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# Immunomodulation by herpesvirus U51A chemokine receptor *via* CCL5 and FOG-2 down-regulation plus XCR1 and CCR7 mimicry in human leukocytes

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Human herpesvirus-6A (HHV-6A) betachemokine-receptor U51A binds inflammatory modulators CCL2, CCL5, CCL11, CCL7, and CCL13. This unique specificity overlaps that of human chemokine receptors CCR1, CCR2, CCR3, and CCR5. In model cell lines, expression leads to CCL5 down-regulation with both constitutive and inducible signaling. Here, immunomodulation pathways are investigated in human leukocytes permissive for infection. Constitutive signaling was shown using inositol phosphate assays and inducible calcium signaling by response to CCL2, CCL5 and CCL11. Constitutive signaling targets were examined using an immune response-related microarray and RT-PCR, showing down-regulation of CCL5 and FOG-2, a hematopoietic transcriptional repressor. By RT-PCR and siRNA reversion, CCL5 and FOG-2 were shown down-regulated, during peak U51A expression post infection. Two further active ligands, XCL1 and CCL19, were identified, making U51A competitor to their human receptors, XCR1 and CCR7, on T lymphocytes, NK and dendritic cells. Finally, U51A-expressing cell lines and infected *ex vivo* leukocytes, showed migration towards chemokine-gradients, and chemokine internalization. Consequently, U51A may affect virus dissemination or host transmission by chemotaxis of infected cells to sites of chemokine secretion specific for U51A (for example the lymph node or lung, by CCL19 or CCL11, respectively) and evade immune-effector cells by chemokine diversion and down-regulation, affecting virus spread and inflammatory pathology.

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## Introduction

Interaction of chemokines and their receptors on leukocytes are essential for mediating inflammatory responses to infection. Blood-borne viruses encode proteins, which have subverted this system to facilitate infection, and some of these may have applications as novel immunomodulators. Human herpesvirus 6 (HHV-6), together with human cytomegalovirus, are members of the betaherpesvirus subgroup that cause widespread, persistent infection. Under immune suppressive/aberrant settings, HHV-6 reactivates and is associated with inflammatory conditions, including encephalitis and transplantation diseases; it has also been linked with multiple sclerosis. HHV-6 homologues of human chemokines and their receptors mimic the inducible inflammatory class of chemokines and thus are major candidates for virulence factors mediating HHV-6-associated inflammatory pathology. HHV-6

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**Abbreviations:** **ECB:** extracellular medium buffer · **HHV:** human herpesvirus · **GPCR:** G protein-coupled receptors · **InsP:** inositol phosphate

exists in two strain groups, termed variant A and variant B (HHV-6A and HHV-6B). HHV-6A has been shown to be more neurotropic and has been linked with neuroinflammatory diseases, including chronic fatigue and multiple sclerosis, as well as more severe pathologies from virus reactivations in immunosuppressed transplantation patients with occasional fatal outcomes [1]. We have also shown that there are population biases with childhood infection with variant A more prevalent in southern Africa, but less frequent in north America or Europe [2–4]. Therefore, neuroinflammatory diseases linked with HHV-6A can present as emergent infections, but little is known of molecular mechanisms by which the virus may modulate immunity and inflammation. Furthermore, due to the efficient infant infection and lifetime persistence of the virus in populations worldwide, HHV-6 viral genes, from either A or B variants, effectively present as alternative human alleles. Moreover, recent results suggest that these viruses may even integrate in germ cells of 0.2 to 0.8% of different populations [5, 6]. Expression of select viral genes from either persistent infections or integrated virus genomes can have potent effects. Our recent studies have shown that the HHV-6A chemokine U83A has a completely different specificity from HHV-6B U83B. U83A has a broad but selective reactivity with receptors present on immune effector cells, including activated T lymphocytes, monocyte/macrophage and dendritic cells, whilst U83B is only specific for basal monocytes [7]. Here, we examine activities and effects of signaling on human leukocytes of the HHV-6A U51A chemokine receptor and show how it can also dysregulate the immune response.

The β-herpesviruses have two conserved sets of G protein-coupled receptors (GPCR) genes defined by their similarities as gene positional homologues and the encoded seven-transmembrane GPCR structure. This gene family can be further subdivided between the homologues in the 'Cytomegaloviruses' or β1-herpesvirus, UL33 and UL78, compared to those in the 'Roseoloviruses' (HHV-6 and HHV-7) or β2-herpesvirus, U12 and U51, respectively. Unlike the CMV gene families, where some members have been shown to signal only constitutively with no known ligands [8–11], the HHV-6 and HHV-7 GPCR genes U12 and U51 encode chemokine receptors, which bind betachemokine ligands [12–15]. HHV-6B U12 binds efficiently CCL2/MCP1, CCL5/RANTES, and CCL4/MIP-1β, while HHV-7 U12 binds CCL19/MIP-3β. Both HHV-6 and HHV-7 U12 also show inducible calcium signaling [12, 14]. In contrast, we have shown that HHV-6A U51A despite only very distant similarity to either viral or cellular chemokine receptors, has a novel specificity for CCL5 with competitive binding to CCL2, CCL11/eotaxin, CCL7/MCP-3 and CCL13/MCP-4. Therefore, U51A is distinct from other viral or cellular chemokine receptors, overlapping the combined activities of CCR1, CCR2, CCR3, and CCR5 [13]. Furthermore, U51A also appears to signal in cell lines of epithelial and hematopoietic origin, showing shape changes and effects on CCL5 expression; however, exact pathways have not been defined [13].

Constitutive signaling is a hallmark of many viral GPCR, including chemokine receptors, distinct from the cellular homologues, which show mainly inducible mechanisms. Moreover, recent studies using model transient expression systems (simian fibroblasts) have shown that HHV-6 U51A can signal by both inducible and constitutive pathways [16]. Constitutive expression in stable cell lines resulted in down-regulation of CCL5 expression [13]. Interestingly, for inducible signaling, the binding of each ligand was shown to sort signals selectively to different G protein-mediated pathways [16].

In this report, these signaling pathways are further investigated in the natural target cell types for HHV-6, in both U51A-expressing stable cell lines and infected leukocyte cultures. The results show both constitutive and inducible signaling, giving rise to both chemokine- and hematopoietic cell-specific regulatory gene modulation. Furthermore, additional ligands are identified, which may affect the chemokine response to infected cells and mediate homing of infected cells as demonstrated by altered chemotaxis of infected *ex vivo* human primary monocytic cells.

The specificity and interactions of the U51A receptor on human leukocytes are characterized. The functionality of these interactions is demonstrated by chemotaxis in expressing cell lines and infected PBL, which may affect virus dissemination and transmission. Interestingly, we show that viral U83A cannot bind U51A, but that binding of human chemokines promotes receptor internalization. Consequences of signaling are investigated by microarray-based studies and examination of effects on movement of infected cells in response to exogenous ligands with implications for immune dysregulation coinciding with effects on virus dissemination, reactivation and host transmission.

## Results

### U51A expression in human leukocytes

HHV-6A infects human leukocytes. To investigate U51A chemokine receptor constitutive or inducible properties in these natural targets, several different cell lines and primary cell systems were used. The human leukocyte cell line K562 (pre-monocytic and pre-erythroid) was used to assess U51A-specific functions (in stable transfectants), as this cell line does not express competing human chemokine receptors. Human $CD4^+$

 

T leukocyte cell lines, JJhan or Hut78, were used to investigate U51 properties during infection time course, as these lines are the most fully permissive for HHV-6A replication, ensuring adequate U51A expression. Hut78 were used to examine effects on CCL5 regulation because JJhans do not express this chemokine gene. Finally, to examine effects of expression on cell migration *in vivo*, human *ex vivo* PBL were used in infection studies, as the appropriate animal models are not available.

## U51A inducible and constitutive signaling in expressing human hematopoietic cells

Previously, initial HHV-6A U51A chemokine receptor specificity was performed by binding studies on stable human cell lines, K562, expressing U51A [13]. These cells of pre-erythroid-myeloid lineage can be infected persistently by HHV-6. Calcium-mobilization experiments were undertaken to directly investigate inducible signaling by this receptor in permissive cells compared to previous results using a transiently expressing model cell system (COS-7 cells, simian fibroblasts), which are non-permissive for this human leukocyte-specific virus [16]. Using a fluorescent digital imaging system (MagiCal, Applied Imaging), responses of individual cells to chemokines CCL2, CCL5 and CCL11 were investigated. The results showed inducible calcium mobilization in the U51A-expressing cell line (Fig. 1A) whereas the cell line transfected with the vector only, pCDNA3, showed no response (CCL11 shown in Fig. 1A). Negative controls were single stimulus with either buffer only or CCL3, which does not bind U51A; neither showed a response (not shown). Inducible signaling was observed in early log phase of the cell cycle at early cell passage, where all cells were responsive (Fig. 1B). Analyses of the U51A cell line using inositol phosphate (InsP) assays indicated that U51A in these human cells could also signal constitutively (Fig. 2) as described previously for the transfected COS-7 cells [16]. Furthermore, stimulation with CCL5 could enhance this signaling in the U51A-expressing cells, but not the vector only cell line (Fig. 2).

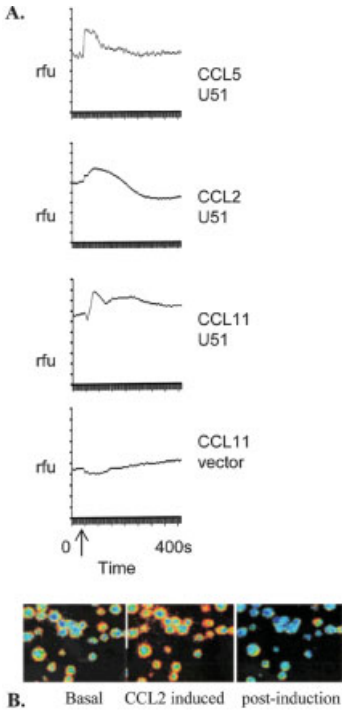


**Figure 1.** Inducible signaling by calcium mobilization in U51A cell line. (A) Scales are y axis expressed in relative fluorescence units (rfu) representing 0 to 400 nM $Ca^{2+}$, x axis 0 to 400 s, chemokine added after 50 s as indicated by the arrow. The first three graphs show stable U51A-expressing K562 cell line transfectants, stimulated with 50 nM CCL5, 50 nM CCL2 and 50 nM CCL11, respectively. The last panel shows vector plasmid only (pCDNA3) K562 cell line transfectant treated with 50 nM CCL11. The responses shown are from representative single cells from similar responses measured for 10 to 20 individual cells per view, all cells surveyed responded. Each panel is an example of four separate experiments. (B) The micrograph shows the cellular response to CCL2 with a color scale representing relative calcium concentrations corresponding to the concentration scales in the graphs (blue-low, yellow-intermediate, red-high), showing basal levels, before ligand addition, induction after ligand addition, followed by desensitization.

## U51A constitutive signaling targets shown by microarray analyses of human hematopoietic cells

To specifically identify targets of constitutive signaling, array analyses were performed on non-induced cells

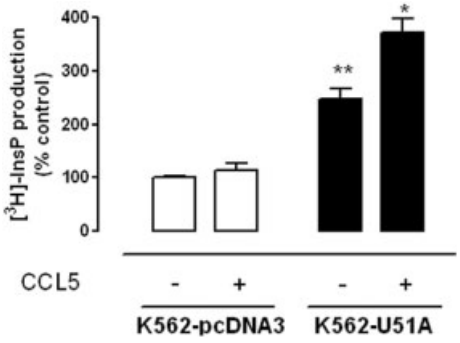


**Figure 2.** Constitutive and CCL5-stimulated InsP. K562-U51A cells showed significantly higher basal (**$p<0.05$, $n = 3$) InsP levels than K562-pcDNA3 (vector only) cells. Further, CCL5 stimulated K562-U51A cells are significantly higher than unstimulated K562-U51A cells (*$p<0.05$, $n = 3$). In contrast, InsP levels in K562-pcDNA3 cells treated with CCL5 were not significantly different from those in untreated cells. Control refers to unstimulated (no CCL5) levels in pCDNA-K562 cells, considered as 100% for normalization procedure.

 

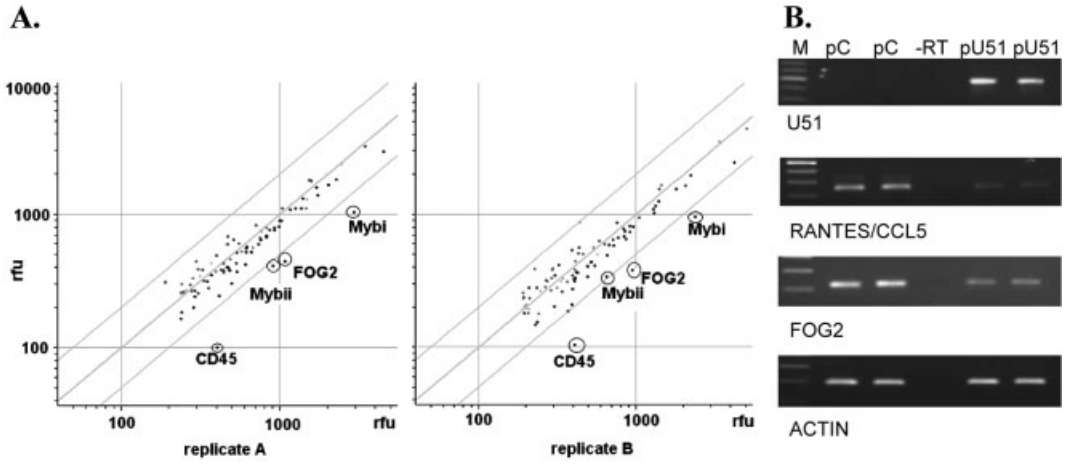


**Figure 3.** Array analyses show constitutive signaling down-regulates immune-response related genes in U51A cell line. (A) Mini-array of 383 immune response-related genes identifies four genes that are down-regulated. The two graphs show two sets of cell cultures each performed in duplicate (separately resuscitated from frozen) and cultured as discussed in *Materials and methods* (for both tests pCDNA3-U51A and control vector only, pCDNA3, cell lines were used). The graphs are representative of four independent experiments (see Table 1), with vector cell line labeled with Cy3 and U51A labeled with Cy5, after hybridization. The x axis represents greater Cy5 fluorescence showing up-regulation and the y axis greater Cy3 showing down-regulation. All genes identified with a positive signal in the replicate (106) are shown. They were normalized using 2, 5 and 10 mg/mL dilutions of ACTB control elements and filtered for detection cut-off signal of mean above and below 3 SD, midline and upper or lower diagonals, respectively. Four genes were outside the cut-off and significantly down-regulated, indicated by open circles (see also Table 1). (B) RT-PCR confirmation of microarray results using the previously determined CCL5 down-regulation in the U51A expressing cell line as a control. pC = pCDNA3 vector cell line cDNA; pU51A = U51A pCDNA-transformed cell line. FOG2 shows a representative result of the further genes that were significantly down-regulated in the U51A cell line; each result was performed in duplicate and is representative of three independent experiments.

using a restricted gene set microarray composed of gene targets relevant to infection and immunity. This array had over 380 genes represented as exon-specific PCR products gridded on glass slides with a design based on minimal cross-hybridization [17] (for a complete V2 gene list, see *Materials and methods*). The U51A data were filtered for all genes with signal above mean + 3 SD of non-homologous (negative) controls, then using a cut-off of twofold or greater changes in gene expression with $p$ values less than 0.05. Four down-regulated targets were identified from three genes in the K562-U51A cells (Fig. 3A, Table 1). Two were from different exons (5 and 13) of the MYB oncogene, the other two from FOG2 (friend of GATA2, zinc finger protein) and PTPRC (protein tyrosine phosphatase receptor-type C, CD45, leukocyte common antigen) genes. These were confirmed by RT-PCR analyses in comparison to CCL5 (Fig. 3B), which was also down-regulated as previously demonstrated only on epithelial-derived U51A cell lines, but was not included on the microarray slide [13]. The results for gene expression of human chemokine receptors and chemokines included on the array were also examined. The array included human chemokine receptor genes CCR1, CCR3, CCR5 and CXCR4. These receptors were not expressed in the K562 cells and there were no changes of expression in K562-U51A cells consistent with previous reports on undifferentiated K562 cells [18]. The chemokine genes included on the array were CXCR2 ligands CXCL8/IL8 or CXCL2/GROβ. Neither chemokine gene showed any change in expression, similar to previous results for CXCL8 assayed by RT-PCR [13].

**Table 1.** Immune response-related gene down-regulation in U51A-expressing cells by array analyses[a)]

| Name | GeneBank | Description | Replicate A Normalized $t$-test $p$-value | Replicate B Normalized $t$-test $p$-value |
|---|---|---|---|---|
| FOG2 | AC015561 | friend of Gata2, FOG-2. | 0.49 (0.40 to 0.53) 4.6 E-08 | 0.48 (0.43 to 0.57) 7.9 E-09 |
| CD45/PTPRC | AL355988 | LCA; CD45 antigen; PTPRC | 0.30 (0.18 to 0.44) 2.3 E-07 | 0.31 (0.20 to 0.43) 0.0006 |
| MYBi | AL023693 | C-MYB oncogene, exoni | 0.54 (0.45 to 0.62) 3.3 E-07 | 0.64 (0.54 to 0.73) 0.024 |
| MYBii | AL023693 | C-MYB oncogene, exonii | 0.41 (0.40 to 0.44) 0.0008 | 0.47 (0.39 to 0.51) 0.01 |

[a)] Two independently resuscitated and cultured replicates (A and B) are shown, each performed in duplicate.

 

## Analyses of infected cells show repression of CCL5 and FOG-2 linked to U51A up-regulation

Expression of U51A constitutively down-regulated genes was further analyzed by semi-quantitative RT-PCR of infected T lymphocytes. The infection time course was conducted 0, 5, 48, 96 and 168 h post infection. Hut78 human $CD4^+$ T lymphocyte cell line was used for an infection time course, as these cells express sufficient levels of CCL5 so that effects on down-regulation of expression could be monitored. However, in uninfected cells, levels of FOG-2 were undetectable, thus here only induction could be assayed. The infection time course was run in duplicates and then analyzed by RT-PCR on tenfold serial dilutions of cDNA for CCL5, FOG2, U51A and U12 in comparison to Actin expression levels. The HHV-6A U12 gene, was used as a supplemental infection control, as it is a chemokine receptor like U51A, but shows different kinetics, with gene expression continuing at late time points post infection. Results for MYB and PTPRC were inconsistent in the infected cells, possibly due to cell type differences, but more likely to conflicting effects on these genes from expression of other viral regulatory genes during the virus infection. However, during infection of the T lymphocytes, effects on both FOG-2 and CCL5 could reproducibly be shown as indicated. Representative RT-PCR results using cDNA dilutions were from the logarithmic phase of the amplification (Fig. 4A). The last endpoint dilution detected at each time point was also plotted (Fig. 4B) although FOG2 levels were too low for plotting, as they were only detected in the undiluted cDNA at late time points post infection. The results show that as U51A expression increased maximally at early times post infection, from 24 to 96 h, CCL5 expression was reduced, and that of FOG-2 undetectable. As U51A expression levels decreased from 96 h post infection, CCL5 and FOG2 were induced.

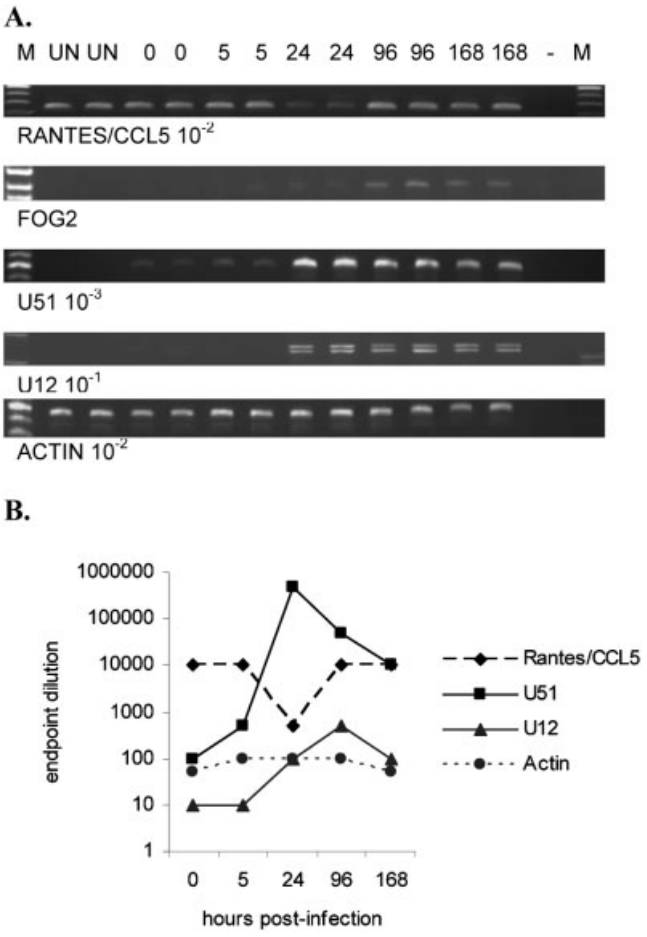


**Figure 4.** Bimodal regulation of CCL5 expression follows U51A expression in infected human T lymphocytes. (A) Dilutions used were in logarithmic phase one or two logs below saturation and above endpoint. M indicates molecular weight ladders, UN stands for uninfected, and numbers indicate times post infection in hours. CCL5 expression is bimodal, repressed at 24 h and then induced from 96 h while FOG-2 is also induced from 96 h. U51A expression peaks at 24 h (early gene) when CCL5 is repressed, then decreases to 168 h. While U12 chemokine receptor (early/late) shows similar levels through to later times post infection. (B) Analysis of kinetics of gene expression by semi-quantitative RT-PCR during the infection time course in cells expressing CCL5, showing that U51A early expression correlates with CCL5 down-regulation. Similar results for FOG2 could not be plotted, as the levels were too low and could only be detected in undiluted cDNA. Each infection was performed in duplicate with less than 10% variation as shown in RT-PCR results, and the results are representative of two independent infection time-course assays.

## Inhibition of U51A constitutive signaling using siRNA reverses FOG-2 and CCL5 down-regulation

In order to further evaluate the role of U51A and its signaling on gene expression, siRNA were designed to knock out U51A expression and compared to effects of control siRNA. The stable U51A-expressing K562 cell line was transfected with the siRNA, RNA extracted and examined by RT-PCR. The results show that siRNA was effective at reducing levels of U51A expression (Fig. 5A). Further, the reduction of U51A expression resulted in increased CCL5 and FOG-2 expression as detected by RT-PCR with similar results shown by analyses of CCL5 secretion by ELISA (Fig. 5A and B). Furthermore, the same results were found using both 27mer blunt-ended and 21mer overhanging-ends forms (U51A-1, Fig. 5A) of 51A siRNA except that the smaller form required a double transfection. Moreover, negative controls using stable cell lines transfected with the empty plasmid (K562-pcDNA3), showed no change in expression of CCL5 or FOG-2 after transient transfections with either control or U51–1 siRNA (Fig. 5A). Overall this confirms that constitutive signaling *via* U51A expression affects

 

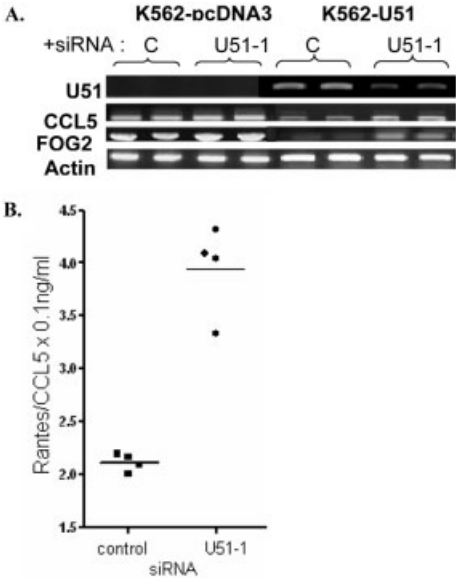


**Figure 5.** U51A inhibition by siRNA correlates with increased CCL5 and FOG-2 expression in cell lines. (A) The stable negative control K562-pcDNA3 (empty plasmid) or K562-U51A cell lines were transfected in duplicate with different siRNA. C lanes show control random siRNA (Ambion), U51–1 lanes show U51–1 siRNA -effective against U51A expression. The RNA was extracted and tested by RT-PCR with primers specific for U51A, CCL5, FOG-2 and Actin. The experiment is representative of three independent experiments. (B) Analyses of CCL5 protein levels in K562-U51A cells transfected with indicated siRNA as determined by ELISA, performed in quadruplicate with mean indicated. Down-regulation of U51A expression using U51A siRNA results in increased CCL5 secretion. Results show replicates of electroporations and are representative of four independent assays.

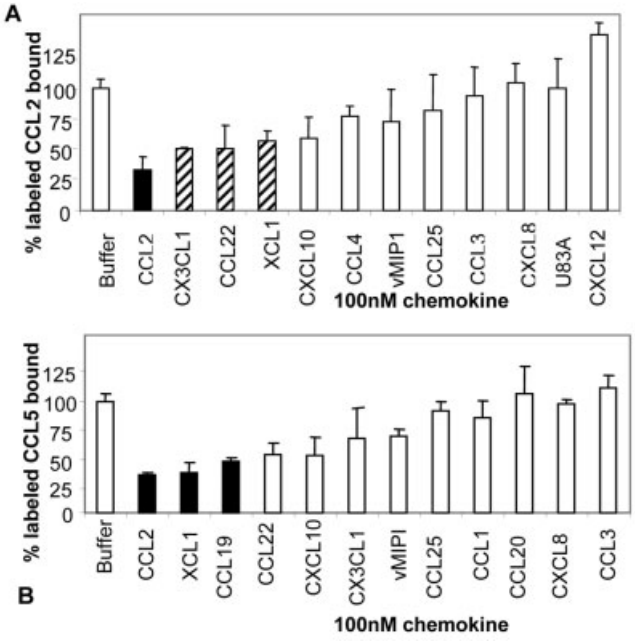


**Figure 6.** Extended specificity analyses of U51A. The graphs indicate chemokines, which displace binding of the labeled ligand at 100 nM. A cut-off of more than 50% displacement (less than 50% bound labeled ligand, $IC_{50}$ less than100 nM) was used to identify possible new high-affinity ligands as indicated in black. Those just at the cut-off level were striped and those above in white. The buffer only control shows no displacement, marking reference 100% bound ligand. Previously defined ligand CCL2 is used as a positive control, and CCL3, which does not bind, as a negative control. (A) Binding against $^{125}$I CCL2 on U51A expressing COS-7 cells. (B) Binding against $^{125}$I CCL5. Each assay was performed in triplicate with standard error shown.

CCL5 and FOG-2 in human leukocyte cell types, HHV-6 target-cell models.

## U51A interacts with lymph node and lymphocyte-specific ligands of CCR7 and XCR1

To assess contributions of inducible signaling, the extended ligand specificity of U51A was examined covering all classes of chemokines, including XC and $CX_3C$ classes, and further CXC (alpha) or CC (beta) chemokines and viral chemokines. Previous results showed high-affinity binding to U51A by CC (beta) chemokines CCL5, CCL11 and CCL2, with displacement by CCL7 and CCL13 [13, 16]. This is representative of CCR1, CCR2, CCR3, and CCR5 cellular receptors present on monocytes and T lymphocytes. This experiment was extended by examining interactions with further ligands with two displacement binding screens against radio-labeled CCL2 (Fig. 6A) or CCL5 (Fig. 6B). Here, COS-7 cells transiently transfected with U51A were used. COS-7 cells are commonly used in binding studies, as they allow a combination of high-level expression with an absence of all human chemokine receptors. New potential high-affinity ligands were identified using a cut-off of 50% displacement with 100 nM of chemokine used (relative affinity less than 100 nM). Of the new viral chemokines tested, vMipI and U83A, both did not displace binding. Two possible high-affinity ligands were identified below the cut-off, XCL1 and CCL19. In the first screen against $^{125}$I CCL2 there were no new ligands below the cut-off comparable to the previously defined CCL2 (Fig. 6A), although three were identified near the cut-off, $CX_3CL1$, CCL22, and XCL1. Thus, a repeat screening was performed against $^{125}$I CCL5 and here XCL1 and CCL19 were selected for further investigations as they were below the cut-off and comparable to CCL2 (Fig. 6B). These were endogenous ligands for the human receptors XCR1, and CCR7, respectively. These two new potential high-affinity ligands, XCL1 and CCL19, were further tested by displacement binding kinetics against labeled CCL5 and CCL19. The results showed high relative affinities of 4 and 37 nM for XCL1 against labeled CCL5 and CCL19 within the limits of the points tested, with high direct and relative affinities of 8 and

 

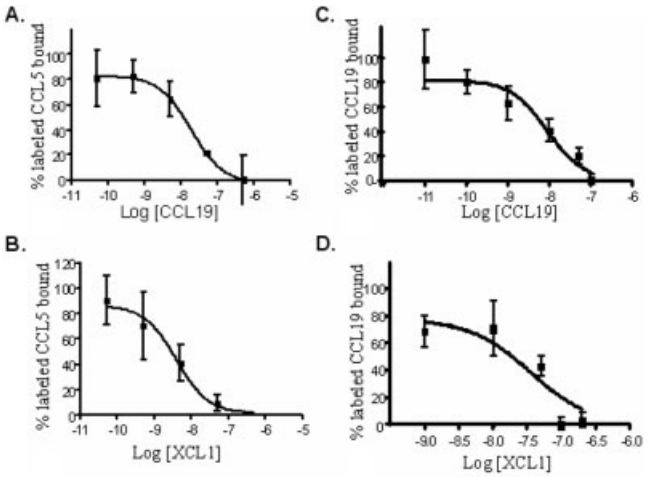


**Figure 7.** Affinity determinations for new ligands CCL19 and XCL1 identified on U51A are similar to affinities for human receptors, CCR7 and XCR1. Ligand displacement kinetics were performed for (A) CCL19 and (B) XCL1 against binding of $^{125}$I CCL5 on U51A expressing COS-7 cells, then (C) CCL19 and (D) XCL1 against binding $^{125}$I CCL19. Relative affinities calculated as 1.9–$10^{-8}$ M for CCL19 and $4.0 \times 10^{-9}$ M for XCL1 against CCL5 and $3.7 \times 10^{-8}$ M for XCL1 against CCL19. Direct affinity calculated as $8.6 \times 10^{-9}$ M for XCL19. Assays were performed in triplicate with standard error shown and are representative of two independent results.

20 nM for CCL19 against labeled CCL19 and CCL5, respectively (Fig. 7A–D). These affinities were similar to those of the human chemokine receptors (Table 2) and showed an overall unique specificity for U51A compared to any human chemokine receptor.

## Inducible activation of U51A cell lines plus infected *ex vivo* human leukocytes to U51A ligands, CCL11 and CCL19

To test the possible role of U51A in sequestering human chemokines and affecting migration of infected cells in the human host, receptor internalization and chemotaxis experiments were undertaken with U51A-expressing human hematopoietic cell lines as well as infected human *ex vivo* leukocytes from different donors. For example, U51A expression on infected or reactivated monocytic/macrophage cells or T cell subsets could aberrantly direct their migration to sites for host transmission as lungs where CCL11 can be secreted by lung epithelia after cell damage. Alternatively, expression could mediate dissemination by directing infected cells *via* interactions with CCL19 secreted by lymph nodes, which contain susceptible cells and are site of infection *in vivo*. Conversely, U51A expression and binding ligands can divert human chemokines from homologous receptors CCR3, XCR1 and CCR7 evading local inflammation and immune priming in the lymph node. *In vitro* studies with an epitope-tagged U51A-expressing hematopoietic cell line, showed that the human chemokines mediated efficient U51A receptor internalization upon binding consistent with sequestration or diversion of human chemokines from their endogenous receptors, as shown for chemokines CCL2, CCL11, XCL1 and CCL19 (Fig. 8A). This was demonstrated at 10 and 100 nM, near or at receptor saturation, while below this, at 1 nM, little internalization was observed (not shown). Moreover, a negative control, 100 nM CCL3, which does not bind U51A, had no effect on receptor internalization (Fig. 8A). Since CCL19 is important for lymph node homing, it was further tested for evidence of direct sequestration or diversion by assay of radiolabeled CCL19 internalization as described [19]. At 4°C only 30% of labeled CCL19 remained bound after acid wash, while shifting to 37°C (to enable internalization) allowed 55% to remain cell associated after the

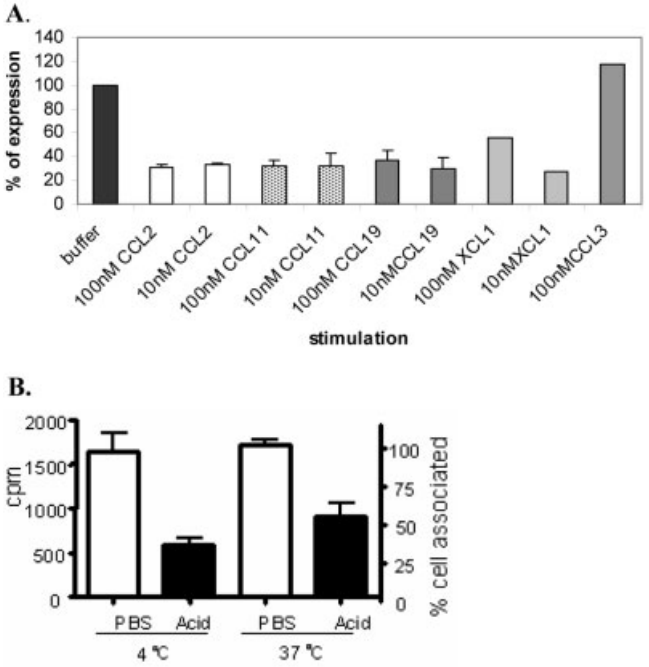


**Figure 8.** (A) Chemokine induced internalization of U51A. K562 cells stably expressing an epitope-tagged (HA) form of U51A were treated with different chemokine at 10 or 100 nM for 15 min at 37°C. Cell surface U51A down-modulation was monitored by staining with an anti-HA antibody and quantified by flow cytometry. CCL2 and CCL3 were used as positive and negative controls, respectively, (previous data show that CCL2 but not CCL3 binds UL51 [13]). The results show the percentage of cells expressing the receptor compared to untreated cells, representative of six independent assays; results in duplicate with error bars indicated. (B) CCL19 internalization by U51A. Cells were incubated with $^{125}$I CCL19 at 4°C for 1 h, then washed in PBS and incubated at 37°C for 5 min. CCL19 internalization was determined as the proportion of cell-associated cpm that became acid-resistant by comparing acid- to PBS-washed cells. The experiments shown were run in triplicate, error bars indicating SD.

 

**Table 2.** Summary ligands for HHV-6A U51A chemokine receptor

| Chemokine | U51A binding[a)] | IC50 | (Ref) | Human receptor[b)] |
|---|---|---|---|---|
| **Human** | | | | |
| Rantes/CCL5 | + | 1nM[c)] | [13, 16] | CCR1,3,5 |
| MCP1/CCL2 | + | 8nM | [13, 16] | CCR2 |
| Eotaxin/CCL11 | + | 8nM | [13, 16] | CCR3 |
| MCP3/CCL7 | + | nd | [13] | CCR2, 3 |
| MCP4/CCL13 | + | nd | [13] | CCR2, 3 |
| Lymphotactin/XCL1 | + | 4 nM | here | XCR1 |
| Mip3beta/CCL19 | + | 8nM[c)] | here | CCR7 |
| Fractalkine/$CX_3CL1$ | +/– | nd[d)] | here | $CX_3CR1$ |
| MDC/CCL22 | +/– | nd | here | CCR4 |
| MIP1alpha/CCL3 | – | | here | CCR1, CCR5 |
| MIP1beta/CCL4 | – | | here | CCR5 |
| MIP3alpha/CCL20 | – | | here | CCR6 |
| TECK/CCL25 | – | | here | CCR9 |
| I309/CCL1 | – | | here | CCR8 |
| IL8/CXCL8 | – | | here | CXCR1, 2 |
| IP10/CXCL10 | – | | here | CXCR3 |
| SDF1/CXCL12 | – | | here | CXCR4 |
| **Herpesvirus** | | | | |
| vMIPII | + | nd | [13] | CCR,CXCR |
| vMIPI | – | | here | CCR8 |
| U83A | – | | here | CCR1,4,5,6,8 |

a) Cut-off for binding was under 50% displacement at 100 nM.
b) Nomenclature and properties reviewed in [37].
c) $EC_{50}$.
d) nd – not determined.

acid wash, indicating that 25% of labeled CCL19 was internalized (Fig. 8B).

To examine inducible chemotaxis, the effects of chemokines on the K562-U51A and negative control K562-pcDNA3 vector cell lines were used. The K562-U51A cell line migrated to CCL11 between 0.01 and 0.1 nM, and to CCL19 between 1 to 100 nM, with only marginal chemotaxis to XCL1 at 100 nM. No migration was observed using the vector control cells (Fig. 9A, B, and C). To test effects on cellular migration during natural infection, *ex vivo* PBL were prepared, infected with HHV-6A, and assayed for migration. CCL11- or CCL19-induced chemotaxis was examined. At 48 h post infection, cells were shown to specifically chemotax toward these ligands, with maximum induced chemotaxis at 0.1 to 1 nM for CCL11 and 10 to 100 nM for CCL19. This is similar to results obtained for the U51A cell line, with marginal migration to XCL1 at 10 nM, while no migration was observed from uninfected cells (Fig. 9D, E, and F). As a positive control, CXCL12-induced CXCR4 chemotactic responses were measured. CXCR4 is abundantly expressed in JJhan T leukocytes and its expression is down-regulated by HHV-6 infection as previously described [20, 21]. Here, both uninfected and infected cells chemotax to the CXCR4 ligand, CXCL12. However, after infection, this response decreases, consistent with down-regulation of CXCR4 post infection. In contrast, as demonstrated on the PBL, these cells do not express CCR3 (before or after infection) and do not chemotax to CCR3 ligand, CCL11, but only chemotax after infection, when U51A is expressed (Fig. 10). Although migration mediated by CXCR4 is higher, all the U51A chemotaxis demonstrated are significant compared to controls (Fig. 9 and 10). Analyses by RT-PCR or flow cytometry of the uninfected compared to infected PBL showed no evidence for post-infection changes in XCR1, CCR3 (undetectable) or CCR7 (low levels by RT-PCR), while U51A was detected.

 

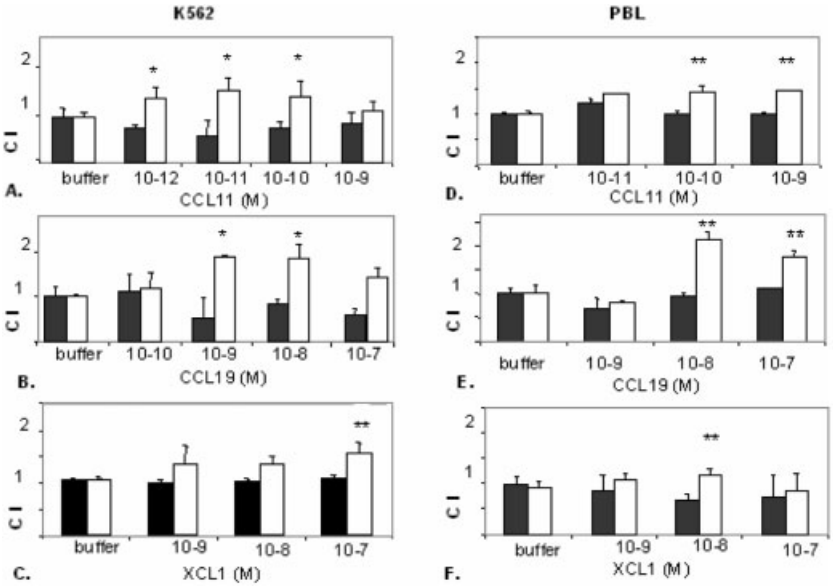


**Figure 9.** Chemotaxis of U51A-expressing human hematopoietic cells and infected *ex vivo* human leukocytes induced by CCL11 and CCL19. In (A–C) filled columns are K562-pcDNA3 control-vector only cells, open columns are K562-U51A cells. Chemotactic index is shown (CI) normalized against a CI of 1 for treatment with buffer only, representing background random migration. Migration of U51A-expressing cells is shown induced by (A) eotaxin/CCL11, (B) Mip3beta/CCL19, and (C) lymphotactin/XCL1. No migration was shown with the K562-pcDNA3 cells, empty plasmid vector negative control. Shown are representative of four independent assays each run in triplicate, with error bars indicating SD. In (D–F) filled columns are uninfected cells, open columns are human PBL 48 h post infection with HHV-6A. No chemotaxis was observed in uninfected PBL. Significant chemotaxis was only shown in infected PBL to CCL11 between 0.1 to 1 nM (C), to CCL19 at 10 to 100 nM (D) and to XCL1 at 10 nM (E). Each assay was performed in triplicate and PBL used were from four separate donors with representative results shown. Dilutions of chemokines from 100 to .01 nM are as indicated. Representative results of two independent assays each performed in triplicate with SD shown in error bars. Significance of chemotaxis analyzed with Bonferroni multiple comparison test is shown ($^{*}p<0.01$, $^{**}p<0.001$)

Only CCR1 and CCR2 with low levels of CCR5 were identified, which are not specific for CCL11 or CCL19. Therefore, at 48 h post infection, human leukocytes can be mobilized, consistent with U51A expression and specificity.

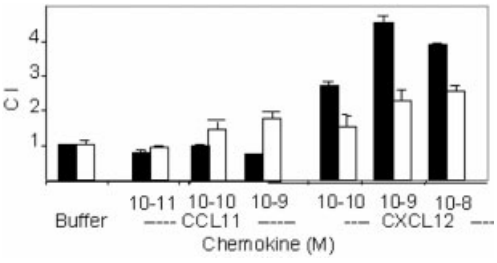


**Figure 10.** Chemotaxis of U51A-expressing infected JJhan T leukocytes induced by CCL11 and CXCL12. Assays run in triplicate, showing chemotactic index as in Fig. 9, with error bars indicating SD. Filled columns are uninfected cells, open columns are infected cells 48 h post infection. No chemotaxis to CCL11 was shown in uninfected cells, while in infected cells chemotaxis was demonstrated at 0.1 to 1 nM. The CCL11 human receptor CCR3 was not expressed in uninfected or infected cells, while U51A was expressed in infected cells. Chemotaxis to CXCL12 is shown in both uninfected and infected cells. It is used as both a negative and positive control, as it does not bind U51A but does bind CXCR4 present on both uninfected and infected cells. Further, CXCR4 is down-regulated by infection as reported previously and accordingly reduced chemotaxis to CXCL12 after infection is shown.

## Discussion

Previous results using model cell-expression systems have shown that HHV-6A U51A has immunomodulatory properties and signals in a unique combination of constitutive and inducible pathways influenced both by extracellular chemokine and intracellular Gα protein levels [13, 16]. Here, signaling is further investigated in human leukocyte cell types, which are permissive for HHV-6 infection and replication. In a stable human hematopoietic cell line, results showed U51A-mediated constitutive signaling, from InsP assay and array analyses of gene expression, as well as inducible signaling, as demonstrated by calcium mobilization and chemotaxis assays and enhanced InsP signaling in response to chemokine stimulation. Interestingly, in a separate study (our unpublished results), the $CD4^{+}$ T lymphocyte cells used for virus replication *in vitro* and *in vivo*, G proteins for both constitutive, $G_q$, and inducible signaling, $G_{\alpha i2}$, were identified and the effects of these pathways are explored here.

 

In the constitutive signaling U51A hematopoietic cell line, analyses of gene expression compared to vector cell line by microarray and semi-quantitative RT-PCR and siRNA analyses revealed down-regulation of CCL5 shown previously in an epithelial cell line [13], but also showed decreased expression of the hematopoietic-specific regulatory gene, FOG-2, both confirmed in an infection time course. The FOG proteins are transcriptional repressors, which interact with GATA proteins to affect hematopoietic or heart/brain-specific development [22–24]. FOG interactions with GATA3 have been shown as master regulators to influence the Th1/Th2 balance, repressing Th2 development [25, 26]. Thus, U51A-induced effects on FOG expression may contribute to the observed influence of HHV-6 infection on gene expression associated with a Th1-like phenotype [27]. Recent studies show that FOG-2 interacting with GATA4 specifically affects heart and pulmonary development [22, 28–30], although biochemical studies show that individual zinc finger domains can interact with multiple GATA proteins [23, 24]. In this regard, HHV-6A has been shown more frequently in congenital infections than early childhood [31]. Furthermore, recent evidence indicates HHV-6 as a common cause of viral myocarditis [32–34], resulting in occasional infant mortality [35, 36]. Therefore, FOG-2 dysregulation may contribute to effects on cardiac development and inflammatory complications as well as immune repertoire.

To investigate further inducible signaling, the fine specificity of U51A was examined. Previously determined U51A-ligand specificity included CCL5, CCL2 as well as CCL7 and CCL13 [13, 16]. This overlaps with the reactivity of the human homologues CCR1, CCR2, CCR3 and CCR5. Here, analyses of the extended specificity covered all chemokine classes and included viral chemokines (see Table 2). First, the responses similar to those observed for CCR1 and CCR5 stimulation were further investigated. These were initially defined by binding of CCL5 and here this was tested with other CCR5 ligands (*i.e.* CCL3 and CCL4). In contrast to CCR5, U51A does not bind CCR1 and CCR5 ligand CCL3, or CCR5-specific ligand CCL4. This suggests that overall the properties of U51A most closely resemble those of human CCR2 and CCR3, as CCR2 binds with high affinity CCL2, CCL7 and CCL13 while CCR3 overlaps by binding CCL7 and CCL13, together with CCL5 and CCL11 [37]. In addition, two new agonist ligands were determined, XCL1 and CCL19, with high relative and direct affinity, respectively. The affinities for interactions of human chemokines with the human receptors mirror these with the viral receptor (Table 2) [38–40].

Lymphotactin/XCL1 normally binds the human receptor XCR1 [41]. XCR1 has restricted specificity for only two ligands, lymphotactins XCL1 and XCL2. This receptor is present on T lymphocytes and, together with the fractalkine receptor, it is one of the few identified on NK cells [37, 42]. As these are increasingly viewed as essential for controlling herpesvirus infections [43], a role for U51A in evading this response may be critical. Interestingly, U51A appears to interact with intermediate affinity with fractalkine also possibly as a sink for this chemokine and XCL1 in interactions with NK cells. NK cells are important as first lines of defense against herpesvirus infections, and can orchestrate chemokine secretion; therefore, a block to their activity can favor viral persistence [43, 44]. U51A binding XCL1 may sequester its activity away from NK cells. Alternatively, U51A-XCL1 binding may allow the infected cell to chemotax to sources of this chemokine secretion, including differentiated T lymphocyte populations, which are susceptible to virus infection [45, 46]. In addition, recent data show that XCL1 binding to XCR1 sends an apoptotic signal to $CD4^+$ T cells, suggesting that diversion of binding to the U51A receptor may prolong survival of the cell to favor the virus replicative cycle in these permissive cell types [47, 48].

The interaction of U51A with CCR7 ligand CCL19, at EC50 of 8 nM and IC50 of 20 nM (competing CCL5), is similar to the affinity to the human receptor (Table 1) [49, 50]. This human receptor CCR7, like XCR1, has restricted specificity. CCR7 has only two known ligands, CCL21/6Ckine and CCL19. However, viral receptors HHV-7 U12 and HHV-7 U51 also appear to have specificity for CCR7 ligands, albeit only low-affinity interactions, and only HHV-7 U12 has been shown to function in chemotaxis while HHV-7 U51 has additional low-affinity interactions to CCR4 ligands, CCL17/TARC and CCL22, giving calcium signaling [14, 15]. Thus, HHV-6A U51A is unique in combining CCR7-like activities with those overlapping XCR1, CCR2 and CCR3 specificities. U51A interactions with ligands CCL19 could modulate responses to infected cells by T lymphocytes bearing these receptors and act to sink the ligands of the lymph node homing CCR7, normally present on mature or semi-mature dendritic cells or lymph node homing T (including naive T cells and some memory T cell subsets) or B lymphocytes. Alternatively, U51A binding CCL19 could aid dissemination of infected cells to sites of this ligand secretion in the lymph node [51], for spread to susceptible cell types concentrated there. This was supported by the chemotaxis assay results. Additional activities of CCR7 ligands include roles in differentiation and maturation [52]. Therefore, U51A may function as a sink for ligands for CCR7, as a decoy to prevent recruitment as well as maturation of cell types for antigen presentation in the lymph node.

Altogether, U51A has combined specificities of CCR2/CCR3/XCR1/CCR7, unique compared to both human and viral chemokine receptors. U51A expression allows responses to ligands normally stimulating

 

eosinophils, basophils, monocytic, T (naive and memory) and B lymphocyte populations, mature dendritic cells and NK cells. Interestingly, this provides a complementary interaction with the activities of the HHV-6A U83A chemokine, which appears to target distinct leukocyte subsets. Here, we show this viral chemokine does not bind U51A, while previously we have shown that it does bind CCR1, CCR4, CCR5, CCR6 and CCR8, which are human chemokine receptors present on $CD34^+$ bone marrow progenitor cells, immature dendritic cells (CCR6), macrophages (CCR1, CCR5), activated T lymphocytes (CCR1, CCR5) and skin-homing T lymphocytes (CCR4, CCR8) [7]. Thus, combining the specificities of U51A and U83A indicates that U51A may mediate evasion of immediate antiviral properties of NK and mature dendritic cells while reutilizing their ligands for dissemination of infected cells. U83A may target chemoattraction of the latent cell type (monocytic/macrophage and bone marrow progenitor), cell types for virus replication (activated T lymphocytes), and those for effective antigen presentation to drive the virus towards latency (immature dendritic cells and activated macrophage and Th2 T lymphocytes). Moreover, U51A down-regulates the CCR5 ligand CCL5 at early time post infection, which is the same window when the spliced U83A chemokine is made that can bind and block the CCL5 receptor CCR5 [7, 53]. This double block on CCR5 activities *via* both U51A chemokine receptor and U83A chemokine highlights the importance of CCR5 in infection, also indicated in recent studies [54].

U51A expression is by early kinetics [55], this property combined with ligand selectivity could also cause chemotaxis of infected primary human leukocytes, hence aiding dissemination, transmission and affecting reactivation. This was demonstrated using cell lines and donor *ex vivo* PBL, where infection allowed migration towards U51A-specific ligands CCL11 and CCL19. Interestingly, binding led to bound receptor internalization, indicating sequestration or diversion of the human chemokines. Thus, there can be both a distortion of available ligands for homing of immune cells to the lymph node as well as re-direction of infected cells expressing the receptor to this site of virus replication. These responses can be important for dissemination of the virus during initial infection *via* the lymph nodes [51]. In addition, they could mediate host to host spread. In the case of CCL11 responses, this chemokine as well as CCL2 also recognized by U51A can be secreted by damaged epithelial lung cells and airway parasympathetic nerves in an infected host [56–59]. Virus reactivation in monocytic cells could direct these cells *via* U51A expression to a site for host transmission *via* respiratory mucosal secretions. The *ex vivo* chemotaxis of infected PBL to these ligands in absence of the human receptors shows possible routes of dissemination *in vivo*.

Secretion of U51A-binding chemokines in the brain may also contribute to recruitment of infected inflammatory cells in the CNS as observed for this lymphotropic and neurotropic virus [1]. Thus, the expression of this gene is not only essential for virus replication *in vitro* [60], but can have profound implications *in vivo* for overall dysregulation of cellular immunity *via* both constitutive and inducible signaling, ultimately supporting virus spread and chronic inflammatory disease.

## Materials and methods

### Cell and virus culture

The human $CD4^+$ T cell leukemic cell line JJhan, Hut78 and stable transfected human myelogenous leukemia K562 ( K562-U51A, K562-U51AD or K562-pcDNA3) cells [13] were cultured in RPMI 1640 medium, supplemented with 10% v/v FCS and 2 mM glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Human blood samples were taken with informed consent from healthy donor volunteers from the institute, and were anonymously coded following procedures from our local ethical review board (London School of Hygiene & Tropical Medicine). PBL were purified from whole blood using Histopaque-1077 (Sigma-Aldrich) following the manufacturer's protocol and used *ex vivo* either uninfected (only HHV-6-negative PBL were used as screened by PCR) or infected in the same media with 5% FCS. Transfected cell lines were maintained in 500 or 750 μg/mL of G418 for K562 lines. COS-7 cells were cultured in DMEM medium, supplemented with 10% v/v FCS and 2 mM glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin. For time-course infections, PBL, JJhan or Hut78 cells were infected with HHV-6A strain U1102 at a MOI of 1 tissue culture infectious dose (TCID)50/cell for 4 days. Then, $1 \times 10^6$ infected and uninfected cells were cocultivated and harvested at different times. Cells were then pelleted by centrifugation at 800 rpm for 5 min, then washed in 5 mL 1 × PBS and re-centrifuged for 5 min, resuspended in 1 mL PBS and centrifuged at 2000 rpm and the PBS removed.

### Calcium measurements in stable cell lines

HHV-6 U51A-K562 or pCDNA3- K562 cells at early log phase (0.2–0.4 mill/mL) were centrifuged, culture supernatant removed, then incubated 45 min in a HEPES buffer (extracellular medium buffer, ECB, with 125 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM $NaH_2PO_4$, 5 mM $NaHCO_3$, 10 mM glucose, and 10 mM HEPES/NaOH, pH 7.4) containing 2 mM Fura2-AM (Molecular Probes, Eugene, OR), with 0.1% BSA and 2 mM $Ca^{2+}$. Fura-loaded cells were then centrifuged, washed in ECB for 45 min, centrifuged again, resuspended in ECB, transferred to a temperature controlled (37°C) recording chamber fitted with a 22-mm glass coverslips on an inverted Nikon Diaphot microscope. After 5 min for adherence, intracellular calcium imaging was undertaken using a MagiCal system (Applied Imaging International, Sunderland, UK) as

 

described [61]. The chemokine dilutions (0.1 mL: CCL2, 3, 5 or 11, R& D systems) were added to 0.4 mL ECB in the cell chamber. Sequences of images at 340 and 380 nm excitation (*via* interference filters from a Xenon light source), 510 nm emission wavelength, were taken for 50 s before chemokine addition and 400 s afterwards, using a 40x objective lens. Emitted fluorescence captured by CCD camera (Photonic Sciences, Turnbridge Wells, UK) was sent to a MagiCal computer hardware digital image analysis system (Applied Imaging). Background images taken at the two excitation wavelengths were subtracted, producing 340/380 ratio images (color coded) used to calculate intracellular calcium concentrations. Between 10–20 cells were interrogated per view, and ratio responses plotted for individual cells. The relative cytoplasmic $Ca^{2+}$ concentration was calculated using a calibration table, as described, using loaded cells exposed to 200 mM EGTA or 10 mM $Ca^{2+}$ in the presence of 10 mM ionomycin [62, 63].

## [$^3$H]Inositol phosphates production

[$^3$H]Inositol phosphates were isolated by anion exchange chromatography and measured essentially as described previously [16]. Log-phase stable transfected K562 cells (K562-pcDNA3 and K562-U51A) were labeled overnight in labeling medium (RPMI 1640 medium containing 10% FCS, 2 mM glutamine, 50 IU/mL penicillin, 50 mg/mL streptomycin, 750 μg/mL of G418 and 1 μCi/mL *myo*-[2–$^3$H]inositol) washed for 10 min with RPMI 1640 medium containing 25 mM HEPES, pH 7.4, 0.5% BSA, and 20 mM LiCl and incubated for 2 h in the same medium with or without the indicated chemokine. InsP were counted by liquid scintillation. Assays were performed in triplicates with $3 \times 10^6$ cells per point. Statistics and data analysis were performed using GraphPad Prism 4.0.

## RNA extraction and RT-PCR

K562-pcDNA3, K562-U51A cell lines or uninfected or infected JJhan, HaCat or PBL were cultured and RNA was extracted as described [13]. Briefly, $10^6$ cells (or less with proportional volumes adjusted) were washed in PBS, then lysed in 1 mL of cold RNA Isolator reagent (Genosys, The Woodlands, TX) or Tri reagent (Sigma) and incubated at room temperature for 5 min. Next, the cells were extracted with chloroform (0.2 mL) after 15 min incubation, followed by microcentrifugation (13 000 rpm for 30 min at 4°C), and RNA precipitation using isopropanol, dried and resuspended in water, then contaminating DNA removed using DNase (Promega; 0.08 U/mL, 2 h at 37°C). DNase-treated RNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol, washed with 70% v/v ethanol, and resuspended in water to 150 ng/mL. Complementary DNA (cDNA) was synthesized from 2 μg of template RNA using 8 U/mL Moloney murine leukemia virus reverse transcriptase (Promega) in 60 μL reactions containing 0.5 mM dNTP, 100 U of RNasin, 10 ng/mL random hexamers, 1 × RT buffer (50 mM Tris-Cl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT). RNA was heated to 65°C for 10 min before cDNA synthesis, chilled on ice for 5 min, and then added to the prepared reaction mix. Parallel reactions, excluding RT, were prepared for each RNA template to confirm the absence of contaminating DNA. Reactions were incubated at 37°C for 2 h, then diluted with an equal volume of RNase-free water (Promega) and stored at –20°C.

PCR was conducted in 25 μL reactions using 0.1 nM primers and GoTaq® Green Master Mix according to manufacturer's instruction (Promega). Primer sequences were as follows (expected product sizes in base pairs are given in parentheses):

β-actin: actin-F, GATGGAGTTGAAGGTAGTTT and actin-B, TGCTATCCAGGCTGTGCTAT (445 bp); HHV-6 U51A: U51A-F, TCGGTCGAGAATACGCTGTG and U51A-B, AGATACGTAGTCACGGTCGA (493 bp); CCL5: RAN-1, TCGCTGTCATCCTCATTGCTACTG and RAN-2, CATCTCCAAAGAGTTGATGTACTC (248 bp); FOG2: FOG2–1, TGCTGGACTATCACGAGTGC; FOG2–2, GCTGGAACCATTCACACCTT (788 bp) [17].

Thermal cycling parameters for β-actin, FOG2 and U51A reactions were 94°C for 1 min, 58°C for 30 s, and 72°C for 1 min, for 40 cycles; for CCL5 reactions the parameters were 95°C for 30 s, 49°C for 30 s, and 72°C for 1 min for 40 cycles.

## Microarray analyses

Synchronized cultures of the vector only cell line (K562-pCDNA3) and the U51A-expressing cell line (K562-U51A) were established by passage at early log phase ($0.2–0.4 \times 10^6$/mL) and seeding at $1–2 \times 10^4$ cells/mL twice weekly (3- or 4-day culture). After identical growth curves were established (2-week culture), cells were seeded at $1.2 \times 10^4$ cells/mL in 3–5 80-cm$^2$ flasks and harvested 3 days later in log phase ($0.2–0.6 \times 10^6$ cells/mL). Cultures were set up in duplicate, and then conditions replicated from independent resuscitations of frozen cells followed by identical culture conditions (replicates A and B). Cells were centrifuged for 5 min at 1000 rpm and RNA extracted from cell pellet using RNA Isolator as above and the RNA concentration determined using an Agilent Bioanalyzer.

Control (K562-pCDNA3) and test (K562-U51A) total RNA were labeled as described [17]. RNA (50 μg) and 3 μg random hexamers in 11 μL RNase-free $H_20$ were first heated at 95°C for 5 min and then snap-cooled on ice for 2 min. Then, dNTP were added (to 0.2 mM A, G, T and 0.1 mM C) plus 0.1 mM Cy-labeled dCTP (Cy3 for control reaction and Cy5 for test reaction) with 10 nM DTT and reverse transcriptase (Promega) together with manufacturers first strand buffer, incubated at 16°C for 10 min followed by 42°C for 90 min in the dark. Microarray slides were pre-printed on glass slides with PCR products amplified from 383 immune response-related genes in a microarray as described [17] together with dilutions of control elements ACTB (SGHMS02 array, http://www.sgul.ac.uk/depts/medmicro/TechSupTranscrptomics.htm#sgul Array). These were incubated at 65°C for 20 min in prehybridization solution (2.5 × sodium saline citrate, SSC; 1x SSC containing 0.15 M sodium chloride and 0.015 M sodium citrate, 0.1% SDS and 10 mg/mL BSA). The slides were rinsed in $H_20$ and isopropanol for 1 min each, and then dried by centrifugation at 1500 rpm for 5 min. The labeled RNA was mixed with hybridization solution (1x SSC, 0.1% SDS) in a 30 μL reaction, and heated at 95°C for 2 min. Next, 40 μL of $H_20$ was added to the hybridization chamber and the

 

microarray slide was added. A LifterSlip glass coverslip was placed over the printed array section and 30 μL of the hybridization solution was added, the chamber sealed, and then incubated 65°C for 16 h. The slide was removed and washed in 1X SSC, 0.05% SDS at 65°C for 5 min, followed by two washes for 2 min each in 0.05X SSC followed by centrifugation at 1500 rpm for 5 min to dry the slides.

The slides were scanned using an Affymetrix 418 scanner and the data transferred for analyses using Imagene 5.5 software. The Cy3 and Cy5 data were superimposed with a grid aligned and the combined intensities quantified. Imagene files outputs were analyzed using GeneSpring 5.1 software to normalize the data, followed by pooling all the individual results (duplicates of replicates) using *t*-tests to demonstrate statistical significance as described [17].

### Electroporation and siRNA

Electroporation of K562-U51A cells was performed essentially as described previously for DNA [13]. Conditions were modified using Cy3 labeled control siRNA (Ambion, siPORT siRNA kit) for 95–100% siRNA transfection efficiency in K562 cells as follows: $1.5 \times 10^5$ cells were resuspended in 75 μL siPORT$^{TM}$ siRNA Electroporation Buffer (Ambion) with 2 μg of siRNA in 1-mm cuvettes and electroporated at 325 V for 0.1 ms on a Bio-Rad Gene Pulser. Two siRNA specific for U51A were designed using the programs available from Ambion and MWG (siRNA design tool) with Tuschl motifs [64–66]. One U51A siRNA blunt-end duplex fit the criteria, 27mer U51A-1: 5′-GCAAUUCUUCUCUACAUCGAUGAUUUU-3′, and was used together with control random siRNA supplied by the manufacturer following their protocols. A smaller 21-mer in the same first region was also synthesized with overhanging ends (MWG): U51A-1A: 5′-UCUUCUCUACAUCGAUGA (dTdT)-3′. Primers to follow effects on RNA expression are the same as described above.

### Quantification of CCL5 secretion

A CCL5 enzyme immunoassay was used according to the manufacturer's instructions (Peprotech) as described previously [13]. Samples of culture supernatant were tested in duplicate in dilution series compared to standards giving a standard curve plot. The sensitivity was 32–3000 pg/mL.

### Binding

Assays for measuring binding affinities were as described [13]. U51A-pcDNA3 transfected COS-7 cells were washed in RPMI and resuspended at $2.5 \times 10^7$ cells/mL in binding buffer (RPMI, 0.1% BSA, and 20 mM HEPES, pH 7.4) on ice. Assays, in triplicate, contained $2.5 \times 10^6$ cells, 166 pM radiolabeled chemokine (specific activity 2000 Ci/mmole $^{125}$I CCL2 , CCL5 from GE Amersham), and diluted concentrations of unlabeled competitor human chemokines (XCL1, CX3CL1, CCL2, CCL19, CCL20/MIP-3*α*, CCL25/TECK and CXCL10/IP-10 from Peprotech and CCL3, CCL4, CCL22/MDC, CXCL12/SDF1 and CXCL8/IL8 from R&D systems) or viral chemokines, vMIP1 from R&D systems, U83A as previously described [7]. After 2 h incubations on ice, cells were separated from the unbound chemokine by microcentrifugation through a phthalate oil cushion [1.5 parts dibutyl phthalate to 1 part bis-(2ethylhexyl)phthalate] with bound radioactivity counted with a gamma counter. Data analyses used Graphpad Prism v.0.1.53.

### Internalization assays

To measure U51A chemokine receptor internalization, K562-U51A-D cells, stably expressing an N-terminal HA epitope-tagged form of U51A, as described [13], were washed into cold FACS buffer (PBS with 0.1% BSA), resuspended in 50 μL of FACS buffer and stimulated with indicated chemokine concentrations for 15 min at 37°C. Cells were then fixed in 3% PFA for 10 min at room temperature. They were washed once in FACS buffer and incubated with human IgG (Sigma) for 10 min at room temperature before a 30 min incubation with anti-HA antibody (Roche). FACS analysis was performed on $10^4$ cells using a FACScan flow cytometer (BD Biosciences) using CellQuest software. U51A internalization was expressed as a percentage of the expression of unstimulated cells. To measure CCL19 internalization, an acid wash-resistance assay was used essentially as described [19]. Briefly, U51A cells ($10^6$) were loaded at 4°C with $^{125}$I-CCL19 in binding buffer as described above for 1 h, then washed in ice-cold PBS, cells were next resuspended in binding buffer, shifted to 37°C for 5 min, then pelleted by centrifugation, followed by washes with ice-cold PBS or acid wash (0.2 M acetic acid, 0.5 M NaCl). Cell-associated cpm were determined using a gamma counter, and ligand internalization was calculated by the ratio of cpm in acid-stripped *versus* PBS-washed cell pellets (or relative percentages).

### Chemotaxis

The chemotaxis assay was carried out as described [7]. Briefly, calcein-labeled K562 (pcDNA3 or U51A) cells or infected PBL ($2 \times 10^6$ cells/mL, 48 h post infection) were allowed to migrate for 2 or 3 h, respectively, at 37°C, 5% $CO_2$, in presence of PBS 0.1% BSA (carrier) or chemokines diluted in carrier (0.01to 1 nM, CCL11 and 1 to 100 nM CCL19) in the bottom well on a 96-well chemotaxis plate (Neuroprobe). After incubation, unchemotaxed cells were carefully wiped off, and the plate read with an excitation wavelength of 485 nm and an emission wavelength of 535 nm in a Wallac Victor$^2$ 1420 multilabel counter (Perkin Elmer, USA). Each set was performed in triplicate, and SD were calculated. Background random migration using only carrier gave the chemotactic index (CI) of 1.

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**Conflict of interest:** The authors declare no financial or commercial conflict of interest.

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