Human cytomegalovirus chemokine receptor US28 induces migration of cells on a CX3CL1-presenting surface

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Human cytomegalovirus (HCMV)-encoded G protein-coupled-receptor US28 is believed to participate in virus dissemination through modulation of cell migration and immune evasion. US28 binds different CC chemokines and the CX3C chemokine CX3CL1. Membrane-anchored CX3CL1 is expressed by immune-activated endothelial cells, causing redirection of CX3CR1-expressing leukocytes in the blood to sites of infection. Here, we used stable transfected cell lines to examine how US28 expression affects cell migration on immobilized full-length CX3CL1, to model how HCMV-infected leukocytes interact with inflamed endothelium. We observed that US28-expressing cells migrated more than CX3CR1-expressing cells when adhering to immobilized CX3CL1. US28-induced migration was G protein-signalling dependent and was blocked by the phospholipase C inhibitor U73122 and the intracellular calcium chelator BAPTA-AM. In addition, migration was inhibited in a dose-dependent manner by competition from CCL2 and CCL5, whereas CCL3 had little effect. Instead of migrating, CX3CR1-expressing cells performed ‘dancing-on-the-spot’ movements, demonstrating that anchored CX3CL1 acts as a strong tether for these cells. At low receptor expression levels, however, no significant difference in migration potential was observed when comparing the migration of CX3CR1- and US28-expressing cells. Thus, these data showed that, in contrast to CX3CR1, which promotes efficient cell capture upon binding to anchored CX3CL1, US28 acts to increase the migration of cells upon binding to the same ligand. Overall, this indicates that infected cells probably move more than uninfected cells in inflamed tissues with high CX3CL1 expression, with soluble chemokines affecting the final migration.

INTRODUCTION

Human cytomegalovirus (HCMV) is a widespread beta-herpesvirus that establishes latent infections in the host. It infects endothelial, epithelial and smooth muscle cells of the upper gastrointestinal, respiratory and urogenital tracts (Landolfo et al., 2003). HCMV infections are often asymptomatic in healthy individuals but may be lethal upon reactivation in immune-compromised patients (Landolfo et al., 2003). Reactivation is believed to occur following differentiation of latently infected monocytes into macrophages, with the subsequent release of infectious virions (Smith et al., 2004; Zhuravskaya et al., 1997). The ability to establish latent infections relies on mechanisms evolved by the virus that allow escape from immune detection through local suppression of immune activation and effective dissemination (Alcami, 2003; Vischer et al., 2007).

The G protein-coupled receptor US28 is one of four receptors (US27, US28, UL33 and UL78) encoded by the viral genome with homology to human chemokine receptors (Chee et al., 1990; Margulies et al., 1996). US28 is known to be important for HCMV infection, and has been proposed to participate in virus dissemination and reactivation. US28 undergoes rapid, ligand-independent, constitutive internalization and recycling (Fraile-Ramos et al., 2001). Thus, one hypothesis is that US28 acts as a decoy receptor that removes danger signals from the infected area by rapid internalization of secreted chemokines, thus negatively interfering with host immune surveillance and activation (Billstrom et al., 1999; Randolph-Habecker et al., 2002). As US28 enhances cell fusions mediated by different viral proteins (Pleskoff et al., 1998), it has also been speculated that US28 participates directly in virus dissemination (Digel et al., 2006). The fact
that the US28 gene is transcribed during latency in infected THP-1 monocytes indicates that it may also play a role in capture and dissemination of latent HCMV (Beisser et al., 2001).

US28 signals in a ligand-dependent as well as a ligand-independent manner through coupling to heterotrimeric G proteins that initiate specific intracellular signalling events depending on the type of G protein contacted. It is known as a broad spectrum chemokine receptor, as it binds many CC chemokines (CCL2, CCL3, CCL4 and CCL5), as well as the CX3C chemokine CX3CL1 (Kledal et al., 1998).

CX3CL1, expressed on activated endothelial cells, plays an important role in immune cell recruitment to sites of inflammation through its interaction with the endogenous CX3CR1 receptor expressed on leukocytes. An important route of systemic virus spread may be through infected leukocytes (Dankner et al., 1990; van der Strate et al., 2003; Smith et al., 2004). As US28 also binds to CX3CL1 but has different properties from CX3CR1, it is likely that HCMV-infected leukocytes behave differently from uninfected cells. Thus, the lower release rate or off-rate of CX3CR1 from CX3CL1 (compared with US28) (Haskell et al., 2000) and the increased signalling properties of US28 are both factors that could influence recruitment and dispersal of infected cells. Knowledge of how cells expressing CX3CR1 or US28 behave when bound to immobilized full-length CX3CL1 may help elucidate the mechanism behind HCMV spread.

In the current study, we investigated the migration of cells expressing the US28 receptor on a CX3CL1-presenting surface as a model of how infected leukocytes may interact with an inflamed endothelium. We used human embryonic kidney (HEK) Flp-In T-Rex-293 cells (Invitrogen) as model cell line to get targeted integration of the receptor constructs, securing persistent tetracycline-inducible gene expression from all clones. These cells express an almost full complement of G protein subunits of each isoform, making them ideal for G protein–coupled receptor expression studies where downstream signalling is important (Atwood et al., 2011). Our study showed that cells expressing the viral US28 receptor migrated on a CX3CL1-presenting surface with significantly higher frequency than cells expressing the endogenous CX3CR1 receptor. CX3CR1-expressing cells had a round shape, whereas US28-expressing cells were elongated and left trails of cytoplasmatic material behind when they migrated. A non-internalized deletion mutant of the US28 receptor, US28Δ300, which is fully capable of signalling, elicited migration to a similar extent to US28, indicating that further increasing the cell-surface receptor concentration in cells already expressing high receptor levels did not stimulate migration further. In contrast, at reduced receptor expression levels (lower tetracycline concentrations), a difference in migration pattern between US28- and CX3CR1-expressing cells was no longer observed. When comparing the migration of cells expressing either receptor, on a tissue culture polystyrene (TCP) surface in the absence of CX3CL1, both cell types migrated equally. Taken together, our data indicated that CX3CL1 acts as a tether for CX3CR1-expressing cells in contrast to its role in inducing migration of US28-expressing cells. The migration of US28-expressing cells was inhibited by the phospholipase Cβ (PLCβ) inhibitor U73122 and the intracellular calcium chelator BAPTA-AM. CCL2 and CCL5 also inhibited migration in a dose-dependent manner, in contrast to CCL3, which did not affect migration. Our results indicate that US28 signalling induces migration of infected cells, but also suggest that the final migration potential of US28-expressing cells depends on the combination and concentrations of ligands in the cellular microenvironment.

RESULTS

Inducible receptor expression

Cloning of genes encoding CX3CR1, its viral counterpart US28 and a C-terminal deletion mutant of US28 (US28Δ300), all fused to an N-terminal haemagglutinin (HA) tag, was carried out to facilitate detection of protein expression by stably transfected HEK-293 cells. Tetracycline-induced protein expression from these cell lines was investigated by Western blotting using antibodies against the HA tag and antibodies recognizing glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal standard. Fig. S1 (a–c; available in JGV Online) shows dose–response curves of HA–CX3CR1, HA–US28 and HA–US28Δ300 expression as a function of tetracycline concentration at a fixed time (24 h) after induction. Tetracycline stimulation at 0.25 μg ml⁻¹ for 24 h resulted in a saturation level of HA–CX3CR1, HA–US28 and HA–US28Δ300 expression. Fig. S1(d–f) shows the time-dependent receptor expression after stimulation with 0.5 μg tetracycline ml⁻¹. At 4–8 h after tetracycline addition, HA–CX3CR1 and HA–US28 expression reached half their respective saturation levels, whilst 12 h of tetracycline stimulation resulted in full induction. HA–US28Δ300 expression did not reach a maximum within 24 h at 0.5 μg tetracycline ml⁻¹.

Receptor localization

Receptor localization was investigated by flow cytometry. Two sets of stainings were carried out: one for detection of total receptor expression (membrane localized and internal) and another for detection of membrane-localized receptors only. Total expression of HA–US28 and HA–US28Δ300 far exceeded the expression of HA–CX3CR1 (Fig. S2, right panel), but the amount of membrane-localized receptors was almost equal when comparing HA–US28 and HA–CX3CR1, reflecting the high constitutive internalization activity of HA–US28. As expected, the internalization-deficient receptor mutant HA–US28Δ300
localized to the membrane in much higher amounts compared with HA–US28 (Fig. S2, left panel).

Receptor function
The ability of CX3CR1, US28 and the US28 deletion mutant US28A300 to bind to CX3CL1 was tested to verify receptor function. Adsorbed capture IgG (anti-His IgG) with bound His-tagged CX3CL1 served as a positive control, PBS as a blank control and adsorbed capture IgG alone as a negative control. Control cells (no receptor induction) did not bind to any of the wells, whereas cells in which receptor expression had been induced (0.5 μg tetracycline ml⁻¹, 24 h) bound to wells with captured CX3CL1 but not to the blank or negative-control wells (Fig. S3). All three receptors, CX3CR1, US28 and US28A300, retained their ability to bind to CX3CL1 in the presence of the HA tag.

US28-expressing cells migrate more than CX3CR1-expressing cells on anchored CX3CL1
Time-lapse experiments revealed a clear difference in the movement of cells expressing CX3CR1 and US28 when seeded on a CX3CL1-modified surface. Only cells induced to express the respective receptors adhered to the surface and only to surfaces with both capture antibody and CX3CL1. US28-expressing cells moved more than CX3CR1-expressing cells (Fig. 1a, d, e), with movement being defined as a mean speed of ≥0.2 μm min⁻¹. Many of the CX3CR1-expressing cells perform ‘dancing-on-the-spot’ movements, i.e. small movements back and forth, without actually migrating away from their original starting point. Including a migration criterion that required the cells to move ≥25 μm (approx. two cell lengths) away from their origin over the period of tracking to count as migrating allowed discrimination between cells that were truly migrating and cells that performed ‘dancing-on-the-spot’ movements. Due to their high migration frequency, US28-expressing cells are sickle shaped.

US28-expressing cells are sickle shaped
The morphology of CX3CR1- and US28-expressing cells seeded on a CX3CL1-modified surface is shown in Fig. 3. At a high tetracycline concentration (0.5 μg ml⁻¹), CX3CR1 cells had a round morphology (Fig. 3a), whereas US28 cells were generally elongated with curvature (Fig. 3d). At decreased receptor expression levels, CX3CR1-expressing cells got more elongated (Fig. 3b), whereas US28-expressing cells started to display a round morphology (Fig. 3e). In the absence of tetracycline, neither cell type adhered to the surface (Fig. 3c, f).

Differential ligand binding controls migration of US28-expressing cells
CX3CR1 and US28 rely on different G proteins for their signalling and thus activate different, partially overlapping, intracellular signalling pathways. CX3CR1 is known to signal through pertussis-toxin (PTX)-sensitive Gαi proteins upon CX3CL1 ligation (Goda et al., 2000; Imai et al., 1997; Volin et al., 2007). In our setup, PTX had no significant effect on the migration of any of the cells, regardless of receptor type expressed (data not shown). The PLCβ inhibitor U73122 is known to influence signalling from many receptors that converge on PLCβ. U73122 completely blocked the migration of US28-expressing cells at a concentration of 0.5 μM (Fig. 4a). The effect of U73122 (0.5 μM) on migration was also tested on all three cell types in parallel. The inhibitor reduced the frequency of migration induced by both US28 and US28A300 by 80–90%, whereas the initially much lower migration frequency
Fig. 1. Time-lapse microscopy recording of cell motility on CX3CL1-modified surfaces. Cells were tracked every 5 min for 13.75 h. (a) US28- and US28Δ300-expressing cells migrate with similar frequency but at a much higher frequency than CX3CR1-expressing cells according to a defined migration criterion of a speed ≥0.2 μm min⁻¹ (P=0.011 and P=0.013, respectively; t-test). All data represent the mean±SEM of three independent experiments. (b) The difference in migration frequency was even more pronounced when applying an additional migration criterion that required the cells to have moved ≥25 μm away from their initial location to count as migrating. (c) Snapshots of US28-expressing cells at different time points. Cells expressing US28 and US28Δ300 cluster upon receptor induction (0 min) but gradually move apart. Thus, the total cell number at the end of the experiment is frequently much higher than that at the start when studying these cell types (see Table S1). (d–f) Typical spiderweb diagrams (the initial position of all analysed cells is located at the origin of coordinates) depicting CX3CR1, US28 and US28Δ300 cell movement (tick mark represents 100 μm), with a detail of a photograph of cells from the respective experiments given below. Bar, 100 μm.
Thus, migration of US28-expressing cells was strongly dependent on PLC\(\beta\) activation.

Ligand-dependent US28 signalling induces transient changes in the intracellular calcium ion concentration, \([Ca^{2+}]_i\) (Stropes \textit{et al.}, 2009). To test whether changes in \([Ca^{2+}]_i\) are important for US28-induced cell migration and thus whether the observed migration is ligand dependent, we used the intracellular calcium chelator BAPTA-AM in time-lapse experiments. BAPTA-AM blocked US28-induced migration from 65 % migration in control cells (treated with DMSO) to 7 % in BAPTA-AM-loaded cells at a concentration of 50 \(\text{mM}\) (Fig. 5), clearly indicating that ligand-dependent signalling was important for the observed migration. BAPTA-AM completely blocked the mobilization of calcium as investigated in cells loaded with Fluo4, a green-fluorescent calcium indicator (data not shown).

As US28 is a broad chemokine receptor with many ligands, the presence of other chemokines with an affinity for US28 might influence the migration of cells already attached to a CX3CL1 surface. In contrast to CCL3, which had no effect on migration, CCL2 and CCL5 inhibited US28-induced migration on a CX3CL1 surface in a dose-dependent manner (Fig. 6), with CCL2 being the most potent. We also tested the effect of adding soluble CX3CL1 (sCX3CL1) to the migration assay. As sCX3CL1 efficiently competed with surface-bound CX3CL1 for binding to the cells, the presence of this ligand greatly affected cell attachment to the substratum, which was not the case for CCL2. At sCX3CL1 concentrations >100 \(\text{ng ml}^{-1}\), the cells detached from the surface, and at a concentration of 10 \(\text{ng ml}^{-1}\), they were only very loosely attached. Thus, in contrast to CCL2, sCX3CL1 seemed to affect migration primarily through inhibition of cell-surface attachment.

**DISCUSSION**

Leukocyte adhesion to the endothelial lining and migration into extravascular tissue are central processes involved in immune surveillance and host defence. Following infection, immune cells (residing in the infected tissue) release substances that activate the endothelial cells of nearby blood vessels. Once activated, the endothelial cells express several molecules that participate in the recruitment of circulating leukocytes into sites of infection. These molecules include selectins, integrins and chemokines such as CX3CL1 (Tarrant & Patel, 2006). CX3CL1 is believed to play a special role in leukocyte recruitment, as it functions as both adhesion molecule and chemokine for circulating leukocytes.
Like other chemokines, sCX3CL1 triggers firm adhesion of leukocytes upon binding to CX3CR1 through inside-out activation of integrins on the same cells (Goda et al., 2000; Kerfoot et al., 2003). In addition, the membrane-anchored version of CX3CL1 (expressed by the endothelium), through binding to CX3CR1, is believed to play a direct role in leukocyte capture independent of integrin activation (Fong et al., 1998, 2002; Goda et al., 2000; Green et al., 2006; Haskell et al., 1999, 2000; Kerfoot et al., 2003; Umehara et al., 2001).

In the current study, we investigated the effect of CX3CR1 and US28 expression on random cell migration on a CX3CL1-modified surface. CX3CL1 was presented in its stalk version to mimic the chemokine form that leukocytes experience when encountering an inflamed endothelium (Bazan et al., 1997). We showed that expression of either of the two receptors, CX3CR1 or US28, mediated cell adhesion to a CX3CL1-modified surface but that only cells expressing US28 migrated to any significant degree on this surface. Interestingly, at reduced receptor expression levels (low tetracycline concentration), there was no statistically significant difference in migration potential between cells expressing CX3CR1 and US28, and the cells may be approaching the intrinsic migration level of HEK293 cells. Similarly, it was shown that US28- and CX3CR1-expressing cells migrated to the same extent on a TCPS surface in the absence of CX3CL1. CX3CR1-transfected cells without induction of receptor expression displayed a migration pattern similar to cells expressing either receptor. Unexpectedly, US28-transfected cells without receptor expression migrated significantly less in the same setup. This might be explained by clonal variation. That the background level of migration was indeed lower in US28-expressing cells only strengthens our data, and the fact that US28-expressing cells migrated more than the same cells without induction of receptor expression in the absence of ligands is in agreement with the ability of the US28 receptor to signal in a ligand-independent manner. As US28 is constitutively internalized, a large proportion of the total receptor pool is stored inside the cell at any given moment. To investigate whether a further increase in receptor concentration at the membrane (above what could be achieved with wild-type US28) could influence migration, either by increasing the number of cell attachment sites or through increased ligand-induced signalling, a non-internalized deletion mutant of the US28 receptor, US28A300, was included. US28A300-expressing cells adhered to and migrated on a CX3CL1-modified surface to the same extent as US28-expressing cells. Thus, the sum of receptor–ligand interactions had no effect on adhesion-dependent migration of transfected cells already expressing high levels of chemokine receptors. We have previously published data on the capture of CX3CR1-expressing cells on a surface-attached gradient of CX3CL1 presented to cells by the same method used here. In this study, it was discovered that the cells that bound to the gradient also internalized the CX3CL1 presented to them; thus, the presentation of His-tagged CX3CL1 via anti-His IgG did not seem to preclude receptor–ligand internalization (Hjortø et al., 2009).

Our study is the first study to investigate the random migration of cells on a surface modified to present full-length CX3CL1, as former studies have used Transwell assays to investigate chemotaxis of CX3CR1- or US28-expressing cells induced by gradients of sCX3CL1. sCX3CL1 is released from the endothelial cells by constitutive and inducible protease cleaving of the membrane-attached stalk version (Tsou et al., 2001), but, as it binds poorly to glycosaminoglycans lining the endothelium, it is probably the membrane-bound stalk version of CX3CR1 that mediates cell capture (Patel et al., 2001). It is well known that sCX3CL1 plays a role in inducing directed migration of leukocytes, although the potency of this chemokine compared with, for example,
CCL2 has been debated. Thus, in a study performed by Imai et al. (1997), only a small percentage of CX3CR1-positive leukocyte subsets migrated towards sCX3CL1, which correlates well with a study performed by Umehara et al. (2001), where sCX3CL1 had little influence on THP-1 cell migration compared with CCL2, despite the high constitutive CX3CR1 expression found in these cells (Shulby et al., 2004). In contrast to sCX3CL1, our data suggested that the primary role of membrane-anchored CX3CL1 is to capture activated immune cells (expressing CX3CR1) on the inflamed endothelium, rather than to mediate migration. Thus, we were able to show that CX3CR1-expressing cells only migrated to a small extent on surface-immobilized CX3CL1. In contrast, US28-expressing cells migrated significantly more, supporting a role of US28 in promoting migration, in addition to providing adhesion. Full-length CX3CL1 is well known to promote stronger adhesion of CX3CR1-expressing cells than US28-expressing cells, due to a slower off-rate of the CX3CR1 receptor from full-length CX3CL1 (Haskell et al., 2000). The fact that US28 expression strongly stimulated cell migration on a CX3CL1-presenting surface compared with CX3CR1 indicated that infected cells that express high levels of US28, possibly competing with CX3CR1 for binding to CX3CL1 on the inflamed endothelium, may be relieved from the capture effect, which could allow infected cells to move more freely.

In our setup, the migration frequency of US28-expressing cells was reduced by the PLCβ inhibitor U73122 to a level of migration similar to the migration frequency observed for CX3CR1-expressing cells. U73122 had no effect on migration of CX3CR1-expressing cells. Like U73122, the intracellular calcium chelator BAPTA-AM also inhibited US28-mediated migration, supporting the suggestion that activation of PLCβ and changes in $[Ca^{2+}]_{i}$ are essential for the US28-mediated migration process. BAPTA-AM was found to completely block mobilization of calcium, as investigated in Fluo4-loaded cells. As US28 only induces changes in $[Ca^{2+}]_{i}$ upon ligand binding and blocking of intracellular calcium signalling by BAPTA-AM inhibits migration, we assume that CX3CL1 binding to US28 plays an essential role in the observed migration of US28-expressing cells.

**Fig. 4.** (a) Concentration-dependent effect of the PLCβ inhibitor U73122 on migration of US28-expressing cells. U73122 significantly blocked migration at 1 μM ($P=0.003$) and 0.5 μM ($P=0.007$) (t-test). (b) Effect of U73122 (0.5 μM) on migration of different receptor-expressing cells. U73122 had no measurable effect on CX3CR1-induced migration ($P=0.97$) but blocked migration of US28A300-expressing cells ($P=0.002$) and US28-expressing cells ($P=0.00002$) to a similar extent. All data points represent the mean ± SEM of three independent experiments.

**Fig. 5.** The intracellular calcium chelator BAPTA-AM inhibits US28-induced migration. There was a tendency towards dose dependency, but at the concentrations tested (1, 10 and 50 μM), migration was only significantly inhibited at a concentration of 50 μM ($P=0.02$). All data points represent the mean ± SEM of three independent experiments.
US28 has been shown by others to strongly influence signalling of infected cells and to be directly responsible for increased migration of smooth muscle cells (SMCs) upon HCMV infection (Streblow et al., 1999). Migration of HCMV-infected SMCs is dependent on endogenous CCL2, produced in high amounts by these cells. Interestingly, deletion of the US28 cassette from the viral genome resulted in a tenfold reduction in the migration of infected cells. Migration could be restored by co-infection with an adenovirus expressing US28 (Ad-US28), but not by co-infection with adenovirus expressing another chemokine receptor, Ad-CCR5 (Streblow et al., 1999). Infection with Ad-US28 itself induced migration to the same extent as that observed in HCMV-infected cells. Interestingly, whereas both CCL2 and CCL5 stimulated US28-mediated migration of SMCs (Streblow et al., 1999; Vomaske et al., 2009), sCX3CL1 did not. sCX3CL1 was actually shown to inhibit CCL5-induced US28-mediated migration (Vomaske et al., 2009), indicating that these ligands stimulate different signalling pathways. In contrast to its effect on SMCs, sCX3CL1 was shown to induce a dose-dependent increase in US28-mediated cell migration in a macrophage cell line of rat origin, infected with Ad-US28 (Vomaske et al., 2009). CCL5 stimulation of migration was small in this setup, and CCL5 was surprisingly found to compete with sCX3CL1 and inhibit sCX3CL1-induced migration. We performed an experiment with increasing amounts of CCL2, CCL3 or CCL5 in the migration assay to test whether other US28 ligands had any effect on US28-induced migration in our setup. CCL3 did not affect migration, whereas CCL2 and CCL5 displayed a dose-dependent inhibitory effect.

Thus, it is possible that HCMV-infected (US28-positive) leukocytes adjust their migration according to changing combinatorial chemokine concentration levels in the surroundings. There is a possibility that high concentrations of immune cell-specific chemokines, indicative of intense immune surveillance, may inhibit migration, with US28 acting more as a decoy receptor than a chemokine receptor under such conditions.

Our results do not rule out a contribution from a ligand-independent effect, as ligand-independent US28 signalling as well as CX3CL1-stimulated, US28-induced calcium signalling are both PLCβ-dependent and PTX-insensitive processes (Casarosa et al., 2001; Stropes et al., 2009). CX3CL1-induced signalling is essential for migration of US28-expressing cells on a CX3CL1 surface, as the process was found to be calcium dependent, and calcium release from intracellular stores is known to be ligand dependent (Stropes et al., 2009). Further studies are needed to determine whether the US28-mediated migration we observed was primarily CX3CL1 driven or was a combined effect of ligand-dependent and -independent signalling events. CX3CR1-expressing cells migrated at a much lower frequency than US28-expressing cells, and migration was not affected by PTX. At decreased receptor expression levels, the difference in migration potential between CX3CR1- and US28-expressing cells was no longer significant.

In summary, our results showed that US28 expression increased the migration of cells on a CX3CL1-presenting surface compared with CX3CR1, indicating that uninfected cells move less and thereby have fewer cell encounters than infected cells. It could be speculated that the increased number of cell encounters experienced by US28-expressing cells might be another mechanism that has evolved to increase virus spread.

**METHODS**

**Vector constructs.** To construct HA–CX3CR1, the gene encoding CX3CR1 was isolated by PCR from pcDNA3.1 with the following primers: forward: 5'-TAGAAGCTTCCATGAGCTAGTCCCT-3' and reverse: 5'-ACCTCTTCATGAGAAGGACAAAGCATCAG-3' (restriction sites underlined). The PCR product was cloned into pcDNA 5/FRT/TO (Invitrogen). CX3CR1 containing an N-terminal HA tag was generated by PCR on the 5/FRT/TO-CX3CL1 plasmid with the following primers: forward: 5'-TAGAAGCTTCCACCGGTACGATCCCT-3' and reverse: 5'-GATGCGGCCGCTACATGATCCCT-3' [the sequence in italics is the ATG start codon, followed by the HA tag sequence encoding YPYDVPDYA, followed by the sequence encoding the first four amino acids after methionine in CX3CR1: DQFP plus the first nucleotide of the fifth amino acid] and cloned into the 5/FRT/TO-CX3CL1 plasmid cut with the same enzymes to remove the old N-terminal part of the CX3CR1 gene.
HA–US28 and HA–US28A300 (generated as described previously; Fraile-Ramos et al., 2001; Waldhoer et al., 2003) were cut out of pTEJ8 and cloned into pcDNA 5/FRT/TO.

**Stable transfections.** Stable, inducible clones of HA–CX3CR1-, HA–US28- and HA–US28A300-expressing cells were generated by co-transfecting Flp-In T-Rex-293 cells (Invitrogen) with pOG44 (Invitrogen) and pcDNAs/FRT/TO receptor constructs. As the T-Rex-293 genome and pcDNA 5/FRT/TO vector both contain an FRT (Flp recombinase target) site, co-transfections of receptor constructs with pOG44 (for transient expression of the Flp recombinase) results in targeted integration of the receptor constructs. Targeted integration at the FRT site brings the receptor gene under the control of the tetracycline repressor/operator system for easy and efficient expression regulation by externally added tetracycline. Stable transfected cells were analysed for expression of the recombiant proteins by Western blot analysis.

**Cell culture.** Cells were grown in a humidified incubator (37 °C, 5 % CO2) in DMEM (Lonza) with 10 % FBS, 10 000 U penicillin ml−1 and 10 000 mg streptomycin ml−1. For the stimulation of gene expression, tetracycline (Invitrogen) was added to the medium 1 day after seeding, and the cells were cultured for varying time periods after induction.

**Western blotting.** Lysates from stable transfected HEK Flp-In T-Rex-293 cells stimulated with various concentrations (0.015–1 μg ml−1) of tetracycline or with 0.5 μg tetracycline ml−1 for increasing time periods were analysed by Western blotting using 4–12 % Bistris Gels (Nupage; Invitrogen). Proteins were transferred to nitrocellulose membranes (Amersham Biosciences, GE Healthcare), probed with antibodies against HA and then against GAPDH, stripping the membranes in between. The primary antibodies used were mouse anti-HA (clone 16B12; Covance) or mouse anti-GAPDH (clone 6C5; BioRad) antibodies against HA and then against GAPDH, stripping the membranes in between. Bands were visualized using chemiluminescence substrate (Pierce) and a cooled CCD camera (BioChem camera and BioSpectrum Darkroom). Digital images were used to quantify the proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by Western blot analysis.

**Flow cytometry.** Control cells (no stimulation) and receptor-expressing cells (0.5 μg tetracycline ml−1 for 24 h) were rinsed in PBS, detached in EDTA solution and pelleted in DMEM, then washed and resuspended in FACS buffer (1 % BSA, 0.05 % sodium azide in PBS). Half of each cell solution was transferred to a new vial for permeabilization. The cells were washed in PBS, fixed in 4 % formaldehyde (in PBS) for 10 min, washed and permeabilized in 0.2 % Triton X-100 (in PBS) on ice for 20 min, and finally washed twice in FACS buffer. All cell solutions were incubated with the mouse mAb against the HA tag (1:1000, 60 min, 4 °C). Unbound antibodies were removed and the cells were washed with FACS buffer and incubated with FITC-conjugated goat anti-mouse IgG (1:1000 dilution, Santa Cruz Biotechnology) (30 min, 4 °C, light protected). After secondary-antibody removal, the cells were washed twice with FACS buffer and analysed for fluorescence (FACSDiva; BD Biosciences).

**Static adhesion assay.** Four wells of an eight-well glass chamber slide (Nunc) were coated overnight with mouse anti-His mAb (clone AD1.1.10; R&D Systems) in PBS (10 μg ml−1, 4 °C). Two wells were incubated with PBS alone. All wells were washed twice with PBS and incubated with 400 μl buffer (RPMI containing 1.0 mg ml−1 BSA, 10 mM HEPES (pH 7.4)) for 1 h at room temperature. After buffer removal, 10 nM of human CX3CL1 with a C-terminal 6×His tag (R&D Systems), was added to two of the wells with pre-adsorbed anti-His antibody (200 μl per well in buffer). The remaining wells were incubated with buffer alone (1 h, room temperature). Finally, all wells were washed twice with buffer. Control cells (no stimulation) and receptor-expressing cells (0.5 μg tetracycline ml−1, 24 h) were rinsed in PBS, detached in EDTA solution and pelleted in DMEM. The cells were then washed and resuspended in buffer at a concentration of 1 × 106 cells ml−1. An aliquot (150 μl) of each cell suspension was added to three wells in an eight-well chamber slide coated as follows: (i) blank (PBS), (ii) capture IgG (anti-His IgG) and (iii) CX3CL1 (C-terminal His-tagged CX3CL1 captured by pre-adsorbed anti-His IgG). The cells were incubated at 37 °C for 30 min in a CO2 incubator. Non-adherent cells were removed by three washes in PBS. Cells were fixed with 1 % glutaraldehyde (Sigma) in Hanks' balanced salt solution (15 min) and stained with 0.1 % crystal violet solution (Sigma) in water (30 min). The cells were washed in water and the number of adherent cells was counted at 50 × magnification at five pre-defined positions in each well.

**Time-lapse experiments.** Eight-well glass chamber slides were coated with the mouse anti-His mAb in PBS (10 μg ml−1, 4 °C, overnight). After washing twice with PBS, human CX3CL1 with a C-terminal 6×His tag was added (final concentration 10 nM in PBS; 200 μl per well). After 1 h at room temperature, the wells were washed twice with PBS. Control cells (no stimulation) and receptor-expressing cells (0.5 μg tetracycline ml−1, 24 h) were rinsed in PBS and detached in EDTA solution. The cells were pelleted in DMEM, washed and resuspended in DMEM at a concentration of ~8000 cells ml−1 before seeding. When using U73122 (Calbiochem), the inhibitor was only present during the time-lapse experiment, and control cells were treated with DMSO. When using BAPTA-AM (Calbiochem), cells were suspended in serum-free medium and incubated with various amounts of BAPTA-AM or DMSO for 1 h at room temperature. The cells were pelleted, resuspended and seeded in fresh medium. sCX3CL1, CCL2, CCL3 and CCL5 were from R&D Systems and were present throughout the experiments when used.

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**REFERENCES**


