were electroporated with 10 μg of β-arrestin2 fused to GFP. 24 h after electroporation, the cells were stimulated with 8 nM CXCL12 during 15 min, or with 800 nM SgG2 or the complex SgG2:chemokine at a 100:1 molar ratio. Prior to treatment, Jurkat cells were ACP-labelled to stain CXCR4. Images of the cells were recorded every 10 sec.

**Analysis of GPCR incorporation into Lck_10mCherry rafts.**

Jurkat cells were lentiviral transduced with Lck_10mCherry during 72 h and electroporated with CXCR4-GFP during 24 h. Cells were stimulated with 8nM CXCL12, 800 nM SgG2 or SgG2:CXCL12 at a 100:1 molar ratio or mock-treated during 5 min at 37°C. Time-lapse was recorded every 31 sec. HEK-293T cells stably expressing Lck_10mCherry grown in 6-well plates were transfected with 1 μg CXCR4-GFP during 48 h. Afterwards the cells were stimulated with 8nM CXCL12, 800nM SgG2 or the complex SgG2:CXCL12 at a 100:1 molar ratio during 15 min at 37°C, or left unstimulated. Subsequently, the cells were recorded taking images every 13 sec.

For the quantification of CXCR4 incorporation into Lck_10mCherry lipid rafts in fixed cells, lentiviral transduced Jurkat cells were stimulated during 30 min with 8nM CXCL12, 800nM SgG2 or PRV-SgG or the vCKBP:CXCL12 complex at a 100:1 molar ratio and then fixed with PFA 4% during 5 min at RT. Subsequently, the cells were stained to detect endogenous CXCR4 using a polyclonal antibody against the receptor (Abcam). The incorporation of CXCL12α-biotin/QDots into Lck_10mCherry rafts was also analyzed. MDCK cells stably expressing the fluorescent lipid raft marker were incubated with the chemokine/QDots (8nM), alone or in the presence of SgG2 (800nM) during 30 min at 37°C, and then fixed with PFA 4%.

For the analysis of β2AR inclusion in rafts, Lck_10mCherry expressing HEK-293T cells were grown in 6-well dishes and transfected with Flag-tagged β2AR (1 μg). After 48 h, the cells were stimulated for different time points with isopreterenol (1 μM) or SgG2 (800nM), and fixed with PFA 4%. The receptor present at the surface was detected using a monoclonal anti-Flag antibody (Sigma) followed by an anti-mouse fluorophore-conjugated Alexa antibody.

**Analysis of lipid raft clustering.**

Jurkat cells were stained with Vybrant DIO plasma membrane marker following manufacturer’s instructions. Afterwards, the cells were washed and incubated with 8nM CXCL12, 800nM SgG2 or PRV-SgG or the vCKBP:CXCL12 at a 100:1 molar ratio during 30 min at 37°C, and subsequently fixed with 4% PFA. ROIs corresponding to areas where the Lck_10mCherry signal was more intense along the perimeter of the plasma membrane were drawn and the intensity corresponding to both fluorescent signals (Lck_10mCherry and Vybrant marker) was obtained and profiled in qualitative intensity histograms. In addition, the ratio between Lck_10mCherry
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and Vybrant intensities in the selected ROIs was calculated and plotted for each experimental condition.

To investigate GM3 lipid raft clustering, Jurkat cells were stained using the plasma membrane marker Vybrant DiD following manufacturer’s instructions. Afterwards, the cells were stimulated during 15 min at 37°C using 8nM CXCL12, 800 nM SgG1, SgG2 or PRV-SgG or the vCKBP in combination with chemokine at a 100:1 molar ratio. Cells were fixed with PFA 4% and GM3 lipid rafts and CXCR4 were detected using a specific antibody against GM3 (Dr. Illa83) and a polyclonal antibody recognizing the receptor (Abcam), respectively. A ROI corresponding the perimeter of the plasma membrane was selected in order to generate the qualitative intensity histograms.

**ACP labelling reaction.**

CXCR4 was engineered with a tag at the N-terminus derived from the acyl carrier protein (ACP). The ACP tag contains the specific consensus for the 4’-phosphopantetheinyl transferase (PPTase) from Bacillus subtilis. Co-enzyme (CoA) covalently labelled with fluorescent dyes is used by the PPTase as substrate to transfer the labelled phosphopantetheine moiety to the serine located within the consensus of the ACP tag234. CoA labelled with the fluorescent dye Atto-647N (CoA-Atto647N) was used as substrate to covalently modify the ACP-tag of CXCR4. Since the reagents used are non-cell permeable, this methodology enables the specific labelling of surface CXCR4, while the pool of receptor located inside the cells when the enzymatic reaction is carried out remains unlabelled. In order to label ACP-tagged CXCR4, cells were washed with D-PBS containing calcium and magnesium and incubated during 15 min at RT with the CoA-Atto dye (5 μM) in the presence of the enzyme (PPTase, 1 μM). Cells were washed to remove the excess of dye and immediately proccessed for time-lapse or immunofluorescence. The ACP-CXCR4 plasmid, the PPTase and the CoA-Atto dye-647 were kindly provided by M. Thelen (IRB, Switzerland).

**Analysis of CXCR4 and CXCL12 localization in HEK-293T cells.**

CXCL12α-biotin was conjugated to Qdots during 15 min at RT prior to the assay. HEK-293T cells expressing ACP-tagged CXCR4 were ACP-labelled and subsequently mock-treated or incubated with 8nM CXCL12α-biotin/Qdots, 800nM SgG2 or the complex SgG2:CXCL12α-biotin/Qdots at a 100:1 molar ratio during 30 min at 37°C. Afterwards the cells were fixed after 30 min at 37°C, time-lapse images were taken at 11 sec intervals.

For the quantification of CXCL12 and CXCR4 colocalization ACP-CXCR4 HEK-293T cells were ACP-labelled and treated with 8nM CXCL12-biotin/Qdots alone or in the presence of 800nM SgG2. The cells were fixed with PFA after 30 min of stimulation to study chemokine and receptor colocalization.

To analyze the localization of CXCL12 outside or inside of the cells, ACP-CXCR4 HEK-293T cells were incubated during 45 min at 37°C with 8nM CXCL12-biotin/Qdots alone or in the presence of SgG2 at a 800nM concentration. Subsequently, the cells were washed with an acidic buffer (glycine-HCl pH 3) during 30 sec at 4°C following fixation with PFA 4%.
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**Videomicroscopy.**
Jurkat cells were plated on untreated μ-Slide 8-well (Ibidi) for videomicroscopy studies. For adherent HEK cells, glass bottom petri-dishes (MatTek cultureware) or μ-Slide 8-well previously coated with poly-D-lysine (Sigma) were used. In both cases, growing medium was replaced with Opti-MEM serum-free medium (Gibco) 1h prior to the start of the assay. Cells were placed on a microscope stage and maintained at 37ºC, and a series of fluorescent and bright-field frames were captured at different time intervals, as indicated in the video legends. Two confocal microscope systems were used, a Zeiss LSM510 META coupled to an Axiovert200 inverted microscope and a LSM710 coupled to an AxioObserver inverted microscope with 63x and 100x PlanApo oil immersion objectives (1.4 numerical aperture). Time lapse of HEK-293T cells shown in videos 4-7 was recorded using a Leica DI6000 microscope stand connected to a SP5 scan head equipped with a temperature controlled chamber (Cube, LIS, Basel). Images and videos were processed using Image J software.

**Immunofluorescence.**
For confocal microscopy of fixed cells, cells were attached to untreated, poly-D-lysine or fibronectin-coated coverslips, as indicated in each assay. After stimulation with the chemokine, the vCKBPs or the combination or both at the ratio indicated in each assay, the cells were PFA-fixed and incubated with PBS containing 2% FBS 2% BSA to reduce unspecific binding. The incubation with the appropriate primary antibodies was routinely performed for 1h at 37ºC. After washing, the incubation with the secondary Alexa-conjugated antibodies was performed during 45min at 37ºC. The cells were incubated during 15min at RT with DAPI reagent or FITC-conjugated Cholera toxin β when needed. Coverslips were washed with PBS, mounted with Prolong Gold antifade mounting medium (Invitrogen), and kept at 4 ºC protected from light for subsequent analysis. Images were taken by an AxioImager M2 microscopy system coupled to a confocal LSM710 (Zeiss), using 63x and 100x PlanApochromat oil immersion objective lens (1.4 numerical aperture). In all cases, images were obtained at a resolution of 1024x1024 pixels. Images were subjected to background and mean filter correction using Image J 1.43u or 1.45 software. Threshold analysis was carried out in order to perform quantitative colocalization analysis (described below), for the analysis of lipid rafts areas, and for the obtention of qualitative intensity histograms. Images of SCG were taken using a Zeiss LSM 510 Confocal Laser Scanning Microscope. Images were subjected to threshold analysis prior to calculation of the proximal/distal axonal ration of the explants using Image J software.

**Isolation of primary mouse neurons and culture.**
Mouse sympathetic neurons from SCG were cultured as described by others. Briefly, ganglia were dissected from newborn mice (postnatal day 0–1), digested in collagenase and trypsin (Worthington, Lakewood, NJ), dissociated by trituration, plated on dishes previously coated with rat tail collagen I (BD Biosciences) in DMEM containing 50 ng/ml NGF (Alomone Labs, Jerusalem, Israel), 10% FBS, and 5 ng/ml aphidicolin (A. G. Scientific, San Diego, CA) for 5–7
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To study the incorporation of TrkA into different subtypes of lipid rafts, neurons were grown on collagen-coated coverslips for 5-7 days. The cultures were NGF-deprived O/N and then stimulated with 1nM NGF, 200 nM NGF or the combination of SgG2:NGF at a 200:1 molar ratio. The stimulation was performed during 2 or 10 min at 37ºC. Cells were washed with ice-cold PBS and then fixed with 4% PFA during 5 min at RT, before labelling with specific antibodies against TrkA (R&D Systems) and GM3 lipid rafts (Dr. Illa) during 1 h at 37ºC. In the case of GM1 lipid rafts staining, FITC-conjugated cholera toxin β was added to the cells during 15 min at RT following incubation of the neurons with the anti-TrkA antibody (R&D Systems). Afterwards the cells were incubated with specific fluorophore-conjugated Alexa antibodies and kept at 4ºC for subsequent analysis.

After O/N deprivation of NGF, the cells were stimulated with 1nM NGF, 200nM SgG2, SgG1 or M3, or the complex vCKBP:NGF at a 100:1 molar ratio during 5 min at 37ºC. The cells were fixed with PFA and then incubated with a mixture of an anti-TrkA (R&D Systems) and an anti-p75 (Abcam) antibodies. Following incubation with the corresponding fluorophore-conjugated Alexa antibodies, the coverslips were kept protected from light at 4ºC.

Culture of SCG explants.

Mouse sympathetic SCG were cultured in a 3D collagen matrix, following the procedure described by Hazen and et al.236. In brief, 340µL of rat tail collagen I (BD Biosciences, San Jose, CA) were mixed 40 µL of 10x MEM, 10µL of HEPES (for mock-treated controls) or the vCKBPs at a final concentration of 50nM, and NGF (Alomone labs, Jerusalem, Israel) at final concentration of 0.25nM (6.5ng/mL). Afterwards, the solution was neutralized using 0.8M sodium bicarbonate (NaHCO3) and immediately placed forming drops where ganglia were included.

Immunogold labelling, replica preparation, and electron microscopy analysis.

Immunogold labelled cell surface replicas were obtained as described previously237, 238. In our study, Jurkat cells lentiviral transduced with ACP-CXCR4 or HEK-293T cells stably expressing ACP-CXCR4 were studied. Jurkat cells were stimulated during 30 min at 37ºC with 15nM CXCL12, SgG2 or SgG2:CXCL12 at a 100:1 molar ratio in suspension, and then fixed in 2% PFA and labelled on ice with an anti-CXCR4 monoclonal antibody (BD Pharmingen) or an isotype control antibody (BD Pharmingen). Afterwards cells were incubated with protein A conjugated to 10 nm gold particles, washed, attached to mica strips coated with poly-D-lysine and post-fixed with 0.1% glutaraldehyde. In the case of HEK-293T cells, cells were cultured O/N on mica strips in DMEM containing 10% FBS. Following washing steps, cells were stimulated during 25 min at 37ºC with 8nM CXCL12, SgG2 or both, and then washed and fixed with 2% PFA. Cells were stained with the monoclonal antibodies anti-CXCR4 (BD Pharmingen) or anti-CD3 (eBioscience) as a control. Subsequently, cells were washed, incubated with 10 nm-conjugated protein A and post-fixed with glutaraldehyde 0.1%. Samples were plunge frozen in liquid propane, and then mounted on the sample table of a Balzers400T freeze fracture (FF) unit under liquid nitrogen. Samples were placed in the FF unit cooled at -150 ºC and etched for 12 min to remove excess of ice, and the stage temperature was modified from -150 ºC to -90
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The samples were shaded with platinum (2 nm at 45 °C angle) and coated with carbon (20 nm at 90 °C angle), removed from the FF unit and floated overnight on a domestic bleach solution to remove the organic material. The replicas were extensively washed in distilled water, mounted on 400 mesh copper grids and examined on a JEM1010 electron microscope (Jeol, Japan) operating at 80 kV. Images were taken with a TemCam-F416 Camera (TVIPS, Gauting, Germany) at 10,000x magnification. Overlapping photos of each cell replica were merged into a single image with TVIPS software (TVIPS, Gauting, Germany) and gold particles were counted using Cell Counter plugin from Image J software 1.45. Particles were counted as part of a cluster if the distance to the next gold particle was less than the diameter of the gold particles (10 nm). The background staining detected was subtracted to all gold particles counts.

Quantification of colocalization.

Pearson’s Coefficient (PC) is a standard statistical analysis designed to measure the strength of a linear relationship between two variables, in this case fluorescent intensities from two images. To calculate PC for a pair of fluorescence images, all the pixels having the same image coordinates are paired. The mean pixel intensity of an image is subtracted from the intensity of each pixel within the image, and the value generated for each pixel is multiplied by the equivalent value from the pixel’s partner in the counterpart image to generate the product of the difference from the mean. The product of the difference from the mean is added for the entire dataset and divided by the maximum possible sum of the product of the difference from the mean239. PC generates a range of values from 1, a perfect positive correlation, to −1, a perfect but inverse correlation, with 0 representing a random distribution. In order to perform a more detailed analysis, we took advantage of a recently developed correlation method termed Intensity correlation analysis (ICA)240. The ICA method is based on the principle that if two proteins are part of the same complex then their staining intensities should vary in synchrony, whereas if they form part of different complexes or structures they will exhibit asynchronous staining. ICA analysis involves generating scatter-plots of stain A or stain B against the product of the difference of each pixel A and B intensities from their respective means. The resulting plots emphasize the high intensity stained pixels and allow us to identify protein pairs that vary in synchrony, randomly, or independently within the cell. Intensity Correlation Quotient (ICQ) was used to provide an overall index of whether the staining intensities are associated in a random, a dependent or a segregated manner240. The outlining of regions in which two probes may distribute is essential to obtain accurate measurements of colocalization241. Therefore regions of interest (ROI) were drawn when necessary. A minimum of three different sections were quantified in the assays shown.

Statistical analysis

Statistical analyses of data were performed with the program GraphPad Prism. The significant value (P value) for the parameters measured in the assays was calculated using a two-tailed unpaired student t-test.
Results
1. SgG2 INCREASES THE EFFICIENCY OF CELL MIGRATION.

We have demonstrated that SgG is the first vCKBP expressed by a human pathogen that enhances the functions of chemokines; nevertheless, the bases beneath this phenomenon are unknown. As a first approach to gain a better understanding of the impact of SgG on the chemotactic process, we analyzed the migration of primary monocytes by time-lapse video microscopy (Fig. R-1). Given that SgGs bind CXCL12 with even higher affinity than its α counterpart, the former is the isoform used throughout this work, unless indicated. CXCL12, SgG2 or SgG2:CXCL12 were released from a micropipette connected to a microinjector system with a constant backpressure. SgG2 did not attract the cells when dispensed on its own (Video 1). On the other hand, SgG2:CXCL12 greatly enhanced chemotaxis of monocytes towards the gradient compared to CXCL12 alone (Video 3 and Video 2, respectively), consistent with the data obtained when monocyte migration was assessed by transwell assays (Fig. R-1F). Analysis of tracks recorded by time-lapse videomicroscopy allowed us to determine other chemotactic...
parameters. An increased number of cells were able to respond to a given CXCL12 concentration and, moreover, they sensed the chemokine from longer distance to the dispensing pipette as compared to CXCL12 dispensed alone (Fig. R-1B). The velocity, the total distance travelled and the forward migration index (FMI), i.e. the ratio between the net distance the cell progressed in the forward direction and the total distance the cell travelled, were calculated during the initial 10-min period. When CXCL12 was dispensed bound to SgG2 monocytes migrated longer distances (Fig. R-1B and R-1E), they did so in a more directional fashion (Fig. R-1D) and displayed higher velocity (Fig. R-1C). Similar results were obtained when using CXCL12αα (not shown).

Our results indicated that SgG2 makes cells more prone to sense threshold concentrations of the chemokine explaining the faster and more directional cell migration observed in the presence of the viral protein.

2. HSV SgGS BIND TO CELL SURFACE GAGS

We have previously shown that soluble recombinant HSV SgG interacts with chemokines through the GAG-binding domain of the chemokine; however and importantly such interaction does not abrogate chemokine binding to its receptor\(^\text{196}\), suggesting that both chemokine and SgG are located at the plasma membrane. To test the possibility of direct binding of SgG to the plasma membrane we performed several experiments. We first decided to test the potential SgG2 interaction with human primary monocytes, a cell type that is highly sensitive to SgG2 modulation\(^\text{196}\). Cells were incubated at 37ºC during 30min with SgG2, PFA-fixed and stained with a polyclonal antibody raised against SgG2 for its subsequent detection by confocal microscopy. As shown in Fig. R-2A, SgG2 was evenly distributed on the surface of monocytes, independently of the presence of the chemokine (Fig. R-2A). Surface CXCR4 was stained to detect changes in the polarization phenotype of monocytes. Upon CXCL12 stimulation, cells polarized and both SgG2 and CXCR4 showed a tendency to accumulate at one of the edges of the cell (Fig. R-2A, top panels). Such changes in cell morphology were not evident when the stimulation was performed at 4ºC (Fig. R-2A, lower panels), indicating the need of membrane trafficking events and cytoskeletal rearrangements for the acquisition of a polarized phenotype. The ability of SgG2 to bind to other cell types, including HEK-293T and MM-1 cells (Fig. R-2B), CHO cells (Fig. R-4) and Molt-4 and Hela cells (not shown) was also demonstrated by flow cytometry and/or immunofluorescence analysis.

We and others have described the ability of other vCKBPs to interact with GAGs\(^\text{177}\,242\); the observation that SgG binds to the surface of all cell types analyzed pointed towards the possibility that such interaction takes place through binding to GAGs, which ubiquitously cover the surface of most cells. In order to analyze whether SgG displayed the ability to interact with GAGs we used several \textit{in vitro} approaches. First, we tested the interaction of SgG and heparin by SPR technology using a BIACore biosensor, as previously described\(^\text{177}\). The use of SPR not only allows the study of real time protein-protein interactions, but also the calculation of the kinetic parameters for the interaction.
between a soluble analyte (in this case, SgG) and a ligand covalently immobilized on the surface of the sensor chip (in this assay, heparin). A schematic view of a prototypical BIAcore sensorgram and the binding and kinetic information that provides is depicted in Figure R-3A. Different amounts of SgG2 were injected through a biotinylated heparin-coated streptavidin sensor chip. As shown in the sensorgrams SgG2 bound heparin with nanomolar affinity (Fig R-3B). Next, the interaction between SgG and the GAG was confirmed by means of a heparin-sepharose bead pull-down assay. Different amounts of SgG1 and SgG2 were incubated with heparin beads for 1 h at RT, and bound protein was collected by centrifugation, extensively washed, and immunoblotted using an anti-His tag antibody to detect the protein eluted from the beads. Purified SgGs bound to heparin-sepharose in a dose-dependent manner and the interaction was competitively inhibited when the incubation was performed in the presence of soluble heparin, showing the specificity of the interaction (Fig. R-3C and D for SgG1 and SgG2, respectively). GAGs are divided into four subgroups depending on their disaccharide composition and degree of sulfation: heparin, heparan sulfate and chondroitin sulfate A and B, which are ubiquitously but differently found in the extracellular matrix and surface of cells.

To characterize whether SgG2 was able to interact with other GAGs we carried out a competition assay by SPR. SgG2 was incubated with increasing concentrations of soluble GAGs and injected over the BIAcore chip coated with heparin. Binding to heparin in the absence of competitor GAG was considered 100%. As shown, all soluble GAGs competitively inhibited SgG2 binding.
### Figure R-3. SgGs are GAG-binding proteins.

(A) Overview of a typical SPR sensorgram obtained by using a BIAcore biosensor. SPR allows the measurement of real time protein-protein interactions between a ligand, immobilized on a sensor chip, and a variety of soluble analytes. Changes in analyte mass next to the surface of the chip where the ligand is covalently coupled are detected as an increase in response units (RUs) in the sensorgram. The chip surface is regenerated after every injection to allow subsequent measurements. The affinity of each interaction is calculated based on the association and dissociation kinetic parameters. (B) Sensorgrams depicting the interaction between SgG2 and heparin. SgG2 was injected at different concentrations over biotinylated-heparin immobilized on a chip. (B, Table) Kinetics parameters of the binding of SgG2 to the heparin artificial surface. The arrow indicates the end of the injection. All curves were analyzed with the BIAEvaluation software and represent the interaction of SgG2 after subtraction of the blank curve. C) Heparin pulldown assay using SgG1. The indicated concentration of SgG1 was incubated with heparin-sepharose beads (lanes 4 and 5) and the eluted protein was subjected to SDS-PAGE and detected using an anti-His tag antibody. Lane 1 shows starting material (SgG1 input). Controls in the absence of SgG1 (lane 2) or the binding to sepharose beads (lane 3) are shown. Excess of soluble heparin was used in lane 6 (10x molar excess) to compete the interaction of viral protein bound to the heparin-sepharose beads. Molecular sizes are indicated in kDa. (D) Heparin-pulldown assay using SgG2. Indicated concentrations of SgG2 bound to heparin-sepharose beads (lanes 2 and 3) or to sepharose beads alone (lane 4). Excess of soluble heparin (10x molar excess) was used to compete the interaction of viral protein bound to the heparin-sepharose beads (lane 5). Lane 6 shows heparin-sepharose beads without protein. The eluted protein was subjected to SDS-PAGE and blotted using a monoclonal anti-His antibody. (E) SgG2 interacts with other sulfated GAGs. SgG2, alone or in combination with increasing concentrations of the soluble GAGs heparin, heparan sulfate (HS) and chondroitin sulfate A (CS A) or B (CS B), was injected over the BIAcore chip coated with heparin. The percentage of SgG2 bound respective to the amount of protein bound in the absence of any soluble GAG (considered 100%) was plotted as a function of the concentration of each competitor GAG.

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to the heparin surface, indicating that SgG2 was capable of interacting with GAGs from all subgroups with variable affinities (Fig. R-3E). These results suggested that binding of SgG to cell surfaces was taking place through GAGs. In order to assess whether that was the case, we took advantage of wt CHO K1 and mutant CHO 618 cells that
are deficient in GAG expression due to the lack of the enzyme galactosyltransferase\textsuperscript{231}. Different amounts of SgG2 were incubated with both cell lines at 4°C, and binding was determined by flow cytometry and immunofluorescence using a polyclonal anti-SgG2 antibody. The binding of SgG2 present in virus-free supernatants from HSV-2 infected cells (HSV-2 SgG2) was also analyzed. Supernatants from insect cells containing similar amounts of VACV B18 and ECTV semaphoring (sema) were used as control for positive binding to GAGs and no binding to the surface, respectively (Fig. R-4A). As previously reported, VACV B18 bound to CHO K1 cells where it showed

![Figure R-4. SgGs bind to the cell surface in a GAG-dependent fashion.](image)

(A) Western blot showing the presence of purified recombinant SgG2, SgG2 present in the supernatants of virus-free infected cells (HSV-2 SgG2), ECTV Semaphorin (sema) or VACV B18 (bottom blot), stained with anti-SgG2 or anti-His antibodies. Molecular sizes are indicated in kDa. (B) Binding of SgG2 and HSV-2 SgG2 to CHO cells. The different proteins were incubated with wt CHO-K1 cells or GAG-deficient CHO-618 cells during 30 min at 4°C, and then stained using specific antibodies to detect surface-bound proteins by flow cytometry. (C-D) Immunofluorescence experiment showing binding of purified SgG and HSV-2 SgG2 (C) or SgG1 (D) to the plasma membrane of CHO-K1 cells. Proteins were stained with an anti-gG2 polyclonal antibody or a monoclonal antibody raised against SgG1 and DNA was stained with DAPI.
a plasma membrane disposition, but it did not interact with CHO 618 cells, whereas ECTV sema did not bind to any of the cell types (Fig. R-4B, left histograms and middle panels). Binding of SgG2 to CHO K1 cells was readily detected by flow cytometry (Fig. R-4B, right histogram). SgG2 present in the supernatant of HSV-2 infected cells also bound to CHO K1 cells, although to a lesser extent consistent with the presence of lower amounts of SgG2 in this sample (Fig. R-4B, upper right histogram). On the other hand, the amount of bound protein significantly dropped when purified SgG2 or SgG2-containing supernatants were incubated with CHO 618 cells (Fig. R-4B, bottom right histogram) confirming our hypothesis that SgG2 binds to the cell surface mainly in a GAG-dependent manner. This result was further confirmed by immunofluorescence analysis of SgG2 and HSV-2 SgG2 bound to the surface of CHO wt and mutant cells (Fig. R-4C). In accordance with its high GAG-binding affinity, SgG1 was also found to interact with CHO K1 cells, showing a clear membrane pattern undetected when the protein was incubated with CHO 618 GAG-deficient cells (Fig. R-4D).

In summary, these results show that HSV SgGs interact with the cell surface in a GAG-mediated fashion. This observation is supported by several biochemical and SPR-based assays showing that HSV SgG is a GAG-binding protein.

Our results indicate that SgG HSV is able to bind to both chemokines and GAGs. The lack of sequence similarity between vCKBPs makes difficult to predict the protein domain involved in chemokine binding only by sequence analysis. Moreover, the fact that HSV SgG constitutes the first example of a vCKBP that increases migration suggests that novel structural features may arise in order to rearrange the bound chemokine so as to enhance its presentation to the receptor. A schematic representation of the SgG1 and SgG2 constructs used during this work is depicted in Fig. R-5A. In an attempt to elucidate both the chemokine and GAG binding domains of SgG2, several N- and C-terminal deletion mutant proteins were engineered and expressed in the baculovirus system (Fig. R-5B). Proteins were purified by affinity chromatography (Fig. R-5C), and subsequently dialyzed against HEPES buffer (Fig. R-5D, Coomassie gel) and detected by western blot using a monoclonal antibody against the His-tag (Fig. R-5D, lower blot). Only SgG2-Nter1, Cter1, Cter2 and Cter3 deleted proteins were expressed, whereas the rest of the mutant proteins (SgG2- Nter2 to 4 and SgG2-Cter4) were hardly detectable by western blotting in the supernatants from cultures of insect cells infected with the corresponding recombinant baculoviruses (not shown and Fig. R-5D). We first checked the ability of the C-terminal deleted versions of SgG2 to bind heparin using a pulldown assay, following the procedure described above. SgG2-Cter1 contained both proposed cleavage sites (Arg 321-Ala 322 and Arg 342-Leu 343) so it probably resulted in SgG2 following processing, therefore retaining full GAG-binding activity. SgG2-Cter2 showed reduced GAG-binding ability, consistent with the lack of some positively charged residues that might be involved in the interaction with GAGs, which are highly negatively charged molecules. SgG2-Cter3 interaction with GAGs was further diminished, indicating that the series of arginine residues present at the C-terminus of SgG2 that are loss in the deleted protein.
Figure R-5. N and C-terminal deleted versions of SgG2 show an impaired binding to GAGs and to chemokines.

(A) Schematic representation of SgG2 (left) and SgG1 (right) constructs used in this Thesis. A fragment of the extracellular domain of both gG1 and gG2 was amplified and cloned into a baculovirus-expression vector. The putative signal peptide from gG was substituted by the honeybee melittin signal peptide (HM). The position of the amino acid residues in the polypeptide and the protease cleavage site (black arrow) are indicated. Abbreviations: SP, signal peptide; TM, transmembrane domain; CD, cytoplasmic domain; HT, His-tag; ED, extracellular domain. (B) Schematic representation of the collection of SgG2 deleted mutant proteins generated, including four versions with increasing deletions in their C-terminus (SgG2-Cter1 to Cter4) and three versions with a shorter N-terminus (only SgG2-Nter1 is represented). The number of the amino acid residues comprised in each mutant is indicated. Consensus GAG-binding sites and clusters of positive residues are highlighted in pink. (C) Coomassie gels showing...
could also be implicated in GAG-binding (Fig. R-5E). In addition, SgG2-Nter1 and SgG2-Cter3 were immobilized on a BIAcore chip to analyze their chemokine-binding capacity. Both truncated SgG2 forms showed a reduced capacity to interact with several chemokines in comparison to the full-length SgG2 (Fig. R-5F). However, such diminished binding could reflect either the real loss of residues involved in chemokine binding or overall defects in SgG2 spatial structure. Indeed, the fact that most N-terminal mutants were not expressed points towards defects in protein folding or secretion. The inherent instability of SgG2 made unfeasible the purification of enough quantity of the truncated proteins to perform additional functional analysis such as chemotaxis. The generation of an array of point mutants of SgG will be required to dissect the structural determinants involved in HSV SgG binding to GAGs and chemokines in future studies.

3. HSV SgGS MODULATE CXCR4 TRAFFICKING AND COUNTERACT CHEMOKINE-MEDIATED INTERNALIZATION

The responsiveness of a cell to a chemotactic gradient strongly depends on the expression level of chemokine receptors on the cell surface. Chemokine receptors constitutively internalize and cycle back to the plasma membrane, where they are exposed to the chemokine that, in turn, promotes the endocytosis and degradation of the cognate receptor rather than its recycling to the surface. Since we had previously demonstrated that SgG enhanced chemotaxis and chemokine-mediated signalling, we next decided to investigate any potential impact of SgG on chemokine receptor trafficking. To do so, we first measured the internalization of CXCR4 in freshly isolated monocytes. Cells were exposed to CXCL12, SgG2 or SgG2: CXCL12 at 37°C for different time points, washed and incubated at 4°C with a fluorophore-conjugated CXCR4 antibody in order to detect surface receptor levels by flow cytometry. A significant increase in the levels of CXCR4 on the surface was observed at all time points tested when monocytes were treated with SgG2 (Fig. R-6A). The increased amount of surface CXCR4 was
Figure R-6. HSV SgG upregulates CXCR4 on the surface of leukocytes and prevents chemokine-induced internalization of the receptor. (A) Time-dependent stimulation of the expression of CXCR4 on the surface of Jurkat cells Jurkat cells MM-1 cells SgG2F:CXCL12 CXCL12 SgG SgG2:CXCL12 PRV-SgG PRV-SgG:CXCL12. Two-tailed unpaired t-test was performed *P<0.05, **P<0.001, ***P<0.0001. Error bars represent standard deviation (SD).

Below each graph show the surface levels of CXCR4 after 30 min stimulation. Plots show two (B) or one (C, D) incubation of the cells with SgGs, CXCL12 or SgG2:CXCL12 at a 100:1 molar ratio during 30 min at 37ºC. SgG2F:CXCL12-CXCR4 in Jurkat T cells and MM-1 cells. The levels of surface CXCR4 were measured by ow cytometry, following /f_l triplicates. Post-stimulation of monocytes with SgG2:CXCL12 vs CXCL12. Plot shows two independent assays performed in ow cytometry. The amount of receptor on the washed, and the amount of CXCR4 on the surface was detected by flow cytometry. The amount of receptor on the surface was represented as a percentage of the amount of surface CXCR4 in mock-treated cells at each time point (A, left). Histogram showing (A, right) the blockade of CXCL12-induced internalization of CXCR4 at early time points post-stimulation of monocytes with SgG2:CXCL12 vs CXCL12. Plot shows two independent assays performed in triplicates. (B-D) Time-dependent effect of SgG1 or SgG2 on surface levels and CXCL12-mediated endocytosis of CXCR4 in Jurkat T cells and MM-1 cells. The levels of surface CXCR4 were measured by flow cytometry, following incubation of the cells with SgGs, CXCL12 or SgGs:CXCL12 at a 100:1 molar ratio during 30 min at 37ºC. SgG2F:CXCL12- and SgG1F:CXCL12-treated cells were incubated during 30 min with SgG2 prior to addition of CXCL12. Bar plots below each graph show the surface levels of CXCR4 after 30 min stimulation. Plots show two (B) or one (C, D) representative assays run in triplicates or three independent assays. Error bars represent standard deviation (SD). Two-tailed unpaired t-test was performed *P<0.05, **P<0.001, ***P<0.0001.
accompanied by a delayed internalization when monocytes were stimulated with the SgG2:CXCL12 complex, in comparison with CXCL12 (Fig. R-6A). As a control, we used PRV-SgG, a vCKBP that binds CXCL12 and inhibits chemotaxis[^30]; PRV-SgG neither affected CXCL12-induced internalization nor CXCR4 surface levels (Fig. R-6A).

The impact of SgG2 on CXCR4 trafficking was also assessed in Jurkat T cells and MM-1 cells. The dynamics of CXCR4 trafficking in cell lines were slightly slower than in freshly isolated monocytes, a fact that correlated with the faster migration and higher responsiveness to SgG displayed by primary cells in our transwell assays (not shown). SgG2 reduced CXCL12-driven CXCR4 endocytosis, with the greatest decrease observed upon incubation of the cells with the SgG2:CXCL12 complex during 30 min (Fig. R-6B). Similar results were obtained when surface CXCR4 was measured in MM-1 cell line (Fig. R-6C), indicating that the impact of SgG on CXCR4 endocytosis was not cell type specific. Furthermore, SgG1 was also capable of partially blocking chemokine-induced internalization in Jurkat cells (Fig. R-6D). Interestingly, both SgG1- and SgG2-mediated impact on receptor internalization was more pronounced when the cells were incubated with the protein prior to stimulation with the chemokine, suggesting that SgG was able to exert an effect on the cell independently of the presence of the chemokine (Fig. R-6C and D).

We next studied CXCR4 trafficking in HEK-293T cells stably expressing Flag-tagged CXCR4. After analyzing CXCR4 expression in three of the cell clones generated (not shown), the one that harboured intermediate levels of the receptor was utilized for further assays to avoid artifacts due to receptor overexpression. HEK-293T cells grown on coverslips were stimulated at 37°C with CXCL12, SgG2 or SgG2:CXCL12, after which they were fixed in order to quantify surface CXCR4 using a monoclonal anti-Flag tag antibody followed by an alkaline-phosphatase conjugated goat anti-mouse immunoglobulin. The amount of surface CXCR4 was upregulated at all time points in the presence of SgG2 or SgG2:CXCL12 (Fig. R-7A). Unlike primary monocytes, in the case of HEK-293T cells the most evident increase of surface CXCR4 was observed upon stimulation with SgG2 in combination with the chemokine (Fig. R-7A). This observation could be explained by cell type differences in the composition of the membrane in the proximity of the receptor and/or by the availability of specific signalling adaptors or endocytic machinery, which would lead to different rates of recycling and internalization. The expression of surface CXCR4 in HEK-293T cells was also checked by flow cytometry using specific anti-CXCR4 antibodies, obtaining similar results (Fig. R-7B). Consistent with results obtained with monocytes, PRV-SgG did not have any effect on surface receptor expression in CXCR4-expressing HEK-293T cells, tested by flow cytometry(Fig. R-7B).

**3.1 SgG2 promotes the presence of an active chemokine-receptor complex at the cell surface**

Since the levels of surface CXCR4 significantly raised upon stimulation of SgG2:CXCL12, we analyzed the fate of
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then cells were washed with an acidic pH buffer to remove any chemokine remaining on the surface and fixed. Following washing, fluorescent signal was detected in cells treated with the chemokine alone, the chemokine following CXCR4 binding, CXCL12-biotin conjugated with fluorescent quantum dots (Qdots)-streptavidin alone or in the presence of SgG2 was incubated with HEK-293T cells during 45 min at 37°C, and then cells were washed with an acidic pH buffer to remove any chemokine remaining on the surface and fixed. Following washing, fluorescent signal was detected in cells treated with the chemokine alone.

Figure R-7. SgG2 enhances the presence of CXCR4:CXCL12 complex at the surface of HEK-293T cells. (A) The effect of SgG2 on CXCR4 levels at the cell surface of HEK-293T cells. HEK-293T cells stably expressing Flag-tagged CXCR4 were stimulated with 1.5 μM SgG2, 15 nM CXCL12 or SgG2:CXCL12 at a 100:1 molar ratio for different time points at 37°C. Cells were PFA-fixed and incubated with a monoclonal anti-Flag antibody followed by an alkaline-phosphatase conjugated goat anti-mouse immunoglobulin. The amount of receptor at the surface is represented as the percentage of the value obtained for mock-treated cells at each time point. Plots represent the mean ± SEM from four independent assays performed in duplicates or triplicates. (B) Histograms showing the effect of SgG2 (left) or PRV-SgG (right) on CXCR4 surface levels in HEK-293T. Cells were mock-treated or incubated with 15 nM CXCL12, 1.5 μM SgG2, 1.5 μM PRV-SgG or with the vCKBPs:CXCL12 in a 100:1 molar ratio during 30 min at 37°C. (C) Immunofluorescence showing the effect of SgG2 on CXCL12 at the surface of HEK-293T cells expressing ACP-tagged CXCR4. HEK-293T cells were ACP-labelled prior to stimulation with CXCL12-biotin conjugated to fluorescent Qdots, alone or in the presence of SgG2, during 45 min at 37°C. Afterwards, chemokine remaining at the surface was washed away with an acidic pH buffer, cells were PFA-fixed and the fluorescent signal corresponding to the chemokine was quantified using Image J software and plotted (right panel). Quantification of the signal (approximately 60 cells for each experimental condition) is represented in the graph. Scale bar=5μM. Two-tailed unpaired t-test was performed, *P<0.05, **P<0.001, ***P<0.0001.
indicating that much of the chemokine had been internalized during the incubation period. On the contrary, a great portion of the fluorescent signal was lost after cell stimulation with SgG2 and CXCL12, indicating the susceptibility of CXCL12 to the acidic wash when SgG2 was present, due to the stabilization of the complex at the cell surface (Fig. R-7C). A quantification of the CXCL12 fluorescent signal is shown (Fig. R-7C).

The dynamics of CXCR4 trafficking were further characterized by time-lapse videomicroscopy of HEK-293T cells. To do so, we took advantage of a HEK-293T cell line stably expressing CXCR4 with a tag at the N-terminus derived from the acyl-carrier protein (ACP) that contains the specific consensus sequence for a phosphopantetheinyl transferase (PPTase). Co-enzyme A (CoA) covalently labelled with fluorescent Atto-647N dye is used by the PPTase as a substrate to transfer the labelled phosphopantetheine moiety to the serine located within the consensus of the ACP, allowing the specific labelling of ACP fusion proteins displayed on cell surfaces. Since the cells are impermeable to the enzyme used for the reaction, only the receptors present at the surface when the reaction is carried out become labelled. The conjugation of the fluorophore to the ACP-tagged CXCR4 was performed, cells were washed to remove the excess of free dye and incubated with CXCL12-biotin/Qdots-Streptavidin, SgG2 or both during 30min at 37℃. Afterwards, cells were recorded for 30 min at 37℃. The receptor constitutively trafficked in mock-treated cells, without evident changes in its surface levels (Video 4). Instead, cells stimulated with SgG2 showed some areas of CXCR4 accumulation at the surface (Video 5). On the other hand, CXCL12 reduced CXCR4 present at the surface over time; accordingly, the fluorescent signal accumulated intracellularly (Video 6). On the contrary, when the culture was incubated with the SgG2:CXCL12 complex the internalization of CXCR4 was delayed leading to a sustained presence of the receptor at the surface (Video 7). Selected video frames showing the localization of CXCR4 after incubation with the different stimuli for the same periods of time are shown in Figure R-8A. Other interesting features could be observed in these videos. Internalizing vesicles carrying CXCR4 and CXCL12 appeared upon stimulation with the chemokine, while they were much more scarce when SgG2 was bound to the chemokine, pointing towards a defect in the CXCL12-mediated endocytic process (Video 8 and Video 9, respectively).

A quantitative analysis of chemokine and receptor colocalization in fixed cells showed a marked increase in the amount of CXCL12 found in the vicinity of CXCR4 when SgG2 was present (Fig. R-8B). The Intensity correlation analysis (ICA) method was utilized to quantify colocalization of fluorescent signals, as described in the Material and Methods section. Two values were obtained: Pearson’s and Intensity correlation quotient coefficient, PC and ICQ respectively. Representative Scatter and ICA plots derived from such analysis are shown in Figure R-8C and Figure R-8D. For subsequent analysis throughout this work only the averaged values for both coefficients are shown (Fig. R-8E).

Immunofluorescence of fixed and permeabilized HEK-293T cells stained for Flag-CXCR4 showed that receptor staining
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Figure R-8. SgG2 impacts CXCR4 trafficking. (A) Analysis of CXCL12 and CXCR4 localization by time-lapse videomicroscopy. HEK-293T cells expressing ACP-CXCR4 were ACP-labelled in order to follow the fate of receptors present in the plasma membrane at the moment when the labelling reaction was carried out. The excess of free dye was washed before incubation of the cells with 8 nM CXCL12α-biotin conjugated to Qdots, SgG2 or SgG2: CXCL12 at a 100:1 molar ratio during 30 min at 37ºC. Afterwards, cells were recorded for 30 min in a confocal microscope connected to a temperature chamber. Selected frames from Videos 4-7 corresponding to the time when the movie started (t30) and 20 min later (t50) are shown. (B) SgG2 induces an increased colocalization of CXCL12 with its cognate receptor. Quantitative colocalization of CXCR4 and CXCL12 was performed by the Intensity correlation analysis (ICA) method, explained in the Materials and Methods section. Representative images and plots generated during the colocalization analysis of different fixed cells (n=12) are shown. The +ves image shows positive product of the difference of the means (PDM) values resulting from areas in which the pixel value for both CXCR4 and CXCL12 staining were above the mean (i.e., red intensity-mean red intensity and green intensity-mean green intensity are both positive), shown pseudocolored white. These results were illustrated by (C) the highly correlated Scatter plot, as well as for (D) the right-shifted ICA plots obtained for cells treated with SgG2:CXCL12 in comparison with CXCL12 alone. (E) Two coefficients were obtained to evaluate the correlation of both fluorescent intensities and their averaged values were plotted: Intensity correlation quotidian (ICQ) and Pearson’s coefficient (PC). Approximately 60 cells were quantified for each condition, and the mean±SD was represented. Scale bar represents 10 µm. Two-tailed unpaired t-test, *P<0.05, **P<0.001, ***P<0.0001.
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Altogether these results show that HSV SgG2 enhances the presence of CXCR4 at the cell surface limiting CXCL12-driven endocytosis. The defects in internalization change the fate of CXCR4 abrogating its accumulation into lysosomes and subsequent degradation, and favour the stabilization of an active signalling complex with CXCL12 at the cell surface.

3.2 Generation of an SPR chip containing CXCR4 in its native lipid environment

Our immunofluorescence and time-lapse data indicate that SgG2 increases the colocalization between CXCL12 and its receptor (Fig. R-8). However, we have accumulated perinuclearly in a time-dependent manner, as it decreased on the surface upon CXCL12 incubation. On the other hand, and in accordance with our previous results, CXCR4 remained at the plasma membrane for a longer period of time in cells treated with SgG2:CXCL12, an stimuli that did not cause the accumulation of CXCR4 in intracellular vesicles until later time points (Fig. R-9A). CXCL12 stimulation provokes a great part of CXCR4 to traffic to the endosomal compartment and then to lysosomes where it is degraded. The reduced endocytosis upon SgG2:CXCL12 stimulation correlated with a decrease in receptor degradation 2 and 4 h post-incubation, as revealed by the analysis of total CXCR4 in HEK-293T cell lysates (Fig. R-9B).

Figure R-9. SgG2 inhibits intracellular CXCR4 accumulation and degradation. (A) Confocal microscopy images showing the subcellular localization of CXCR4 after chemokine or SgG2 stimulation. Flag-CXCR4 HEK-293T expressing cells were stimulated with 8nM CXCL12, 800nM SgG2, SgG2:CXCL12 at a 100:1 molar ratio, or mock-treated during 30 min and 60 min at 37ºC and then fixed and permeabilized to detect total CXCR4 and DNA (DAPI). Scale bar=10 μM. (B) Inhibition of CXCR4 degradation by SgG2 at later time points. Total CXCR4 was analyzed in cell lysates by western blot. Graph below shows the results obtained after performing a densitometer analysis of three independent experiments. The densities obtained from each of the lanes were normalized to the loading controls and later to the mock sample.
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increase the local concentration of the chemokine in the proximity of the receptor or alter receptor trafficking. As an additional attempt to better understand chemokine-receptor interactions in the presence of SgG we carried out an SPR-based approach (Fig. R-10). The effect of GAG presentation of chemokines on the interaction with

not observed a significant increase in the total amount of chemokine bound to the surface in the presence of the viral protein, suggesting that SgG2 may impact chemokine function through a different mechanism. Among other possible scenarios, SgG2 could improve chemokine presentation to the receptor,

Figure R-10. Immobilization of CXCR4-lentiviral particles on a BIAcore chip for the analysis of CXCR4 and CXCL12 interactions by SPR. (A) Lentiviral particles enriched in CXCR4 were produced in HEK-293T cells, analyzed by electron microscopy and covalently coupled to a F1 (CM3) sensor chip. Negative staining of the virus preparation bound to the chip is shown in the inset (scale bar, 100 nm). (B) Binding of the indicated concentrations of CXCL12 to the CXCR4-lentiviral surface. (C) Analysis of the binding of a collection of CXCL12 mutant chemokines, injected at 100 nM, to the chip surface. The degree of interaction of the mutant chemokines with CXCR4 and GAGs is indicated. All curves were analyzed with the BIAEvaluation software and represent the interaction of SgG2 after subtraction of the blank curve. Arrow indicates the end of the injection.
their receptors using a lentiviral surface has been previously assessed, showing that low concentrations of heparin modulate CXCL12 and CXCR4 interaction, whereas high concentrations abrogate it\(^2\). Lentiviral particles highly enriched in CXCR4 were purified and immobilized on the surface of a BIAcore sensor chip, similarly to previously described\(^3\). To analyze their integrity, viral particles were attached to a niquel grid, negatively stained with uranyl acetate and visualized by electron microscopy. CXCR4-lentiviral particles were successfully coupled to a CM3 sensor chip (Fig. R-10A). The incorporation of a membrane-spanning protein to a virus envelope avoids the need for detergent solubilization and reconstitution, and provides a native lipidic environment that allows the accurate calculation of kinetic parameters. As such, this approach would allow the calculation of the CXCL12 binding affinity to CXCR4 in the presence or absence of SgG2. Sensorgrams showing the association and dissociation kinetics of different amounts of CXCL12\(^\beta\) sequentially injected over the chip surface showed that CXCL12\(^\beta\) binds in a dose-dependent manner to CXCR4 present in the lentivirus particle (Figure R-10B). In addition, we screened the binding of different CXCL12\(\alpha\) mutant chemokines in order to confirm the proper conformation of CXCR4 on the chip as well as the specificity of the interactions measured. Deletion of the N-terminal four residues of the chemokine (mutant 4-67) impairs receptor interaction without affecting GAG binding. A CXCL12 mutant carrying a P2G substitution binds to CXCR4 but does not activate it whereas the mutation of a cluster of basic residues in the CXCL12 3/6 variant abrogates chemokine interaction with GAGs\(^5\). Wt CXCL12\(\alpha\) showed the highest binding, closely followed by the P2G mutant. On the contrary, the amount of bound chemokine significantly dropped when the mutant CXCL12 unable to bind CXCR4 was injected. Interaction of the GAG-binding defective version of CXCL12 was also diminished, indicating the contribution of GAGs for the overall interaction with the chip lentiviral surface (Fig. R-10C). SgG2 strongly bound to the chip (not shown). Lentiviral particles incorporate cellular plasma membrane during their egress from the packaging cell, carrying among other molecules GAGs and explaining the ability of SgG2 to interact with the chip surface. Moreover, the fact that such interaction was hardly dissociable with the mild conditions needed to regenerate the chip lentiviral surface made unfeasible both the calculation of CXCL12 binding affinity in the presence of the vCKBP and the determination of SgG2 binding kinetics to the surface. Nonetheless, these results provided an interesting tool for future studies, and further confirmed the high affinity interaction of SgG with the cell surface. The utilization of mutant version of SgG able to bind chemokines but unable to interact with GAGs may be of help to overcome these drawbacks.

### 3.3 SgG2 interaction with primary neurons increases surface CXCR4 levels

As mentioned in the Introduction, the chemokine network and CXCL12/CXCR4 in particular, plays essential roles in the nervous system. Since the mechanisms beneath HSV neurotropism are not well understood, we decided to test whether
the observed gG-mediated modulation of the CXCL12/CXCR4 axis occurred in neurons too. As a first step towards the understanding of the implications of SgG2-chemokine modulating activity in the nervous system, we analyzed cultures of mouse primary neurons obtained from the superior cervical ganglia (SCG). SgG2 was detected at the surface of nervous cells (Fig. R-11A). More importantly, immunofluorescence analysis of CXCR4 expressed at the plasma membrane of neurons revealed that SgG2 was able to disregulate CXCL12-induced endocytosis as well as to upregulate surface CXCR4 in such context (Fig. R-11B). This observation points towards the involvement of SgG2-mediated regulation of chemokine receptor trafficking in neurons. The functional consequences of such regulation are currently unknown.

Figure R-11. SgG2 interaction with primary neurons upregulates surface CXCR4 and reduces CXCL12-induced internalization. (A) Confocal microscopy images showing the interaction of SgG2 with the surface of neurons. 400ng of recombinant SgG2 were incubated with the neurons during 30min at 37ºC and then stained using a polyclonal antibody raised against gG. (B) Cultures of primary mouse neurons were mock-treated or stimulated with 8 nM CXCL12, alone or in the presence of SgG2 at a 100:1 molar ratio, or with 800 nM SgG2. Cells were PFA-fixed at different time points after stimulation at 37ºC and stained to detect endogenous CXCR4 present on the surface on the nervous cells. Scale bar=5μm.
4. THE INTERNALIZATION OF OTHER CHEMOKINE RECEPTORS IS MODIFIED UPON SgG INTERACTION WITH LEUKOCYTES

SgGs bind several human chemokines and enhance the chemoattractant properties of all target chemokines tested\(^{196}\). We next decided to assess whether SgG2 specifically modulated CXCR4 or whether exerted similar effects on other chemokine receptors. We have previously shown that SgG increases CXCL13-driven chemotaxis of m300-19 B cells stably transfected with CXCR5. The internalization of CXCR5 in this cell line was assessed by flow cytometry, following incubation with CXCL13, SgG2 or SgG2:CXCL13 complex during different periods of time at 37ºC. SgG2 increased the amount of CXCR5 on the surface when compared to mock-treated cells, and decreased CXCL13-mediated internalization of CXCR5 (Fig. R-12A), a difference that was significant at early time points. We next assessed whether SgG2 had any role in the internalization of CXCR7, the second cognate receptor for CXCL12. Interestingly, SgG2 markedly counteracted CXCR7 endocytosis, with an effect that was more evident late time points (Fig R-12B). Similarly to CXCR4, cell pre-stimulation with SgG2 (SgG2F-CXCR7 condition) led to a higher decrease of CXCR7 internalization upon CXCL12 stimulation (Fig. R-12B). We also wondered whether CCR2 internalization was affected in MM-1 cells. CCR2 is the receptor for CCL2, a chemokine not bound by SgG and whose chemotactic function is not modified in the presence of the viral protein. Surprisingly, SgG2 significantly increased the surface levels of CCR2, and decreased CCR2 internalization during the first 15min of stimulation with CCL2 (Fig. R-12C). Similarly to CXCR4 and CXCR7, incubation of the cells with SgG2 prior to addition of chemokine further reduced the CCL2-induced internalization, indicating that the effect on receptor level at the surface is independent on ligand binding to SgG (Fig R-12C).

We next decided to analyze whether the SgG2-induced increase of surface CXCR4 triggered and enhanced migration of primary monocytes. Cells were preincubated with SgG2 and then washed and placed on the upper chamber on transwell plates. The increase of surface CXCR4 induced by SgG was not sufficient to trigger an increased migration towards the low doses of CXCL12 tested (Fig. R-12D). Different amounts and incubation times with the cells and SgG2 were analyzed, obtaining similar results (not shown). The use of Transwell assays to perform these assays may not allow the formation of the SgG2:CXCL12 complex unless the viral protein is directly incubated with the chemokine in the lower chamber. This observation would suggest that the chemokine needs to bind to SgG2 to enhance migration which may be mediated by an improved presentation to the GPCR or by the promotion of chemokine oligomerization. This hypothesis was further supported by our previous observation that CCL2 migration is not affected, in spite of the enhanced presence of its cognate receptor on the surface.

4.1 The trafficking patterns of non-related receptors are differentially modulated by SgG2

We analyzed the endocytosis of non-
chemokine related receptors. We first measured the internalization of a well-studied GPCR, the β2-adrenergic receptor (β2AR). HEK-293T cells expressing Flag-tagged β2AR were incubated with its cognate ligand isoproterenol or with SgG2, and the surface levels of the receptor were measured using an anti-Flag antibody as described earlier for HEK-293T Flag-CXCR4 cells. As expected, isoproterenol treatment induced the internalization of β2AR. However, contrary to what was observed for chemokine receptors, the surface amount of β2AR was further reduced by

![Image](https://via.placeholder.com/150)

Figure R-12. SgG2 increases the surface levels of other chemokine receptors. (A) Expression of CXCR5 on the cell surface. B cells stably expressing CXCR5 were incubated with 15 nM CXCL13, 1.5 μM SgG2 or SgG2:CXCL13 at a 100:1 molar ratio for 15, 30 and 45 min, and receptor levels at the surface were measured by flow cytometry using a fluorophore-conjugated anti-CXCR5 antibody. (B) CXCR7 expression on the surface of MM-1 cells. Cells were stimulated with SgG, 15 nM or SgG2:CXCL12 at a 100:1 molar ratio, and CXCR7 expression at the surface was measured by flow cytometry using a fluorophore-conjugated specific antibody. The SgG2:CXCL12 condition represents cells that were incubated during 30min at 37°C with SgG prior to stimulation with the chemokine. (C) CCR2 expression at the surface of MM-1 cells. Cells were incubated with 5 nM CCL2, 500nM SgG2 or SgG2:CCL2 at a 100:1 molar ratio for different time points and stained for surface CCR2 using a specific antibody. The SgG2F:CCL2 condition represents cells that were incubated with SgG2 30 min before CCL2 stimulation. (D) Migration of primary monocytes measured by transwell assays. Chemotaxis of freshly isolated monocytes towards a low range of CXCL12 alone or in the presence of SgG2 at a 50:1 molar ratio. The CXCL12:SgG2 conditions represent the migration of monocytes that were preincubated with SgG2 the indicated time points prior to assess migration towards the chemokine. The expression of each chemokine receptor at the surface was represented as the percentage of the value obtained for mock-treated cells at each time point. The plots in A and B represent the average values (±SEM) from two independent assays performed in triplicates; the plot in C correspond to the mean (±SD) is one representative assay performed in triplicates out of two. Graph in D represents the mean (±SD) of one representative assay performed in triplicates out of three independent assays. Two-tailed unpaired t-test, *P<0.05, **P<0.001, ***P<0.0001
Figure R-13. The dynamics of non-chemokine related GPCRs and other types of receptors are differentially affected by SgG2. (A) Expression of β2AR on the cell surface. HEK-293T cells stably expressing Flag-tagged β2AR were stimulated with the agonist of the GPCR, isopretanol, or SgG2 for different time points at 37°C. Cells were PFA-fixed and incubated with a monoclonal anti-Flag antibody followed by an alkaline-phosphatase conjugated goat anti-mouse immunoglobulin. The amount of receptor at the surface is represented as the percentage of the value obtained for mock-treated cells at each time point. The plot represents the mean ± SEM from five independent assays performed in duplicates or triplicates. (B) Visualization by immunofluorescence of Flag-β2AR in permeabilized HEK-293T cells, incubated for 30 min in the presence of isopretanol and SgG2. (C) Trafficking of CD71, the receptor for transferrin, is not significantly modulated by SgG2. Scale bar represents 5 μm. (D) MM-1 cells were incubated with transferrin at 1 mg/ml, alone or in the presence of 1.5 μM SgG2, and CD71 expression on the surface was measured by flow cytometry using an anti-CD71 antibody. The plot represents mean (±SEM) of four independent assays performed in triplicates. Two-tailed unpaired t-test was performed in all cases, *P<0.05, **P<0.001, ***P<0.0001. (D) Expression of the adhesion receptor CD44 on the surface of Jurkat cells. Cells were attached to fibronectin-coated coverslips and stimulated with 8 nM CXCL12, 800nM SgG1 or SgG2 and the vCKBP: CXCL12 at a 100:1 molar ratio during 30 min at 37°C. Subsequently, cells were fixed and stained for the lipid raft marker GM3, and for endogenous CD44 and CXCR4. Scale bar, 2 μm.
Results

binding to CCR5. Moreover, CD44 cooperates with CXCL12 in the trafficking of CD34+ progenitors to the bone marrow through the enhancement of chemokine-dependent transendothelial migration that requires CD44 concentration at the leading edge and crosstalk with CXCR4-mediated signalling. Immunoﬂuorescence analysis of CD44 location in non-permeabilized Jurkat T cells seeded on ﬁbronectin-coated coverslips showed that SgG1 and SgG2 seemed to upregulate CD44 at the surface (Fig. R-13D). Moreover, cell stimulation with SgGs, in the presence or absence of CXCL12, seemed to cause some relocalization of CD44 to form patches at the cell surface (Fig. R-12D). This was evident in sites enriched in both CXCR4 and GM3, a marker of leading edge lipid rafts. Due to time constraints, the functional consequences of these observations have not been investigated, but they are likely connected to the increase in chemokine responsiveness induced by SgG.

In summary, we have shown that SgG2 modulates the trafficking patterns of all chemokine receptors assayed increasing their surface levels, regardless of the cognate chemokine being a ligand of SgG2 or not, whereas it differentially modiﬁes other GPCR and non-GPCR receptors. CD44, a GAG-attached adhesion receptor involved in chemokine migration is among the surface molecules increased by HSV SgGs. Importantly, SgG2 also modulated the trafficking of CXCR4 expressed in neurons, indicating that modulation of chemokine function might play a role in the viral access to the nervous system.
5. SgG UNBALANCES LIPID RAFT CONTENT OF THE PLASMA MEMBRANE, REDISTRIBUTES SURFACE CXCR4 AND ENHANCES CELL POLARIZATION.

Lipid rafts have been implicated in many cell functions, including trafficking of cholesterol and a number of transport processes such as endocytosis and transcytosis, establishment of cell polarity and location and tuning of the cell signalling machinery next to the surface\(^\text{65, 85, 247, 248}\). Interestingly, endocytic processes mediated by lipid rafts have been commonly associated with slower rates of internalization, compared to the highly efficient clathrin-dependent pathway\(^\text{68, 249, 250}\). Although CXCR4 has been defined as a non-raft protein, the interaction of CXCR4 with lipid raft components has been related to changes in receptor and chemokine functionality\(^\text{74, 90, 94}\).

5.1 SgG2 relocates CXCR4 towards lipid rafts

Having in mind the impact of SgG2 on chemotaxis and receptor trafficking, we next asked whether SgG2 could modulate the incorporation of CXCR4 into specific microdomains of the plasma membrane. To test this hypothesis we first utilized the fluorescent lipid raft marker that comprises the N-terminal 10 amino acids of the Lck protein that confers myristoylation and double palmitoylation on a mCherry fusion protein driving its incorporation into detergent resistant membranes\(^\text{251, 252}\). Jurkat cells were transduced with lentiviral vectors expressing Lck\(_{10}\)mCherry and electroporated with EGFP-CXCR4, and the location of the receptor was examined and visualized by time-lapse videomicroscopy. SgG2 promoted a strong relocalization of CXCR4 to lipid rafts (Video 11 and Fig. R-14) that was not observed in mock- or CXCL12-stimulated leukocytes (Video 10 and 12 and Fig. R14). Colocalization of both fluorescent signals was also observed following SgG2:CXCL12 stimulation (Video 13 and Fig. R-14). Moreover, it is very interesting to note

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**Figure R-14. CXCR4 localizes to lipid rafts upon SgG2 stimulation of leukocytes.**

Panels show selected frames from Videos 8-11. Jurkat cells were lentiviral transduced with the fluorescent lipid raft marker Lck\(_{10}\)mCherry and transfected with EGFP-CXCR4. Cells were mock-treated or stimulated with 8 nM CXCL12, 800 nM SgG2 or the complex SgG2:CXCL12 for 10 min at 37ºC before the time lapse was started. White circles mark double-positive vesicles suggesting the existence of CXCR4 lipid raft-mediated trafficking processes. Scale bar = 5 \(\mu\)m.
the appearance of double positive vesicles containing CXCR4 and Lck<sub>10</sub>mCherry, which could suggest the onset of lipid raft-mediated recycling and/or endocytic events (Video 13 and Fig. R-14).

The incorporation of CXCR4 into rafts was further studied by a quantitative colocalization analysis of fixed cells. Lentiviral transduced Lck<sub>10</sub>mCherry Jurkat cells were mock-treated or stimulated with SgG2, CXCL12 or both at 37°C, fixed and stained to detect endogenous CXCR4 to rule out any nonspecific effect due to receptor overexpression. Neither mock-treated nor chemokine-stimulated cells showed evident colocalization between CXCR4 and Lck<sub>10</sub>mCherry lipid rafts (Fig. R-15A). On the contrary, the presence of CXCR4 into lipid rafts was significantly increased upon SgG2 or SgG2:CXCL12 stimulation of the T cell. The enrichment of CXCR4 into rafts occurred independently of CXCL12, as SgG2 on its own promoted a strong incorporation of the receptor into lipid raft microdomains (Fig. R-15A). Contrary to SgG2, PRV-SgG did not cause a higher incorporation of CXCR4 in Lck<sub>10</sub>mCherry rich domains, indicating that the shift of the chemokine receptor to rafts was specifically triggered by SgG2 (Fig. R-15A). The averaged colocalization coefficients (PC and ICQ) obtained from the analysis are shown in the plots below the images.

5.2 SgG2 clusters rafts, where the chemokine-receptor complex is enriched

CXCR4 was specifically found in Lck<sub>10</sub>mCherry accumulations along the plasma membrane upon SgG2 stimulation. In order to distinguish proper Lck<sub>10</sub>mCherry clusters from membrane ruffling we probed the cell plasma membrane with the lipophilic dye Vybrant-DiO. The ratio corresponding to Lck<sub>10</sub>mCherry signal intensity vs Vybrant staining intensity was calculated for selected regions of interest (ROIs) along the plasma membrane where the Lck<sub>10</sub>mCherry marker clustered (Fig. R-15B). The ratios were plotted and profiled in qualitative intensity histograms (Fig. R-15C). Focal clusters of Lck<sub>10</sub>mCherry lipid rafts in regions where no membrane ruffling was apparent were indeed observed upon stimulation of the cell with SgG2 (Fig. R-15B and C). Accumulation of lipid rafts in the presence of SgG2:CXCL12 was higher than in the case of CXCL12 alone, although it did not reach statistical significance. PRV-SgG did not mediate any clustering of lipid rafts (Fig. R-15D); the absence of effect in raft clustering was not due to the lack of interaction with the cell, since PRV-SgG binds to the cell surface (Viejo-Borbolla A. and Martinez-Martin N., unpublished data). Both SgG2-induced clustering of lipid rafts and enrichment of CXCR4 into these domains were dependent on the cholesterol content of the plasma membrane since both effects were abrogated when cells were pretreated with the cholesterol-sequestering reagent MβCD (Fig. R-15E). Similar results were obtained when colocalization of EGFP-CXCR4 and Lck<sub>10</sub>mCherry lipid rafts was measured in HEK-293T cells expressing both constructs. SgG2, alone or in the presence of CXCL12, strongly enhanced the presence of CXCR4 into Lck<sub>10</sub>mCherry enriched areas, in comparison to mock- and chemokine-treated cells respectively (Videos 14-17). An increase in lipid raft area mediated by SgG2 was also evident in these movies.
Figure R.15. SgG2 promotes clustering of lipid rafts and improves CXCL12 and CXCR4 interaction in those microdomains. (A) Quantitative analysis of lipid rafts and CXCR4 colocalization. Jurkat T cells were transduced with the fluorescent lipid raft marker Lck_10mCherry and stained for endogenous CXCR4 (shown in green). Cells were mock-treated, stimulated with 8 nM CXCL12 or incubated with the vCKBPs SgG2 and PRV-SgG alone or in combination with CXCL12 at a 100:1 molar ratio for 30 min at 37°C. Representative confocal microscopy images are shown. Scale bar 5 μm. Bar plots below show PC and ICQ values (means±SEM) obtained from 14-20 cells for a region of interest (ROI) corresponding to the plasma membrane. Two-tailed unpaired t-test *p<0.05, **p<0.01, ***p<0.0001. (B) Lck_10mCherry T cells were stained with the lipophilic plasma membrane marker Vybrant-DiO before stimulation with the vCKBPs or the chemokine. Representative confocal images are shown. The fluorescent intensity for both markers was measured in ROIs along the membrane where the Lck_10mCherry signal was more intense. Typically 2-3 ROIs were analyzed in most of the cells. (C) Fluorescence intensity histograms corresponding to the selected ROI indicated by a white bar in the images on the left. The y-axis represents the fluorescent intensity of Lck_10mCherry (red line) and that of Vybrant DiO plasma membrane staining (blue line); x-axis represents the distance around the periphery of the cell. (D) The average ratio between Lck_10mCherry vs Vybrant DiO staining for all ROIs quantified is shown in the plot (n=29-46 Lck_10 clusters from 14-20 cells). Lck_10mCherry clusters promoted by SgG2 are indicated by asterisks in the intensity profile. (E) SgG2-induced focal clustering of lipid rafts and CXCR4 incorporation into these domains upon cholesterol extraction with MβCD.
These results indicate that SgG2 triggers lipid raft clustering and association with CXCR4 not only in leukocytes, but also in epithelial-like cells. In additional experiments, we observed that in the presence of SgG a higher amount of biotinylated-CXCL12 was found in Lck<sub>10</sub>mCherry domains, very likely due to the increased inclusion of CXCR4 and to raft-related conformational/post-translational modification of such relocated receptor that would make it more suitable to interact with the chemokine (Fig. R-16). The preferential binding of CXCL12 to raft-associated CXCR4 has been previously suggested by others<sup>90</sup>.

In addition, we analyzed the localization of β<sub>2</sub>AR, a GPCR whose internalization increases upon SgG2 binding to the cell (Fig. R-13). We analyzed β<sub>2</sub>AR location in Lck<sub>10</sub>mCherry-expressing HEK-293T cells that were transiently transfected with Flag-tagged β<sub>2</sub>AR. Resting receptor was partially associated with rafts, as described, whereas it moved away from such domains upon isopreterenol stimulation for 5 min at 37°C (Fig. R-17). On the contrary, SgG2 triggered a more extended incorporation of β<sub>2</sub>AR into lipid rafts (Fig. R-17). It has been well established that cholesterol is included into β<sub>2</sub>AR transmembrane helices and that it regulates receptor conformation stabilizing the formation of homodimers<sup>253, 254</sup>. The differential association of β<sub>2</sub>AR with cholesterol as a consequence of the imbalance of lipid raft composition in the presence of SgG2 might be beneath the altered trafficking of the receptor that we have observed.

5.3 SgG enhances CXCR4 polarization and responsiveness to chemotactic gradients

Cell migration is a highly coordinated process that involves the segregation of membrane components to form two functionally different cell poles, the leading edge and the uropod<sup>83, 255, 256</sup>. CXCL12 stimulation of leukocytes triggers the redistribution of CXCR4 and other chemosensory receptors to the

![Figure R-16. SgG2 promotes the binding of CXCL12 to lipid rafts. MDCK cells stably expressing the fluorescent lipid raft marker were incubated with CXCL12-α-biotin conjugated to Qdots alone or in the presence of SgG2. The colocalization of both fluorescent signals was analyzed. PC values (mean±SD) from CXCL12 or SgG2:CXCL12 correspond to 20 and 30 cells, respectively. *P<0.05, **P<0.001, ***P<0.0001 (two-tailed unpaired t-test). Scale bar = 20 μm.](image-url)
RESULTS

leading edge, enriched in the ganglioside GM3 (termed GM3 or L-rafts), while the ganglioside GM1 and adhesion molecules localize at the uropod (GM1 or U-rafts)\(^{83, 256}\).

As previously described for Lck\(_{10}\)mCherry rafts, we analyzed GM3 raft clustering by normalizing the intensity of the signal by the fluorescent intensity corresponding to the bulk plasma membrane in Jurkat cells (Fig. R-18A). The fluorescent of CXCR4 was analyzed and profiled around the perimeter of the plasma membrane of each cell (Fig R-18B). Both SgGs, contrary to PRV-SgG, promoted the clusterization of GM3 lipid rafts, which was accompanied by an enrichment of CXCR4 in the these microdomains (Fig. R-18B). Moreover, stimulation of primary monocytes with SgG1 and SgG2 led to an increase in the area of different subtypes of lipid rafts (Fig. R-18C). To further characterize the interaction established between CXCR4 and lipid rafts mediated by SgG2, we monitored the relative relocalization of CXCR4 to GM3 vs GM1 rafts in human primary monocytes (Fig. R19A and B, respectively). As previously described, upon CXCL12 incubation, monocytes acquired a polarized phenotype characterized by the accumulation of CXCR4 in the GM3-enriched leading edge (Fig. R-19A). Treatment of cells with CXCL12 and SgG2 resulted in most CXCR4 localized in GM3 and a higher exclusion from GM1 rafts. Importantly, SgG2 on its own significantly increased the inclusion of CXCR4 into GM3 rafts which was also accompanied by a slightly decrease in colocalization with GM1 rafts (Fig. R-19A and B). Monocytes did not show a clear polarization in the presence of PRV-SgG (Fig. R-19), consistent with our previous findings that PRV-SgG impairs chemokine binding to its receptor and thereby migration\(^{194}\). However, it is interesting to note that PRV-SgG increased the presence of the receptor in GM1 rafts. It is plausible that freshly isolated monocytes cultures contain and/or produce a background level of chemokines, including CXCL12 that trigger a basal incorporation of CXCR4 into GM3 rafts in our mock condition. PRV-SgG, which binds CXCL12 and other chemokines with subnanomolar affinity could sequester them preventing any sign of a chemotactic stimuli in the monocyte culture. Colocalization coefficients were calculated and plotted for all experimental conditions (Fig. R-19C and D for GM3 and GM1 rafts, respectively).

Figure R-17. \(\beta_2\)AR is incorporated into lipid rafts upon SgG2-stimulation of cells. HEK-293T cells stably expressing the Lck\(_{10}\)mCherry marker and transiently transfected with Flag-\(\beta_2\)AR were stimulated with isopreterenol or with SgG2 during 5 min, PFA-fixed and stained using a monoclonal anti-Flag antibody. A detail of the plasma membrane of each cell, corresponding to the area marked with a white square, is shown in the right panels. Scale bar = 10 \(\mu\)m.
Figure R-18. HSV SgG unbalances the lipid raft and non-raft disposition of the plasma membrane. (A) Jurkat cells were mock-treated or stimulated with 8 nM CXCL12, 1.5 μM SgG1, SgG2, or PRV-SgG, or the indicated vCKBPs to the chemokine at a 100:1 molar ratio, during 15 min at 37°C. Cells were fixed and stained for endogenous CXCR4 and GM3 lipid rafts using specific antibodies. Total plasma membrane was detected using Vybrant-DiD lipophilic marker. (B) The intensity of GM3 raft and Vybrant-DiD staining along the periphery of the plasma membrane is represented in the fluorescent intensity histograms. Arrows indicate areas of the plasma membrane corresponding to GM3 clustered. Scale bar 2 μm. (C) The area (square μm) corresponding to GM3 lipid raft staining in primary monocytes was measured using Image J software. Bars represent mean±SD values for 10 monocytes. (D) The area corresponding to GM1 lipid rafts after stimulation of monocytes. Data (mean±SD) from one experiment out of two are represented. In all cases, a two-tailed unpaired t-test was performed *P<0.05, **P<0.001, ***P<0.0001
Results

A B
GM1 CXCR4 merge BF

mock
SgG2
SgG1
PRV:SgG
CXCL12
SgG2: CXCL12
SgG1: CXCL12
PRV:SgG CXCL12

Figure R-19. HSV SgG increases primary monocyte polarization. (A) Freshly isolated monocytes were mock-treated, stimulated with 8nM CXCL12 or incubated with 400nM SgG2, SgG1 or PRV:SgG alone or in the presence of CXCL12 at a molar 50:1 molar ratio. Cells were then fixed and stained for endogenous CXCR4 and GM3 rafts using specific antibodies. (B) In an additional set of experiments, same stimulations were performed and monocytes were stained for CXCR4 and GM1 rafts using an anti-CXCR4 monoclonal antibody and FITC-conjugated cholera toxin B, respectively. Representative images obtained by confocal microscopy are shown. Scale bar 5 µm. (C) ICA analysis of CXCR4 and GM3 colocalization. PC and ICQ mean±SD values for 10 monocytes are shown in the plots. (D) Average numerical values (mean±SD) for PC and ICQ corresponding to ICA analysis of CXCR4 incorporation into GM1 rafts (n=25 cells). Two-tailed unpaired t-test was performed *P<0.05, **P<0.001, ***P<0.0001
RESULTS

6SgG2-MEDIATED RELOCALIZATION OF CXCR4 AT THE CELL SURFACE PROMOTES DISTINCT REARRANGEMENTS IN THE RECEPTOR THAT DIVERT CXCR4 INTERACTION WITH β-ARRESTIN2

To date there is no evidence supporting the influence of the extracellular loops of chemokine receptors on their cholesterol-binding affinities. Other receptor types, such as the epidermal growth factor factor, are incorporated into rafts due to interactions between the extracellular part of the receptor and GM1 gangliosides. Intracellular loops and the carboxy terminal tail of chemokine receptors have been involved in receptor targeting to lipid rafts by means of different addressing signals, mainly acylation and protein-protein interactions. Molecular modelling of GPCRs has indicated the participation of the transmembrane α-helices of the GPCR in the formation of cholesterol binding sites. The importance of cholesterol binding and palmitoylation has been well studied for β2AR whose X-ray crystal structure showed that receptor dimers organize in supramolecular complexes that are assembled by interactions through palmitate residues bridged by cholesterol molecules. As the conformation of α-helices depends on the activation state of GPCRs, it is possible to speculate that agonist binding may affect their localization in lipid rafts by means of the same molecular mechanism that lead to receptor activation, that is, by transmembrane rearrangements. Similarly, receptor oligomerization also modifies the orientation of transmembrane helices and may thus modulate cholesterol binding and raft localization.

The recently solved X-ray structures of CXCR4 in combination with several small peptide antagonists revealed the ubiquitous presence of CXCR4 homodimers, with a dimer interface including helix V and VI. The authors concluded that the nature of the interactions established in the CXCR4 dimer would facilitate the ability of the receptor to heterodimerize with other chemokine receptors, a fact that has indeed been reported by others. The use of fluorescence and bioluminescence resonance energy transfer methods (FRET and BRET respectively) has lead to the conclusion that CXCR4 homodimerizes in the absence of chemokine and that chemokine binding induces conformational changes within preformed homodimers rather than promoting dimer assembly.

6.1 SgG2 triggers conformational rearrangements in pre-existing CXCR4 homodimers

We next wanted to analyze whether SgG2 modulation of location and functioning of CXCR4 correlated with changes in the conformation receptor homodimers. To do so we took advantage of BRET technique, using the CXCR4-luciferase (Rluc) and CXCR4-GFP pair. BRET allows the measurement of protein-protein interactions in live cells, by the transference of bioluminescent energy from an excited donor (luciferase fusion protein) to a fluorescent acceptor (GFP fusion protein). BRET is measured after the addition of DeepBlueC (DBC), the substrate for luciferase. An increase in BRET signal serves as a readout for molecular proximity (<100 A) and it also depends on
the relative orientation of the two proteins (Fig. R-20A). BRET \(_2\) measurements were performed as described previously\(^{70}\). Initially, BRET titration curves were obtained by transfecting the cells with different ratios of the acceptor CXCR4-GFP\(_2\) protein to luciferase-tagged donor CXCR4 that was constant. In contrast to “bystander BRET” that results from random collision and increases linearly, the BRET ratio obtained increased hyperbolically and rapidly saturated, being indicative for specific protein-protein interactions between CXCR4-Rluc and CXCR4-GFP\(_2\) (not shown). All measurements were corrected by subtraction of the signal obtained for the pcDNA-luciferase empty vector expressed alone. Two internal controls were carried out in all experiments. BRET ratios corresponding to the pairs CXCR4-GFP\(_2\) and \(\beta_2\)-AR-Rluc or CXCR4-Rluc and a constitutively active form of \(\beta\)-arrestin\(^{227}\) (termed \(\beta\)-arrestin\(2\)) tagged to GFP\(_2\) were used as negative and positive control, respectively (Fig. R-20B). HEK-293T cells were cotransfected with CXCR4-Rluc and CXCR4-GFP\(_2\) and the BRET signal was measured at different time points after cell stimulation with CXCL12, SgG2 or SgG2 in complex with CXCL12. The BRET ratio for each experimental condition was expressed over the BRET ratio obtained for mock-treated cells. Different concentrations of CXCL12 triggered different BRET ratios suggesting a dose-dependent effect on

Figure R-20. SgG2 induces conformational rearrangements in CXCR4 homodimers. (A) Principle of BRET\(_2\). BRET allows the study of protein-protein interactions, expressed as an Rluc- and GFP\(_2\)-fusion proteins. If the two monomers interact (<100A apart) the energy generated by the Rluc after oxidizing its substrate DBC (\(\lambda\_^{400nm}\)) is transferred to the GFP\(_2\)-tagged protein, which emits light at a longer wavelength (\(\lambda\_^{510nm}\)). (B) \(\beta\)-AR-luc and CXCR4-GFP\(_2\) were used as a negative control in the assays, whereas the interaction between CXCR4 and \(\beta\)-arrestin\(2\) was used as a positive control. (C) BRET ratio obtained after cell stimulation with the indicated concentrations of CXCL12. (D) Plot represents the BRET ratio calculated for each indicated experimental condition over the ratio obtained for mock-treated cells at each time point. Three independent assays performed in triplicates are represented. Two-tailed unpaired t-test, *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\).
the rearrangement of CXCR4 dimers (Fig. R-20C). Interestingly, BRET variation was higher upon SgG2 stimulation indicating that CXCR4 homodimers adopted a conformation that is different from that promoted by the chemokine (Fig. R-20D). The rearrangement of receptor dimers was especially evident when cells were stimulated with SgG2 in comparison to mock-stimulated cells, indicating that the conformation acquired by CXCR4 was substantially altered respective to that of the resting receptor (Fig. R-20D).

Although a higher BRET cannot be unequivocally assigned to an increased functionality, we have shown that SgG2 relocalization of CXCR4 at the surface is accompanied by a structural reorganization in receptor homodimers, which indeed correlates with an increased CXCR4/CXCL12 function. These results suggest that the stabilization of a different conformation of CXCR4 homodimers by the SgG2:CXCL12 heterocomplex would be associated to the enhanced signalling properties of the receptor.

6.2 Relocated CXCR4 presents a different phosphorylation status that modifies its interaction with β-arrestin2 and with the endocytic machinery

Besides conformational changes, the function and location of CXCR4 depends on post-translational modifications. One of such modifications is the phosphorylation of the receptor’s C-tail. Following CXCL12 stimulation, agonist-occupied CXCR4 is phosphorylated by GRKs, leading to desensitization of the receptor and recruitment of β-arrestins, that subsequently target the receptor to clathrin-coated pits. Moreover, the coordinated action of specific GRKs acting on particular Ser and Thr residues within the C-tail is necessary for proper receptor regulation by dictating specific interactions through alternative phosphorylation patterns, so termed the “phosphorylation code”. We took advantage of two phospho-specific antibodies against phospho Ser residues 324/325 (pSer324/325) and phospho Ser300 (pSer330) present at the C-terminus of CXCR4. HEK-293T cells stably expressing Flag-CXCR4 were stimulated at different time points and total cell extracts were analyzed by western blotting. Interestingly, the phosphorylation of Ser330 residue was significantly reduced upon SgG2 stimulation of cells, and dropped below the levels found for the inactive receptor in a time-dependent manner (Fig. R-21A). Furthermore, incubation with SgG2:CXCL12 significantly inhibited CXCL12-induced phosphorylation of CXCR4 pSer330 at 15 and 30 min after stimulation (Fig. R-21A). On the other hand, SgG2 increased the phosphorylation of CXCR4 at Ser324/325 residues in comparison to mock-treated cells, and it also augmented the phosphorylation induced by CXCL12 at early time points after stimulation (Fig. R-21B). Interestingly, the phosphorylation of a protein migrating with ~34 kDa was clearly decreased upon SgG2 stimulation (Fig. R-21B, arrow head). Such band could correspond to another phosphorylated protein with a similar sequence to that recognized by the anti-pSer324/325 antibody, suggesting that the activation state of other molecules potentially involved in signalling is also modified by
SgG2. Previous mutagenesis studies have shown that mutation of pSer324/325 to alanine modulates CXCL12-mediated internalization and degradation of CXCR4 respectively, while substitution of pSer330 reduces degradation of the receptor with no apparent changes in internalization70. Therefore, the alterations observed in the “phosphorylation code” of CXCR4 upon SgG2 stimulation likely mediate a differential recruitment of adaptors to the C-tail of CXCR4, accounting in part for the alterations in receptor internalization, degradation and function mediated by SgG2.

**Figure R-21.** The phosphorylation code of CXCR4 C-tail is modified upon SgG2-triggered redistribution of the receptor at the cell surface. Western blots showing the phosphorylation of CXCR4 at different residues of the receptor C-terminus. (A) Analysis of the phosphorylation of pSer330 residue upon stimulation with CXCL12, SgG2 or SgG2:CXCL12 for different time points. (B) Analysis of the phosphorylation status of CXCR4 C-tail at pSer324/325 residues. Blots were stripped and incubated with an anti-tubulin antibody as a loading control. Molecular masses in kDa are indicated. Graphs below each plot depict the results obtained after performing a densitometer analysis of three independent assays. The densities obtained from each of the lanes were normalized to the loading control and later to the mock sample. Bars represent standard error.

\(\beta\)-arrestin2 is a key mediator of CXCR4 internalization whose interaction highly depends on the phosphorylation status of the receptor. The distribution of \(\beta\)-arrestin2 and CXCR4 was analyzed by videomicroscopy of Jurkat cells expressing ACP-CXCR4 and \(\beta\)-arrestin2-GFP. The levels of surface CXCR4 decreased due to electroporation of the \(\beta\)-arrestin2 construct. Nevertheless, important differences were observed upon stimulation with SgG2, CXCL12 or SgG2:CXCL12. CXCR4 staining was more evident at the surface of SgG2-stimulated cells than in mock-treated cells, in accordance with our previous data (Video 18). Moreover, \(\beta\)-arrestins were not scattered through the cytosol but concentrated...
Results

Incubation with the chemokine caused CXCR4 to colocalize with β-arrestin2 in vesicles that internalized and dissipated upon internalization (Video 20). The presence of double positive vesicles was also observed in SgG2:CXCL12 cells, however, the formation of vesicles containing only β-arrestin2 was evident as well as the presence of β-arrestin-free CXCR4 at the cell surface and CXCR4 enriched vesicles (Video 21). Selected frames from these videos are show in Fig. R-22.

β-arrestin binding to CXCR4 targets the receptor to AP-2, a component of CCPs through direct interaction between AP-2 and β-arrestin. In order to assess whether the diminished recruitment of β-arrestin to the C-tail of CXCR4 had any impact on its internalization we performed immunofluorescence analysis of HEK-293T stained for Flag-CXCR4 and α-adaptin, a subunit of AP-2. Mock-treated cells showed low levels of colocalization of α-adaptin with CXCR4 whereas stimulation of the cells with CXCL12 during 5 min at 37°C increased the association of the receptor with α-adaptin next to the cell surface (Fig. R-23). On the contrary, both SgG2 and SgG2:CXCL12 significantly hindered the incorporation of CXCR4 to clathrin-coated vesicles.

In conclusion, we suggest that SgG2-induced partition of CXCR4 at the cell surface promotes conformational and post-translational modifications in the receptor, which lead to alterations in the endocytic and signalling machinery associated to CXCR4 at least at the onset of the internalization process.

7. LIPID RAFTS INTEGRITY, AND NOT CLATHRIN-COATED PIT FORMATION, IS A REQUIREMENT FOR HSV SGG-INDUCED ENHANCEMENT OF CHEMOKINE FUNCTION

Although clathrin-dependent mechanisms govern CXCR4 canonical internalization, the inclusion of the receptor in lipid rafts and further modifications induced by SgG modulate CXCR4 fate and increase the functionality of the CXCL12/receptor pair. We next analyzed the relative
importance of lipid raft- and clathrin-associated signalling pathways during SgG potentiation of CXCL12 function by measuring cell migration using a panel of selective inhibitors. Chemotaxis of freshly isolated monocytes and MM-1 cells was analyzed by transwell assays in the presence of drugs targeting CCP formation (chlorpromazine), lipid rafts disrupting or sequestering reagents (filipin, nystatin and MJCD), an inhibitor of PI3K activation (LY294002) and PP2 as an inhibitor of Src kinase function. All compounds were used at low doses that did not affect cell viability. Cells were mock-treated or incubated with the different drugs for 30 min before starting chemotaxis, which was subsequently analyzed towards a range of low concentrations of CXCL12, in the presence or absence of SgG2. When primary monocyte migration was evaluated, SgG2 enhanced the process by 2 to 3 fold, increasing the potency of CXCL12 as indicated by the displacement of the bell-shaped chemotactic curve towards lower concentrations of the chemokine (Fig. R-24A). PRV-SgG inhibited CXCL12-directed migration at all chemokine doses (Fig. R-24A), as described for MM-1 cells and in accordance with our previous results showing the absence of monocyte polarization upon CXCL12 stimulation (Fig. R-19). Perturbation of lipid raft formation by filipin slightly affected migration towards CXCL12 but it completely abrogated SgG2-induced increase of monocyte migration (Fig. R-24B). On the contrary, the inhibition of CCP formation by chlorpromazine did not affect the increase in monocyte chemotaxis mediated by SgG2 (Fig. R-24C). Similar results were obtained when analyzing MM-1 cell migration. The drugs sequestering or impairing cholesterol disposition in the cell membrane abrogated enhancement of migration (Fig. R-24D and E). The blockade of clathrin-pit formation, however, did not completely impair SgG2 function (Fig. R-24F). We confirmed that the dose of chlorpromazine used was functional by analyzing CD71 internalization in the presence of the drug, since CD71 internalization upon transferrin binding fully depends on clathrin. As shown in Fig. R-24G, treatment of MM-1 cells with
Results

Figure R-24. HSV SgG2-mediated enhancement of chemokine function depends on lipid raft integrity but it does not require clathrin-coated pit formation. (A-C) Analysis of human primary monocyte migration by transwell towards a range of concentrations of CXCL12, alone or in combination with a 50:1 molar ratio of SgG2 or PRV-SgG and CXCL2. The number of total migrated monocytes was determined using a light microscope. Migration in the absence of drugs (A) or in the presence of the cholesterol-binding drug filipin (B) or chlorpromazine, a compound that blocks the formation of clathrin-coated pits (C). The induction of migration is depicted from one assay performed in triplicate (plot in A) or two assays in triplicate (plots in B and C) Error bars represent SD and SEM, respectively. (D-I) MM-1 cell migration towards a range of low concentrations of CXCL12 or SgG2:CXCL12 in a 100:1 molar ratio was assessed in the absence or presence of different chemical inhibitors. Absorbance at 492nm indicates the number of migrated cells quantified using CellTiter solution. Impact of the perturbation of lipid raft assembly by (D) the cholesterol-binding reagent nystatin, (E) cholesterol extraction using MβCD or (F) inhibition of clathrin-coated pit formation on the function exerted by SgG2. (G) Blockade of CD71 internalization in the presence of chlorpromazine at the concentrations used in the migration assays. (H) Migration in the presence of the selective inhibitor of Src kinases, PP2, or (I) in the presence of Ly294002, an inhibitor of PI3K activity. Plots show two independent experiments performed in triplicate representative of at least three independent assays. Error bars represent SEM. Statistic significance was calculated by an unpaired two-tailed t-test, *P<0.05, **P<0.01, ***P<0.001.
the inhibitor blocked transferrin-induced CD71 endocytosis after 30 and 60 min of incubation with transferrin, indicating that the drug dose used was active.

To further dissect the signalling pathways involved in the potentiation of chemokine function by SgG2, the migration of MM-1 was tested in the presence of additional inhibitors. The correct function of Src tyrosine kinases is essential for the migration of normal hematopoietic cells towards CXCL12 besides, the association of Src-related kinase Lyn with CXCR4 in the lipid raft microenvironment controls the migration of chronic myeloid leukemia cells to bone marrow stroma. PI3K is one of the main players activated upon CXCL12 binding to CXCR4, since it directs NFκB-mediated induction of gene expression as well as the activation of adhesion molecules as FAK. Interestingly, clustering of Src- kinases, small G protein Rac and PI3K with CXCR4 in lipid rafts has been related to a better sensitization to CXCL12 gradient. Inhibitors targeting the activation of Src kinases and PI3K activity caused a significant reduction of HSV SgG function (Fig. R-24H and I, respectively), indicating that the association of CXCR4 with these signalling routes in the context of lipid rafts is important for the enhancement of chemokine function by SgG.

8. THE INCREASE IN LOW ORDER OLIGOMERS OF CXCR4 AT THE CELL SURFACE IS BENEATH THE ENHANCEMENT OF CHEMOKINE RECEPTOR FUNCTION

The simplest view of chemokine receptor activation by chemokines assumes the binding of a monomeric chemokine to a single unit of receptor that then becomes activated. Although some GPCRs fit in this model, this does not seem to be the case for chemokine receptors, a field where emerging data indicate the existence of dimers and higher-order arrays of receptor. There is convincing evidence showing that CXCR4 forms constitutive homodimers soon after biosynthesis, and the presence of chemokine-free receptors in clusters of at least three molecules has been reported. Nonetheless, neither the aggregation status of steady-state or chemokine-activated receptor nor the functional relevance of chemokine receptor oligomerization are well understood. There are data indicating that GPCRs, including CXCR4, coexist in equilibrium between transient inactive and active states. In such context, chemokine binding stabilizes an active conformation shifting that equilibrium. Rather than just one single active conformation, an array of flexible conformations all capable of activating G proteins exists, as it has been demonstrated for CXCR4.

SgG2 primes the partition of CXCR4 into lipid rafts, a process that is accompanied by conformational rearrangements in receptor constitutive homodimers, and changes in the intracellular machinery associated to them. Therefore, we investigated whether the oligomerization status of the receptor was influenced by SgG2. To address this
Results of dimers (Fig. R-26C and A, respectively). In striking contrast, there was a significant increase in the total number of small clusters (up to 6 gold particles) of CXCR4 on the surface of T cells stimulated with SgG2 or SgG2:CXCL12 compared to the mock- or CXCL12-treated cells, respectively (Fig. R-26). Interestingly, SgG2:CXCL12 also induced the accumulation of larger CXCR4 clusters (9 gold particles) (Fig. R-26D). The quantification of gold particle number and cluster distribution at the cell surface is shown in Fig. R-26E. Moreover, and in agreement with our previous data, we found a significant increase in the total number of gold particles in SgG2 treated cells, indicating that the surface levels of CXCR4 rose upon SgG2 interaction (Fig. R-26E, inset 1). Since the amount of surface CXCR4 was upregulated in the presence of SgG2, we calculated the percentage corresponding to gold particles forming clusters of the

Figure R-25. Preparation of surface replicas by the label-fracture technique for its analysis by electron microscopy. Schematic overview of the procedure followed to obtain cell surface replicas for electron microscopy analysis. (A) T cells were stimulated with CXCL12, SgG2, SgG2:CXCL12 or mock-treated in suspension, following PFA-fixation before they were labelled on ice with an anti-human CXCR4 monoclonal antibody followed by 10 nm gold-conjugated protein A. (B) Labelled cells were adhered to poly-L-lysine-coated mica strips and post-fixed with glutaraldehyde. (C) Samples were shaded with platinum and coated with carbon in order to obtain the replicas. (D) Replicas were washed with distilled water and mounted on copper grids for their examination by electron microscopy. In the case of HEK-293T, the cells were attached O/N and treated with the different stimuli directly on the mica strips. After stimulation, the cells were extensively washed with PBS, PFA-fixed and processed as described for T cells.
**Results**

Figure R-26. CXCR4/CXCL12 functional enhancement is associated with an increase in receptor oligomers at the surface of Jurkat T cells. Distribution of CXCR4 at the cell surface of SgG2-stimulated T cells. Jurkat cells were lentiviral-transduced with ACP-CXCR4 and stimulated with 8 nM CXCL12, 800nM SgG2, SgG2:CXCL12 at a 100:1 molar ratio or mock-treated. Cell surface replicas were prepared and the number and size of clusters present in the replicas of individual cells was determined by electron microscopy. Selected small field images (10,000x) showing the distribution of gold particles in cell surface replicas of (A) mock-treated cells, and (B) SgG-, (C) CXCL12- or (D) SgG2:CXCL12-stimulated cells. (E) Quantification of gold particle distribution for each experimental condition. Data for 8 mock-treated cells (4,380 gold particles), 17 SgG2- (26,291), 18 CXCL12- (14,476) and 17 SgG2:CXCL12-stimulated cells (22,683) from two independent assays are represented. Inset shows the average total number of gold particles per cell (means±SEM). Scale bar = 200 nm. Statistical analysis was performed by a two-tailed unpaired t-test, *P<0.05, **P<0.01, ***P<0.001.
Figure R-27. SgG2-mediated modulation of CXCR4 at the surface of HEK-293T cells correlates with an enhanced formation on receptor oligomers. Selected images taken at 10,000x magnification for (A) mock-, (B) SgG2-, (C) CXCL12- and (D) SgG2:CXCL12-stimulated cells are shown. (E) Quantitative analysis of the distribution of gold particles between clusters of the indicated sizes at the surface of HEK-293T ACP-CXCR4 expressing cells (mean±SEM). Averaged numbers represent the values obtained for an area of 6 x 8 μm² of the cell surface. Inset in E shows the average number of total gold particles for each experimental condition. The plot shows the average values obtained from 29 to 37 cells from 3 independent assays. Error bars represent SEM. Two-tailed unpaired t-test, *P<0.05, **P<0.001, ***P<0.0001. Scale bar=500nm.
9. HSV PARTICLES ARE COVERED BY CHEMOKINES

HSV gG1 was engineered as a soluble secreted protein in order to assess its chemokine binding properties by SPR\(^ {196} \) (Fig. R-5). Nonetheless, gG1 is a structural, transmembrane protein anchored into the viral envelope and into the membrane of the infected cell. HSV-2 gG on the contrary, has two forms, SgG2 and mgG2, which similarly to gG1 is a membrane-anchored protein\(^ {12, 19} \). We have previously reported the ability of gG1 found at the plasma membrane and of SgG2 present in the supernatant of infected cells to bind radio-iodinated chemokines\(^ {196} \). Currently there is no data on chemokine binding to the HSV virion or the HSV-2 infected cell neither. In order to address these questions, we adapted the use of the BiAcore sensor to analyze potential interactions of the whole HSV particle with chemokines by SPR (Fig. R-28). HSV virion stocks were prepared by Ficoll purification that allowed us to obtain highly purified preparation of viral particles (Fig. R-28A). The integrity and purity of the viral preparations was assessed by direct visualization of uranyl acetate negatively-stained samples by electron microscopy (Fig. R-28B). A higher degree of purity was achieved by Ficoll-purification (Fig. R-28B) in comparison to that obtained by routinely sucrose-cushion preparation (Fig. R-28C). Removal of low levels of cellular debris such as traces of damaged cell membranes from virion preparations was a relevant step for this procedure, since these materials would preferentially attach to the chip during the coupling procedure, interfering with subsequent results.

SPR allows the detection of interactions that occur within the first 100 nm on the gold surface of the chip, resulting in sensorgrams where the interactions are visualized by changes in response units (RU). In order to improve the sensitivity of the technique when working with ligands as large as viruses, we selected F1 (CM3) sensor chips, which carry shorter dextran chains that favour that the interactions occur closer to the chip gold surface. Wt HSV-1 and HSV-2 virions were covalently immobilized through amino groups to the activated dextran matrix of CM3 chips. Coupling of an HSV-1 mutant in gG (ΔgG HSV-1) was used as a control\(^ {21} \). Small amounts of viruses were coupled (typically around 3000
Results

Figure R-28. Generation of HSV surfaces for the analysis of virus particle-chemokine interactions by SPR. 

(A) Purification of HSV virions by a Ficoll-gradient, as exemplified by wt HSV-1. The fractions collected from the Ficoll gradient were analyzed by western blot with a polyclonal antibody raised against gB/gD (Austral Biologics). Molecular size markers are indicated in kDa. (B) The integrity of the viral stocks assessed by electron microscopy and compared with that of virions obtained by sucrose cushion (C). (D) Generation of viral chip surfaces for the analysis of soluble chemokine interactions by SPR. Sensorgram depicts the immobilization of HSV-1 on a F1 (CM3) sensor chip.

RU of viruses were covalently attached) in order to generate low density surfaces that would be suitable to measure the kinetic parameters of the virus-chemokine interactions (Fig. R-28D). The coupling of viral particles (ligands) allows the analysis of the interaction between a series of soluble analytes (in our case, chemokines) and the gG present in its native environment, surrounded by glycoproteins and other viral envelope components that may modulate the final outcome of the interaction. All human chemokines commercially available were screened for the binding to each virus (Fig. R-29). A representative sensorgram showing association, dissociation and regeneration of the wt HSV-1 surface after chemokine injection is shown in Fig. R-29A. Reproducible binding was achieved and the viruses remained intact, as observed by the monitoring of the same RU after several injections of a given concentration.
of chemokine, without significant changes in the base line after successive cycles of binding and regeneration (not shown). Wt HSV-1 virus interacted with chemokines (Fig. R-29B) whereas chemokine binding dramatically dropped or disappeared when the HSV-1 ΔgG virion surface was screened (Fig. R-29C). Therefore, these results indicate that HSV virion binds chemokines and that virion-anchored gG1 is the main glycoprotein mediating such interaction.

Interestingly, gG1 in the context of the viral particle showed the same binding specificity that we had previously defined for SgG1<sup>196</sup>.

Surprisingly, we were able to detect binding of chemokines to the HSV-2 virion chip (Fig. R-30). A monoclonal antibody directed against SgG2 detected a band of 100 kDa in the purified virions that may correspond to the full-length gG2 (Fig. R-30B). We hypothesized that, at least

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**Figure R-29.** HSV virions bind chemokines through gG present at the viral envelope. (A) Sensorgram depicting the interaction of HSV-1 with CXCL13. Association and dissociation after injection of a given chemokine concentration are marked by a gradual increase and decrease in RU, respectively. The viral surface was regenerated by 2-3 pulses of HBS buffer pH 4.5-5.5 in order to allow subsequent measurements. Changes in buffer are marked by sharp changes in RU due to changes in buffer density. Sensorgrams showing the interaction between the indicated human chemokines (100 nM) and wt HSV-1 (B) or gG-deficient mutant HSV-1 (C). The arrow indicates the end of the injection. All curves were analyzed with the Bia Evaluation software and represent the interaction of the chemokine after subtraction of the blank curve.
in Vero cells where the viral stocks were produced, the proteolytic cleavage of gG2 is not complete and that a portion of the gG2 precursor protein remains virion-anchored, thus conferring chemokine-binding capacity to the viral particle. The fact that a great amount of SgG2 remains associated to the virion may also importantly contribute to chemokine-binding in that context, and it could have relevant functional consequences during the viral infection that have not been characterized yet. We have demonstrated that gG1 present at the plasma membrane of infected cells is the only viral glycoprotein that shows the ability to interact with chemokines; however, although very unlikely, the presence of additional proteins with that capacity in the HSV-2 envelope has not been unequivocally discarded yet. It has been shown that gB exerts some chemokine-binding Nevertheless, the affinity calculated was 1000-fold lower than those we measured for SgG. The potential binding to chemokines mediated by gB did not contribute to the interactions we detected by our SPR approach given the low chemokine concentrations we tested (10nM to 500nM as the highest concentration injected). The study of an HSV-2 ΔgG2 mutant will unequivocally address such questions.

A series of concentrations of each of the chemokines that showed positive binding were injected over the viral surface in order to measure the kinetics of the interaction, and affinities were calculated using the BLAevaluation software. A representative sensorgram showing association and dissociation phases corresponding to several concentrations of CXCL12 injected over the HSV-1 chip is shown in Figure R-31A. Wt HSVs interacted with all chemokines with nanomolar affinities, with HSV-2 showing higher affinities than HSV-1, in agreement with results for SgG1 and SgG2 (Table
presence of the chemokine bound to the virions was detected by immunogold labelling using an anti-CXCL12 antibody followed by incubation with 10nm gold-conjugated protein A and subsequently visualized by negative staining. The quantification of more than 100 virions showed that the number of CXCL12-gold particles attached to the virus was 5-fold higher for the wt virus in comparison with its ΔgG counterpart (Fig. R-32E and F, respectively). The same procedure was repeated for CXCL12b, and similar results were obtained (not shown).

In summary, we have adapted SPR to the analysis of virus-chemokines interaction. We demonstrated the capacity of a complex human DNA virus to directly interact with chemokines, and identified gG as the only glycoprotein in the virion that exerts such binding activity. Also, we were able to calculate the relative affinities for the interaction between gG present in the virion and chemokines. Furthermore and importantly, we found that HSV-2 virion interacts with chemokines. Such
Table R-1. Interaction affinities between SgG1, SgG2 with HSV-1, HSV-2 and chemokines.

<table>
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<th>Chemokine</th>
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**Results**

Table R-1: Interaction affinities between SgG1, SgG2 with HSV-1, HSV-2 and chemokines.
Results

A B
C D
E F
wt HSV-1 delgG HSV-1
anti-gG1
anti-gB/gD
anti-CXCL12
1.47 (n=151 virions) 0.28 (n=169 virions)

Figure R-32. Detection of CXCL12 bound to the viral envelope by immunogold labelling. Analysis of purified virions by electron microscopy using specific antibodies to detect (A) gG1 in wt HSV-1 or (B) in ΔgG HSV-1; and (C) gB/gD (Austral Biologicals) in the wt virus envelope or in (D) ΔgG HSV-1. Similar experiments were performed following pre-incubation of the virions with CXCL12 to detect CXCL12 at the surface of (E) HSV-1 virions or (F) ΔgG HSV-1. The average number of gold particles associated to a virion in panels E and F is indicated below the images. Some of the gold particles are marked by yellow arrows.

chemokine-binding ability may be mediated by a precursor virion-anchored form of gG2 resulting from an incomplete proteolytical processing, together with SgG2 associated to the viral particles. The application of SPR to the analysis of virus-ligand interactions provides a powerful tool that may serve to detect virus binding to other unknown ligands relevant for the anti-viral immune response or viral pathogenesis, as well as for the kinetic analysis of such interaction.

9.1 SgG further enhances the inherent chemoattractant ability of HSV virions

The fact that the HSV particle displays the ability to bind chemokines with such a high affinity suggests that chemokine-binding and modulation plays a role during early stages of the HSV-cycle and/or right after the viral progeny is released, when virions would immediately be covered
**Figure R-33. Initial viral entry in HaCat cells is not significantly modified by chemokine binding.** Keratinocyte-like HaCat cells grown on coverslips were infected with (A) HSV-1 alone or with (B) HSV-1 pre-incubated with CXCL12 at a low multiplicity of infection (0.1 pfu/cell). Virus attachment to the cells was performed at 4ºC, after which cells were incubated at 37ºC. Cells were fixed at the indicated times post-infection, permeabilized and stained with an anti-ICP0 antibody to detect viral replication by immunofluorescence. A detail of selected cells is shown. Scale bar 20 µm.

Results

with chemokines in the chemokine-rich environment of the infected mucosa.

We analyzed whether chemokine binding by the viral particles had any impact on viral entry into the cell. To address that issue we used HaCat cells, a cell line routinely used to study different aspects of HSV infection given that they share many properties with primary keratinocytes, the initial target of infection in vivo. Adsorption of HSV-1, alone or preincubated with CXCL12, to HaCat cells was carried out during 1h at 4ºC. Afterwards, cells were extensively washed to remove unbound virus and transferred to 37ºC to allow virus entry. Cells were fixed at different time points post-entry, and stained for the immediate-early ICP0 protein in order to detect viral replication. ICP0 was detected in distinctive punctate structures in the nucleus that accumulated in a time-dependent manner (Fig. R-33A). The presence of the chemokine seemed to cause a higher accumulation of ICP0 nuclear staining, suggesting a slightly faster progression of the viral infection when the virion was pre-incubated with chemokines (Fig. R-33B). Next, to address whether chemokine binding could have an effect on viral production, HaCat cells were infected following the same procedure, and supernatants containing viral progeny were harvested at different time points after infection (from 10h to 48 h) and titrated by plaque assays on Vero cells. There were no significant differences in the virus titres obtained when HaCat cells were infected in the absence or presence of CXCL12 (not shown). Therefore, we cannot conclude that CXCL12 binding to virions benefits viral replication, at least under the conditions tested. The growth of HaCat cells in specific transwell filters in order to induce full polarization of the monolayer could be required to ascertain the effect of chemokine binding on the infection through the apical surface.
RESULTS

Virion chemokine binding could have an effect in aspects other than viral entry. We analyzed whether gG present in the viral envelope could modulate chemotaxis in a similar manner as SgG. The number of virus particles in viral stocks was quantified by incubating an aliquot of each preparation with a solution of a known concentration of latex beads, which was used as a standard to perform counts. Stocks containing similar number of viral particles were used to measure MM-1 cell migration by transwell assays (Fig. R-34). A constant amount of the concentrated virus preparation (containing between $10^9$ and $10^{10}$ particles/ml) was added to the lower compartment of the transwell plate, alone or with increasing concentrations of CXCL12 or CCL2, and cell migration towards these stimuli was analyzed (Fig. R-34B and C, respectively). Some interesting observations were made. First, HSV particles resulted slightly

![Figure R-34. HSV virions synergize with chemokines triggering an enhanced cell migration. (A) Cell migration towards HSV virions and chemokines was studied by transwell assays. MM-1 chemotaxis was assessed in the presence of increasing concentrations of (B) CXCL12 or (C) CCL2, incubated with a constant amount of wt HSV-1 or HSV-1 ΔgG particles. Viral particles were quantified by electron microscopy in order to ensure the comparable amounts of both viruses were used in the assays. (D) HSV-2-mediated increase of CXCL12-dependent migration.](image)

Plots show data from 4-5 assays performed in triplicate or quadruplicate except for D where data from two assays performed in triplicate are shown. Errors bars represent SEM. Two-tailed unpaired t-test, *p<0.05, **p<0.001, ***p<0.0001.
Results

Figure R-35. HSV virions increase chemokine-mediated signalling. (A) Western blots showing the effect of HSV-1 virions on the activation of CXCL12-dependent signalling pathways. The indicated concentration of CXCL12 was incubated with no virus (mock), wt HSV-1 or ΔgG HSV-1. From top to bottom, the activation of pERK and loading control total ERK; activation of p38 and JNK, and phosphorylation of JNK. As a loading control the blots were stripped and rebotted using an anti-tubulin antibody. (B) The graphs depict the results obtained from densitometer analysis of the blots. Plots show the results from two or three independent assays.

These observations suggest that the overall effect occurs by an uncharacterized crosstalk of independent yet-related pathways, virion-induced and chemokine-induced, which converge leading to an increased cell migration. Wt HSV-1 further enhanced CXCL12-mediated response in comparison to the ΔgG mutant (Fig. R-34B). The fact that the wt virus particles did not trigger such increase over the deletion mutant when CCL2-driven chemotaxis was analyzed (Fig. R-34C), emphasizes the contribution of gG-mediated chemokine

chemotactic on their own. Furthermore, virions showed a synergistic effect with the chemokine-induced migration, as seen by the strong increase of chemotaxis when cells migrated towards chemokines combined with viruses, in comparison to chemokines alone. Such HSV-1-mediated synergic increase of chemokine function was observed for both, CXCL12 and CCL2 (Fig. R-34B and C, respectively), the latter a chemokine that did not bind to HSV virions (Table 2). Similarly, HSV-2 enhanced CXCL12-induced migration (Fig. R-34D).
interaction with virions in the enhancement of CXCL12 function. In addition, HSVs displayed similar synergistic effects when the migration of m300-19 CXCR5 expressing cells towards CXCL13 was assayed (not shown). The results presented were obtained with viral purifications prepared independently. When migration towards a higher range of CXCL12 concentrations was assessed, we did not observed differences between the wt and the deletion mutant virus. These results were consistent with our previous data regarding SgG function, and suggested that gG found in the viral envelope may play a role at a moment of the infection were chemokine levels are still low, such as the very beginning of the primary infection.

9.2 HSV increases the activation of chemokine-triggered signalling pathways

Activation of MAPKs is a hallmark of chemokine-induced signalling and is also a requirement for early HSV replication. Indeed, we have observed that SgGs enhance CXCL12-induced phosphorylation of MAPKs. We next assessed whether the effect of HSV particles correlated with an increased activation of MAPKs, alone or in the presence of CXCL12 (Fig. R-35). Different concentrations of CXCL12 were incubated with virions to stimulate MM-1 cells at the same ratio virus/cells ratio used for the chemotaxis assays, and the reaction was stopped with cold PBS 2 min after stimulation at 37°C. Cells were lysed and the activation levels of MAPKs ERK, JNK, p38 and AKT, a signalling signature of CXCR4 activation, were analyzed by western blotting using specific antibodies. In accordance with the observed increase in migration in response to HSV particles or virions: CXCL12, we observed a clear upregulation of the phosphorylation status of all signalling molecules, with the exception of MAPK p38 (Fig. R-35A). Blots were subjected to densitometer analysis using tubulin or total ERK levels as a loading control (Fig. R-35B). Wt HSV-1 virions triggered a higher activation of ERK and AKT at lower concentrations of CXCL12 (0.5 nM and 2 nM) compared to the phosphorylation achieved upon stimulation with HSV-1 ΔgG virion: CXCL12 (Fig. R-35A and B), an observation that is in agreement with the higher impact on CXCL12 chemotaxis (Fig. R-34). These observations suggested that the binding of chemokines to the virion-anchored gG1 contributes to enhance cell chemotaxis at low levels of the chemoattractant, a phenomenon that is accompanied by an increased MAPK activation.

In conclusion, we have shown for the first time that HSV virions are chemotactic. Moreover, it appears that a crosstalk between virus-induced migration and chemokine-triggered chemotaxis synergistically enhance cell movement towards site of infection where both chemokines and viral particles are enriched. Furthermore, we have determined that virion-gG1 contributes to such increased cell recruitment, which is less potent when chemokines interplay with a gG1 deletion mutant. The contribution of HSV gG interaction with chemokines in the viral cycle deserves further investigation.
1. HERPESVIRUSES ARE EXPERTS IN IMMUNE REGULATION

1.1 The immune system and the biology of HSV

Chemokines are essential for both the immune and nervous system\(^{138}\). An uncontrolled unorganized chemokine response is beneath the development of immunopathologies and is associated with many neuronal disorders. HSV-1 and HSV-2 are neurotropic human viruses among the most prevalent pathogens worldwide. Reaching seroprevalences that range from 15 to 90% and virtually everybody is infected with at least one of the human herpesviruses in certain geographical regions\(^{5}\). Latency in neurons is a major evolutionary advantage of HSV that allows the virus to persist in a so-called immune privileged niche, the nervous system. The capacity to manipulate multiple aspects of the immune and nervous system biology is based on the evolution of a complex network of exquisitely organized regulatory and evasion strategies by HSV. Such finely tuned co-existence is manifested by the lack of dramatic collateral damages to the host during most HSV infections. Nevertheless, HSV infection can result in severe and even fatal consequences that usually involve spreading of the virus throughout the nervous system and are more often associated with an exacerbated immune response than with the virus itself\(^{157}\). The causes for the distinct pathological manifestations of HSV infection are not sufficiently understood.

Since chemokines regulate trafficking and activation of immune cells, being responsible for some immunopathologies as well as neuroinflammation and intercellular communications in the nervous system, and they are also primarily responsible for immunopathologies, such different HSV outcomes very likely depend, up to a certain extent, on the interplay between the virus and the chemokine system. However, the modulation of the chemokine system by HSV is not well understood.

1.2 HSV gG exerts a novel strategy of immune modulation

One of the immunoregulatory strategies evolved by herpesviruses consists of the expression of vCKBPs\(^{170}\). These chemokine modulatory proteins have been identified mainly in viruses, with only a couple of such proteins being identified in eukaryotes: Evasins in ticks and smCKBP in the human parasite Schistosoma mansoni\(^{189, 190}\). Not a single soluble chemokine receptor or a molecule similar in sequence to CKBPs have been identified in humans so far. Our previous work contributes to clarify the importance of chemokine modulation during HSV infection. We have reported that HSV gG is the first vCKBP encoded by a human pathogen that enhances chemokine-dependent responses\(^{196}\). Contrary to what was expected and to the rest of vCKBPs, HSV has developed a strategy to increase chemokine functionality that may represent an evolutionary advantage for this virus.

Among the Herpesvirales order, the gG gene is only present in the genome of Alphaherpesviruses, suggesting that gG originated relatively recently, after the divergence of this subfamily. gG probably arose independently, not being the result of a gene capture, as indicated by the lack of gG homologues in other species. The
origin of gG1 and gG2 has been proposed to occur from a duplication event involving gD, which shares some amino acid similarity with gG that is more pronounced in the case of gG2. gG is expressed early during the viral cycle and, in the case of gG2, a domain is rapidly secreted (in the case of SgG2) into the extracellular medium, suggesting the implication of gG in immune modulation. The function of gG during HSV infection is, however, the less well characterized among all viral glycoproteins. Moreover, whether the sequence and structural differences between gG1 and gG2 have any functional consequence for HSV replication is currently unknown.

The fact that gG only exists in Alphaherpesviruses suggests that this glycoprotein could play a role in the biological properties that characterize these subfamily members. gG encoded by non-human Alphaherpesviruses has been proven to be relevant for viral pathogenesis and immune modulation. Although the exact function of gG during HSV infection is currently unknown, three independent reports have shown that lack of gG expression in HSV-1 leads to different degrees of virus attenuation. Importantly, all studies showed defects in viral replication in the CNS and a delayed induction of neurological symptoms, supporting the involvement of HSV gG in viral pathogenesis in the nervous system of mice. In addition, gG1 has been implicated in the entry, but not in the initial attachment, to polarized epithelial cells. Although not assessed, chemokines bound to gG1 in that context could contribute to shape the polarization of the cell. These observations suggest that gG could also play a role in neuron infection, since neurons are prototypical examples of highly polarized cells. The lower pathogenesis of gG1 mutant viruses could be explained by the dysregulation of the chemokine system, a function that we have recently attributed to HSV gG. There are currently no data on the role of HSV-2 gG on pathogenesis, since a gG2 deficient mutant has not been generated yet.

2. THE INTERACTION OF HSV PARTICLES WITH CHEMOKINES MAY PROVIDE AN ADVANTAGE TO THE VIRUS

2.1. HSV particles are covered with chemokines

As mentioned earlier, gG1 is present as a membrane-anchored protein at the surface of infected cells and in the envelope of HSV-1 particles; gG2, in turn, is processed by cellular proteases giving rise to two forms of gG2, the membrane-anchored mgG2 and the extracellular SgG2. We have previously demonstrated that both, gG1 present at the surface of infected cells and SgG2 secreted to the extracellular medium of HSV-infected cells bind chemokines. However, there are no reports on the role of gG present in the viral envelope. As part of the work performed during this Thesis, we have adapted the SPR technology to analyze the interaction between soluble chemokines and whole viral particles that were immobilized on a sensor chip using a BIACore biosensor. We have described for the first time the capacity of HSV-1 virions to interact with human chemokines with high affinity. Moreover, we have identified gG as the
main viral envelope glycoprotein involved is such interaction, as demonstrated by the lack of binding to a HSV-1 ΔgG mutant virus. Unexpectedly, we detected chemokine binding to HSV-2 virions, which theoretically should not contain the chemokine binding domain present in SgG. A monoclonal antibody directed against SgG2 detected the presence of a ~100 kDa band in purified virions, that may correspond to a precursor, full-length form of gG2 resulting from an incomplete proteolytical processing that remains at the viral particle, conferring the virion the capacity to bind chemokines (Fig. 1-7). Nonetheless, the SgG2-specific antibody detected large amounts of SgG2 that remained associated to the viral particle, probably anchored through the interaction with GAGs expressed at the virus envelope, and accounting for a great part of the chemokine binding activity detected. The functional consequences of SgG2 stably association to the viral envelope are unknown, but they could be of great importance during the initial steps of the infection, and therefore deserve further investigation. Interestingly, the same ~100kDa gG2 species was detected by the SgG2-specific antibody in the plasma membrane of infected cells (not shown) suggesting that the precursor form of gG2 may bind chemokines in the infected cells, similarly to gG1.

The possibility that mgG2, lacking the protein domain that is cleaved and constitutes SgG2, may also contribute to chemokine binding is unlikely but cannot be completely ruled out, although there are no data assessing such possibility yet. The importance of mgG2 for the viral infection is highlighted by the fact that mgG2-negative isolates are hardly detected in clinical settings. mgG2 has also been implicated in cell attachment through GAG-binding and it constitutes a promising candidate for HSV-2 vaccination, since mgG2-immunized mice present significant lower titres in vagina and spinal cord. The generation of an HSV-2 mutant devoid of SgG2 or mgG2 are needed to unequivocally understand the importance of each protein form for viral pathogenesis.

The utilization of SPR to analyze virus-chemokine interactions allowed us to calculate the binding affinities for the interaction between each of the human chemokines and the viruses. HSV bound chemokines with high affinities that were in the nanomolar range in all cases. The affinities obtained for the different chemokines correlated with those previously measured for recombinant secreted gG with several exceptions (Table R-1). Such disparity can be explained by the presence of other components within the HSV envelope that may modify chemokine binding to the virion-anchored gG. HSV envelope is acquired from the membrane of the infected cell during the egress of viral progeny, and thus it displays a complex composition including GAGs that may tune the binding of the chemokine to the viral particle and thus modify the affinity values. Moreover, the native conformation of the viral protein located at the virion may differ from that of the soluble, recombinant protein. Therefore, rather than absolute affinities, the affinities calculated can be understood as relative binding measurements. The relative affinities calculated for the binding to HSV-2 were higher than those measured for HSV-1, consistent with our previous results for gG2 and gG1.

Although a comprehensive analysis of
chemokines expressed during and HSV infection and their functions is lacking, data from several groups support the relevance of chemokines, including some of those bound by secreted and virion-anchored gG on HSV pathogenesis. Our observation that virions strongly interact with chemokines further supports that notion. As discussed earlier, many chemokines are upregulated upon HSV infection and play a role in the tissues where viral replication and spread takes place, including the CNS. Such upregulation may be as pathogenic as viral infection. The fact that the viral particles bind with high affinity to 12 out of 45 human chemokines underscores the necessity of the virus to interfere with a particular subset of chemokines, suggesting the existence of a selective and specific modulation of the immune response mediated by HSV.

2.2 HSV virions synergize with chemokine functions

The only gG previously reported to bind chemokines in its virion anchored-form is FeHV-1 gG. Although it was proposed that chemokine-virion interaction could play a role in the virus attachment to cells, no function was ascribed for such binding by means of in vitro assays. Nevertheless, the involvement of the interaction during an infection by FeHV-1 or HSV may be completely different given the opposite functionality of both vCKBPs. The relevance of chemokine interaction with HSV particles was assessed. We were not able to detect a significant effect for chemokine binding to HSV-1 on the virus entry into cells. During an infection, HSV first encounters the apical membranes of epithelial or mucosal surfaces. These cells are highly polarized, presenting a protein composition that strongly differs between the apical and basolateral membranes, so that different viral entry pathways have evolved for each surface. gG1 has been, in fact, implicated in the viral infection of polarized epithelium through apical cell surfaces. Interestingly, a gG1-deficient virus was able to infect cells only through their basal membranes and to spread into surrounding cells. The gG-dependent step in apical entry occurred after attachment, suggesting that the ability of gG to bind GAGs described in this thesis is not sufficient to account for the effect on polarized cell infection. A different experimental approach must be set up in order to ascertain the importance of chemokine interaction for the early stages of viral entry and/or replication.

The interaction between chemokines and HSV virions could play a role in other aspects of the viral infection. Indeed, we have shown that HSV particles are slightly chemotactic on their own. More importantly, HSV virions strongly increased the migration of cells towards chemokines. Such increase on chemotaxis was independent of the high affinity binding of chemokines to the viral particles, since it was observed for CXCL12 and CCL2, the latter not a ligand of HSV-1 according to our Biacore data. Besides, wt HSV-1 promoted CXCL12-mediated migration further than its gG-deficient counterpart, indicating that viral envelope gG does contribute to the modulation of chemokine function. These results suggest the existence of nonlinear virus- and chemokine-induced signalling pathways that converge in a synergistic-fashion triggering cell activation and leading to an enhanced cell recruitment to the infection.
site. These attracted cells could constitute the target for HSV infection facilitating virus spread. The characterization of this mechanism requires further investigations.

3. INSIGHTS INTO HOW SgG TURNS ON THE CHEMOKINE RECEPTORS

The main focus of this Thesis was to decipher the molecular basis beneath HSV gG function, that is, the enhancement of chemokine-dependent signalling and migration. The insights provided are needed for a comprehensive understanding of HSV pathogenesis, and they also shed light into basic concepts of the chemokine system functionality.

3.1 SgG modifies chemokine receptor trafficking

We have shown that gG interaction with cells triggers an upregulation of chemokine receptor levels at the surface, including the GPCRs CXCR4, CXCR5, CXCR7 and CCR2. Focusing on the axis CXCR4/CXCL12, we found that SgG2 modulates CXCR4 trafficking leading to an increase in surface CXCR4 and to a delayed CXCL12-induced receptor internalization in all cell types assayed: freshly isolated human monocytes and the cell lines, including different leukocytes types and HEK-293T cells. Moreover, SgG2 upregulated the expression of CXCR4 at the surface of primary neurons, both alone or upon chemokine stimulation, suggesting that the regulation of chemokine receptor function could go beyond immune function. Monocytes, one the immune cell types primarily involved in the anti HSV response, appear to be more prone than other cells to the alterations in chemokine receptor trafficking mediated by gG. Although intracellular chemokine receptor expression has been detected in nearly all primary monocytes in the literature, the amount of receptor at the surface is quite variable and strongly depends on the cytokine environment. Since the expression of CXCR4 was typically measured during short periods of time (<60min) in all cell types, a change in the mRNA levels of the receptor is very unlikely. Rather, the increase of surface receptor levels by SgG argues for the mobilization of pre-existing intracellular CXCR4 pools, the blockade in the constitutive internalization of the receptor, for an increase in recycling processes or all of the above.

The correlation between an improved chemotactic response and an augmented presence of surface CXCR4 has been previously stated by other studies. WHIM (for warts, hypogammaglobulinemia, infections and myelokathexis syndrome) receptor, whose C-terminus is truncated, shows refractoriness to desensitization upon CXCL12 stimulation that associates to the retention of neutrophils in bone marrow reducing their peripheral circulation. Not only genetic defects, but also several physiological situations have been involved in an increased CXCR4 function. For instance, anaphylatoxin C3a increased CXCL12-mediated chemotaxis and adhesion to VCAM-1 by an undetermined mechanism. L-selectin stimulation with specific ligands during leukocyte extravasation blocked CXCL12-induced internalization of CXCR4, leading to a higher adhesion and transendothelial migration of lymphocytes. cAMP and cAMP-inducing
Indeed, the heterodimerization of CXCR7 and CXCR4 at the surface of HEK-293T cells has been related to an enhanced responsiveness towards CXCL12 gradients. Although we have not proven it, the increased presence of both receptors at the surface upon SgG2 stimulation might be associated with heterodimerization events that would contribute to the impact on receptor functionality observed. Since emerging data indicate that CXCR7 is an active-signalling β-arrestin-biased receptor, the observation that β-arrestin looses contact with CXCR4 at the surface might also explain in part the modulation of CXCR7 function mediated by SgG2. A more detailed analysis of CXCR7 trafficking dynamics and surface localization is needed to better understand SgG-modulation of CXCR7/CXCR4/CXCL12 function.

Surprisingly, the delayed internalization of CCR2 in the presence of SgG2 does not correlate with an increased migration towards the cognate chemokine, CCL2 (not a ligand of SgG), whereas the effect on other chemokine receptors whose ligands are bound by gG (CXCR4, CXCR5) does. Interestingly, these observations suggest that, besides the stabilization of receptors on the cell surface, the chemokine needs to be presented to its receptor bound to SgG in order for the enhancement of migration to occur.

3.2 HSV gG behaves as a viral GAG

GAG binding to chemokines goes beyond cell-surface retention, regulating aspects such as chemokine oligomerization, processing or even receptor specificity. In fact, differential receptor recognition
by free or GAG-bound chemokines has been reported\(^6^9\). HSV gG interaction with chemokines may give rise to novel structural rearrangements in the complex, in comparison with those triggered by non-human Alphaherpesviruses gGs. The resolution of the spatial structure of SgG will provide for the first time an explanation for the mechanism of chemokine function potentiation from a molecular point of view. We propose a model in which HSV SgG acts similarly to GAGs. The SgG:CXCL12 complex may alter the quaternary structure of the chemokine, increasing the local concentration in the proximity of the cognate GPCR. Alternatively, SgG could rearrange the chemokine, maybe bringing two molecules together that would be presented with an optimal spatial conformation to CXCR4 dimers, eventually increasing the effective activity of CXCL12. A variety of chemokines and inflammatory molecules are concomitantly produced at the site of infection to regulate inflammation. Chemokines, including some of those bound by gG, form heterotrimeric complexes consisting of agonistic and synergy-inducing molecules that show enhanced chemotactic-triggering abilities compared to concentrations of the individual chemokines that would be inactive on their own\(^{278,279}\). Such chemokine synergistic-complexes, either by formation of heterocomplexes with other chemokines or by the interaction with other regulatory molecules\(^1^{14}\), seem to represent a novel amplification system in chemokine-rich tissues that sensitize immune cells to respond to threshold migratory stimuli. We propose that, in the complex environment of the inflammatory foci, SgG could behave as a synergy-inducing agent, leading to the formation of an SgG2:CXCL12 complex with an enhanced biological activity and chemotactic potency.

Chemokine receptor sustainment on the surface was more pronounced when the cells contacted SgG prior to chemokine stimulation. A physiological situation where SgG interacts with the cell before the chemokine may be plausible: SgG2 would immediately bind to the surface of the infected cell, or neighbouring cells, promoting the redistribution of chemosensory receptors and therefore modifying the subsequent interaction with chemokines enriched in the infected mucosa. We have indeed observed the presence of significant amounts of SgG2 bound to the surface of HSV-2-infected cells (not shown). This observation is in agreement with our data showing that SgG reorganizes the receptors at the plasma membrane altering their interaction with the intracellular machinery and probably with their ligands. Nonetheless, SgG2-mediated partitioning and stabilization of chemokine receptors at the surface is not sufficient to enhance migration, supporting our proposed model, according to which the chemokine must be presented to its receptor by SgG acting as a viral GAG. The synchrony of both SgG-triggered effects, at the chemokine and at the cellular level, finally results in the functional optimization of cell responses that render immune cells more competent to efficiently respond to chemokine cues.
3.3 Remodelling of plasma membrane microdomains by HSV SgG

The fact that unrelated receptors are subjected to SgG2-regulation of cell trafficking, suggests that the viral protein may affect the overall organization of the plasma membrane microdomains, which is distributed in lipid rafts and non-raft domains. SgG2 promoted, rather than decreased, the internalization of the non-chemokine related GPCR β2AR whose basal association to lipid rafts decreases upon agonist stimulation280, 281. On the other hand, SgG2 did not significantly modify the kinetics of the transferrin receptor, a receptor with very low affinity for cholesterol that is commonly found outside lipid rafts84. GPCRs are seven-spanning integral membrane proteins, and therefore it is conceivable that they are highly dependent on structural and functional regulation by lipid and lipid-associated components of the plasma membrane. In fact, cholesterol has been demonstrated to modulate GPCR function either by a direct interaction with the receptor that is associated to conformational changes254, 282, 283, by altering the plasma membrane environment in which the GPCR is embedded or by a combination of both284-286. Such cholesterol-mediated regulation seems to be receptor-specific, and therefore, the fate of different types of receptors after SgG2 activation of cells would depend on the affinity and grade of dependency for cholesterol and related elements enriched in lipid rafts. The exact regulation exerted by cholesterol in the oligomerization and activity of chemokine receptors is not well understood. Many chemokine receptors reside mostly outside rafts and establish transitory interactions with raft components that are stabilized upon stimulation with their agonists, indicating that rafts importantly tune the activation of chemokine receptors89, 91, 287. A number of studies have shown that CXCR4 incorporation into and interaction with lipid raft components improve the signalling properties of the receptor. The inclusion of cholesterol derivatives increases binding of CXCL12 to CXCR490, 288. Products released during inflammatory responses and platelet activation sensitizes hematopoietic stem/progenitor cells to migrate to threshold concentrations of CXCL1294, 289. The ability of progenitor B cells to respond to CXCL12 gradients depends on the segregation of FAK, Src kinases and PI3K to lipid rafts; the lack of such reorganization of signalling elements in mature B cells is associated with the absence of response to CXCL12 in these cells93, 94. Disruption of raft formation by cholesterol sequestering drugs decreases HIV replication in vitro, and indicates that CXCL12 preferentially binds to lipid raft-associated CXCR4, likely due to cholesterol-mediated conformational changes in the receptor90, 287. Although non-specific effects of cholesterol-depleting drugs cannot be completely ruled out, altogether these results point towards a role for membrane cholesterol content and raft association in the regulation of CXCR4 function. Cholesterol regulates different aspects of CXCR4 function, either by promoting more active conformations of the receptor, or by creating a platform for the association with a specific set of signalling proteins.

Here, we present evidence that SgG2 restructures the organization of plasma membrane microdomains and induces the focal clustering of lipid rafts. Moreover,
we show that SgG2 targets CXCR4 to GM3 rafts or L-rafts, a subtype of lipid rafts that are found at the front of migrating leukocytes, while no such increase in CXCR4 incorporation to GM1, known as uropod rafts, was observed. SgG2-induced redistribution of CXCR4 promotes receptor coupling to specific pathways contributing to the sensitization of the leukocyte to threshold concentrations of chemokines that would otherwise be insufficient to trigger cell responsiveness. Upon SgG-stimulation, the chemokine receptor partition to lipid rafts optimizes gradient sensing, a process that would account for the faster and more directional movement observed in primary monocytes. Besides, the increased incorporation of CXCR4 into lipid rafts could impact CXCR4 trafficking and functions on at least two additional aspects. Caveolae and other rafts components are characterized by a very slow diffusion rate in the cell membrane, a property that contributes to the slower kinetics of the caveolae/lipid raft-dependent route of endocytosis, compared to the highly efficient clathrin-dependent pathway. Thus, an improved interaction with raft components could provide the basis for the delayed endocytosis observed in the presence of SgG. The internalization of a given receptor may preferentially switch to raft-dependent over clathrin-dependent (or vice versa) depending on the specific cellular setting. For instance, cholesterol sequestration by nystatin increases endostatin uptake in endothelial cells by shifting endostatin internalization to the clathrin-mediated pathway. Moreover, the segregation of TGF-β receptor to raft/caveolar or clathrin-enriched compartments regulates signal transduction and receptor turnover, respectively, highlighting the relevance of each endocytic route for receptor functionality. Concerning chemokine receptors, there are controversial reports on the relative degree of dependence for each pathway, which may be due in part to cell type differences in the utilization of lipid rafts vs. clathrin. A direct link between lipid raft-dependent endocytosis and CXCR4 internalization has not been reported yet. However, we have observed the formation of vesicles enriched in lipid rafts and CXCR4 in cells stimulated with the complex SgG2:CXCL12, suggesting the association of CXCR4 with lipid raft-mediated transport processes. In addition, the recycling rate of receptors could be also modulated. Recycling endosomes are highly enriched in lipid rafts components, suggesting that the presence of the receptor in lipid-rafts enriched vesicles could correlate with an increased recycling.

3.4 SgG2-induced partitioning of CXCR4 triggers a conformational rearrangement that is transmitted to the cytoplasmic tail of the receptor

Several groups have reported the constitutive formation of CXCR4 homodimers by different techniques, including direct evidence obtained by X-ray crystallography. There is evidence indicating that chemokine engagement drives conformational changes in pre-established homodimers, rather than inducing the formation of new dimers. By using BRET technique, we have observed that SgG stimulation of cells triggers different conformations.
in CXCR4 homodimers compared to non-stimulated or CXCL12-treated cells. Such conformational reorganization may be in part related to the differential recruitment of CXCR4 to lipid rafts, which is very likely associated to spatial rearrangements of transmembrane helices due to increased association with cholesterol moieties. Modifications on the receptor C-tail are beneath differences in receptor localization on the membrane and initial interaction with a variety of signalling molecules, importantly contributing to determine receptor fate\cite{253}. In fact, a chimeric CXCR4 bearing the C-tail of CCR5 switched its trafficking fate towards that of CCR5, thus showing an increased association with rafts and a slower internalization rate upon CXCL12 exposure\cite{88}. We propose that the rearrangements in receptor dimers upon SgG2-induced relocalization to cholesterol-rich surface microdomains is transmitted to the cytosolic part of the GPCR, modifying the receptor’s C-tail flexibility and leading to a differential recruitment of signalling molecules. In fact, we have shown that CXCR4 “phosphorylation code” is altered, an observation that could explain in part the diminished recruitment of β-arrestin2 to the receptor\cite{135,291}. The impairment of β-arrestin2 interaction with the receptor greatly abrogates the interaction with the canonical clathrin-mediated endocytic machinery, importantly contributing to the overall effect on internalization observed. In addition, a less stable interaction of GPCRs with β-arrestins has been associated with higher recycling rates\cite{135,292}, again supporting the idea that recycling might be increased upon SgG stimulation. In accordance with our results, others have shown that CXCR4 changes its G-protein coupling specificity when the receptor localizes to the T cell immune synapse\cite{10,293}, an structure characterized by the formation of clusters of rafts of several micrometers in size, similarly to the structures induced by SgG.

## 4. The Formation of Receptor Oligomers at the Surface Is Beneath the Enhancement of Chemokine Function

Although the oligomerization of some chemokines has been proven important for receptor activation\cite{61,99}, whether chemokine receptors are active as monomers, dimers or higher order oligomers remains poorly characterized\cite{98}. Further investigations are needed to define the relevance and functional implications of different chemokine:receptor stoichiometries and structures. Current CXCR4 structures are compatible with emerging concepts of signalling diversity induced by alternative binding modes of the chemokine. It seems that the non-stimulated receptors exist in an equilibrium of inactive and active conformations, which is shifted towards specific conformations depending on the ligand triggering the response\cite{98,103,113,294}.

Given that the SgG-mediated increase of chemokine function was associated with altered receptor functionality and enhanced presence at the surface, cell surface replicas were prepared to analyze the clusterization of CXCR4 by EM. The distribution of CXCR4 significantly shifted towards the formation of small CXCR4 oligomers at the surface of SgG-stimulated cells. The inefficiency of the gold technique procedure used does not allow to determine the absolute size of
CXCR4 oligomers. Thus, we cannot conclude that the preponderance of CXCR4 clusters unequivocally means that the receptor forms monomers, dimers, trimers and so on. We can, however, provide relevant data in a comparative manner. We show that CXCR4 distribution in small clusters significantly increases in SgG2-stimulated cells compared to cells that harbour a steady-state receptor. Importantly, a similar receptor rearrangement occurs upon stimulation with the heterocomplex SgG2:CXCL12 in comparison to CXCL12-treated cells. Similar results were obtained for HEK-293T cells stably expressing CXCR4. However, it is interesting to note that Jurkat cells formed larger clusters of CXCR4 as compared to HEK-293T cells in response to SgG, an observation that could be related to the natural predisposition of the T cell to migrate.

The formation of trimers of CXCR4 in the absence of chemokine has been previously demonstrated. However, although the authors hypothesized that such distribution served to improve chemotactic gradient sensing, no functional assays were performed. We have, on the contrary, clearly demonstrated that SgG increases CXCL12-mediated chemotaxis and signalling, as readouts for an enhanced function of the CXCR4/CXCL12 pair. Chemokine receptors, including CXCR4, form heterodimers with other chemokine receptors. Such heterodimers carry out different functions in comparison to the ones exerted by each chemokine receptor forming the complex. Although we have not addressed that issue during this work, it is tempting to speculate that SgG2-induced CXCR4 oligomers are accompanied by other chemokine receptors, and that in such clusters a novel form of receptor crosstalk occurs, modulating cell responsiveness that would eventually benefit viral infection. Such possibility is supported by the fact that SgG modulates the function and trafficking of different chemokine-receptor pairs, so that a simultaneous effect over several receptors is likely to take place during infection.

Our proposal is that SgG2 induces clustering of lipid rafts, promoting the segregation of GPCRs to these microdomains. The driving forces for such inclusion into rafts might be mediated by the interaction between cholesterol and the GPCR domains. Differences in the intrinsic avidity of each receptor to interact with lipids would account for a differential recruitment to rafts. Consistent with previous evidences published, we did not observe a significant increase in the oligomerization of the receptor after stimulation with low doses of chemokine. In our experiments, CXCL12 neither triggered raft clustering nor inclusion of CXCR4 into these microdomains, whereas SgG did. These observations support the idea that SgG-induced imbalance of the plasma membrane architecture is beneath the conformational rearrangements of GPCRs, predisposing the receptors to establish a differential and/or more efficient interaction with the signalling machinery leading to increased cell responsiveness to threshold chemotactic gradients.

The role of gG during a viral infection is not well characterized. We can, however, hypothesize diverse situations where gG could impact viral pathogenesis in the light of our former and current findings. Enhancement of chemokine function
by SgG could impact several scenarios relevant for HSV spread and pathogenesis. The observation that HSV viral particles bearing gG bind chemokines contributing to the increase of chemoattractiveness, suggests that gG could help in the initial attachment and entry into chemokine receptor-expressing host cells. Furthermore, membrane remodelling by gG may serve as a mechanism to increase the availability of HSV entry receptors. In addition, it is likely that the positive effect of HSV gG on chemokine and receptor function facilitates HIV infection by enhancing the level of viral coreceptors on the surface of target cells. The increase in GPCR signalling could aid viral replication; in fact MAPK activation is required for efficient HSV replication. gG could increase the level of infiltrating leukocytes in the mucocutaneous zone, and maybe the composition of such infiltrate skewing the immune response to favour viral replication and immune evasion. Moreover, leukocytes recruited to the site of infection could be infected by the virus, facilitating viral spread and increasing viral loads. The recruited leukocytes could in turn favour a particular cytokine environment at the site of infection, further promoting gG-mediated effects or altering the functionality of other immune cell types, such as NK or T cells.

Viral immunology is a dynamic area of research, and it will certainly uncover undiscovered mechanisms of viral pathogenesis and immune modulation, leading to a comprehensive understanding of our immune system. Our findings reveal the molecular basis beneath a novel process of immune modulation exerted by a glycoprotein whose function was unknown.
Conclusions
1. HSV SgGs attach to the cell surface through a high affinity interaction with GAGs.

2. SgGs modify chemokine receptor trafficking and prevent chemokine-induced internalization, promoting higher levels of receptor signalling. The impact of SgG on receptor fate is not restricted to chemokine receptors, but it differentially affects other GPCRs.

3. SgG alters the lipid raft content and distribution of the plasma membrane, causes raft clustering and triggers the incorporation of GPCRs into specific microdomains. As a result, chemokine receptors are distributed in a more polarized fashion at the surface, making the cell more prone to sense chemotactic gradients.

4. SgG2 optimizes cell responsiveness leading to a faster and more directional cell movement towards the chemokine.

5. SgG2-mediated redistribution of receptors at the surface is associated with conformational rearrangements in CXCR4 dimers. In addition, SgG promotes alterations in the endocytic machinery associated to the receptor.

6. Interaction of SgG2 with the cell membrane promotes the reorganization of surface CXCR4 into small-size oligomers of receptors.

7. HSV viral particles bind to chemokines through gG present at the viral envelope. HSV virions enhance cell migration in a synergical manner with chemokines, and virion-anchored gG contributes to the chemotaxis enhancement.
Conclusions

1. La SgG del virus herpes simplex (HSV) se une a la membrana plasmática a través de una unión de alta afinidad con los glicosaminoglicanos.

2. La SgG modifica el tráfico de los receptores de quimiocinas y evita los procesos de internalización inducidos por éstas, promoviendo un incremento en las vías de señalización asociadas a estos receptores. El efecto de gG no está restringido a receptores de quimiocinas, sino que afecta a otros receptores acoplados a proteína G.

3. La SgG altera la disposición de las balsas lipídicas en la membrana plasmática, causando el agrupamiento de éstas y aumentado la incorporación de receptores acoplados a proteína G en microdominios específicos. Como resultado, los receptores de quimiocinas se distribuyen de una manera más polarizada en la superficie, incrementando la capacidad de la célula para sentir el gradiente quimiotáctico.

4. SgG optimiza la respuesta celular, de modo que la migración es más rápida y direccional.

5. SgG2 promueve una reorganización estructural en los dímeros de CXCR4 estabilizados en superficie y modifica la maquinaria endocítica asociada al receptor.

6. La interacción de SgG2 con la membrana celular promueve la reorganización de CXCR4 en oligómeros de pequeño tamaño en la superficie celular.

7. Las partículas virales de HSV interaccionan con quimiocinas a través de la gG presente en la envuelta viral. Los viriones de HSV incrementan la migración celular mediante una acción sinérgica con las quimiocinas que depende parcialmente de gG.
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Enhancement of Chemokine Function as an Immunomodulatory Strategy Employed by Human Herpesviruses

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Abstract

Herpes simplex virus (HSV) types 1 and 2 are highly prevalent human neurotropic pathogens that cause a variety of diseases, including lethal encephalitis. The relationship between HSV and the host immune system is one of the main determinants of the infection outcome. Chemokines play relevant roles in antiviral response and immunopathology, but the modulation of chemokine function by HSV is not well understood. We have addressed the modulation of chemokine function mediated by HSV. By using surface plasmon resonance and crosslinking assays, we show that secreted glycoprotein G (SgG) from both HSV-1 and HSV-2 binds chemokines with high affinity. Chemokine binding activity was also observed in the supernatant of HSV-2 infected cells and in the plasma membrane of cells infected with HSV-1 wild type but not with a gG deficient HSV-1 mutant. Cell-binding and competition experiments indicate that the interaction takes place through the glycosaminoglycan-binding domain of the chemokine. The functional relevance of the interaction was determined both in vitro, by performing transwell assays, time-lapse microscopy, and signal transduction experiments; and in vivo, using the air pouch model of inflammation. Interestingly, and in contrast to what has been observed for previously described viral chemokine binding proteins, HSV SgGs do not inhibit chemokine function. On the contrary, HSV SgGs enhance chemotaxis both in vitro and in vivo through increasing directionality, potency and receptor signaling. This is the first report, to our knowledge, of a viral chemokine binding protein from a human pathogen that increases chemokine function and points towards a previously undescribed strategy of immune modulation mediated by viruses.

Introduction

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2, respectively) and varicella zoster virus (VZV) are the three human members of the Alphaherpesvirinae subfamily, which establish latency in the sensory ganglia of the peripheral nervous system. Both HSV-1 and -2 are highly prevalent viruses with values around 90% for HSV-1 and 12–20% for HSV-2 in adult populations of industrialized countries, reaching up to 80% for HSV-2 in developing countries [1,2]. Infection by HSV can be either asymptomatic, show mild symptoms in localized tissues or cause severe diseases such as stromal keratitis or herpes simplex encephalitis (HSE), with high mortality and neurologic morbidity [3]. HSV infection of neonates can result in disseminated disease including infection of the central nervous system or involve several organs with mortality reaching 80% [4]. The causes of such different outcomes following HSV infection or reactivation are unknown but involve the interplay between the virus and the immune response.

Chemokines are essential elements of the antiviral response. They constitute a family of chemotactic cytokines that orchestrate leukocyte migration to sites of injury or infection [5]. Chemokines also play relevant roles in the developing and mature nervous system [6]. The chemokine network contains more than 45 chemokines and around 20 G-protein coupled receptors (GPCR). There are 4 subfamilies of chemokines classified on C, CC, CXC and CXC. All chemokines are secreted. CXCL16 and CX3CL1 are also present as membrane-anchored forms. The chemokine network is complex, highly regulated and promiscuous, with some receptors interacting with more than one chemokine and some chemokines binding to more than one receptor. Alterations in the chemokine network are responsible for inflammatory, autoimmune diseases and the establishment of chronic pain [7,8]. Binding of chemokine to glycosaminoglycans (GAGs) is relevant
HSV Glycoprotein G Potentiates Chemokine Function

Author Summary

Chemokines are chemotactic cytokines that direct the flux of leukocytes to the site of injury and infection, playing a relevant role in the antiviral response. An uncontrolled, unorganized chemokine response is beneath the onset and maintenance of several immunopathologies. During millions of years of evolution, viruses have developed strategies to modulate the host immune system. One of such strategies consists on the secretion of viral proteins that bind to and inhibit the function of chemokines. However, the modulation of the chemokine network mediated by the highly prevalent human pathogen herpes simplex virus (HSV) is unknown. We have addressed this issue and show that HSV-1, causing cold sores and encephalitis and HSV-2, causing urogenital tract infections, interact with chemokines. We determined that the viral protein responsible for such activity is glycoprotein G (gG). gG binds chemokines with high affinity and, in contrast to all viral chemokine binding proteins described to date that inhibit chemokine function, we found that HSV gG potentiates chemokine function in vitro and in vivo. The implications of such potentiation in HSV viral cycle, pathogenesis and chemokine function are discussed.

for chemokine function. GAGs promote chemokine oligomerization, mediate retention of chemokines onto the cell surface allowing chemokine recruitment in tissues, increase their local concentration in the microenvironment surrounding the GPCR, and modulate receptor recognition [9]. Interaction of the chemokine with the GPCR triggers a signal cascade that includes stimulation of mitogen activated protein kinases (MAPKs) such as Janus-N-terminal kinase 1 and 2 (JNK1-2), extracellular signal-regulated kinase 1-2 (ERK1/2) and p38 [10]. The proper function of chemokines is essential to trigger an appropriate and effective antiviral response. An exacerbated immune response, often triggered or maintained by chemokines, may lead to immunopathology. Patients suffering from HSE present higher level of chemokine expression in the cerebrospinal fluid than healthy individuals suggesting a relevant role for chemokines in the pathogenesis of HSE [11].

Both pox- and herpesviruses express proteins that interfere with chemokine function playing relevant roles in viral cycle, immune evasion and pathogenesis [12]. One of the strategies of chemokine interference involves the expression of secreted viral proteins that bind chemokines and inhibit chemokine function [13]. These proteins have been termed viral chemokine binding proteins (vCKBP). They lack amino acid sequence similarities among themselves or with host chemokine receptors, making difficult the detection of such proteins by sequence analysis.

We, and others, have previously shown that secreted glycoprotein G (gG) from non-human alphaherpesviruses binds to chemokines and inhibits chemokine function. Examples of such viruses are bovine herpesvirus 5 (BHV-5), equine herpesvirus 1 and 3 (EHV-1 and EHV-3) [14,15], pseudorabies virus (PRV) [16] and infectious laryngotracheitis virus [17]. Chemokine-binding activity was not observed when supernatants of cells infected with the human viruses VZV, HSV-1 and HSV-2 were tested using different radio-labeled chemokines [14]. In the case of VZV, the gene encoding for gG is not present within its genome. However, both HSV-1 and HSV-2 contain the open reading frame us4 encoding gG. HSV-1 and HSV-2 gG (gG1 and gG2, respectively) are present on the viral particle and on the plasma membrane of infected cells [18–20]. gG2 is further processed and an N-terminal fragment is secreted to the medium of the infected cells [19,20]. On the contrary, gG1 is not secreted, similarly to the rest of HSV glycoproteins. The functions of HSV-1 and HSV-2 gGs are not well understood. Two reports point to a role of the HSV gGs in the initial steps of entry. HSV-1 gG seems to be important for the infection of polarized epithelial cells [21]. The non-secreted portion of HSV-2 gG binds heparin and the cellular plasma membrane [22]. Deletion or disruption of us4 attenuates HSV-1 in vitro, indicating that gG is a virulence factor, although the mechanism(s) behind such phenotype are unknown [23–25].

The main aim of this study was to investigate the modulation of the immune system by HSV. We focused initially on identifying the function of HSV gG and its possible interaction with chemokines. We show here that secreted, soluble HSV gG (SgG) binds both CC and CXC chemokines with high affinity through the GAG-binding domain of the chemokine. Moreover, we could detect chemokine-binding activity in the plasma membrane of HSV-1 infected cells and in the supernatant of HSV-2 infected cells. Further experiments indicate that HSV-1 full-length gG and secreted, soluble HSV gG (SgG) are responsible for this activity. In complete contrast to all previously described vCKBPs, HSV-1 and HSV-2 SgGs are not inhibitors of chemokine function. Instead, they increase chemokine-mediated cell migration both in vitro and in vivo through a mechanism that involves GPCR signaling and phosphorylation of MAPKs. HSV SgGs increase the potency of the chemokine, and the directionality of cell movement. This constitutes, to our knowledge, the first description of a chemokine binding protein expressed by a human pathogen that potentiates chemokine function. The data presented here suggest the existence of a novel viral mechanism of immune modulation and provide tools to investigate the pathways controlling chemotaxis. Given the relevant roles played by chemokines in both the immune and nervous systems, enhancement of chemokine function by HSV gG may be important for HSV-mediated immunopathogenesis.

Results

Recombinant SgG from HSV-1 and HSV-2 binds CC and CXC chemokines with high affinity

To test whether HSV gGs bind chemokines, we expressed soluble, secreted forms of gG1 and gG2 (SgG1 and SgG2, respectively), lacking the transmembrane and cytoplasmic domains, in insect cells infected with recombinant baculovirus vectors (Figure 1A; Protocol S1; Text S1). Following infection, SgG1 and SgG2 were purified from the supernatant of Hi-5 insect cell cultures by affinity chromatography and the purity of the preparation was determined by Coomassie staining (Figure 1B). We routinely obtained two separate bands when SgG1 was expressed in insect cells, probably due to different levels of SgG1 glycosylation. A monoclonal antibody raised against gG1 [18] reacted with purified SgG1 but not SgG2 (Figure 1C, left panel) whereas a monoclonal anti-SgG2 [26] recognized SgG2 only (Figure 1C, right panel). The anti-His antibody reacted with both proteins (Figure 1C, left panel).

Both purified SgG1 and SgG2 were covalently coupled to BiAcore CM5 chips and tested for chemokine binding by Surface Plasmon Resonance (SPR). A screening with 44 commercially available human (h) chemokines (Protocol S2) was performed by injecting each chemokine in a BiAcore X biosensor. Both SgG1 and SgG2 bound with high affinity hCCL18, hCCL25, hCCL26, hCCL28, hCXCL10, hCXCL11, hCXCL12a, hCXCL12b, hCXCL13 and hCXCL14, and SgG2 also bound hCCL22 with high affinity (Figure 2A and Table 1). As negative controls for chemokine binding we used the cysteine-
rich domain (CRD) of ectromelia virus cytokine response modifier B (CrmB), previously shown to lack chemokine-binding activity [27] (not shown). The affinity constants of the interactions between SgG1, SgG2 and the different chemokines were calculated using the SPR technology (Table 1). Both SgG1 and SgG2 interacted with chemokines with high affinity, in the nanomolar range. The interaction between HSV SgGs and chemokines was also observed by cross-linking assays (Protocol S3; Text S1) using radio-iodinated recombinant hCCL25, hCXCL10, and hCXCL12 (Figure 2B–D). As a negative control we employed CrmB-CRD (Figure 2C). Competition assays with \[^{125}\text{I}\]-hCXCL12 and increasing concentrations of cold hCXCL12 showed the specificity of SgG2-chemokine interaction (Figure 2D).

Chemokine binding activity is present in HSV-infected cells

We addressed whether chemokine-binding activity was present in the HSV-1 infected cells. To this end we infected BHK-21 cells (Protocol S4 and S5; Text S1) with HSV-1 wt and an HSV-1 virus where expression of gG had been disrupted by the insertion of the \(\beta\)-galactosidase gene [23] and determined binding of \[^{125}\text{I}\]-hCXCL12 to the cells 14 to 16 hours post infection (h.p.i.). We could detect chemokine binding to HSV-1 wt-infected cells (Figure 3A; Protocol S5). Binding was not observed when the deletion mutant HSV-1\(\Delta gG\) was used. We also obtained supernatants from mock- or HSV-2 infected Vero cells 36 h.p.i., and performed a crosslinking assay with \[^{125}\text{I}\]-hCXCL12. Two bands could be detected in the crosslinking assay (Figure 3B) that could correspond to the high mannose 72 kDa precursor and the 34 kDa secreted protein produced during gG2 expression and processing [19,20]. Another possibility is that the higher molecular weight band observed corresponds to an SgG2 dimer complexed with chemokine.

Binding of SgG to chemokines takes place mainly through the heparin-binding domain of the chemokine

To function properly, chemokines need to interact with both GAGs and GPCRs. We investigated the chemokine domain involved in the interaction with HSV SgGs using two experimental approaches.

First, to address whether HSV SgGs could affect chemokine-receptor interaction, we performed binding assays of \[^{125}\text{I}\]-hCXCL12 and \[^{125}\text{I}\]-hCCL25 with MOLT-4 cells (Protocol S4 and S6) expressing endogenous hCXCR4 (the receptor for hCXCL12) and hCCR9 (the receptor for hCCL25) in the presence of SgG-containing supernatant (not shown). We also performed binding assays of \[^{125}\text{I}\]-hCXCL12 to MonoMac-1 cells expressing endogenous hCXCR4 (not shown). As a positive control, addition of supernatant containing BHV-5 SgG inhibited \[^{125}\text{I}\]-hCXCL12 binding to MOLT-4 cells [14] (not shown). However, similar amounts of SgG1 or SgG2 did not decrease \[^{125}\text{I}\]-hCXCL12 binding to MOLT-4 cells, MonoMac-1 cells or \[^{125}\text{I}\]-hCCL25 binding to MOLT-4 (not shown) compared to the mock sample. Thus, SgGs do not inhibit binding of the chemokines to their receptors.
Second, to determine the implication of the GAG-binding domain of the chemokine in the interaction with HSV SgGs we utilized the SPR technology. The amount of chemokine binding to SgGs, covalently bound to a BIAcore chip, in the absence of heparin was considered 100% of binding (Figure 4). Competition experiments showed that increasing concentrations of heparin impaired chemokine binding to both SgG1 and SgG2 in a significant manner (Figure 4). As a control, each of the different heparin concentrations used were injected independently to confirm that no direct heparin binding to the chip occurred (not shown).

In summary, these results indicate that SgG1 and SgG2 interact preferentially with the GAG-binding domain of the chemokine and do not block the binding of chemokines to cell surface specific receptors.

Interaction of HSV SgGs with chemokines enhances chemokine-mediated cell migration

We, and others, have previously shown that gG encoded by several non-human alphaherpesviruses inhibits chemotaxis [14–17,28]. To examine the functional role of the interaction between HSV SgGs and chemokines we performed cell migration experiments. First we addressed whether the chemokine-binding activity observed in the supernatant of HSV-2 infected cells could have any effect on chemotaxis. We incubated CXCL12 with supernatant from mock- or HSV-2-infected cells and performed a chemotactic assay with MonoMac-1 cells (monocyte-like), a cell line that expresses hCXCR4, the receptor for hCXCL12. The supernatant from HSV-2-infected cells significantly enhanced chemokine function in a dose dependent manner when compared to the supernatant from mock-infected cells (Figure 5A). To address whether this effect could be due to SgG, we performed chemotactic experiments using several cell lines and recombinant protein. Incubation of SgG1 with hCXCL12 resulted in higher MOLT-4 migration (Figure 5B). A similar result was obtained with SgG2 whereas BHV-5 SgG inhibited hCXCL12 migration (not shown). We then incubated SgG1 and SgG2 with hCXCL13 and tested their effect on mouse B cells (m300-19) stably transfected with hCXCR5, the receptor for hCXCL13 (Figure 5C, Protocol S4). Inhibition of migration was observed with the vCKBP M3, as expected [29,30] (Figure 5C). However, SgG1 and SgG2 required the presence of the chemokine and were not able to induce HSV Glycoprotein G Potentiates Chemokine Function
chemotaxis on their own (Figure 5C). The parental m300-19 cells, which do not express hCXCR5, did not respond to the hCXCL13 stimulus (not shown). To test whether binding to the chemokine was necessary for the enhancing effect, we performed chemotaxis experiments using MonoMac-1, a cell line expressing hCXCR4 and hCCR2, the receptor for hCCL2, a chemokine not bound by HSV SgGs (Figure 2 and Table 1). The enhancement in chemotaxis mediated by SgGs required SgG-chemokine interaction since SgG2 did not have any effect on the chemotactic properties of hCCL2 (Figure 5D), whereas it was able to potentiate hCXCL12. A similar result was obtained with SgG1 (not shown).

In all cases, the enhancement in chemotaxis was dose dependent and significant.

The effect of SgGs on chemotaxis was dependent on G protein activation since addition of pertussis toxin (PTX) inhibited both hCXCL12-mediated cell migration and its enhancement mediated by SgGs (Figure 5E). Finally, we examined the effect of SgG1 and SgG2 on hCXCL12-mediated cell migration utilizing increasing concentrations of hCXCL12 and a constant molar ratio (1:100) between the chemokine and SgG (Figure 5F). The effect of hCXCL12 on in vitro cell migration had the characteristic bell-shaped curve (not shown). As a control we used PRV-SgG, which inhibited chemokine-mediated migration [16]. However, both SgG1 and SgG2 enhanced the potency of

<table>
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The derived kinetic parameters and the affinity constants for the interactions between HSV SgGs and chemokines are shown. Abbreviations: n.b., not bound. doi:10.1371/journal.ppat.1002497.t001

Figure 3. HSV-1 and HSV-2 gG expressed during infection bind chemokines. (A) Graph showing binding of radio-iodinated hCXCL10 to the surface of HSV-1 infected cells. Binding is observed at 14–16 h.p.i., only when cells are infected with wt HSV-1 but not when infected with a HSV-1ΔgG mutant. (B) Crosslinking assay showing the interaction between [125I]-hCXCL12α and HSV-2 gG in the supernatant of HSV-2 infected cells. The arrows point to the crosslinked complex. Abbreviations: h.p.i., hours post-infection. **P<0.01. doi:10.1371/journal.ppat.1002497.g003
hCXCL12, displacing the chemotactic bell-shaped curve towards lower concentrations of the chemokine.

HSV SgG enhances chemokine efficiency and directionality

To analyze the impact of HSV SgG on different aspects of chemotaxis in real time we performed time-lapse video microscopy using freshly isolated human monocytes and hCXCL12β. The chemokine, alone or in combination with SgG2, was released from a micropipette with constant backpressure. Analysis of tracks recorded by time-lapse video microscopy from cell cultures stimulated with CXCL12β (Video S1) or CXCL12β-SgG2 (Video S2) clearly showed that chemotaxis in the presence of the viral protein was enhanced, compared to the migration towards the chemokine alone (Videos S1, S2 and Figure 6B). SgG2 was not able to trigger migration in the absence of the chemokine (Video S3). Consistent with our data from transwell assays (Figure 5), SgG2 greatly enhanced the number of human monocytes that moved towards a given concentration of the chemotactrant (Figure 6). The cells sensed the chemokine gradient from longer distance to the dispensing pipette than when chemokine was dispensed alone. Chemotactic parameters, i.e. velocity, FMI and distance traveled, were calculated during an initial 10-min period. The velocity of the cell movement and the Forward Migration Index (FMI), i.e. the ratio of the net distance the cell progressed in the forward direction to the total distance the cell traveled, were significantly increased when SgG2 was bound to CXCL12β (Figure 6C, D). Moreover, the cells travelled a longer distance when the chemokine and SgG2 were dispensed together than when the chemokine was dispensed alone (Figure 6E). Similar results were obtained when using CXCL12α (not shown). Transwell experiments performed in parallel with freshly isolated human monocytes confirmed the SgG2-mediated enhancement of CXCL12β chemotaxis observed by video microscopy (Figure 6F).

Interaction of HSV SgGs with chemokine increases chemokine-mediated signaling

MAPKs are involved in several cellular processes including cell migration [31]. Binding of chemokine to its receptor activates a signaling cascade that involves phosphorylation and, thereby, activation of MAPKs. Incubation of MonoMac-1 cells with low doses of hCXCL12β resulted in low activation of MAPKs (Figure 7). Pre-incubation of different concentrations of hCXCL12β with a constant molar ratio (1:200) of SgG1 enhanced the phosphorylation of ERK (Figure 7A and B). The SgG1-mediated increase in the phosphorylation of JNK1-2 was dose-dependent (Figure 7C and D). Similar results were obtained with SgG2 (not shown). Densitometer analysis of the blots shows a dose-dependent enhancement of MAPK activation in the range of 5 fold for both ERK and JNK at the highest chemokine concentration. These results showed, using a different biological assay, a similar enhancement of chemokine activity mediated by HSV SgGs. Activation of CXCR4 results in the dissociation of GDP from the Gαβγ heterotrimer followed by association of GTP to the Gα subunit. In order to measure the effect of HSV SgG on receptor occupancy we performed a [35S]-GTPγS binding assay. The results show that the incubation of CXCL12β with SgG results in higher levels of [35S]-GTPγS incorporation (Figure 7E).

HSV-2 SgG increases chemotaxis in vivo

We tested the functional relevance of SgG2-chemokine interaction in vivo using the mouse air pouch model, by performing injections of chemokine alone or in combination with SgG2. Injection of 0.2 μg of mCXCL12α or mCCL28 induced the migration of leukocytes into the air cavity (Figure 8). The presence of 2 μg SgG2 enhanced CXCL12α-mediated migration (Figure 8A) of total leukocytes (top panel, P<0.001), lymphocytes (middle panel, P<0.001) and granulocytes (bottom panel, P<0.05). As a control, we used 2 μg recombinant secreted gG from PRV (PRV-SgG), a vCKBP shown to inhibit chemotaxis [16]. PRV-SgG significantly inhibited CXCL12α-mediated chemotaxis of total leukocytes (top panel, P<0.001) and granulocytes (bottom panel, P<0.05). CCL28-mediated chemotaxis (Figure 8B) of total leukocytes (top panel) and lymphocytes (middle panel) was significantly increased by SgG2 (P<0.05), whereas the migration of granulocytes (bottom panel) was not affected by SgG2. This could be explained by the specificity of CCL28 in driving T cell chemotaxis. In contrast to the inhibition observed when CXCL12 was used, PRV-SgG did not significantly inhibit CCL28-mediated chemotaxis. This may be due to uncontrolled factors such as the stability of the PRV-SgG-CCL28 complex in vivo or the indirect effect of SgG on the chemokine function.
Figure 5. HSV SgGs enhance chemokine-mediated cell migration. MonoMac-1 (A, E, F), MOLT-4 (B), m300-19-hCXCR5 (C) cells were incubated with the specified chemokine in Transwell plates. The effect of mock- or HSV-2-infected supernatant (Mock SN or HSV-2 SN, respectively) (A), purified SgG1 (B, C, E, F), SgG2 (C–F), M3 (C) and PRV-SgG (F) was analyzed. The number of migrated cells or the fold activation of migration is depicted. (C) SgG1 or SgG2 require the presence of the chemokine to enhance migration since addition of either of them without chemokine did not have any effect on chemotaxis. (D) Binding of HSV SgGs to the chemokine is necessary for the enhancement in chemotaxis. Representation of the
activation of other chemoattractants that may also induce migration. Injection of SgG2 or PRV-SgG alone, in the absence of chemokine, did not result in differences in leukocyte chemotaxis when compared to PBS injection.

Discussion
HSV glycoproteins play relevant roles in the viral cycle and pathogenesis, and constitute promising vaccine candidates [32,33]. Among all HSV glycoproteins, gG is the least well characterized and its function has not been fully elucidated. A role for HSV-1 gG on virus entry has been suggested. HSV-1 gG seems to be important for the infection of, but not initial binding to, polarized cells through the apical surface [21]. The non-secreted domain of HSV-2 gG could participate in initial interaction of the virion with the cell surface [21,22]. A synthetic peptide encompassing amino acids 190–205 from the secreted domain of HSV-2 gG was found to have a proinflammatory role in vitro when bound to the formyl peptide receptor [34]. However, until present, no function has been attributed to the full-length secreted portion of HSV-2 gG. Here, we have investigated the function of secreted forms of gG from HSV-1 and HSV-2. We show for the first time a chemokine-binding activity both in HSV-1 infected cells and in the supernatant of HSV-2 infected cells. Disruption of the HSV-1 gG expression abrogated chemokine binding suggesting that HSV gG is the protein responsible for the interaction. We could indeed show that both HSV-1 and HSV-2 SgG bind with high affinity, in the nanomolar range, CC and CXC chemokines. This interaction was demonstrated by the use of two different experimental approaches: crosslinking assays and SPR. Finally, and more importantly, we describe the first vCKBP, to our knowledge, with the ability to increase chemotaxis both in vitro and in vivo by enhancing the potency of the chemokine and the directionality of cell migration. HSV SgGs enhancement of chemotaxis required the interaction with the chemokine through the chemokine GAG-binding domain and involved signaling through the GPCR and activation of MAPKs. We confirmed that supernatant containing gG secreted following...
HSV-2 infection enhances chemokine-mediated migration of leukocytes. Moreover, in preliminary experiments we have found that membrane-anchored gG expressed during HSV-1 replication in cell culture also enhances chemokine activity (N.M.-M. and A.V.-B., unpublished data).

During evolution, viruses have developed strategies to modulate the host immune response. Inhibition of chemokine function through the expression of vCKBP is a common strategy in members of the Poxviridae family [12,35] indicating the importance of chemokines in antiviral defense. In the Herpesviridae family,
however, there are only three examples of vCKBP reported to date, two of them expressed by animal viruses -gG from alphaherpesviruses and M3 from murine herpesvirus 68- and one expressed by a human pathogen, pUL21.5 encoded by human cytomegalovirus [14,36]. In addition, interaction of HSV gB with a reduced number of chemokines has been reported [37]. However, this interaction was of low affinity, in the micromolar range [37] compared to the nanomolar range observed for all vCKBP [14,16,17,29,30,36]. Moreover, gB did not seem to have an effect on chemotaxis [37]. Nearly all previously described vCKBP have been shown to inhibit chemotaxis either in vitro or in vivo. As a general rule, vCKBPs inhibit chemokine function through impairing chemokine-receptor interaction or chemokine presentation by GAGs [38]. For instance, gG from some animal alphaherpesviruses blocks chemokine interaction with its receptor [14,28] and with GAGs [14] inhibiting chemotaxis [14,16,17]. To date, there are no reports of a vCKB that potentiates chemokine function either in vitro or in vivo. HSV SgG is, therefore, the first vCKBP described, to our knowledge, which enhances chemokine function both in vitro and in vivo.

Our studies with SgG1 and SgG2 show that these viral proteins interact with the GAG-binding domain of the chemokines and enhance the chemokine activation of GPCRs. Chemokine-GAG interaction is required for correct chemokine function in vivo [9]. Several reports show that GAG-binding deficient chemokines are functionally impaired in vivo and when in vitro migration and invasion assays are performed [39,40]. GAGs also modify chemokine quaternary structure and this seems to be required

![Figure 8. HSV-2 SgG enhances chemokine-mediated cell migration](https://www.plospathogens.org/content/pathtogether/article/8/2/e1002497/figure EV/8)

**A** CXCL12

**B** CCL28

**Figure 8. HSV-2 SgG enhances chemokine-mediated cell migration in vivo.** CXCL12 (A) or CCL28 (B) were injected into dorsal air pouches in mice alone or in combination with HSV-2 SgG or PRV SgG. Cell migration into the air cavity was monitored. Cells were extracted and identified by flow cytometry with specific markers. The number of total leukocytes (top), lymphocytes (middle) and granulocyte cells (bottom graph) is represented. Data are mean and SEM from 5-6 mice per group and are representative of 2-3 separate experiments. *: P<0.05; **: P<0.001. doi:10.1371/journal.ppat.1002497.g008
for chemokine function [39,41]. We propose a model in which SgG1 and SgG2 act similarly to the GAGs, maybe by increasing the local chemokine concentration, modifying the chemokine quaternary structure or improving chemokine presentation to the receptor so that signaling is enhanced. This would cause the observed activation of chemokine signaling at lower doses of chemokine when gG is present. This contrasts with the related gGs encoded by non-human herpesviruses, which have been shown to inhibit chemokine-mediated signal transduction and cell migration [14–17]. It appears that HSV-1 and HSV-2 have evolved a vCKBP to enhance, rather than to inhibit, chemokine function, and this may represent an advantage to these human herpesvi-

The functional relevance of chemokine enhancement in HSV life cycle and pathogenesis is unknown. The role of alphaherpesvi-

rus gG in vivo is not fully understood. Results presented in several reports indicate that gG from animal alphaherpesviruses is relevant for pathogenesis and immune modulation [15,17]. There are currently no data on the role of HSV-2 gG on pathogenesis. Three independent reports show that lack of gG expression in HSV-1 leads to different degrees of virus attenuation [23–25]. Thus, lower viral titers were detected in mouse tissues infected through scarification of the ear with an HSV-1 mutant lacking gG [23]. A double us3/us4 deletion mutant (with us3 encoding a kinase and us4 encoding gG) was attenuated following intracranial injection [24]. However, the relative contribution of either protein in that animal model could not be defined. Mutation of the us4 gene by the use of transposon Tn5 resulted in an HSV-1 mutant that was less pathogenic, which was sufficient in its ability to replicate in the mouse central nervous system and caused a delay in encephalitis induction [25]. The mechanisms of attenuation of HSV-1 gG mutant viruses are unknown, but the discovery that HSV-1 gG enhances chemokine function points to a role of HSV gG on deregulation of chemokine function that could explain the lower pathogenicity observed with the mutant viruses.

Although there are not yet systematic analyses on the expression of all known chemokines on the tissues relevant for HSV infection, the information obtained by several laboratories supports the relevance of chemokines on HSV infection and pathogenesis. The expression of some chemokines is upregulated upon HSV-1 and HSV-2 infection [42,43] leading to leukocyte infiltration, which may be as pathogenic as viral infection [44]. In fact, chemokines are important in HSE pathogenesis in humans [11]. Deficiency in CXCR3 or CCR5 increases susceptibility to genital HSV-2 infection although through different mechanisms [43,45]. Interest-

ingly, the lack of CXCR3 does not result in lower leukocyte recruitment. On the contrary, CXCR5 excess mice show an increase in viral titers, infiltrating cells and neuropathology accompanied by a higher level of cytokine and chemokine expression in brain and spinal cord [46]. Differences were observed between CXCL10 and CXCL13 (the receptor for CXCL10) mice when challenged with ocular HSV-1 infection [47,48]. However, CXCR3 responded like CXCL9 or CXCL10 in a gestational model of HSV-2 infection [46]. There are also differences in susceptibility depending on the route of infection and the nature of the pathogen employed. The redundancy of the chemokine network may be beneath some of these differences and discrepancies.

The chemokines bound by SgG1 and SgG2 are expressed in tissues relevant for HSV infection, replication and spread. Among other cell types, mucosal epithelial cells express CXCL5, CXCL28 and CXCL13: (1) CXCL25 expression is upregulated during oral wound healing [49]; (2) CXCL28 is expressed in airway epithelial cells [50]; and (3) CXCL13 is required for the organization and function of the nasal-associated lymphoid tissue [51]. Human corneal keratinocytes express CXCL15, CXCL10 and CXCL11, expression that can be further induced by proinflammatory cytokines [52]. CXCL14 expression in taste-bud cells is remarkably high and secreted to the saliva [53]. Among other tissues, CXCL12 is expressed in nervous tissues where it has been suggested to play a role in leukocyte extravasation [54]. CXCL12 also induces migration of neural progenitors, is required for axonal elongation and pathfinding, is relevant for neurotoxicity and neurotransmission in the adult nervous system and contributes to chronic pain [6,8]. Thus, modulation of the activity of chemokines mediated by gG1 and gG2 could occur in tissues infected by HSV and play a role in HSV biology.

Enhancement of chemokine function by HSV SgGs could impact at least four different scenarios relevant for HSV spread and pathogenesis. First, enhancement of GPCR signaling could aid in the early steps of infection and in viral replication. In fact, MAPK activation is required for efficient HSV replication [55]. In this scenario gG1, due to its presence in the viral particle and at the plasma membrane of the infected cells, may play a more relevant role than gG2, which is processed secreting its chemokine-binding domain. Second, increase in the level of infiltrating leukocytes, or differences in the composition of such infiltrate, could skew the immune response and favor viral replication. The fact that HSV SgGs only bind 11–12 out of 45 human chemokines with high affinity suggests the existence of a selectivity and specificity in the modulation of the immune response. Third, enhancement in the migration of a particular leukocyte population could recruit cells that may be subsequently infected by HSV, enhancing viral load. Fourth, modulation of chemokines present in the nervous system, such as CXCL12, could play a role in the initial infection of the ganglia, sites of HSV latency, and increase the ability of HSV to persist and cause disease. The impact of HSV gG-chemokine interaction on HSV biology requires further characterization.

In summary, this is the first report of a vCKBP that enhances chemokine function and suggests a novel mechanism of immune modulation mediated by a highly relevant and prevalent human pathogen. The findings reported here shall foster further investigations on the role of HSV gG on pathogenesis and immune modulation and will allow the design of novel immunomodulators, antiviral drugs and tools to study chemokine function.

Materials and Methods

Ethics statement

All animal experiments were performed in compliance with Irish Department of Health and Children regulations and approved by the Trinity College Dublin’s BioResources ethical review board. Human peripheral blood monocytes were prepared from buffy coats obtained from the local donor bank (Servizio Trasfusione, Svizzera Italiana, CH-6900 Lugano, Switzerland), with oral consent from the donors according to Swiss regulations. The use of buffy coats was approved by the institutional review board “Comitato Etico Cantonale, CH-6500 Bellinzona, Switzerland” and the experimental studies were approved by the “Dipartimento della Sanita e della Socialita’”.

Determination of SgG-chemokine binding specificity and affinities using SPR technology

The interactions between chemokines and SgGs and their affinity constants were determined by SPR technology using a Biacore X biosensor (GE Healthcare) as previously described [16].
Both proteins were dialyzed against acetate buffer pH 5.0 for SgG1 and pH 5.5 for SgG2, prior to amine-coupling of the recombinant proteins in CM5 chips. Chemokines that did not bind under kinetic conditions were considered negative and not taken into further consideration for the study. In competition experiments with heparin the chemokine was injected at 100 nM alone or with increasing concentrations of heparin in HBS-EP buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, 0.005% (vol/vol) surfactant P20, pH 7.4) at a flow rate of 10 µl/min, and association and dissociation were monitored. All Biacore sensorgrams were analyzed with the software Biaevaluation 3.2. Bulk refractive index changes were removed by subtracting the reference flow cell responses, and the average response of a blank injection was subtracted from all analytic sensorgrams to remove systematic artifacts.

**Competition of chemokine binding to cells**

Competition experiments were carried out incubating 0.5 pmol of [125I]-hCCL25 or [125I]-hCXCL12 with or without different concentrations of SgGs (or baculovirus supernatants) at 4°C in binding medium (RPMI 1640 containing 1% FBS and 20 mM HEPES pH 7.4) during 1 h at 4°C. Then, 3×10⁶ MOLT-4 or MonoMac-1 cells were added to the mixture and incubated for further 2 h at 4°C with gentle agitation, subjected to phalhulate oil centrifugation, washed twice with PBS, and cell-bound chemokine was determined using a gamma-counter.

**Chemotaxis assays**

Chemokines were placed in the lower compartment of 24-well transwell plates (Costar) or in 96-well ChemoTx System plates (Neuro Probe Inc., MD, USA) with or without recombinant SgGs in RPMI 1640 containing 1% FBS, MOLT-4, MonoMac-1, m300-19, or m300-19-hCXCR5 cells were placed on the upper compartment (3×10⁵ cells) in the 24-well transwell plate and 1.25×10⁶ cells in the 96-well ChemoTx System plate, with the exception of m300-19-hCXCR5 where 2.5×10⁵ cells were used. To test the effect of supernatant from mock- or HSV-2-infected cells in chemotaxis, the cells were injected in the presence of OptiMEM (Gibco) and the supernatants were collected 36 h p.i. These supernatants were inactivated with psoralen as previously described. Both chambers were separated by a 3 µm filter. Chemokine alone or in combination with SgGs was added to the upper compartment, and chemotaxis was measured.

**Isolation of human monocytes from blood and time-lapse video microscopy**

Monocytes were isolated from blood of healthy donors by negative selection using Monocyte Isolation kit II (MACS Miltenyi Biotec). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll (Lymphoprep) gradient centrifugation. Cells were resuspended in MACs buffer and incubated with FcR blocking reagent at 4°C. Monocytes were purified by negative selection according to the manufacturer’s protocol. Time-lapse video microscopy analysis of chemotaxis was performed immediately with a Leica DMR000 microscope stage connected to a SP5 scan head equipped with a temperature controlled chamber (Cube, LIS, Basel). Freshly isolated monocytes were placed in a humidified and CO₂-controlled incubator, which was mounted on the microscope stage (Brick, LIS, Basel). Cells were resuspended in D-PBS containing calcium and magnesium (Invitrogen) supplemented with 1% FBS, Pen/Strep, 0.04 mM sodium pyruvate, 1 mg/ml fatty acid free BSA (Sigma), 1 mg/ml glucose (Fluka). Cells were plated on glass bottom petri-dishes (MatTek culture-ware) which were coated previously with D-poly-lysine (5 µg/ml) and subsequently overlaid with 3 µg/ml VCAM-1 (BD Biosciences) at 4°C overnight. Before plating the cells, coated-dishes were treated with PBS containing FBS and BSA to block non-specific binding. Chemokine was dispensed with a micropette (Femtotip II, Eppendorf) controlled by a micromanipulator (Eppendorf) at a constant backpressure of 30 hPa (Femtojet, Eppendorf).

**Actuation of mitogen activated protein kinases**

Chemokine alone or in combination with SgGs was added to 10⁵ MonoMac-1 cells and incubated during 1 h at 37°C. Cells were lysed in lysis buffer (20 mM triethanolamine pH 8.0, 300 mM NaCl, 2 mM EDTA, 20% glycerol, 1% digitonin and proteinase inhibitors). The lysate was analyzed by western blotting using anti-phospho-ERK, anti-phospho-P38 (Cell Signaling Technology) or anti-phospho-JNK1/2 polyclonal antibodies (Abcam). Blots were scanned and the densities of the bands were analyzed and compared with the ImageJ 1.43 software normalizing the densities obtained from each band from the MAPK blots to their respective loading controls.

**Air pouch model**

Age-matched female C57BL/6 mice from Harlan (Bicester, U.K.) were housed in a specific pathogen-free facility in individually ventilated and filtered cages under positive pressure. All animal experiments were performed in compliance with Irish Department of Health and Children regulations and approved by the Trinity College Dublin’s BioResources ethical review board.

Dorsal air pouches were induced in mice as described [57]. In brief, 5 ml of sterile-filtered air was injected subcutaneously into the dorsal skin of mice, with air pouches re-inflated with 3 ml of sterile air 3 days later. The dorsal air pouches of groups of 5–6 mice were injected 2 days later with 0.2 µg chemokine alone or in combination with 2 µg SgG. Mice were killed and air pouches were lavaged with PBS 3 h later. The air pouch aspirate was centrifuged and total leukocytes cells were counted. Cells were stained with a panel of mAbs for surface markers for flow cytometric cell characterization as described [58]. mAbs used were from BD Biosciences; PerCP anti-CD4 (RM4-5), PerCP-Cy5.5 anti-CD19 (1D3), PerCP anti-CD16a (56-6.7), PerCP anti-CD11b (M1/70) and eBioscience: PE anti-Ly6G (RB6/8C5). mAbs used were from BD Biosciences; PerCP anti-CD4 (RM4-5), PerCP-Cy5.5 anti-CD19 (1D3), PerCP anti-CD16a (56-6.7), PerCP anti-CD11b (M1/70) and eBioscience: PE anti-Ly6G (RB6/8C5). Cells were defined as lymphocytes (CD4+CD16+CD19+) and Ly6G+CD11b+ granulocytes (neutrophils). Data were collected on a CyAn (Beckman Coulter) and analyzed using FlowJo (Tree Star). Quadrants were drawn using appropriate isotype-controls and data plotted on logarithmic scale density- or dot-plots.

**Statistical analysis**

Statistical analyses of data were performed with the program GraphPad Prism. The significant value (P value) for the parameters measured in all assays was calculated using the
student’s t-test with the exception of the ones obtained in the air-pouch model experiments which was calculated using the one-way analysis of variance (ANOVA).

Supporting Information

Protocol S1 Generation of recombinant baculoviruses and purification of recombinant proteins. Description of the procedure employed to generate recombinant baculoviruses and purify recombinant proteins.

Protocol S2 Recombinant chemokines. Relation of recombinant chemokines used in this report.

Protocol S3 Cross-linking experiments. Explanation of the method used to perform cross-linking.

Protocol S4 Cells and viruses. Cells and viruses utilized in this report.

Protocol S5 Chemokine binding to infected cells. Description of the method employed to analyze binding of radiolabeled chemokine to infected cells.

Protocol S6 Competition of chemokine binding to cells. Explanation of the procedure performed to determine the effect of HSV SgG on radiolabeled chemokine binding to cells.

Text S1 Includes references used in Protocol S1, Protocol S3, Protocol S4.

Video S1 Migration of human monocytes towards CXCL12b. Freely isolated monocytes from blood of healthy donors were plated on glass bottom cover slips coated with Poly-D-lysine and VCAM-1. CXCL12b (100 nM) was dispensed from a micropipette with constant backpressure. Time-lapse video was recorded at 10 seconds interval with DIC optics at 63× magnification. 1 representative video of three is shown. DVX software should be used to open and play this video.

Video S2 Migration of human monocytes towards CXCL12b-SgG2. Freshly isolated monocytes from blood of healthy donors were plated on glass bottom cover slips coated with Poly-D-lysine and VCAM-1. CXCL12b (100 nM) was pre-incubated with SgG2 in a molar ratio 1:50 during 30 min at RT. CXCL12 and SgG2 complex was dispensed from a micropipette with constant backpressure. Time-lapse video was recorded at 10 seconds interval with DIC optics at 63× magnification. 1 representative video of three is shown. DVX software should be used to open and play this video.

Video S3 Migration of human monocytes towards SgG2. Freshly isolated monocytes from blood of healthy donors were plated on glass bottom cover slips coated with Poly-D-lysine and VCAM-1. 5 μM SgG2 was dispensed from a micropipette with constant backpressure. Time-lapse video was recorded at 10 seconds interval with DIC optics at 63× magnification.

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Author Contributions

Conceived and designed the experiments: AVB NMM HJN PGF AA. Performed the experiments: AVB NMM HJN PGF PR. Analyzed the data: AVB NMM HJN PGF AA. Contributed reagents/materials/analysis tools: AVB FAS MT RM SB. Wrote the paper: AVB AA. Corrected and approved the manuscript: AVB NMM PGF FAS MT AA.

References


Toll-like receptor-mediated recognition of herpes simplex virus

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1. ABSTRACT

Herpes simplex virus type 1 and 2 (HSV-1 and HSV-2, respectively) are two important human pathogens that belong to the genus simplex within the subfamily alpha of the Herpesvirinae. Toll-like receptors (TLRs) constitute a family of conserved sensors that play a prominent role during the early anti-viral response, including that against herpesviruses. Although substantial progress has been made, central questions remain to be solved to figure out how TLRs modulate viral pathogenesis. The aim of the present report is to review the current knowledge about TLR recognition and signaling of herpesviruses, focusing on HSV infection. The relative contribution of the TLR-mediated immune responses to antiviral immunity versus viral pathogenesis will be discussed as well.

2. INTRODUCTION

The host innate immune response is critical in providing the first line of defense against pathogens, including viruses. This response, although not antigen specific, is able to discriminate among a great variety of microorganisms. Early recognition of microorganisms occurs through a limited number of germ line-encoded pattern-recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPs). PAMPs are invariant motifs distinct from those of the host, which are essential for the pathogen to survive and thus are unlikely to be altered. Toll-like receptors (TLRs) form a highly conserved group of transmembrane PRRs that are able to recognize a wide range of PAMPs found on pathogens, playing a relevant role in the innate immune response (1-3). TLRs also play a pivotal role in the initiation of the adaptive immune response through the induction of cytokine and chemokine expression involved in leukocyte recruitment or direct T cell activation by TLR agonists (4-7). Each TLR has the ability to recognize different PAMPs derived from pathogens, including bacteria, fungi, protozoa and viruses. Viral glycoproteins and nucleic acids constitute the main viral PAMPs recognized by TLRs. A list of the motifs recognized by each TLR is presented in table 1. The participation of TLRs in immune protection was first observed in Drosophila, where Toll, a protein involved in dorso-ventral patterning, was found to regulate the expression of the antifungal peptide drosomycin (8). One year later, the group of Charles Janeway, reported the cloning and characterization of a human homolog of Drosophila TLR, termed hToll (9). Soon afterwards, Poltorak et al., identified loss-of-function mutations in the gene tlr4, coding for the mouse homolog of hToll, to be the cause for the hypo responsiveness of mice to bacterial lipopolysaccharide (LPS) (10). A significant portion of the TLR biology has been elucidated by studying this receptor, now termed TLR4. Since those pioneer studies came to light, 10 TLRs have been described in human, 9 of which are conserved in mouse (1, 3, 11).

Herpes simplex virus 1 and 2 (HSV-1 and -2, respectively) are important human pathogens. The prevalence of HSV-1 is estimated to be more than 90% and that of HSV-2 around 22% in the adult U.S. population, reaching up to 80% in sub-Saharan Africa and developing countries (12, 13). Primary infection with HSV takes place in the mucosa. In general, HSV-1 preferentially infects through the oro-labial mucosa and establishes latency in the trigeminal ganglia whereas HSV-2 does so through the genital mucosa, establishing latency in the sacral ganglia. However, both viruses can infect either mucosa. Disease caused by HSV is normally mild in immuno-competent individuals. However, under certain circumstances, HSV infection can have devastating consequences. Thus, HSV-1 infection of the cornea can cause stromal keratitis lesions, termed herpes simplex keratitis (HSK), that represent the most common cause of infectious blindness. Moreover, HSV infection of the central nervous system (CNS) can lead to herpes simplex encephalitis (HSE), an outcome more often observed in non-immunocompromised patients. Infection of the neonate with either HSV-1 or -2 may be limited to the skin, eye or mouth but it can also affect the CNS. Furthermore, HSV disseminated infection in neonates involves the lung, liver and the adrenal gland. These symptoms resemble
those characteristics of bacterial sepsis. Finally, several reports suggest a synergistic effect of HSV-2 on the infection by human immunodeficiency virus (HIV) and progression towards acquired immunodeficiency syndrome (14, 15).

The host immune system contains several mechanisms to counteract herpesvirus infection. Despite the existence of an organized and orchestrated immune response, HSV and other herpesviruses are able to persist in the organism during the lifetime of the individual. HSV, like other herpesviruses, contains several PAMPs that are recognized by TLRs, resulting in the expression of cytokines and chemokines with antiviral activity. Cells targeted by HSV, both in the mucosa and the CNS, express TLRs. The context of the interaction between the different TLRs and HSV might determine the outcome of HSV infection, including the clearance or spread of the virus and the absence or presence of disease. In this regard, recent data suggest that HSV recognition by certain TLRs may play a role in HSV-mediated pathogenesis. Finally, several groups are investigating the use of TLR agonists to inhibit HSV replication and progression to disease.

3. INDUCTION OF AN ANTIVIRAL RESPONSE BY TLRs

3.1. Basis of TLR structure and signaling

The structure of TLRs resembles that of the IL-1 receptor (IL-1R). TLRs consist of an N-terminal extracellular domain containing variable leucine-rich-repeat (LRR) motifs, followed by a transmembrane region and a C-terminal cytoplasmic signaling domain homologous to that of IL-1R, and hence called the Toll/IL-1R homology (TIR) domain. Ligand binding to the receptor induces its dimerization, and causes a series of conformational changes that lead to the recruitment to the TIR domain of TIR-domain-containing proteins. There are four adaptor proteins bound by TIR: Myeloid differentiation primary response protein 88 (MyD88), TIR-associated protein/MyD88-adaptor-like (TIRAP/MAL), TIR-domain-containing adaptor protein-inducing interferon-beta/TIR-domain-containing molecule 1 (TRIF/TICAM1) and TRIF-related adaptor molecule (TRAM). The core of TLR signaling relies on MyD88. MyD88 is used by all TLRs with the exception of TLR3, which signals exclusively through TRIF (1, 3), and TLR4, which is able to signal through both MyD88 and TRIF, the latter in combination with TRAM (16). The use of different combinations of adaptors by each TLR permits the initiation of different responses by a given TLR.

Signaling through the MyD88-dependent pathway results primarily in the activation of nuclear factor kappa B (NF-kappaB) and mitogen activated protein kinases (MAPK), inducing the expression of proinflammatory chemokines and cytokines. The TRIF-dependent pathway mainly activates NF-kappaB and interferon responsive factor 3 (IRF3). Overall, either signaling pathway ultimately results in the expression of cytokines with antiviral activity such as interleukin (IL)-6, IL-12, tumor necrosis factor-alpha (TNF-alpha) and interferon (IFN). Most studies showed type I IFN expression upon stimulation with TLR3, TLR4, TLR7, TLR8 and TLR9 (1, 3, 17-24). Recently, induction of IFN-gamma in T helper 1 effector cells by TLR2 ligands was described (5). Prompt expression of this immune mediator is essential to control viral replication and therefore many mechanisms work in a cell-type and time-dependent manner to trigger IFN response. A detailed description of TLR signaling is beyond the scope of this report; however, a number of interesting reviews concerning different aspects of TLR signaling are available (1, 2, 11, 25).

Most TLRs are found on the cell surface, except for TLR 3, 7, 8 and 9, that are localized in endosomes (1). The relative importance of the subcellular localization of these receptors in the generation of anti-viral responses has not been fully clarified yet. Compartmentalization of TLR and TLR-mediated expression of co-stimulatory signals in dendritic cells (DCs) allows discrimination of non-self from self ligands (26). Intracellular localization is required to favor TLR and viral nucleic acid interactions. Endosomal localization may limit access to "self" nucleic acids (18, 19, 27). Thus, targeting of TLR9 to the plasma membrane abrogated TLR9-mediated recognition of viral nucleic acids whereas it allowed recognition of self nucleic acids (27). On the other hand, TLR4, TLR2/TLR1 and TLR2/TLR6 recognize viral glycoproteins and are expressed on the cell surface (1-3, 11).

Although the first TLRs discovered in mammals were identified due to their anti-bacterial role, the prominent role of TLRs in the generation of an early immune response against viruses became clear in the last few years (18, 20, 22, 28-31). The discovery that vaccinia virus expressed two proteins (A46R and A52R) that suppressed TLR signaling strongly suggested that TLRs could play a role in the antiviral response (32). This was soon confirmed with the first evidence for the recognition of the fusion protein of respiratory syncytial virus (RSV) by TLR4 (33). Since then, a role for several TLRs in the antiviral response has been established. Together with RSV, mouse mammary tumor virus (MMTV)-induced activation of TLR4 (34), while measles virus (MV) stimulated the production of cytokines such as IL-6 in a TLR2-dependent manner (35). Additionally, the ability of MMTV to activate cells also through TLR2 has been proposed (36). Initial studies on TLR7 and TLR8 implicated them in the response to RNA viruses, such as H1N1 influenza virus and vesicular stomatitis virus (VSV) (19, 37). TLRs recognize viral proteins or viral nucleic acids in the form of ssRNA, dsRNA or unmethylated CpG motifs present within the viral genomes. As shown in table 1, TLR3 recognizes dsRNA, TLR7 and 8 ssRNA and TLR9 unmethylated CpG motifs. Viral proteins, in turn, are recognized by TLR2, which forms a heterodimer with TLR1 and TLR6, or by TLR4 (1, 3, 11). For excellent reviews on the complex interplay between viruses and the TLR system see Boehme and Compton (25) or Finberg et al. (38).

4. THE INTERPLAY BETWEEN HERPESVIRUSES AND TLRs
Herpesviruses turn on a complex innate immune response, which includes IFN production, multiple cytokine synthesis and the secretion of chemokines that recruit and activate inflammatory cells (39-41). Nevertheless, not all the mechanisms used by the immune system to trigger the inflammatory response against these viruses are fully understood. The TLR network constitutes one of the main players in the early detection and response against herpesviruses. Recent data demonstrated the recognition of different human pathogens of this family by TLRs (Table 2). Compton et al. showed for the first time the ability of human cytomegalovirus (hCMV) to trigger inflammatory cytokine production through CD14- and TLR2-dependent activation of NF-kappaB (42). Later on, this group identified two envelope glycoproteins of hCMV that were implicated in TLR2 engagement (43). The impairment of TLR3 and TLR9 signaling, in turn, had a dramatic effect on the progression of murine CMV-induced pathogenesis (22). More recently, the recognition of Varicella-Zoster virus (VZV) by TLR2 in human monocytes was described (44). The work of Gaudernault and colleagues showed that infectious and UV-inactivated Epstein-Barr virus (EBV) virions activated NF-kappaB specifically through TLR2. Furthermore, EBV infection of human monocytes induced the release of the monocyte chemotactic protein 1 (MCP-1), and such chemokine response was significantly reduced after treatment with small interfering RNA (siRNA) targeting TLR2 (45). The second human gamma-herpesvirus, Kaposi’s sarcoma-associated herpesvirus (KSHV), the causative agent of KS, is the first herpesvirus reported to be detected by TLR4 (29). The antiviral activity of TLR4 had been previously demonstrated mainly against RNA viruses, such as RSV (33).

As mentioned previously, herpesviruses are able to persist lifelong in the infected host despite the immune response triggered against them. This indicates that herpesviruses have evolved strategies to modulate and evade the immune system, including the TLR network. For instance, EBV manipulates the TLR7 pathway for its own benefit (46). This pathway provides signals that drive naïve B cells, the target of EBV infection, to proliferate. Thus, it was suggested that during the earlier steps of infection, EBV manipulates TLR7 signaling to promote the initial phase of B-cell activation and expansion. However, later during infection, the virus induces negative regulators of the TLR7 pathway, necessary for the establishment of latency (46). Another example of a herpesvirus capable of modulating the TLR system can be found in KSHV. A rapid drop of TLR4 expression was reported during KSHV primary infection of endothelial cells (29). Macrophages lacking the tlr4 gene and cells treated with anti-TLR4 siRNA, appear to be more susceptible to KSHV infection, since impaired cytokine production and higher viral gene expression is observed (29). Thus, the authors suggest that the downregulation of TLR4 could be a mechanism employed by the virus to escape the control of the immune system. The fact that a mutation in a tlr4 allele predisposes KSHV infected individuals to suffer from multicentric Castleman’s disease, a lymphoproliferative disease associated with KSHV, is an additional piece of evidence for the involvement of TLR4 in the innate immunity against the virus (29). Moreover, KSHV seems to induce TLR3 signaling during primary infection (47). As a result of TLR3 activation, a series of cytokines and chemokines that act as potent chemotaxants and angiogenic factors are produced (47). The authors speculate that the modulation of TLR3 signaling by KSHV aids the virus to spread and to establish latency before the acquired immune response starts (47).

4.1. Recognition of HSV by TLRs

The oro-labial, genital mucosa and the cornea constitute the initial sites of HSV infection. Following local replication, HSV reaches a peripheral nerve and thereby the trigeminal or sacra ganglia where latency is established. In some instances, HSV infects the CNS and other organs. Cells targeted by HSV in the mucosa, the cornea and the CNS express TLRs that recognize and trigger an immune response against HSV. HSV displays PAMPs recognized by several members of the TLR network, and the role of such interactions is yet to be totally defined. Experiments carried out using knockout mouse models lacking either one or two TLRs, or MyD88 suggest that innate resistance to HSV is achieved by the regulated activation of several TLRs (21, 30, 31, 48, 49). In fact, HSV is recognized by at least TLR2, TLR3 and TLR9 and experiments carried out both in vitro and in vivo support the notion of an orchestrated response against HSV (21, 30, 31, 48, 49). It is well established that PAMP recognition by means of the TLRs expressed in DCs is required for the induction of T cell responses just after an infection (2). DCs present a distinct TLR expression profile depending on the cell subtype. In humans, plasmacytoid DCs (pDCs), also termed IFN producing cells (IPC s), only express TLR7 and TLR9 (50, 51). Upon infection, pDCs express high levels of all subtypes of type I IFN. IFN induces the expression of cytokines involved in monocyte maturation, antigen cross-presentation to T-cells, leukocyte differentiation and activation (50-52). This process seems to be independent of viral replication, since UV-inactivated HSV triggered identical IFN production (21). HSV contains a great amount of unmethylated CpG motifs in its genome, that serve as ligands for TLR9 (53). In fact, pDCs produce extremely high amounts of type I IFN following recognition of HSV-2 via TLR9 (20, 21, 51). However, mice lacking TLR9 can still control HSV replication in a cutaneous infection model, suggesting that TLR9-independent mechanisms can compensate the impaired pDC response observed in these animals (20). Hochrein et al., using pDCs isolated from different tissues of wt and Tlr9−/− or Myd88−/− mice, showed that IFN-alpha response after HSV infection was generated by TLR9-dependent and independent mechanisms, and mediated by different cell types other than pDCs (54). Thus, fibroblasts, macrophages and other non-pDCs have been shown to produce IFN-alpha in response to HSV infection in a TLR9 or MyD88 independent manner, but dependent on viral entry and replication (55). In contrast, HSV-induced expression of other cytokines, such as TNF and CCL5 by macrophages is mainly dependent on TLR9 (55). Thus, the existence of two waves of type I IFN production during HSV infection has been suggested (55). The synthesis of IFN-alpha/beta shortly after infection would be performed by pDCs mainly in a TLR9-dependent manner, whereas the subsequent IFN production would be mediated by other cell types using mainly TLR9-independent mechanisms (55).

The existence of synergy between TLR2 and TLR9 was observed using single knockout mice for either receptor or double knockout mice (56). Following HSV-2 infection, there were no major differences regarding cytokine response and viral
load between wt and TLR2−/− or TLR9−/− (56). Importantly, the TLR2−/−/TLR9−/− mice had impaired cytokine production and higher viral loads in the brain but not in the liver (56). Interestingly, a recent report described a previously uncharacterized mechanism of viral detection, that functions through the sequential recognition of HSV by TLR2 followed by TLR9, within the same DC (57). This serial activation of multiple TLRs in a given cell, could represent an evolutionary trick that allows the immune system to more precisely identify a pathogen, in order to mount the optimal response against it (57). Monocyte/macrophages constitute another cellular compartment infected by HSV that plays a relevant role in HSV recognition and control (58). Infection of macrophages by HSV results in the production of IFN-alpha/beta, TNF-alpha and chemokines through different signaling pathways (55). TLR2 mediates IL-15 expression upon HSV-1 infection of human monocytes (58). Inhibition of TLR9 signaling impaired TNF-alpha expression whereas IFN-alpha/beta was not affected (58). HSV infection affects TLR signaling through mechanisms that are not fully understood. One of them seems to occur through the interaction between HSV ICP0 and ubiquitin specific protease 7 (USP7). ICP0 induces the translocation of USP7 to the cytoplasm where it deubiquitinates the TNF-associated factor (TRAF)-6 and the inhibitor of kappa B kinase (IKK)-gamma thereby abrogating TLR-mediated NF-kappaB and JNK activation (59). Moreover, HSV-1 infection of monocytes in vitro downregulated TLR2, TLR4, and monocyte activation markers such as CD38 and CD69 whereas it increased the presence of necrosis and apoptosis markers (60). Atopic dermatitis patients are particularly susceptible to HSV infection. Pro-inflammatory monocytes obtained from these patients showed affected TLR2-mediated TNF-alpha and IL-1beta production (61).

The main TLR playing an anti-HSV role in the CNS is TLR3. This TLR recognizes dsRNA produced during HSV infection and has been shown to control HSV-1 infection, particularly in the CNS. Studies carried out in vitro using human monocyte cell lines suggest that HSV US3 controls TLR3-mediated signaling against the virus at least in this context (62). The importance of TLR3 in HSV-mediated pathogenesis is supported by the findings that a dominant-negative point mutation in this receptor may predispose to HSE (49). Monocyte-derived DCs, CD8+ T cells, natural killer (NK) cells and fibroblasts obtained from patients carrying this mutation are impaired in IFN production upon stimulation with polyinosinic-polycytidylic acid (poly I:C), a ligand of TLR3, and are not able to control HSV-1 or VSV infection (49). The importance of TLR signaling in HSE is supported by the finding that mutations in UNC-93B, a protein required for the signaling of TLR3, 7, 8, and 9, correlate with a higher prevalence of HSE in humans (63).

4.2. Immunopathological consequences of TLR activation by HSV

Many of the symptoms observed in the pathogenesis associated with herpesviruses are linked to the presence of inflammatory and angiogenic factors. Several herpesviruses encode chemokines and chemokine receptor homologs that provide strong inflammatory signals and induce angiogenesis (64). These viral homologs of cytokines recruit inflammatory cells that in turn may transport the virus to other potential sites of infection, or can serve for the establishment of virus latency. According to the majority of mouse models of infection used to date, the signals transmitted by PRRs are essential for pathogen clearance and host survival. However, several reports suggest that herpesviruses are able to modulate the TLR network, suggesting a role for TLR signaling in herpesvirus-mediated immunopathogenesis.

Regarding HSV, several reports indicate the involvement of the TLR system in the immunopathogenesis observed. The study by Kurt-Jones demonstrated that TLR2 recognition of HSV-1 mediates an excessive inflammatory response, which was associated with viral encephalitis rather than with a protective response (65). Animal experiments carried out by this group using wt and TLR2−/− mice, showed that TLR2 mediates the induction of multiple proinflammatory cytokines and chemokines in response to HSV (65). The attenuated cytokine response found in TLR2−/− mice correlated with absence of brain inflammation, and was followed by an important reduction of the mortality rate, compared to wt or TLR2−/− mice (65). Importantly, the differences observed in TLR2−/− mice were not due to viral titers, which were equivalent to those found in wt animals. Since the same trend was observed in neonatal animals, the authors proposed that neonate susceptibility could be due to an excessive TLR2-dependent response, rather than being associated with the inability of their immature immune system to contain the infection. Indeed, the production of higher levels of IL-6 and IL-8 by peripheral blood mononuclear cells (PBMCs) isolated from human neonates, compared to that from adults, has been reported in response to the challenge with HSV-1 or HSV-2 (66). A similar detrimental response is observed during certain bacterial infections in neonates, particularly to those antigens that activate TLR2 (67).

Azavalli et al., using microglial cells isolated from wt and TLR2−/− mice, reported the requirement of this receptor for the production of many proinflammatory cytokines and chemokines in response to HSV infection (68). Microglial cells are known to play a key role in neuroimmune responses, once they are TLR stimulated (69). The implication of TLR2 signaling in the induction of apoptosis in HSV-infected microglial cells has been suggested (70). These examples point towards a potential role for TLR2-dependent signaling in HSV-induced neuropathology. Additionally, a recent report found an association between two TLR2 haplotypes and an increase in shedding and lesional rates in HSV-2 infected patients. Therefore, polymorphisms in this receptor may be in part responsible for the observed differences in the severity of HSV infections in humans (71). A role for TLRs on HSK has been proposed (31, 72). Using a corneal scarification disease model, the pathological assessment of MyD88 and TLR knockout mice was examined. Mice lacking MyD88 do not suffer from such pathology but die from encephalitis, indicating the role played by TLR signaling in the immunopathology observed in wt mice and in control of virus spread (31). Deletion of single TLRs pointed to a role of TLR2 and, to a lesser extent, TLR9 in the onset of keratitis. Interestingly, mice lacking TLR4 had more pronounced lesions than wt mice (31). Corneas suffering from active HSK express higher levels of TLR4 mRNA than healthy corneas, in particular those of TLR4, 7, 8, and 9, whereas TLR7 was the sole upregulated TLR in non-active HSK (72). Thus, TLR2, TLR4, 8 and 9 seem to be involved in the pathogenesis observed in active HSK (31, 72).
Taken together, the results presented here provide strong evidence that TLR-mediated responses, in particular those mediated by TLR2, play a prominent role in HSV-associated immunopathology. However, it is important to bear in mind that the context of the interaction between the different TLRs and HSV could determine the outcome of HSV infection. Factors such as age, immunological status and genetic background may have an impact on TLR-related immunopathogenesis.

5. POSSIBLE THERAPEUTIC IMPLICATIONS OF TLR AGONISTS AGAINST HSV-MEDIATED PATHOGENESIS

Due to the pivotal role of TLRs in the immune response several groups are investigating the potential use of TLR agonist as prophylactic or therapeutic agents. The observations presented above open the door to the use of TLR ligands as a promising approach to treat HSV infections in the clinic. Each year there are about 500,000 new cases of HSV-2 infection in the USA (13), and there is currently no vaccine available against the virus. Initial studies examined the use of TLR ligands as adjuvants in mucosal immunization strategies against HSV-2, and more recent reports suggested a protective effect of direct local delivery of these agents. Genital epithelial cells (GEC), which constitute the first line of mucosal defense, were shown to express mRNA for all TLRs, although at different levels (73, 74). A number of studies have shown the efficacy of intra-vaginally (IVAG) delivery of TLR9 and TLR3 agonists to elicit potent innate immune responses dependent on IFN-beta against HSV-2 (73, 75-80). Furthermore, this protection correlated with the production of nitric oxide, IFN-beta and other cytokines, following addition of TLR3, TLR5 and TLR9 ligands to polarized GEC (73). The relevant role of IFN-beta was confirmed using knockout mice and by the IVAG administration of IFN-beta (77). The activation of TLR4 failed to provide any protection (73, 77), while the use of TLR2 ligands had no or little effect against HSV-2 infection (73, 77). Despite these reports indicating lack of efficiency when TLR2 agonists were used solely, immunization studies showed that fusion of a TLR2 agonist to a HSV-2 CD8 T cell epitope resulted in the recruitment of specific memory CD8 cytotoxic T cells both in the genital tract, the lymph nodes and spleen that conferred protection against HSV-2 challenge (81). The response was diminished when Thr2 and Myr88’ mice were immunized, showing less HSV-2-specific T cell responses and higher viral titers, disease and death rates than wt mice (81).

The use of imiquimod and resiquimod, ligands with TLR7/8 mixed agonist activity, has been tested in preclinical and clinical studies. Although with varied success, these studies suggested the ability of these agonists to promote a Th1 specific response that controlled HSV-2 mucosal infection (82, 83). CpG oligodeoxynucleotide (CpG ODN), a TLR9 ligand, is more efficient than resiquimod (R-848, TLR7/8) when administered IVAG in mice and causes a local immune response against HSV-2 (84). The IVAG delivery of CpG ODN protects against IVAG HSV-2 challenge in mice (75-78), however this treatment is associated with local inflammation and splenomegaly (85). IVAG delivery of dsRNA, in turn, induced protection without those symptoms (76). Similarly, the TLR3 ligand, poly I:C, also protects against genital HSV-2 infection without apparent local immunotoxicity (79).

Regarding HSV-1, the use of a mouse model for HSE indicates that the activation of TLR3 through intranasal delivery of poly I:C triggers the expression of TLR3, TNF-alpha, IL-1beta, IL-12, IFN-gamma and CXCL10; reduces HSV loads and increases survival (86). Higher survival rates were also observed after treatment with a TLR9 agonist, compared to those of wt or TLR4’ mice (86). The relevance of TLR3 in protection against HSE is supported by the finding that individuals with naturally-occurring mutations causing lack of TLR3 function, have higher susceptibility to suffer from lethal encephalitis as a result of HSV-1 infection (49).

Only the use of TLR agonists has been investigated so far. However an issue that arises from the works outlined in this review, is the possibility to use inhibitory compounds of certain TLRs to restrain the excessive antiviral inflammatory response that results harmful to the host.

6. CONCLUDING REMARKS

Our knowledge of the TLR-virus relationship has improved dramatically in the last few years. The list of TLR viral partners will expand as more TLR pathways are discovered in the years to come. Research on this field has only started to unravel how TLRs impact on virus-mediated pathogenesis. The finding that several herpesviruses belonging to different subfamilies, together with HSV, MMTV and measles virus, can activate innate responses in a TLR-dependent manner, confirms the main role played by the TLR system in antiviral immunity. Additional support comes from the observation that some viruses have evolved mechanisms to modify or counteract the TLR network.

HSV recognition by TLRs is essential for the initiation of prompt innate immune response and the coordination of the adaptive immune response. TLR activation results in the secretion of high amounts of type I IFN and other cytokines such as IL-12, providing a link between innate and adaptive immunity. TLR3 stimulation, in turn, seems to reinforce an immune mechanism of neuroprotection against the virus (86). On the other hand, TLR-mediated responses can also have detrimental consequences for the host. This is clearly illustrated by the fact that TLR2 recognition of HSV in a particular context causes serious immunopathological lesions, responsible for a significant portion of the morbidity and mortality associated with the infection (31, 65). Therefore, defining the individual role of each TLR alone and in combination is crucial to understand how TLRs modulate
pathogenesis. This is particularly interesting in the case of herpesviruses, to date, the only viral family known to interact with up to five TLRs.

Analysis of the molecular basis of TLR and viral PAMP interactions will be decisive in clarifying the cellular mechanisms by which the immune response is regulated. Understanding how TLRs and viruses interact will provide insight into our own immune system and into viral and host co-evolution, and will be of great help in developing more efficacious antiviral strategies.

7. ACKNOWLEDGEMENTS

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Table 1. PAMPs recognized by human TLRs

<table>
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<th>PAMP</th>
<th>Organism</th>
<th>TLR involved</th>
<th>References</th>
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<tbody>
<tr>
<td>LPS</td>
<td>Gram-negative bacteria</td>
<td>TLR4</td>
<td>10, 29, 33, 34, 87-89</td>
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<tr>
<td>Viral envelope glycoproteins</td>
<td>Viruses</td>
<td>TLR2</td>
<td>35, 42-45, 58, 65, 68, 70, 90-93</td>
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<tr>
<td></td>
<td>(TLR1/TLR2 Or TLR6/TLR2)</td>
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<tr>
<td>Viral envelope glycoproteins</td>
<td>Viruses</td>
<td>TLR3</td>
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<td>Bacteria</td>
<td>TLR5</td>
<td>97, 98</td>
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<tr>
<td>ssRNA</td>
<td>Viruses</td>
<td>TLR7 and TLR8</td>
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<td>TLR9</td>
<td>20-22, 28, 51, 54, 56, 57, 99, 100</td>
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<td>Undetermined</td>
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Table 2. Herpesviruses recognized by TLRs

<table>
<thead>
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<th>Viral PAMP</th>
<th>TLR involved</th>
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<tr>
<td>alpha</td>
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<td>Unknown viral protein</td>
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<td>Cpg motifs</td>
<td>TLR9</td>
<td>18, 52-56, 63</td>
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<td></td>
<td>VZV^4</td>
<td>Unknown viral protein</td>
<td>TLR2</td>
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<tr>
<td>beta</td>
<td>CMV^5</td>
<td>gh and gh glycoproteins</td>
<td>TLR2</td>
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<td></td>
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<td>dsRNA</td>
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<td>Cpg motifs</td>
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<td>22, 28</td>
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<td>gamma</td>
<td>EBV</td>
<td>Unknown viral protein</td>
<td>TLR2</td>
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<td></td>
<td>KSHV^6</td>
<td>Envelope protein</td>
<td>TLR4</td>
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Abbreviations: ^1lipopolysaccharide

Figure 1. Induction of cytokine expression upon TLR-dependent recognition of herpesviruses. TLR-mediated recognition of herpesviruses is essential for the correct development of an antiviral response, and for the coordination of the adaptive immunity. Members from all subfamilies within the Herpesviridae family activate TLRs, triggering a potent immune response. TLR2, present at the cell surface as a dimer with TLR1 or TLR6, recognizes HSV, VZV, CMV and EBV. TLR4, also found at the plasma membrane, interacts with the gammaherpesvirus KSHV. HSV and CMV engage both TLR3 and TLR9 expressed within the endosomal compartment, via recognition of dsRNA and Cpg motifs present on the viral genome respectively. All TLRs contain a cytosolic signaling motif (TIR domain) where specific downstream adapters (MyD88, TIRAP/MAL, TRIF) and signaling transducing proteins are recruited. MyD88 is used by all TLRs, with the exception of TLR3 that signals exclusively through TRIF, and TLR4 that is able to act through MyD88 or TRIF (in combination with TRAM). TLR signaling through MyD88 pathway leads to the activation and nuclear translocation of NF-kappaB, inducing proinflammatory cytokine and chemokine production. TRIF-dependent pathway activation upon stimulation of TLR4 or TLR3 results in inflammatory cytokine expression via NF-kappaB as well as in type I IFN production through IRF activation. In pDCs, stimulation of TLR9 upon viral DNA recognition, causes the expression of high levels of IFN and cytokines through a MyD88- and TRIF-dependent mechanism (discontinuous line).

Running title: TLRs and herpesviruses
Video 1. Migration of primary monocytes towards CXCL12. Freshly isolated monocytes from blood of healthy donors were plated on glass bottom cover slips coated with Poly-D-lysine and VCAM-1. CXCL12b (100 nM) was dispensed from a micropipette with constant backpressure. Time-lapse video was recorded at 10 seconds interval with DIC optics at 63x magnification. 1 representative video of three is shown.

Video 2. Migration of primary monocytes towards SgG2:CXCL12. Freshly isolated monocytes from blood of healthy donors were plated on glass bottom cover slips coated with Poly-D-lysine and VCAM-1. CXCL12b (100 nM) was pre-incubated with SgG2 in a molar ratio 1:50 during 30 min at RT. CXCL12 and SgG2 complex was dispensed from a micropipette with constant backpressure. Time-lapse video was recorded at 10 seconds interval with DIC optics at 63x magnification. 1 representative video of three is shown.

Video 3. Migration of primary monocytes towards SgG2. Freshly isolated monocytes from blood of healthy donors were plated on glass bottom cover slips coated with Poly-D-lysine and VCAM-1. 5 mM SgG2 was dispensed from a micropipette with constant backpressure. Time-lapse video was recorded at 10 seconds interval with DIC optics at 63x magnification. 1 representative video of three is shown.

Video 4. Videomicroscopy of CXCR4 trafficking in mock-treated cells. HEK-293T cells stably expressing ACP-CXCR4 were grown on glass bottom cover slips coated with Poly-D-lysine, and ACP-labelled prior to the stimulation. Cells were mock-treated during 30 min at 37ºC and then recorded at 11-sec intervals, 63x magnification.

Video 5. Videomicroscopy of CXCR4 trafficking in SgG2-treated cells. HEK-293T cells expressing ACP-CXCR4 were grown on glass bottom cover slips coated with Poly-D-lysine, and ACP-labelled prior to the stimulation. Cells were treated with SgG2 for 30 min at 37ºC, and then images were acquired at 11-sec intervals, 63x magnification.

Video 6. Videomicroscopy of CXCR4 trafficking in CXCL12-stimulated cells. HEK-293T cells expressing ACP-CXCR4 were grown on glass bottom cover slips coated with Poly-D-lysine, and ACP-labelled prior to the incubation with the chemokine. The culture was stimulated with 8nM CXCL12 biotin-conjugated, that was previously incubated with Qdots Streptavidin 488 for 10 min RT, in a 1:4 molar ratio. After incubation with the chemokine during 30 min at 37ºC, time lapse was recorded at 11-sec intervals, 63x magnification.

Video 7. Videomicroscopy of CXCR4 trafficking in SgG2:CXCL12-stimulated cells. HEK-293T cells expressing ACP-CXCR4 were grown on glass bottom cover slips coated with Poly-D-lysine, and ACP-labelled. Cells were stimulated with a 100:1 molar ratio of SgG2:CXCL12 biotin-conjugated bound to QDots Streptavidin 488 for 30 min 37ºC. Time lapse was recorded at 11-sec intervals, at 63x magnification.

Video 8. Detail from Video 6.

Video 9. Detail from Video 7.
**Video 10. Videomicroscopy of mock-treated Jurkat cells.** Cells were transduced with Lck<sub>10</sub>mCherry lipid raft marker and electroporated with EGFP-CXCR4. Cells were mock-treated, and images were immediately captured at 31-s intervals at 100x magnification. The movie is displayed at 2 frames s<sup>-1</sup> and features a single confocal section of the three sections that were recorded.

**Video 11. Videomicroscopy of SgG2-stimulated Jurkat cells.** Cells were transduced with Lck<sub>10</sub>mCherry lipid raft marker and electroporated with EGFP-CXCR4. Images were captured at 31-s intervals at 100x magnification. The movie is displayed at 2 frames s<sup>-1</sup> and features a single confocal section out of three that were recorded. CXCR4 clustered into Lck<sub>10</sub>mCherry enriched domains upon SgG2 stimulation.

**Video 12. Videomicroscopy of CXCL12-stimulated Jurkat cells.** Cells were transduced with Lck<sub>10</sub>mCherry lipid raft marker and electroporated with EGFP-CXCR4. Images were captured at 31-s intervals at 100x magnification. The movie is displayed at 2 frames s<sup>-1</sup> and features a single confocal section out of three that were recorded. The intracellular accumulation of CXCR4 was evident in the presence of the chemokine. On the other hand, no apparent incorporation of CXCR4 into Lck<sub>10</sub>mCherry rafts was observed.

**Video 13. Videomicroscopy of SgG2:CXCL12-stimulated Jurkat cells.** Cells were transduced with Lck<sub>10</sub>mCherry lipid raft marker and electroporated with EGFP-CXCR4. Images were captured at 31-s intervals at 100x magnification. The movie is displayed at 2 frames s<sup>-1</sup> and features a single confocal section although different sections were captured at each time to facilitate the identification of the vesicles.

**Video 14. Video microscopy of HEK-293T cells stably expressing Lck<sub>10</sub>mCherry and transiently transfected with EGFP-CXCR4.** Cells were mock-treated during 15 min at 37ºC, and then time-lapse was recorded at 13-sec intervals. Images were taken at 63x magnification.

**Video 15. Video microscopy of SgG2-stimulated HEK-293T cells stably expressing Lck<sub>10</sub>mCherry and transiently transfected with EGFP-CXCR4.** Cells were incubated with SgG2 during 15 min at 37ºC, prior to start recording the cells. Images of the culture were taken every 13 sec at 63x magnification. CXCR4 accumulated in areas where the Lck<sub>10</sub>mCherry lipid raft marker accumulated. Note the presence of double positive spots.

**Video 16. Video microscopy of CXCL12-stimulated HEK-293T cells stably expressing Lck<sub>10</sub>mCherry and transiently transfected with EGFP-CXCR4.** Cells were incubated with 8nM CXCL12 during 15 min at 37ºC, and then time lapse was recorded at a 13-s interval at 63x magnification. No evident colocalization between CXCR4 and the Lck<sub>10</sub>mCherry marker was observed upon stimulation with the low dose of CXCL12.

**Video 17. Video microscopy of SgG2:CXCL12-stimulated HEK-293T cells stably expressing Lck<sub>10</sub>mCherry and transiently transfected with CXCR4-EGFP.** Cells were incubated with SgG2:CXCL12 at a 100:1 molar ratio during 15 min at 37ºC, and then, images were acquired every 13 sec at 63x magnification. Not only the coalescence of CXCR4 and Lck<sub>10</sub>mCherry was evident in the plasma membrane, but also the appearance of double positive vesicles could be observed.
**Video 18. Time lapse analysis of CXCR4 and β-arrestin2 interaction in mock-treated cells.** Jurkat cells were transduced with ACP-CXCR4 and electroporated with GFP-β-arrestin2. Cells were ACP-stained prior to starting time lapse that was recorded at 10sec intervals, 100x magnification. The levels of extracellular CXCR4 were highly decreased due to β-arrestin2 overexpression, which in turn showed a diffuse cytoplasmatic location.

**Video 19. Time lapse analysis of CXCR4 and β-arrestin2 interaction in SgG-stimulated cells.** Jurkat cells were transduced with ACP-CXCR4 and electroporated with GFP-β-. Cells were ACP-stained prior to starting time lapse that was recorded at 10sec intervals, 100x magnification. The levels of extracellular CXCR4 were highly decreased due to β-arrestin2 overexpression. Cell stimulation with SgG2 slightly increased surface CXCR4 in comparison to mock-treated cells while β-arrestin2 cytosolic pattern was altered in comparison to mock-treated cells.

**Video 20. Time lapse analysis of CXCR4 and β-arrestin2 interaction in CXCL12-stimulated cells.** Jurkat cells were transduced with ACP-CXCR4 and electroporated with GFP-β-arrestin2. Cells were ACP-stained prior to starting time lapse that was recorded every 10sec, 100x magnification. Chemokine-stimulation of the cell triggered CXCR4 endocytosis into β-arrestin2 vesicles (indicated by arrows), that disassembled after internalization.

**Video 21. Time lapse analysis of CXCR4 and β-arrestin2 interaction in SgG2:CXCL12 cells.** Jurkat cells were transduced with ACP-CXCR4 and electroporated with GFP-β-arrestin2. Cells were ACP-stained prior to starting time lapse that was recorded at 10 sec intervals, 100x magnification. Note that upon SgG2:CXCL12-activation, individual β-arrestin2 and CXCR4 vesicles that did not co-internalized together were observed (indicated by arrows).