Activation of CCR2+ human proinflammatory monocytes by human herpesvirus-6B chemokine N-terminal peptide

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Human monocytes expressing CCR2 with CD14 and CD16 can mediate antigen presentation, and promote inflammation, brain infiltration and immunosenescence. Recently identified roles are in human immunodeficiency virus infection, tuberculosis and parasitic disease. Human herpesvirus 6B (HHV-6B) encodes a chemokine, U83B, which is monospecific for CCR2, and is distinct from the related HHV-6A U83A, which activates CCR1, CCR4, CCR5, CCR6 and CCR8 on immune effector cells and dendritic cells. These differences could alter leukocyte-subset recruitment for latent/lytic replication and associated neuroinflammatory pathology. Therefore, cellular interactions between U83A and U83B could help dictate potential tropism differences between these viruses. U83A specificity is maintained in the 38-residue N-terminal spliced-truncated form. Here, we sought to determine the basis for the chemokine receptor specificity differences and identify possible applications. To do this we first analysed variation in a natural host population in sub-Saharan Africa where both viruses are equally prevalent and compared these to global strains. Analyses of U83 N-terminal variation in 112 HHV-6A and HHV-6B infections identified 6/38 U83A or U83B-specific residues. We also identified a unique single U83A-specific substitution in one U83B sequence, ‘U83BA’. Next, the variation effects were tested by deriving N-terminal (NT) 17-mer peptides and assaying activation of ex vivo human leukocytes, the natural host and cellular target. Chemotaxis of CCR2+ leukocytes was potently induced by U83B-NT, but not U83BA-NT or U83A-NT. Analyses of the U83B-NT activated population identified migrated CCR2+, but not CCR5+, leukocytes. The U83BA-NT asparagine-lysine14 substitution disrupted activity, thus defining CCR2 specificity and acting as a main determinant for HHV-6A/B differences in cellular interactions. A flow-cytometry-based shape-change assay was designed, and used to provide further evidence that U83B-NT could activate CCR2+CD14+CD16+ monocytes. This defines a potential antiviral target for HHV-6A/B disease and novel peptide immunomodulator for proinflammatory monocytes.

INTRODUCTION

Human monocytes can be classified into at least two distinct groups, classical and non-classical based on CD14 and CD16 expression. These monocyte subsets express chemokine receptors CCR2 and CX3CR1, respectively, which direct specific tissue migration toward sites of selective chemokine secretion during infection (Ziegler-Heitbrock et al., 2010). Recent studies have defined an intermediate monocyte group with intermediate CD14 and CD16 expression as well as intermediate levels of chemokine receptor expression (Balboa et al., 2011; Buckner et al., 2011; Chimma et al., 2009; Lentz et al., 2011; Williams et al., 2012; Ziegler-Heitbrock et al., 2010). Transcriptome profiling of this group has characterized these as antigen-presenting cells with unique proinflammatory properties (Merino et al., 2011; Wong et al., 2011; Zawada et al., 2011; Ziegler-Heitbrock et al., 2010). Therefore, the inflammatory response is fine-tuned depending on activation of specific monocyte subsets. Recent evidence demonstrates that monocytes in blood expressing CD14, CD16 and CCR2, unlike classical or non-classical monocytes, have roles in mediating human immunodeficiency virus infection (HIV) migration across the blood–brain barrier and in increasing the severity of tuberculosis and cardiovascular disease (Balboa et al., 2011; Buckner et al., 2011; Lentz et al., 2011; Williams et al., 2012; Ziegler-Heitbrock et al., 2010). These proinflammatory roles are combined with increased antigen presentation in pleural
effusions for tuberculosis, improved parasite inhibition and increased MHC class II and accessory molecules in donor gene expression studies (Balboa et al., 2011; Chimma et al., 2009; Wong et al., 2011; Zawada et al., 2011). Moreover, it has been demonstrated that CD14, CD16 and CCR2 expressing monocytes are senescent monocytes, with shortened telomeres and increased chemokine receptor expression; they may characterize increased inflammatory disease in the elderly, including disposition to cardiovascular disease (Merino et al., 2011).

Human herpesvirus 6 (HHV-6) is a ubiquitous pathogen in many populations, yet virus reactivations from latent infection can be associated with severe inflammatory disease in immunosuppressed patient populations, including post-transplant acute limbic encephalitis (PALE), and cognitive impairment in haematopoietic stem cell transplantation as well as myocarditis (Kühl et al., 2005a, b; Noutsias et al., 2011; Schmidt-Hieber et al., 2011; Seeley et al., 2007; Zerr et al., 2011). By 2 years of age, over 75 % of infants have acquired this pathogen and in adults, HHV-6 seroprevalence is 83–100 % worldwide (Hall et al., 2006). Primary infections of infants result in a usually self-limited fever and 10–24 % develop a skin rash; exantheme subitum, also called roseola (Hall et al., 2006, 1994; Zerr et al., 2005). Recent evidence shows that approximately 0.1–1 % of populations examined have integrated HHV-6 genomes in the germline, giving inherited, chromosomally integrated virus, cHHV-6, with the potential to express virus genes as human alleles in every cell (Arbuckle et al., 2010; Arbuckle & Medveczky, 2011; Morissette & Flamand, 2010), with implications for inflammatory disease.

HHV-6 comprises two variants, HHV-6A and HHV-6B, which have been recently classified as distinct species (Adams & Carstens, 2012). They are highly similar in terms of genome size, composition and structure, although there are differences in pathology, cell tropism and geographical distribution. Primary infant infections in Europe, USA and Japan are predominantly HHV-6B (97–100 %), while in Africa, the reverse has been found: 86–100 % of healthy infants acquire HHV-6A as their primary HHV-6 infection (Bates et al., 2009; Hall et al., 2006; Kasolo et al., 1997; Sjahril et al., 2009). HHV-6A and HHV-6B lytically infect CD4+ T lymphocytes and undergo latency in monocytic bone marrow progenitor cells (Luppi et al., 1999; Lusso et al., 1988). In addition, it has been shown in vitro that there are differences in cell tropism between HHV-6A and B. HHV-6A has been reported to infect in vitro CD8+ T cells, NK cells, γδT cells, astrocytes and oligodendrocytes (Ahlqvist et al., 2005; Donati et al., 2005; Lusso et al., 1991; 1995).

HHV-6 encodes a specific chemokine, U83, which can mediate chemoattraction for latent infection and dissemination in monocytes, but specificity and activity in subsets is not defined. HHV-6B U83B, like the human chemokine CCL2 (formerly monocyte chemotactic protein-1, MCP-1), is monospecific for CCR2, a chemokine receptor expressed on monocytes (Lüttichau et al., 2003). Therefore, U83B can both chemoattract cellular populations for establishing latency yet also compete with CCL2 for chemokine receptor activation, thereby diverting the host’s cellular responses. This specificity is in contrast to properties of the homologous chemokine U83A, encoded by HHV-6A. The U83A chemokine has broad chemokine receptor specificity: CCR1, CCR4, CCR5, CCR6 and CCR8, yet does not include CCR2 (Catusse et al., 2007; Dewin et al., 2006). The properties of CCL2 have been shown to be essential in a number of systems, including HIV, where it is critical for mediating monocyte movement across the blood–brain barrier and for subsequent correlates to neuroinflammatory disease (Buckner et al., 2011; Lentz et al., 2011; Williams et al., 2012). During this infiltration, cells mature and become susceptible to HIV infection. HHV-6B is linked with status epilepticus and subsequent temporal lobe epilepsy (Epstein et al., 2012), where there is evidence for a role for CCL2-CCR2 signalling (van Gassen et al., 2008). Therefore, we hypothesize that monocytes which express CCR2 together with CD14 and possibly CD16 are targets for HHV-6B, with U83B a key candidate for this selectivity. In this report the specificity of this chemokine is addressed and the effects on ex vivo human leukocytes examined. In contrast, U83A is specific for chemokine receptors, CCR5 or CCR6, which are present on dendritic cells and may affect antigen presentation by different pathways. Experiments with a spliced version of U83A, which encodes a truncated version of U83A, U83A-N, show the chemokine binding specificity is retained in the N-terminal half of the molecule (Catusse et al., 2007; Dewin et al., 2006). Here, this is further explored by analysing strain variants in order to define U83B specificity for CCR2. Based on this variation, synthesized peptides covering U83B-N are described to test specificity and activities in mediating migration, using ex vivo human leukocytes to test possible effects on inflammation. Specificity is defined as well as monocyte subset activation. Since chemoattraction of cellular subsets can be a precursor to latent or lytic infection, this specificity difference also defines a putative determinant of cellular tropism differences between HHV-6A and B.

RESULTS AND DISCUSSION

U83 sequence variability and peptides

Prototype sequences for mature, spliced forms of U83A and U83B chemokines encoded by laboratory reference strains of HHV-6A and HHV-6B were compared (termed U83A-N and U83B-N) (Fig. 1). Additionally, comparisons of this region were made with sequences derived from clinical samples. This included 38 sequences described here from clinical strains in Zambia which were compared to 74 available on GenBank, from Japan, Germany, USA, DR Congo and Uganda (total 112). This comparison showed U83 variation between HHV-6A and B species, as previously identified (Bates et al., 2009; Dewin et al., 2006; French et al.,
specificity. To investigate this, peptides were synthesized from HHV-6B clinical strain N3 peptide was labelled U83BA-NT. HHV-6B U83, U83B-NT had a Gly-Cys3 substitution. The HHV-6A strain had a single substitution Asn-Lys14 only in the N-terminal region and defines Asn-14 as a key component. This substitution is marked with an *.

The 17-mer peptides were derived from U83A and U83B in the mature full-length proteins. Seven clinical strains analysed here in comparison to 74 strains from a full-length purified, mature U83B effectively displaced radiolabelled CCL2 from binding to CCR2, with an EC50 of 10 nM. This suggests signalling is modulated by conformation of the mature virus chemokine and exposure of the N termini of prototypes U83A and U83B (laboratory strains U1102 and Z29 respectively) and from clinical strain N3. These 17-mer peptides span four of the six differing residues between spliced U83A and U83B and the corresponding peptides were termed NT (N-terminal): U83A-NT, U83B-NT and U83BA-NT from the clinical strain N3 (Fig. 1).

**U83B-NT induces chemotaxis in ex vivo CCR2 positive human leukocytes**

U83B-NT, U83A-NT and U83BA-NT were measured for functional activity using a chemotaxis assay in comparison to human chemokines, including physiological ranges between 0.1 and 10 nM. Chemotaxis was first tested in a human monocyte cell line, THP-1, which expresses CCR2 to establish the assay (Fig. 2a). Then chemotaxis assays were conducted on ex vivo human leukocytes expressing CCR2, derived from multiple independent healthy donors and the combined results analysed (Fig. 2b). CCR2 and CCR5 specific chemokines, CCL2 and CCL4 respectively, were used as controls. Both CCL2, 10 nM positive control and U83B-NT, 1–100 nM, induced chemotaxis in the CCR2 expressing THP-1 cell line (Fig. 2a), similar to that reported for a mature U83B-Fc fusion protein (Zou et al., 1999). In the ex vivo human PBMCs, maximal migration was similar for CCL2 and U83B-NT; significant migration was induced by U83B-NT (0.1 and 10 nM), with a bell-shaped response curve to dilution gradients typical of chemokines. In contrast, stimulation with either U83A-NT or U83BA-NT did not induce chemotaxis of ex vivo PBMCs which had shown positive migration to the CCL2 control (Fig. 2c). No chemotaxis was observed with U83A-NT, U83BA-NT or U83B-NT when donor cells had levels of CCR2 or CCR5 surface expression which were undetectable by flow cytometry and no response to positive control chemokines CCL2 or CCL4, specific for CCR2 or CCR5, respectively (Fig. 2d). Efficient chemotaxis by U83B-NT, but not by U83A-NT or U83BA-NT, shows that specificity for CCR2 by U83B resides in this N-terminal region and defines Asn-14 as a key component.

Full-length purified, mature U83B effectively displaced radiolabelled CCL2 from binding to CCR2, with an EC50 of 79 nM, compared to IC50 of 0.08 nM for CCL2 (Lütichau et al., 2003). In chemotaxis assays, mature U83B, vCCL4, was similarly efficient to CCL2 in promoting migration of CCR2-transfected murine L1.2 cell lines, albeit at higher concentrations of 100–1000 nM, compared to effective concentrations for CCL2 of 0.1–10 nM (Lütichau et al., 2003). U83B-NT could not displace CCL2 up to 100 nM (not shown), suggesting interactions at a distinct site, while the chemotaxis mediated by U83B-NT was as potent as that induced by the human chemokine CCL2, at 0.1–10 nM. This suggests signalling is modulated by conformation of the mature virus chemokine and exposure of the U83B N-terminal peptide is important for potency. Human chemokines which bind CCR2 or CCR5 can induce rapid internalization of the receptor within minutes.
The effects of CCL2 and U83B-NT were compared (Fig. 3). Although CCL2 induced some reduction in surface CCR2 staining, indicative of internalization as described (Arai et al., 1997) (Fig. 3a, b), there was no effect by U83B-NT by 10 min (Fig. 3c, d, in duplicate at 50 nM and in quadruplicate at 1, 5, 10 and 10 nM). This resembles effects of U83A on CCR5, which also induces efficient chemotaxis, but with a similar delayed receptor internalization different from the rapid internalization/recycling induced by human chemokines (Catusse et al., 2007, 2009). Interestingly, CCL2 induced chemotaxis has also been reported independent of CCR2 internalization (Arai et al., 1997). The mature U83B is monospecific for CCR2 and shows no interaction with CCR5 or other human chemokine receptors including CX3CR1 (Lütichau et al., 2003). This suggests specificity for classical or intermediate monocytes.

**Migrated ex vivo PBMC induced by U83B peptide are enriched in CCR2**

In the chemotaxis assay donor PBMCs bearing CCR2 could be specifically stimulated by U83B-NT (and also CCL2) and positive chemotaxis could only be observed in donors where there was a relatively high overall prevalence of CCR2-bearing cells in the PBMC population. In order to further investigate this specificity, the phenotype of the actual migrated population of *ex vivo* PBMCs was characterized. Since this assay phenotypes the migrated population, it could be performed on donor PBMCs in which the relative prevalence of CCR2+ leukocytes in the starting population from donor PBMCs was lower or even a minor group. The chemotaxed cells were collected and examined by flow cytometry.

*Ex vivo* PBMCs stimulated immediately after isolation with either U83B-NT or CCL2 in transwell chemotaxis assays showed enrichment for CCR2+ cells post-migration.
relative to the original PBMC population (stock cells) as well as the post-migration buffer control (Fig. 4, top panel). Similar CCR2 enrichment post-migration was shown in three further donors tested after U83B-NT and CCL2 stimulation compared to buffer-only treatment (Fig. 4a, c, respectively). In these, the starting population had low CCR2 expression as shown by background migration to buffer only. Even with lower CCR2 expressing subsets, U83B-NT could selectively enrich the CCR2 population. Full-length, mature U83A did not increase the prevalence of CCR2 expressing cells (Fig. 4b). When cells were cultured to increase CCR5 expression, as shown previously (Catusse et al., 2009; Catusse et al., 2007), treatment with U83B-NT did not increase migration of CCR5 bearing PBMCs. In contrast, there was enrichment of CCR5, but not CCR2, bearing cells after migration towards full-length mature U83A (Fig. 4d), which has specificity for CCR5, but not CCR2 (Catusse et al., 2007; Dewin et al., 2006). Since the migration of CCR2 (and not CCR5) bearing PBMCs was increased with U83B-NT relative to the buffer-only treatment, this indicates CCR2 specificity rather than general leukocyte activation.

Shape-change assay for cellular activation induced by U83B peptide

Shape change is an indicator of cellular activation and can involve alterations in cell size, granularity or overall morphology as shown for CCL4 and U83A treatment of CCR5 expressing human leukocytes by confocal microscopy and on eosinophils treated with CCL11 by flow cytometry (Catusse et al., 2007; Sabroe et al., 1999; Signoret et al., 2005). CCR5 cells interacting with CCL4 show internalization of CCR5 and a decreased cellular morphology with reduced size and forward scatter, whereas CCR3 expressing eosinophils treated with cognate ligand CCL11 react with morphological changes leading to increased forward scatter in flow cytometry. A similar flow-cytometry-based assay was established using ex vivo PBMCs to further investigate the CCR2 specificity of U83B-NT activation and the affected monocyte subsets (Fig. 5). Shape change in response to chemokine stimulation was first compared between CCR2 and CCR5 expressing cells using a gating strategy as shown in Fig. 5a–d. PBMCs were stimulated with chemokine or buffer-only treatment control then either CCR2 or CCR5 expressing cells gated on (Fig. 5a, b) and CD3 expressing and/or dead

Fig. 3. CCL2 but not U83B-NT induces reduction in surface CCR2. Internalization assay was conducted by measurement of any reductions in CCR2 surface staining after chemokine stimulation. THP-1 cells were treated with chemokine or buffer-only control for 10 min then surface expression of CCR2 was assayed by flow cytometry. (a) Control experiment without any incubation. The dotted line shows the no staining control. The grey shading indicates staining with the isotype control for the CCR2-PE antibody. The solid black line shows staining with CCR2-PE antibody indicating CCR2 expression on almost all cells. (b) Cells were treated with CCL2, thick black solid and thick dotted lines, or buffer-only control, thin grey and thin dotted lines, for 10 min. CCL2 treatment resulted in lower levels of CCR2 staining. Results shown in duplicate of two independent experiments. (c) Cells were treated with buffer-only control (thin grey line) or with U83B-NT 50 nM (thick black line), which had no effect on CCR2 surface staining. (d) In a duplicate assay, cells were treated with different dilutions of U83B-NT, 1, 5, 10, 20 and 50 nM, and compared to buffer-only control. There is no evidence of a reduction in surface CCR2 at any concentration of U83B-NT.
cells gated out (lineage gating, Fig. 5c, d). Chemokine stimulated changes in forward scatter, either increased for CCR2 or decreased for CCR5, were then analysed as compared to the no-treatment buffer-only control (Fig. 5e).

The effects of U83B-NT were compared with those induced by stimulation with human chemokines specific for CCR2 or CCR5, CCL2 or CCL4 respectively, in relation to buffer-only treatment (Fig. 5e). Both CCL2 and U83B-NT showed similar shape-change effects in increasing forward scatter relative to the buffer control in the CCR2+/CD3− population (Fig. 5e). In contrast, CCL4 stimulation had little effect on these CCR2+/CD3− cells, while in the CCR5+/CD3− cells, CCL4 stimulation resulted in a decrease in forward scatter, consistent with alterations in cellular morphology previously observed. There was no effect on CCR5+/CD3− PMBCs of either CCL2 or U83B-NT. This further shows the specificity of the effect of U83B-NT in activating CCR2+ PMBCs. Monocytes are a major population expressing CCR2. T-lymphocyte subsets may also express CCR2, but since CD3-expressing cells were also gated out here, it is likely that monocytes are the main population responding. Although NK or dendritic cells may also be present, these generally express CCR5 in activated forms.

**U83B-NT stimulation of CCR2+CD14+CD16+ monocytic cells**

CCR2 expression has been identified in CD14+CD16− classical monocytes, and is decreased or absent in non-classical CD14lowCD16+ monocytes. However, CCR2 is also expressed in the recently defined intermediate monocyte subset which is CD14+CD16+. Therefore, the monocyte subsets activated by U83B-NT in the shape-change assay were examined. In the donor used for the experiment in Fig. 5, there were sufficient CCR2+ cells to enable analysis of the relative contribution of the different monocyte subsets to the response. Therefore, cells activated after stimulation with chemokines as indicated by the shape-change assay were further analysed for monocyty markers, LPS receptor, CD14 and the FcγIII receptor, CD16. CCR2+/CD3− cells and CCR5+/CD3− cells which changed shape (increase or decrease in forward scatter profile, respectively) relative to the buffer median were assessed by flow cytometry to enable the frequency of CD14+, CD16+ populations in the increased (CCR2+ CD3−) or decreased (CCR5+CD3−) forward scatter gates to be compared between chemokine and buffer-only treatments (Fig. 5e). Both the U83A-NT and negative control human chemokine CCL4 (CCR5-specific) stimulation of CCR2+CD3− cells, showed no difference to the buffer-only treatment (Fig. 5e and Table 1), while both U83B-NT and the positive control human chemokine CCL2 (CCR2-specific) induced shape change (increased forward scatter gate) and the responding population contained a higher frequency of both CD14+ and CD16+ cells, as well as dually expressing cells, showing that the activated cells were predominantly (>83%) CD14+ or CD16+ monocytes, with >77% of this population showing dual staining. This was not seen in the CCR5+CD3− CCL4-activated population, with only 0.14% CD14+CD16+ cells being present in the shape-changed population (reduced forward scatter gate) (Table 1). This indicates U83B-NT can activate specifically CCR2+CD14+CD16+ intermediate monocytes.

Intermediate monocytes and non-classical monocytes, which express both CD14 and CD16, have been increasingly defined as an intrinsic subset for virus interactions and some other intracellular pathogens (Balboa et al., 2011; Buckner et al., 2011; Chimma et al., 2009; Lentz et al., 2011; Williams et al., 2012). With lower expression of CD14, human CD14dim monocytes have roles in local tissue surveillance to detect nucleic acids and viruses via innate TLR7 and TLR8 pathways and appear to correlate with motile monocytes which patrol the vasculature (Cros et al., 2010). In intermediate monocytes, CCR2 expression is also intermediate, yet U83B-NT can activate this subset, despite the increased CCR2 expression in the classical CD14+CD16− subset, possibly indicating different CCR2 conformation or signalling in this subset. The transition to CD16 expression from classical monocytes does appear to coincide with expression of genes giving increased motility, so this could also explain this finding. In the donor used for the experiments in Fig. 5/Table 1, CCL2 also activated this subset, so this could be a donor-specific finding, but still demonstrates that U83B peptide can activate the intermediate subset. Monocytes generally comprise 10% of ex vivo human PBMCs, and of these 85% are classical monocytes with CD14+CD16−, while the intermediate monocyte subset CD14+CD16+ is approximately 5% (Ziegler-Heitbrock et al., 2010), so 0.5% of starting input PBMCs collected could have properties of this subset susceptible to U83B activation. This limited the number of cells available for analyses of activation, particularly as CCR2 is induced in proinflammatory conditions, therefore low or not detected in healthy donors. The conditions were only available for ex vivo analyses of the shape-change phenotype assay in the donor indicated. It would be of interest to extend these observations to patient cohorts with inflammatory disease. The other flow cytometry and chemotaxis assays were all replicated in multiple healthy donors. A major strength of these analyses is the use of ex vivo cells that have not been influenced by cytokine-activated culture, therefore are most likely to represent physiologically active circulating subsets and native interactions with the virus chemokine.

The CCR2+CD14+CD16+ phenotype has also been characterized as increased in senescent cells and can explain the increase in chronic inflammatory conditions in ageing populations including those with cardiovascular disease (Merino et al., 2011; Rogacev et al., 2011; Shantsila et al., 2011). HHV-6B is associated with inflammatory conditions, including encephalitis and myocarditis, where it is the most frequent virus identified together with parvovirus
19 (Kühl et al., 2005a). Therefore, the U83B specificity further defined here, provide a mechanism for modulation of the inflammatory response.

Since both HHV-6A and HHV-6B have also recently been identified as integrated genomes in between 0.1 and 1.0 % of global populations, up to 70 million people are potentially exposed to effects of these virus genes (Arbuckle et al., 2010; Arbuckle & Medveczky, 2011; Morissette & Flamand, 2010). Evidence suggests the integrated HHV-6 is primarily in a latent state, but there are reports of reactivation giving placental infection (Hall et al., 2010). Moreover, in the absence of other virus gene expression both HHV-6A and HHV-6B U83N, can be expressed, encoding the spliced-truncated version which includes the U83B-NT peptide (French et al., 1999). This immediate early profile suggests U83 is competent to be expressed from the genome and could be expressed from every cell, thereby enhancing chemokine activities in addition to CCL2 in inflammatory disorders. Notably, CCL2 has been described in both neuroinflammatory and cardiovascular pathologies and U83B with similar properties, but potentially wider cellular distribution as an integrated gene, could contribute to this.

Therefore, properties of U83B shown here are relevant both to the virus and as a virus gene expressed independently as a ‘human’ gene. Furthermore, to our knowledge, U83B-NT, as characterized here, is the smallest CCR2 specific peptide which can function potently in chemotaxis. Given its small size and efficacy, it could be used as a selective agent to stimulate intermediate monocytes, as a novel adjuvant for increasing vaccine efficacy due to the antigen-presenting features, including MHC class II expression, of this cellular subset. It may also have particular applicability to recently defined ‘prime- and chemokine-pull’ vaccination strategies (Shin & Iwashaki, 2012).

METHODS

Chemokine and peptide reagents. Chemokines CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) were purchased in lyophilized form from Peprotech (Rocky Hill) and reconstituted according to the manufacturer’s instructions. Aliquots (10 mM) of the chemokines were prepared, diluting the reconstituted peptide in PBS, pH 8. These aliquots were stored at −80 °C. Working stocks (10 μM) were prepared when required from these aliquots in HEPES buffered saline solution (HBSS, Sigma) with 0.1 % BSA (BSA, Sigma) and were stored at −20 °C. Working stocks were discarded after two freeze–thaw cycles. Viral chemokine peptides were synthesized by Sigma-Genosys and reconstituted using the manufacturer’s instructions. Briefly, chemokine was reconstituted with DMSO (Sigma) to make a 5 mM stock concentration and then aliquots of 100 μM and 10 μM were prepared using PBS/0.1 % BSA and stored at −20 °C. After two freeze–thaw cycles, aliquots were discarded.

PCR amplification and nucleotide sequencing. HHV-6 DNA was isolated from infant sera samples collected in Zambia as part of a nutrition intervention study as described previously (CIGNIS Study Team, 2010). HHV-6 U83 was PCR amplified using Gotaq green mastermix (Promega) or Pfu polymerase (New England Biosciences), as described previously (Bates et al., 2009; French et al., 1999). Outer primers U83OF/OR were used followed by a nested set, U83IF/IR: U83OF 5′AGTTAACACGACGGGAACAC3′, U83OR 5′TTGGGATTTATTGGCAACT3′, U83IF 5′GTAGGAAAAAGACTTGTGCAAA3′, U83IR 5′AACCAGTATTATGTCTTGCA3′. Gel purified DNA PCR products were sequenced using Big dye terminator 3.1 (Applied Biosystems) and run on an ABI3730 (Applied Biosystems). Sequences were analysed using Chromas pro (Technelysium) and compared to chemokine sequences on GenBank using NCBI BLAST. Alignments were prepared using CLUSTAL W (Chenna et al., 2003) and Jalview v2.4 (Waterhouse et al., 2009).

Human ex vivo PBMC purification and culture. Whole blood was collected with 5 mM EDTA (final concentration) from anonymously coded healthy adult human blood donors (LSTHM, UK), with written consent, following local phlebotomy guidelines. PBMCs were separated from whole blood using Histopaque-1077 (Sigma). PBMCs for culture were resuspended in RPMI 1640 with 10 % autologous human serum, 2 mM Glutamax (Fisher), 50 U mL−1 penicillin (Sigma), 50 μg mL−1 streptomycin (Sigma), plated in an ultralow adherence flask (Corning, Corning, NY) and incubated at 37 °C with 5 % CO2 for 72h as described (Catusse et al., 2007).

Chemotaxis assay. PBMCs at a density of 2 × 106 mL−1 were incubated in HBSS with 0.1 % BSA and 1.7μM Calcium-AI-M (Invitrogen) for 30 min at 37 °C, 5 % CO2. Cells were washed in chemotaxis buffer (HBSS/0.1 % BSA), then 1.5 × 106 cells, at a density of 3 × 106 mL−1, were plated out on a Neuroprobe ChemoTX™ microchemotaxis chamber (Receptor Technologies, UK) on the filter above the lower chambers containing chemokine, peptide or buffer. The assay was then run for 90 min by incubation of the cell and chemokine/peptide-filled microchemotaxis chamber at 37 °C, 5 % CO2. Excess cells were removed from the filter and cells migrated into the lower chamber were assayed using calcine fluorescence measured with a Wallac Victor2

Fig. 4. Cells chemotaxed by U83B-NT are enriched for CCR2-bearing cells. Human ex vivo PBMCs, stock cells expressing CCR2, which migrated through a 5 μm filter during a 90 min incubation with chemokine or buffer-only treatment were collected post-migration and stained separately for CCR2 or CCR5 using antibodies conjugated with fluorescent tags (PE and FITC respectively). The top panel demonstrates the assay set up with ex vivo PBMCs from a representative donor. The four graphs show the prevalence of the CCR2 staining of the starting PBMC population compared to the cells that had undergone migration after treatment with chemokines U83B-NT, CCL2 or buffer only. The light grey histograms show the staining with an isotype control antibody and the solid line, clear histogram shows staining with an antibody to CCR2. Y-axes show cell counts (expressed as % max, the percentage of the maximum number of cells). (a–d) Ex vivo PBMC from three further donors were then tested and the prevalence of CCR2- or CCR5-bearing cells which have migrated after chemokine stimulation (solid line, clear histogram) was compared to that of background migration after buffer only treatment (dark grey histogram). (a) U83B-NT 1 nM, (b) U83A 1 nM, and (c) CCL2 10 nM incubation. In (d) PBMCs were cultured for 3 days in non-adherent flasks to induce CCR5 expression, then treated with U83A as above.
Fig. 5. Shape-change assay further defines U83B-NT CCR2 specificity. Cells were stimulated with human chemokines, CCR2-specific CCL2 or CCR5-specific CCL4, virus chemokine U83B-NT or buffer for 90 min and then stained for cell markers. In this assay, cells were stained for: CCR2 (PE, for gating CCR2-expressing cells), CCR5 (FITC, for gating in CCR5-expressing cells), CD3 (pacific blue, PB, for gating out T cells), Dead cell stain (PB analogue, for gating out dead cells), CD14 (APC, for gating monocytic cells), CD16 (PE-Cy7, for gating cells bearing this Fc receptor). In order to define chemokine specific responses to chemokine receptors on the cell surface, cell populations were first defined by chemokine receptor expression, either CCR2 or CCR5, which were gated in separately to be able to compare their responses. Next, the CD3+/dead cells were gated out. Finally, the shape-change effect on chemokine-stimulated cells was evaluated by changes in forward scatter relative to the no treatment buffer only control. The flow cytometry gating strategy is summarized in (a–d). The grey histogram shows the fluorescence of unstained cells, and the black line cells stained cells. (a) Identification of chemokine receptor positive cells, CCR2 or CCR5, within the total PBMC population (shown here for CCR2-PE). The gate used to delineate CCR2+ cells is indicated. CCR5+ cells were separately gated for comparison (CCR5-FITC). (b) Dot plot showing the CCR2+ gated population. (c) Next, within either the CCR2 or CCR5 gated populations, cells which stained for CD3 or were dead (CD3- PB) were gated out. Similar gating was also performed with the CCR5+ population. (d) Dot plot showing the live CCR2+CD3- gated population. (e) Effects on forward scatter of chemokine treatment (black line) compared here to no treatment buffer only control (grey) on cells expressing CCR2+CD3- or CCR5+CD3- cells identified as described above. Cells were stimulated with chemokines as indicated U83B-NT 1 nM, CCL2 10 nM, CCL4 10 nM, or buffer only. In the left panels, cells expressing CCR2 respond to specific chemokine stimulation by increases in forward scatter and a larger, more granular morphology, as shown for both CCL2 and U83B-NT stimulation relative to the buffer. In the right panels, cells expressing CCR5 respond only to specific chemokine CCL4 stimulation shown by decreases in forward scatter and acquisition of a smaller, less granular morphology, relative to buffer. Cells were then gated (black bracketed lines) on the increased or decreased forward scatter profiles relative to the buffer control histogram median and the relative prevalence of CD14 or CD16 cell surface marker in the shape-changed population was further determined (Table 1).
Table 1. Frequency of CD14+ CD16+ monocytes in chemokine stimulated CCR2+ CD3− or CCR5+ CD3− ex vivo human PBMCs relative to buffer in shape-change assay

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>CCR2 + CD3−</th>
<th>CCR5 + CD3−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD14⁺ %</td>
<td>CD16⁺ %</td>
</tr>
<tr>
<td>Buffer</td>
<td>60.9</td>
<td>61.9</td>
</tr>
<tr>
<td>CCL4</td>
<td>65.3</td>
<td>65.8</td>
</tr>
<tr>
<td>CCL2</td>
<td>85.1</td>
<td>88.3</td>
</tr>
<tr>
<td>U83A-NT</td>
<td>67.5</td>
<td>68.4</td>
</tr>
<tr>
<td>U83B-NT</td>
<td>83.3</td>
<td>84.0</td>
</tr>
</tbody>
</table>

Flow cytometry. PBMC or THP-1 cells were stained with combinations of anti-human receptor antibodies directly conjugated to fluorescent labels. These included CCR2-FITC (FA1251P, R&D systems), CCR5-PE (FA1282F, R&D systems), CD3-Pacific blue, PB, (BD Pharmagen #558124), CD4-allophycocyanin (APC; BD 553399) or CD16-PE-Cytoan7 (PE-Cy7; BD 557744) antibodies, as well as ‘Dead’ stain-violet with the same spectrum and detected in the same channel as PB (Invitrogen, L34955). PBMCs were incubated with labelled antibodies, isotype or buffer-only controls for 30 min at 4 ºC, then washed with FACS buffer (PBS/0.1 %BSA) followed by fixing with 2 % paraformaldehyde for 15 min prior to analysis on a FACS Calibur (Becton Dickinson). Data were analysed and compensation applied, where multi-colour staining was used, with FlowJo software (Treestar).

Shape-change assay. Ex vivo PBMCs were incubated with or without positive control human chemokines (CCL2 or CCL4), virus chemokine peptide (U83B-NT, U83A-NT or U83B-NT) or buffer for 90 min at 37 ºC, as for the chemotaxis assay. This was followed by centrifugation, buffer wash and then staining for multi-colour flow cytometry, using incubation with dead stain-PB analogue and the following conjugated antibodies: CD3-PB, CCR2-PE, CCR5-FITC, CD14-APC and CD16-PE.Cy7. Samples were run on a FACS CyAn flow cytometer (Beckman Coulter). For analysis, events were gated on flow cytometry markers (dead stain−/CD3−/CCR2+ or dead stain−/CD3−/CCR5+) and forward and side scatter profiles of these cells examined. CCR2+ or CCR5+ cells with changed forward scatter, relative to the buffer, were gated after chemokine or peptide stimulation and then further analysed for CD14 and CD16 composition by gating for CD14-APC or CD16-PE.Cy7 staining.

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