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The role of the alternative coreceptor GPR15 in SIV tropism for human cells

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ABSTRACT

Many SIV isolates can employ the orphan receptor GPR15 as coreceptor for efficient entry into transfected cell lines, but the role of endogenously expressed GPR15 in SIV cell tropism is largely unclear. Here, we show that several human B and T cell lines express GPR15 on the cell surface, including the T/B cell hybrid cell line CEMx174, and that GPR15 expression is essential for SIV infection of CEMx174 cells. In addition, GPR15 expression was detected on subsets of primary human $CD4^+$, $CD8^+$ and $CD19^+$ peripheral blood mononuclear cells (PBMCs), respectively. However, $GPR15^+$ PBMCs were not efficiently infected by HIV and SIV, including cells from individuals homozygous for the defective Δ32 *ccr5* allele. These results suggest that GPR15 is coexpressed with CD4 on PBMCs but that infection of $CD4^+$, $GPR15^+$ cells is not responsible for the well documented ability of SIV to infect $CCR5^-$ blood cells.

Introduction

The human immunodeficiency viruses, HIV-1 and HIV-2, cause acquired immunodeficiency syndrome (AIDS) in humans and the simian immunodeficiency virus from macaques (SIVmac) induces an AIDS-like illness in experimentally infected Asian macaques (Clavel et al., 1987; Gallo et al., 1984; Letvin et al., 1985). In contrast, more than 40 African non-human primate species are naturally infected with SIVs and usually do not develop disease (Sharp and Hahn, 2010), with the exception of SIVcpz infected chimpanzees (Keele et al., 2009). HIV-1 is responsible for the AIDS pandemic, while HIV-2 is endemic in parts of Western Africa and is phylogenetically and antigenically closely related to SIVmac (Cohen et al., 2008; de Silva et al., 2008).

Infectious host cell entry of HIV and SIV is mediated by the viral envelope protein (Env), which consists of a surface subunit, gp120, and a transmembrane subunit, gp41 (Tilton and Doms, 2010). The Env proteins of all HIV and SIV isolates characterized to date use CD4 as the primary receptor for host cell entry, although several HIV-2 and SIV isolates can infect target cells in a CD4-independent fashion (Edinger et al., 1997b; Endres et al., 1996; Martin et al., 1997; Reeves et al., 1999) and CD4-independent HIV-1 variants have been generated in vitro (Dumonceaux et al., 1998; Haim et al., 2011; Hoffman et al., 1999; Kolchinsky et al., 1999). Binding of gp120 to CD4 induces conformational changes in gp120, which lead to the creation or exposure of a conserved coreceptor binding site (Rizzuto et al., 1998). Coreceptor engagement subsequently activates gp41 mediated fusion of the viral envelope and the cellular membrane, thereby allowing delivery of the viral genetic information and proteins into the host cell cytoplasm (Harrison, 2008; Steffen and Pöhlmann, 2010).

Coreceptor tropism varies between HIV and SIV: HIV-1 and HIV-2 can use CCR5 (R5-tropic viruses), CXCR4 (X4-tropic viruses) or both (R5 × 4-tropic viruses) for entry into primary human T cells (Alkhatib et al., 1996; Chen et al., 1997; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996; Feng

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et al., 1996; Rucker et al., 1997; Sol et al., 1997), while SIV isolates employ CCR5 but not CXCR4 (Chen et al., 1997; Edinger et al., 1997a; Hill et al., 1997; Kirchhoff et al., 1997; Rucker et al., 1997), with rare exceptions (Chen et al., 1998; Schols and De Clercq, 1998). In addition, many HIV-2 and SIV isolates can use the orphan receptor GPR15/BOB and other 7-transmembrane G-protein-coupled receptors for efficient infection of cell lines transfected to ectopically express these receptors (Blaak et al., 2005; Chen et al., 1997; Deng et al., 1996; Edinger et al., 1997a; Elliott et al., 2012; Farzan et al., 1997; Hill et al., 1997; Morner et al., 1999; Rucker et al., 1997). Usage of GPR15 and other alternative coreceptors has also been documented for some HIV-1 isolates (Deng et al., 1996; Farzan et al., 1997; Pöhlmann et al., 1999a; Rucker et al., 1997; Zhang et al., 2001), but robust usage of alternative coreceptors is less frequent among HIV-1 compared to HIV-2 and SIV.

Selective knock-out of GPR15 usage by a point mutation in the SIVmac V3-loop is compatible with efficient viral spread and pathogenesis (Pöhlmann et al., 1999b), suggesting that usage of GPR15 in addition to CCR5 is not important for SIVmac infection of rhesus macaques. However, SIVs can infect PBMCs from human donors homozygous for the Δ32 *ccr5* defect allele (Dean et al., 1996; Huang et al., 1996; Samson et al., 1996), suggesting that engagement of alternative coreceptors can support CCR5- independent SIV spread in primary target cells (Forte et al., 2003; Zhang et al., 2000). Whether GPR15 is expressed by $CD4^+$ human blood cells and contributes to CCR5-independent SIV infection of human PBMCs is currently unknown.

We report that human B and T cell lines express GPR15 and that GPR15 usage is essential for SIVmac infection of the T/B hybrid cell line CEMx174. GPR15 was also detected on a subset of primary human $CD4^+$ PBMCs. However, only a fraction of the SIVmac infected PBMCs expressed GPR15, indicating that the contribution of $GPR15^+$ cells to SIV amplification in human PBMCs is minor.

Results

SIVmac239 uses human GPR15 and CXCR6 with similar efficiency as CCR5 for entry into transfected cells

Previous studies showed that SIVmac239 can use the human alternative coreceptors GPR15 and CXCR6 for efficient host cell entry into coreceptor transfected cells (Deng et al., 1997; Farzan et al., 1997). To confirm and extend these results, we analyzed SIVmac239 utilization of a panel of alternative coreceptors. For this, 293T cells, which are $CD4^-$ and non-permissive to HIV or SIV infection, were transiently cotransfected with plasmids encoding the human CD4 receptor and either one of the potential coreceptors CXCR4, CXCR6, CCR2, CCR3, CCR5, GPR1 or GPR15, all of human origin. The transfected cells were then infected with luciferase-encoding env-deficient HIV-1 NL4–3 pseudovirus bearing the Env proteins of murine leukemia virus (MLV), HIV-1 NL4–3, SIVmac239 or no envelope protein (Fig. 1A). Pseudotypes bearing the MLV Env infected all cells with comparable high efficiency, independent of coreceptor expression (Fig. 1A). The X4-tropic HIV-1 NL4–3 Env protein facilitated efficient entry into cells transfected to coexpress CD4 and CXCR4, as expected, but inefficient infection of all other cells tested was also detected, an observation in line with the previously documented endogenous expression of low amounts of CXCR4 by 293T cells (Wilen et al., 2011) (Fig. 1A). Finally, the SIVmac239 Env mediated robust and comparable infection of cells coexpressing CD4 and either CCR5, CXCR6 or GPR15. Coexpression of GPR1 also allowed for SIVmac239 driven entry, although with markedly reduced efficiency compared to CCR5, CXCR6 and GPR15, while infection of cells coexpressing the CCR2, CCR3 and CXCR4 coreceptors was close to or within background levels (Fig. 1A).

The efficient SIVmac239 Env-driven entry into GPR15 and CXCR6 transfected cells might be due to particularly high expression of these receptors in transfected cells. To investigate this possibility and to confirm our results with authentic SIVmac239, we constructed CCR5, GPR15 and CXCR6 variants with an N-terminal AU-1 tag and compared expression and coreceptor function of these receptors. All constructs were expressed to roughly comparable levels (Fig. 1B), as determined by FACS analysis, and allowed entry of SIVmac239, encoding luciferase in place of nef, with similar efficiency as the wt coreceptors (Fig. 1C), indicating that the N-terminal tag did not interfere with protein folding. In contrast, none of the other coreceptors tested facilitated efficient SIVmac239 entry and all cells were comparably susceptible to infection with an env-defective SIVmac239 vector pseudotyped with the G-protein of VSV (VSV-G) (Fig. 1C). Thus, GPR15 and CXCR6 support efficient SIVmac239 entry into transfected cells, in keeping with published work (Chen et al., 1997; Edinger et al., 1997a; Feng et al., 1996; Kirchhoff et al., 1997; Rucker et al., 1997), and efficient coreceptor activity is not due to exceedingly high expression of these coreceptors compared to CCR5.

GPR15 is expressed on lymphoid cell lines

The robust usage of GPR15 by SIVmac239 for entry into transfected cells prompted the question whether the virus can employ endogenous GPR15 for infection of susceptible cell lines. Expression of GPR15 protein in cell lines has previously not been investigated and was assessed employing a commercially available mouse monoclonal antibody. In order to assure specificity of GPR15 detection, we first analyzed potential cross-reactivity of the anti-GPR15 antibody with other 7-transmembrane G-protein coupled receptors. For this, we transiently transfected 293T cells with different coreceptors and subjected the transfected cells to a two-step antibody staining with either mouse anti-GPR15 or an isotype matched control (IgG2b) antibody and a Cy5-labeled anti-mouse secondary antibody. Reactivity of the anti-GPR15 antibody with the cells transfected to express CXCR4, CXCR6, CCR2, CCR3, CCR5 or GPR1 was within the background range observed for the isotype matched control antibody (Fig. 2A). In contrast, staining of GPR15-transfected 293T cells resulted in strong fluorescent signals and a shift from 5 to 186 in geometric mean channel fluorescence (Fig. 2A).

We next determined if GPR15 is expressed on the human CD4-positive T cell lines C8166-SEAP (Means et al., 1997), Jurkat, PM-1, CEM-T4 and Sup-T1 CD4-negative. In addition, the T/B hybrid cell line CEMx174 (Salter et al., 1985), which expresses GPR15 mRNA (Farzan et al., 1997; Kirchhoff et al., 1997), and a CEMx174 derivate, engineered to express CCR5 (CEMx174 R5, (Hsu et al., 2003)), were examined for the presence of GPR15 protein. Robust GPR15 expression was detected on CEMx174, CEMx174 R5 and PM-1 cells, all of which are susceptible to SIVmac infection (Lusso, 2006; Marzi et al., 2007; Pöhlmann et al., 1999b), but not on the other T cell lines tested. One exception were C8166-SEAP T cells which yielded signals slightly above background in four independent experiments. In order to analyze if GPR15 is also expressed by other lymphoid cell lines, we expanded our analysis to the B cell lines Raji, NC37, BCBL-1, DG75, BJAB, LL58 and Ramos. No evidence for GPR15 expression on Ramos, Raji, BCBL-1, LL58 and DG75 cells was obtained, although the high levels of unspecific antibody binding to Raji cells precluded a definite conclusion regarding this cell line. In contrast, positive results were obtained

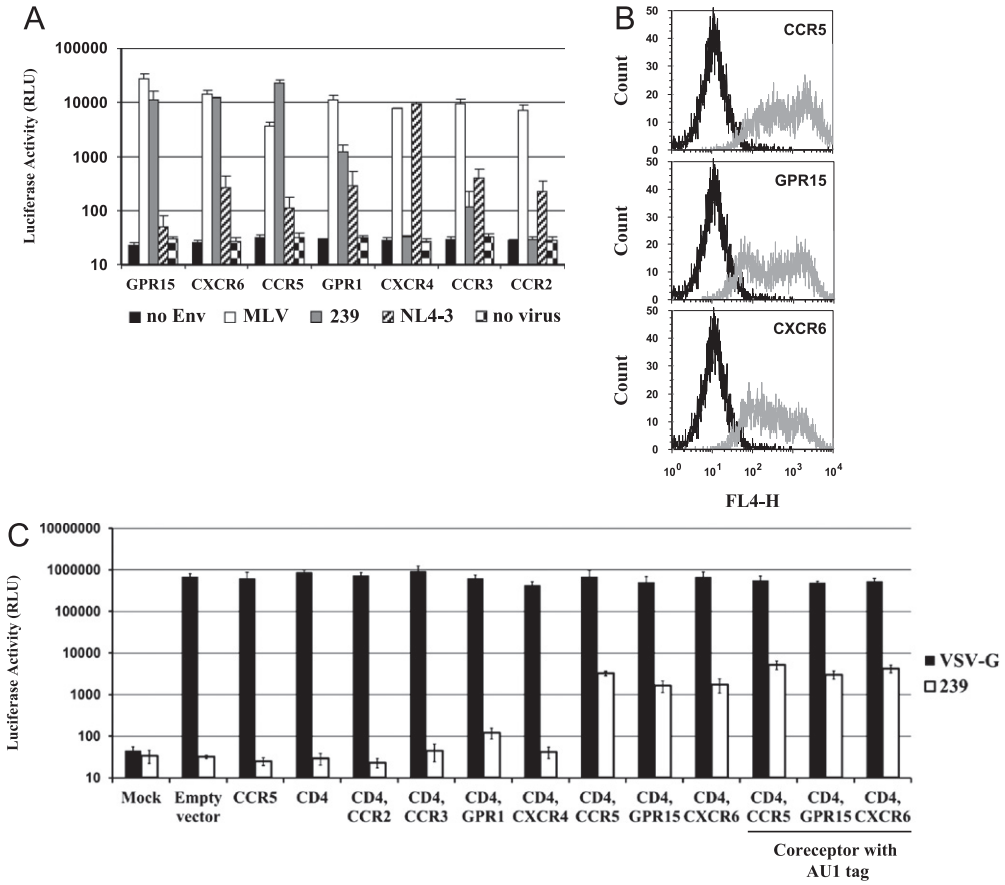

Fig. 1. SIVmac239 uses GPR15 with high efficiency for entry into transfected cells. (A) 293T cells were transfected with expression plasmids for CD4 and the indicated coreceptors, both of human origin. At 48 h post transfection the cells were infected with HIV-1 pseudotypes bearing the indicated envelope proteins. Luciferase activities in cell lysates were determined at 72 h post infection. The experiment was repeated twice and a representative experiment, performed in triplicates, is shown. Error bars indicate standard deviations (SD). (B) 293T cells were transfected with the indicated coreceptors containing an N-terminal AU1 antigenic tag and expression was analyzed by FACS employing anti AU-1 antibody. (C) The experiment was carried out as described for (A) except that the cells were infected with a replication competent SIVmac239 variant encoding luciferase or with env defective, luciferase-encoding SIVmac239 pseudotyped with VSV-G. The results ± SD of a representative experiment carried out in triplicates are shown and were confirmed in a separate experiment. MLV, murine leukemia virus Env; 239, SIVmac239 Env; NL4-3; HIV-1 NL4-3 Env; VSV-G, vesicular stomatitis virus G-protein; RLU, relative light units.

with NC37 B cells, indicating that certain T and B cell lines express GPR15 on the cell surface.

Engineered expression of CCR5 rescues CEMx174 cell infection by SIVmac239 variant P321S, which has a selective defect in GPR15 utilization

The cell line CEMx174 is frequently used for propagation of SIV and we next asked if GPR15 expression is required for SIVmac239 infection of these cells. The SIVmac239 V3 loop mutant P321S was previously shown by us to have a selective defect in GPR15 utilization (entry via CCR5 and CXCR6 was only slightly diminished) and in CEMx174 cell infection (Kirchhoff et al., 1997; Pöhlmann et al., 1999b). In order to ascertain that the reduced CEMx174 cell infection by variant P321S was indeed due to reduced usage of an alternative coreceptor, we analyzed if this defect can be rescued by engineered expression of CCR5. For this, we compared the ability of the SIVmac239 wt Env and P321S Env to fuse with CEMx174 cells engineered to express CCR5 (CEMx174 R5) and with parental CEMx174 cells (CEMx174-SEAP, (Means et al., 1997)), which are CCR5$^-$ (Kirchhoff et al., 1997). CEMx174 R5 cells and CEMx174-SEAP cells harbor LTR-luciferase and LTR-SEAP cassettes, respectively, which allow convenient quantification of cell–cell fusion. To measure cell–cell fusion, 293T effector cells were transiently cotransfected with Env expression plasmids and an expression plasmid for the HIV-1 NL4–3 Tat transactivator or an empty plasmid as control. The effector cells were then cocultured with CEMx174 R5 cells or CEMx174-SEAP cells and reporter activity in the cocultures was measured. Robust activation of reporter gene expression was only observed upon the presence of Tat and an Env protein in effector cells, indicating that the signals specifically reflected cell–cell fusion events, as expected (Fig. 3A). The wt Env protein mediated robust fusion with both cell lines tested, in agreement with its ability to use both CCR5 and GPR15 as coreceptors. In contrast, the P321S Env mediated efficient fusion with CEMx174 R5 cells but not with CEMx174-SEAP cells, in accordance with the previous finding that this mutant can employ CCR5 but not GPR15 for robust host cell entry (Pöhlmann et al., 1999b) (Fig. 3A).

We next determined if the rescue of P321S mediated cell–cell fusion by engineered expression of CCR5 is also reflected on the level of virus–cell fusion. For this, HIV-1 pseudotypes bearing MLV Env, SIVmac239 wt Env and P321S Env were normalized for comparable infectivity on TZM-bl indicator cells and subsequently used for parallel infection of CEMx174-SEAP or CEMx174 R5 cells. In accordance with the cell–cell fusion data, the P321S Env failed to mediate entry into CCR5-negative CEMx174-SEAP cells but facilitated robust infection of CCR5-expressing CEMx174 R5 cells (Fig. 3B). In contrast, the SIVmac239 wt Env allowed efficient infection of both cell lines. Thus, the defect of the P321S variant in mediating cell–cell and virus–cell fusion with parental, CCR5-negative CEMx174 cells can be rescued by engineered

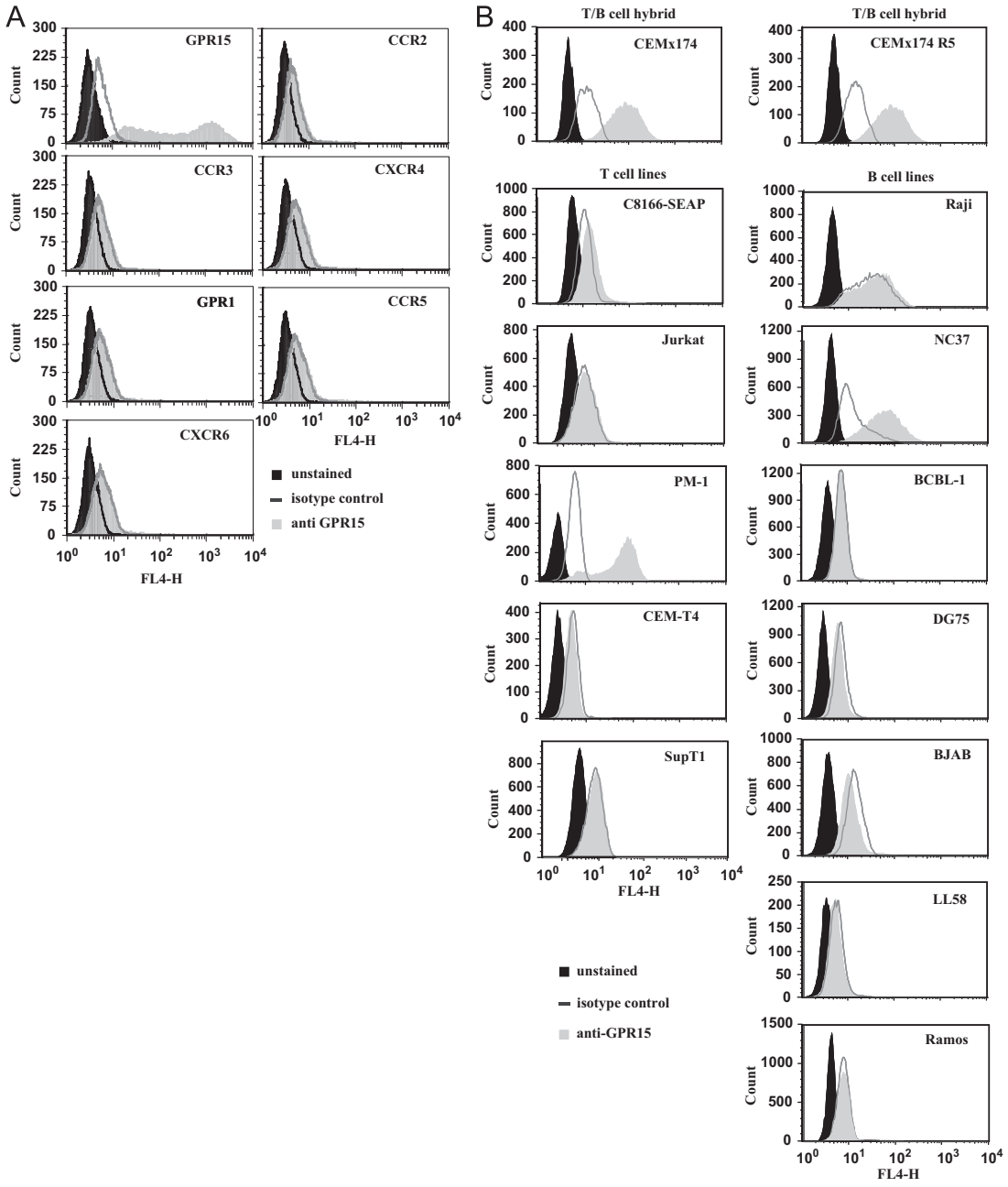

Fig. 2. Lymphoid cell lines express GPR15. (A) Antibody specificity was tested by FACS staining of 293T cells transfected with the indicated coreceptors. At 48 h post transfection the cells were stained with mIgG2b isotype control or GPR15-specific antibody and Cy5-conjugated anti-mouse secondary antibody. Staining was analyzed by FACS and similar results were obtained in two separate experiments. (B) The T/B hybrid cell lines CEMx174 and CEMx174R5 as well as the T cell lines Jurkat, C8166-SEAP, SupT1, CEM-T4, and PM-1, and the B cell lines Raji, Ramos, DG75, BJAB, BCBL-1, LL58 and NC37 were stained for surface expression of GPR15 as described in (A) after blocking the cellular Fc receptors with human IgG or normal donkey serum. Staining was measured by FACS analysis. The experiment was repeated at least three times for each cell line.

expression of CCR5 and is thus due to abrogated usage of an alternative coreceptor, most likely GPR15.

shRNA knockdown of GPR15 in CEMx174 cells inhibits SIVmac239 infection

For direct evidence that GPR15 facilitates SIVmac239 infection of CEMx174 cells, we performed short hairpin RNA (shRNA) mediated knock-down (the anti-GPR15 antibody did not block infection). To this end, a shRNA was identified that efficiently reduced expression of transfected GPR15 but not CCR5 in 293T cells (not shown). Subsequently, CEMx174 cells were electroporated with a shRNA expression plasmid encoding either the GPR15-specific shRNA or a scrambled, non-sense control shRNA. In addition to the shRNA, the plasmids encode for GFP, which allowed convenient identification of transfected cells. CEMx174 cells that had taken up plasmid encoding the GPR15-specific shRNA showed reduced GPR15 expression compared to cells that had received the plasmid encoding the control shRNA (Fig. 3C), indicating specific GPR15 knock-down by the shRNA used. Due to inefficient delivery of plasmid DNA into CEMx174 cells (11% of the cells were transfected, Fig. 3C), transfected and thus GFP-positive cells were sorted by fluorescence-activated cell sorting (FACS) before infection with pseudotypes bearing the MLV Env or the SIVmac239 Env. Infection of CEMx174 cells by MLV pseudotypes was not modulated by GPR15 knock-down. In contrast,

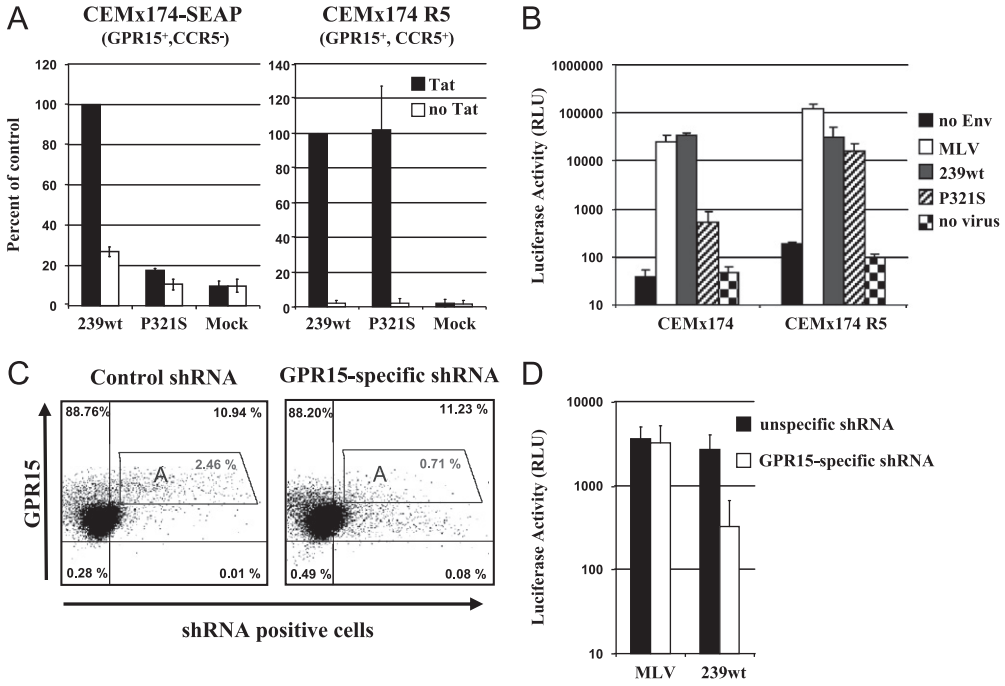

Fig. 3. GPR15 is required for SIVmac239 entry into CEMx174 cells. (A) 293T effector cells were cotransfected with plasmids encoding HIV-1 Tat and SIVmac239 Env (239wt) or SIVmac239 P321S Env, an envelope mutant that has a selective defect in GPR15 usage. The effector cells were then cocultured with CEMx174-SEAP target cells (left panel), which are $CD4^+$, $GPR15^+$ and $CCR5^-$ and contain a secreted alkaline phosphatase (SEAP) expression cassette under control of the viral LTR, or CEMx174 R5 target cells (right panel), which are $CD4^+$, $GPR15^+$ and $CCR5^+$ and contain a luciferase expression cassette under control of the viral LTR. Luciferase and SEAP-activity in cocultures was measured at 48 h post cocultivation. The average of three independent experimens is shown. Error bars indicate standard error of the mean (SEM). (B) CEMx174 or CEMx174 R5 cells were infected with HIV-1 pseudotypes bearing the indicated envelope proteins. Luciferase activities were measured 72 h post infection. The experiment was repeated twice and the results of a representative experiment performed in triplicates are shown. Error bars indicate SD. (C) CEMx174 cells were electroporated with plasmids encoding control shRNA (left dot plot) or GPR15-specific shRNA (right dot plot), stained for GPR15 expression and analyzed by FACS. The shRNAs encoding plasmids also contain an expression cassette for GFP, allowing detection of transfected cells. (D) CEMx174 cells, sorted for expression of the GPR15-specific shRNA (as judged by GFP expression), were infected with HIV-1 pseudotypes bearing the indicated envelope proteins. Luciferase activity in cell lysates was determined at 72 h post infection. The experiment was repeated two times and a representative experiment, performed in triplicates, is shown. Error bars indicate SD. RLU, relative light units.

GPR15 knock-down markedly reduced infection by pseudotypes bearing the SIVmac239 Env (Fig. 3D). These results demonstrate that SIVmac239 utilizes the alternative coreceptor GPR15 for infection of the CCR5-deficient T/B hybrid cell line CEMx174.

GPR15 is expressed on subsets of primary human T cells and B cells

We next explored if GPR15 is expressed on primary $CD4^+$ PBMCs, the major HIV and SIV target cell type. PBMCs were isolated from whole blood of healthy human donors, cultivated in the presence or absence of phytohemagglutinin (PHA) for 3 to 5 days and subjected to antibody staining for GPR15 surface expression as described above. Additionally, PBMCs were co-stained for either CD4, CD8 or CD19 to identify T cell and B cell populations, respectively. An isotype matched antibody control was included to determine the level of background staining. Although ratios varied between donors, PHA-stimulated PBMC populations consisted of approximately 80% $CD4^+$ T cells and 10% B cells (Fig. 4A), while unstimulated PBMCs were composed of approximately 70% $CD4^+$ T cells and 25% B cells. GPR15 expression on PBMCs was readily detectable independent of cellular activation but varied between donors, ranging from approximately 2 to 25% of all stimulated PBMCs, with an average of 9% (Fig. 4B). GPR15 expression appeared to be moderately higher on unstimulated relative to stimulated PBMC (Fig. 4A). However, unspecific binding of the isotype control antibody was also increased and higher background levels could account for the observed difference. Notably, a variable portion of the $GPR15^+$ PBMCs coexpressed CD4 (avg. 11%) or CD19 (avg. 26%), respectively (Fig. 4A and B), suggesting that particular lymphocyte subsets are $GPR15^+$. The activation status of the PBMCs had no major effect on GPR15 expression on these lymphocyte subsets (Fig. 4A). In addition, a subset of the $CD8^+$ cells among the stimulated PBMCs also expressed GPR15 (avg. 6%), demonstrating that GPR15 expression among T cells is not limited to the helper cell compartment (Fig. 4C, unstimulated, $CD8^+$ PBMCs could not be unequivocally assessed because of high background staining). Since $CD4^+$ helper T cells are the main target cells for HIV and SIV infection, we finally investigated which subsets of $CD3^+$ $CD4^+$ cells expressed GPR15. We found that the coreceptor was mainly expressed on central memory cells (CM) and to a lesser degree on effector memory cells (EM), with substantial donor variability (Fig. 4D). In contrast, naïve T cells were largely $GPR15^-$ with the exception of one donor (Fig. 4D). Thus, various lymphoid cells express GPR15, including a subset of effector memory $CD4^+$ T cells, which are the preferential targets of R5-tropic HIV-1 and SIV isolates (Gondois-Rey et al., 2002; Grivel et al., 2000; Mehandru et al., 2004).

GPR15-positive human PBMCs are not efficiently infected by SIV

SIVmne, SIVmac and SIVrcm can infect PBMCs from human donors homozygous for the Δ32 ccr5 allele (Forte et al., 2003; Zhang et al., 2000), indicating that various SIVs can use alternative coreceptors for infection of human PBMCs. We determined if $GPR15^+$ cells are targeted by SIVmac. PBMCs from *ccr5* wt/wt, Δ32/wt and Δ32/Δ32 donors were prepared, PHA-activated and infected with replication competent SIVmac239 encoding GFP. Infection efficiency (as determined by GFP expression) and GPR15 expression were subsequently analyzed by flow cytometry.

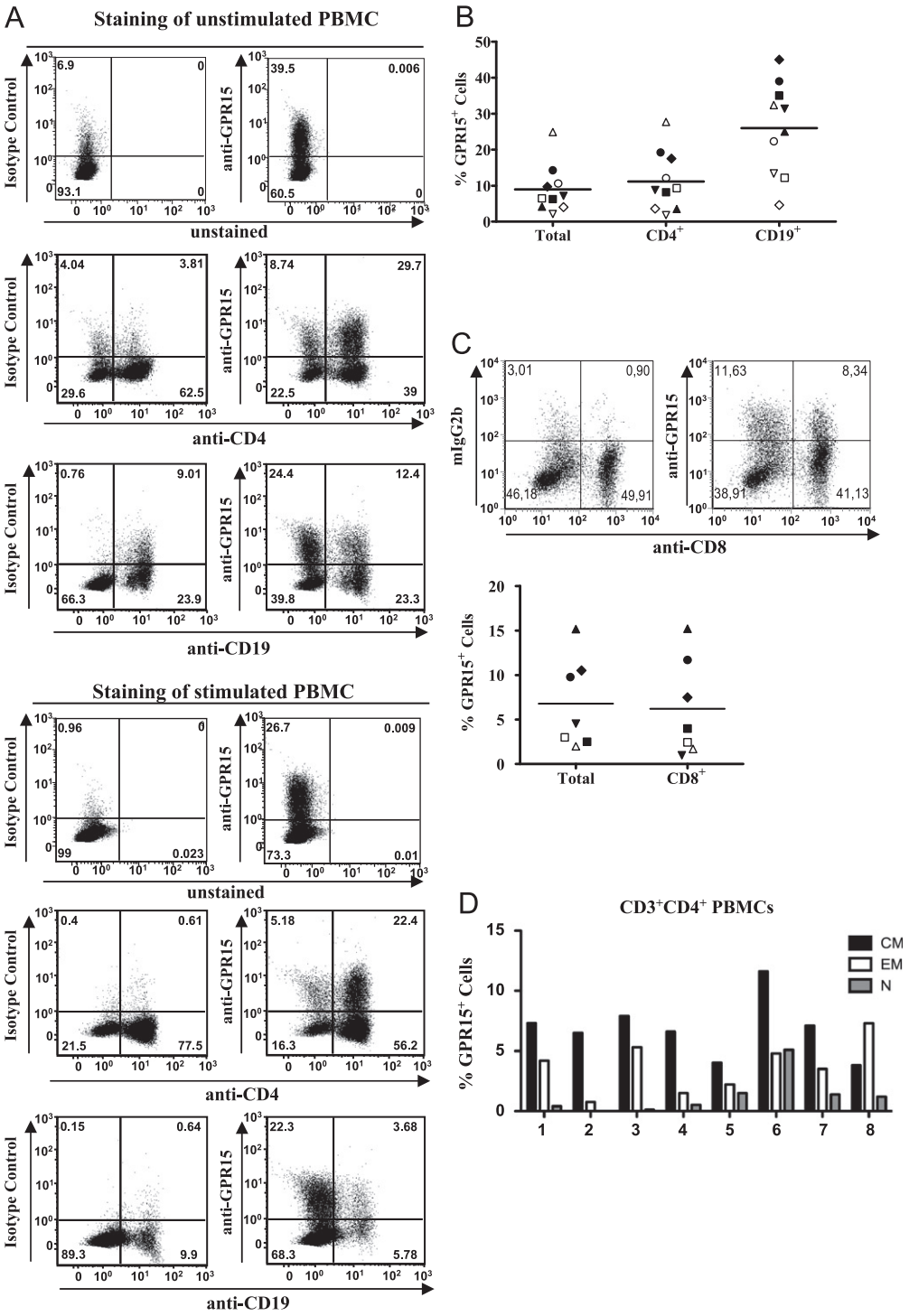

Fig. 4. Subsets of primary human lymphocytes express GPR15. (A) PBMC were isolated from whole blood by Ficoll gradient centrifugation and were either stimulated with PHA for 3 days (stimulated PBMC) or cultured in the absence of PHA for 3 days (unstimulated PBMC). Subsequently, cells were stained for surface expression of GPR15, as described in Fig. 2A. Additionally, stimulated PBMC were co-stained with FITC-conjugated population markers anti-CD4 and anti-CD19 (or respective FITC-conjugated isotype matched controls) to identify T and B cells. Staining was analyzed by flow cytometry. The experiment was repeated 10 times for stimulated PBMCs and 3 times for unstimulated PBMCs to compensate for donor variation. The results of a representative experiment are shown. (B) Donor variation of GPR15 expression on total stimulated PBMC or $CD4^+$ or $CD19^+$ subsets. The percentage of $GPR15^+$ cells in the respective subpopulation or total PBMC was plotted for 10 different donors to reflect the donor dependence of GPR15 expression. (C) PBMC were isolated and stimulated as described in (A) and stained for surface expression of GPR15 and CD8. A representative experiment is shown as dot plots in the upper panel and GPR15 expression on total PBMC or $CD8^+$ cells is shown for 7 different donors in the lower panel. (D) PBMCs were isolated from whole blood by Lymphoprep gradient centrifugation and directly costained for CD3, CD4, CCR7, CD45RA and GPR15. Cells were gated for $CD3^+$, $CD4^+$ cells and GPR15 expression on the subsets was analyzed via FACS. CM: central memory ($CCR7^+$, $CD45RA^-$), EM: effector memory ($CCR7^-$, $CD45RA^-$), N: naïve ($CCR7^+$, $CD45RA^+$). Expression was analyzed for eight different donors to account for donor variation.

Infection of PBMCs by SIVmac239 was detectable independent of the *ccr5* genotype, as expected, although infection of Δ32/wt and particularly Δ32/Δ32 PBMCs was reduced compared to wt/wt PBMCs (Fig. 5A). However, none of the infected cells expressed GPR15, indicating that $GPR15^+$ cells are unlikely to contribute to SIVmac amplification in $CCR5^-$ human PBMCs.

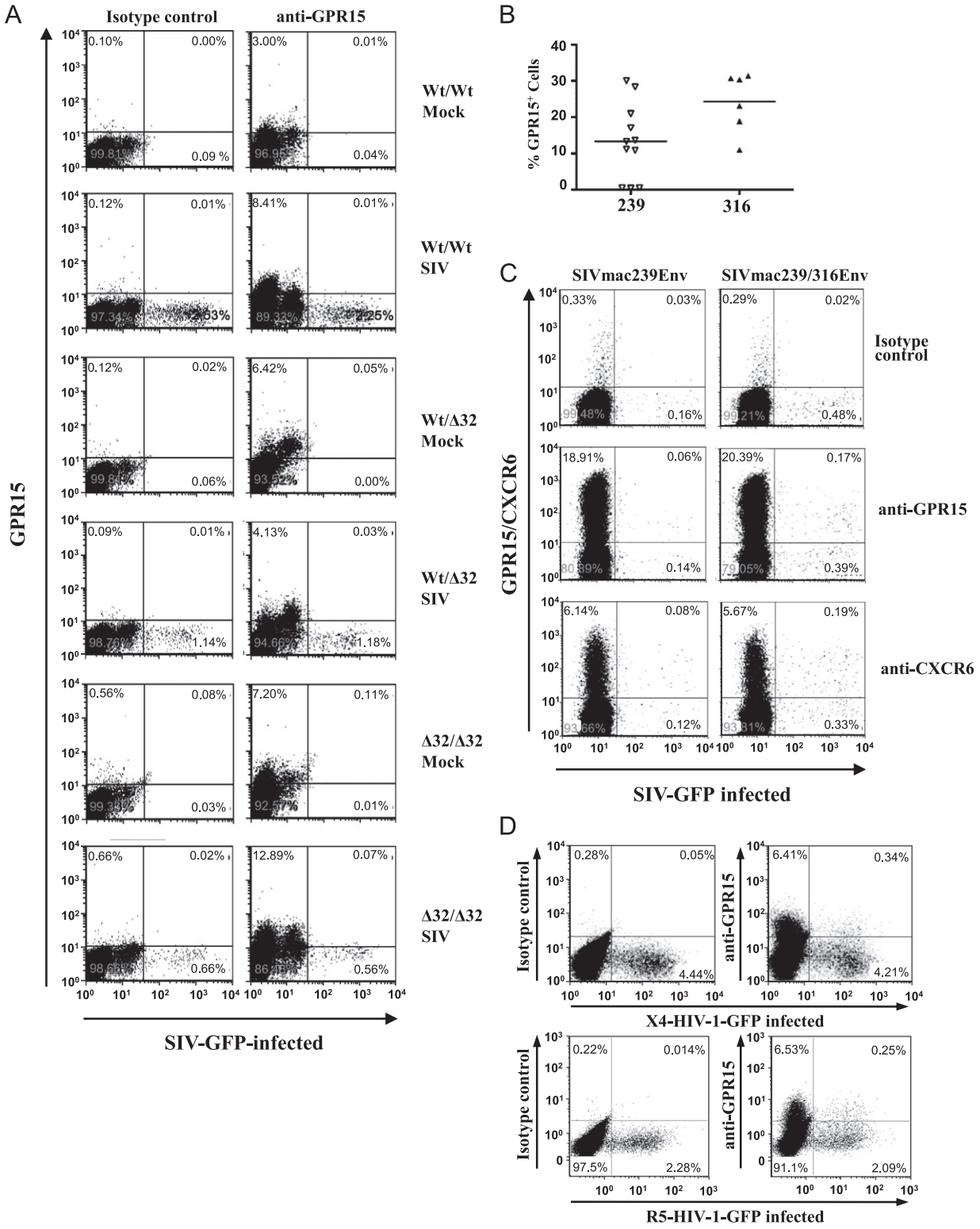

Fig. 5. GPR15$^+$ and CXCR6$^+$ human PBMCs are not preferentially infected by SIV. (A) PBMCs from human donors with the indicated *ccr5* genotypes were isolated, PHA stimulated and infected with replication competent SIVmac239 containing a nef-IRES-GFP cassette. At three days post infection, GPR15 and GFP expression were analyzed by FACS. Similar results were obtained in two separate experiments. (B) Human PBMCs were isolated from 9 different donors, PHA stimulated for 3 to 5 days and infected with GFP-encoding, SIVmac239 or SIVmac239/316Env bearing viruses. At three days post infection, expression of GPR15 and GFP was analyzed. The percentage of the infected cells, which expressed GPR15, is shown. (C) Human PBMCs were isolated and stimulated with PHA for five days prior to infection with GFP encoding, replication-competent SIVmac239 or SIVmac239/316Env, respectively. Three days post infection the expression of GPR15 and CXCR6 as well as GFP were analyzed by flow cytometry. Similar results were obtained in a separate experiment. (D) Human PBMCs were isolated, stimulated with PHA and infected with GFP-encoding HIV-1 NL4-3 (X4-tropic) or HIV-1 NL4-3 encoding the V3-loop of 92TH014-2 (R5-tropic) and stained for expression of GFP and GPR15. Similar results were obtained in two independent experiments.

In order to analyze the susceptibility of GPR15$^+$ cells to SIVmac infection in more detail, we next examined infection of PBMC from multiple donors, including individuals with a high percentage of GPR15$^+$ cells. In addition to SIVmac239 also SIVmac239/316Env, a macrophage tropic variant of SIVmac239 (Mori et al., 1992), was used for these experiments, in order to exclude envelope specific effects. Finally, we asked if infected cells might express CXCR6, which facilitates SIVmac239 and SIVmac239/316Env entry into transfected cells (Fig. 1 and (Sharron et al., 2000)). Infection of GPR15$^+$ cells by SIVmac239 Env bearing viruses was detectable in the majority of donors but was generally inefficient, with on average $13.6 \pm 3.1\%$ of the infected cells expressing GPR15 (Fig. 5B). Similar results were obtained for SIVmac239/316Env, although infection of GPR15$^+$ cells was more efficient ($24.3 \pm 3.1\%$ of the infected cells expressed GPR15). Nevertheless, for a donor with high GPR15 expression up to 30% of the infected cells were GPR15$^+$ and roughly 40% were positive for CXCR6 (Fig. 5C), indicating that GPR15$^+$ and CXCR6$^+$ cells could contribute to viral spread in some individuals. In this context it should be noted that SIVmac239 infection only modestly reduced GPR15 expression on CEMx174 R5 cells (20–40% reduction of geometric mean channel fluorescence), with all infected cells remaining in the GPR15$^+$ gate (not shown), indicating that efficient coreceptor down-regulation was unlikely to account for the absence of GPR15 on the majority of the infected PBMCs. Finally, PBMCs infected by GFP-encoding, X4- and R5-tropic HIV-1 were also largely devoid of GPR15, indicating that GPR15$^+$ cells are poorly susceptible to HIV-1 infection (Fig. 5D). Thus, GPR15$^+$ PBMCs are infected with relatively low frequency by SIVmac and HIV-1 and are thus unlikely to substantially contribute to viral amplification.

Discussion

SIV isolates frequently use GPR15 with similar efficiency as CCR5 for infection of transfected cell lines (present study and (Deng et al., 1997; Farzan et al., 1997)) but the role of endogenous GPR15 in SIV cell tropism is largely unknown. We showed that a subset of human T and B cell lines and primary human T and B cells express GPR15 on the cell surface. Expression of GPR15 was essential for SIVmac infection of the cell line CEMx174. In contrast, GPR15$^+$ PBMCs were not preferentially infected by SIVmac, indicating a minor contribution of GPR15$^+$ cells to SIVmac amplification in human PBMCs.

Shortly after the discovery of CCR5 as major coreceptor for HIV and SIV it was appreciated that CCR5 expression does not account for all aspects of SIV cell tropism. Subsequently, more than ten alternative coreceptors were identified which supported HIV and/or SIV infection, at least under conditions of high expression (Choe et al., 1998; Deng et al., 1997; Dumonceaux et al., 1998; Edinger et al., 1998a,b; Farzan et al., 1997; Kanbe et al., 1999; McKnight et al., 1998; Neil et al., 2005; Rucker et al., 1997; Shimizu et al., 1999). GPR15 and CXCR6 were shown to allow SIV infection with high efficiency (Deng et al., 1997; Farzan et al., 1997), a finding confirmed by the present study, and CXCR6 was subsequently demonstrated to be expressed on cell lines and PBMCs susceptible to HIV and SIV infection (Sharron et al., 2000). Similar studies were not conducted for GPR15, probably because of the lack of a suitable reagent for detection of GPR15 expression.

A mouse monoclonal anti-GPR15 (IgG2b) antibody became commercially available in the meantime and has been used in the present study to characterize GPR15 expression on cell lines and human PBMCs. The antibody reacted efficiently with GPR15 transfected cells but did not bind to CCR5, CXCR4, CXCR6, GPR1, CCR2 and CCR3 transfected cells, indicating that the staining was specific for GPR15. GPR15 expression was detected on the T cell line PM-1, the T/B cell hybrid CEMx174 and the B cell line NC-37. In contrast, GPR15 was absent from Jurkat and Sup-T1 T cells, which are known to be CD4$^+$, CXCR4$^+$, CCR5$^-$ and poorly, if at all, susceptible to SIV infection (Del Prete et al., 2009; Lee et al., 2001). PM-1 cells are commonly used to propagate HIV and SIV and, due to their endogenous expression of CCR5 (Lusso et al., 1995; Pöhlmann et al., 1999b; Steinberger et al., 2000), coexpression of GPR15 might not be required for SIV infection of this cell line. Indeed, the SIVmac239 variant P321S, which uses CCR5 but not GPR15 for efficient cellular entry (Kirchhoff et al., 1997; Pöhlmann et al., 1999b), was previously shown to replicate efficiently in PM-1 cells but not in CEMx174 cells (Pöhlmann et al., 1999b), which are CD4$^+$, CCR5$^-$ and GPR15$^+$, as discussed below. Intriguingly, the P321S variant also failed to replicate in C8166 cells (Pöhlmann et al., 1999b), an HTLV transformed human T cell line susceptible to SIV infection (McKeating et al., 1989), and only trace amounts of GPR15 were detected on these cells. While these low levels of GPR15 might be sufficient to allow entry of wt SIV, it cannot be excluded that the P321S substitution abrogates usage of GPR15 and one or more so far uncharacterized coreceptors, which are engaged by wt SIV for infection of C8166 cells.

The cell line CEMx174 and its derivatives CEMx174-SEAP and CEMx174 R5 are frequently used for the propagation of SIV. An early study demonstrated that this cell line is CD4$^+$, CCR5$^-$ but expresses at this point an unknown coreceptor which is used by wt SIVmac239 but not by the SIVmac239 V3 loop mutant P321S (Kirchhoff et al., 1997). Subsequently, it was shown that GPR15 allows SIV infection of transfected cells and that endogenous GPR15 is expressed in CEMx174 cells (Deng et al., 1997; Farzan et al., 1997). However, it has not been demonstrated that GPR15 is indeed used by SIV for infection of CEMx174 cells. We closed this gap by two approaches. First, we demonstrated that the defect in CEMx174 cell infection exerted by the substitution P321S can be rescued by engineered expression of CCR5 (in CEMx174 R5 cells), which confirms that the P321S phenotype is due to lack of usage of an alternative coreceptor. Second, we showed that shRNA-mediated knock-down of GPR15 expression reduces virus–cell fusion driven by the SIVmac Env but not the MLV Env. These results demonstrate that SIVmac engages GPR15 for infection of CEMx174 cells and provide, to our knowledge, the first formal demonstration that endogenous GPR15 exerts coreceptor function for SIV.

Given the ability of endogenous GPR15 to support SIV entry into CEMx174 cells, we next examined whether endogenous GPR15 is expressed on primary human lymphoid cells. FACS analysis demonstrated that a subset of CD4$^+$ human PBMCs, the major targets of HIV and SIV infection, indeed expresses GPR15. Similarly, a portion of CD8$^+$ and CD19$^+$ PBMCs, respectively, was found to be GPR15$^+$. However, variability between preparations and donors were noted for at present unclear reasons. Differences in the cellular activation status might play a role, although GPR15 expression was detected at roughly comparable levels on unstimulated and PHA stimulated PBMCs. The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway was shown to regulate GPR15 surface expression and activation of this pathway might be donor dependent or might proceed with variable efficiency under the culture conditions chosen (Chung et al., 2009). Effector memory cells are preferentially depleted by CCR5-tropic HIV and SIV (Mehandru et al., 2004; Spina et al., 1997; Veazey et al., 2000a; Veazey et al., 2000b), while infection with X4- or R5X4-dualtropic SIV/HIV hybrid viruses (SHIVs) results in pan CD4$^+$ T cell depletion (Grivel et al., 2000; Nishimura et al., 2004). Our analysis of CD4$^+$ T cell subsets revealed that GPR15 is expressed on effector memory cells and

thus on potential viral targets. The proportion of GPR15$^+$ cells was higher on central memory compared to effector memory cells, while GPR15 was largely absent from naïve CD4$^+$ T cells. How GPR15 expression is linked to the acquisition of the memory phenotype remains to be established.

Homozygosity for the Δ32 *ccr5* allele is associated with marked resistance against sexually acquired HIV-1 infection (Dean et al., 1996; Huang et al., 1996; Samson et al., 1996) and a panel of R5-tropic viruses was found to be unable to replicate in cultured PBMCs from *ccr5* Δ32 homozygous donors, despite efficient usage of alternative coreceptors for entry into transfected cells (Zhang et al., 2000; Zhang et al., 2001). Although evidence for infection of *ccr5* Δ32/Δ32 PBMCs was reported by some studies (Cilliers et al., 2005; Jiang et al., 2011; Willey et al., 2003), these results indicate that most HIV-1 isolates cannot use alternative coreceptors for infection of PBMCs. In stark contrast, various SIV and some HIV-2 isolates were shown to infect *ccr5* Δ32/Δ32 PBMCs in the absence of CXCR4 usage (Forte et al., 2003; Willey et al., 2003; Zhang et al., 2000), indicating that these viruses can use alternative coreceptors for entry into PBMCs. However, the identity of the responsible alternative coreceptors remained largely unclear. Our analysis of infected PBMCs from multiple donors demonstrates that GPR15$^+$ cells are targeted by SIVmac239 with low efficiency, independent of the *ccr5* genotype, indicating that lack of functional CCR5 on target cells does not skew infection towards GPR15$^+$ cells. Nevertheless, when analyzing PBMCs from a donor with a high proportion of GPR15$^+$ cells, roughly 30% of the infected cells were positive for GPR15 and 40% expressed CXCR6, which is used with similar efficiency as GPR15 for SIV entry into transfected cells (Deng et al., 1997; Farzan et al., 1997). Similar results were obtained with a macrophage tropic variant of SIVmac239, SIVmac239/316Env (Mori et al., 1992), in agreement with the previously reported SIV infection of CXCR6$^+$ PBMCs (Sharron et al., 2000). Thus, a fraction of the GPR15$^+$ and CXCR6$^+$ cells in human PBMC preparations can be susceptible to SIVmac infection, although they do not constitute preferred viral targets. Finally, infection of GPR15$^+$ cells by X4- and R5-tropic HIV-1 was inefficient, suggesting that the contribution of GPR15$^+$ cells to the amplification of HIV-1 in human PBMCs is minor.

While our results argue against an important role of GPR15 in the hematogenic spread of HIV-1 and SIV they clearly do not exclude a role of this coreceptor in viral pathogenesis. In fact, recent studies revealed that GPR15 might be involved in enterocyte (Clayton et al., 2001; Li et al., 2008; Maresca et al., 2003) and neutrophil (Elbim et al., 2008) apoptosis in the context of HIV-1 and SIV infection, indicating that HIV/SIV interactions with GPR15 merit further analysis.

Material and methods

Cell culture

The human embryonal kidney cell line 239T and TZM-bl cells (obtained from the NIH AIDS Research and Reference Reagent Program, #8129) were cultured in DMEM (Invitrogen) containing 10% fetal calf serum (FCS) and antibiotics penicillin/streptomycin. CEMx174 (Salter et al., 1985), CEMx174-SEAP (Means et al., 1997), CEMx174 R5 (Hsu et al., 2003), C8166-SEAP (Means et al., 1997), Jurkat, SupT1, CEM-T4, PM-1, LL58, Raji, Ramos, NC37, BCBL-1, DG75, and BJAB cells were cultured in RPMI 1640 (PAA) containing 10% FCS and antibiotics penicillin/streptomycin. Peripheral blood mononuclear cells (PBMC) were isolated via Ficoll (Biomol) or Lymphoprep (Axis shield PoC As) gradient centrifugation and were cultured in RPMI 1640 (PAA) containing 10% FCS and antibiotics penicillin/streptomycin supplemented with 100 units/ml interleukin-2 (IL-2, Roche). Prior to infection PBMC were stimulated with 5 μg/ml phytohemagglutinin (PHA, Sigma-Aldrich) for 3 days.

Plasmids

The plasmids encoding wt SIVmac239 and SIVmac239 variants containing a nef IRES GFP cassette or the luciferase gene in place of nef were described previously (Laguette et al., 2012; Pöhlmann et al., 2001). The SIVmac239/316Env variant encoding a nef-IRES-GFP cassette was generated by insertion of a vpx–vpr–env fragment derived from pSIVmac316 (Mori et al., 1992) into pBRSIVmac239_-nef+_IRES_eGFPd5 (Laguette et al., 2012). The presence of the 316 mutations was confirmed by sequence analysis. The plasmids encoding HIV-1 NL4–3 (X4-tropic) and HIV-1 NL4–3 with the V3 loop of 92TH014–2 (R5-tropic), both encoding EGFP in place of nef, were described previously (Chaipan et al., 2010; Papkalla et al., 2002). The HIV-1 NL4–3 R$^-$E$^-$ Luc lentiviral vector (Connor et al., 1995), the plasmids encoding MLV env, HIV-1 NL4–3 env, SIVmac239 env, and SIVmac239 P321S env (Pöhlmann et al., 1999b; Simmons et al., 2003) and the plasmids encoding CCR2, CCR3, CCR5, CXCR4, CXCR6, GPR1, and GPR15 were also previously described (Pöhlmann et al., 1999a; Pöhlmann et al., 1999b; Pöhlmann et al., 2000) or were obtained via the NIH AIDS Research and Reference Reagent Program. For shRNA-mediated GPR15 knock-down, the oligonucleotides 5-siGPR15-pos48-BamHI/MluI GATCCAGCCCAAACTCTGACATCATTCAAGAGATGATGTCAGAGTTTGGGCTTTTTTTACGCGTG and 3-siGPR15-pos48-EcoRI/MluI AATTCACGCGT AAAAAAAGCCCAAACTCTGACATCATCTCTTGAATGATGTCAGAGTTTGGG were annealed and inserted into pSirenRetro-Q-EGFP (Clontech) employing the BamHI/EcoRI restriction sites. The following oligonucleotides were used to generate coreceptors with N-terminal AU1-antigenic tag: p5-CCR5-AU1-HindIII CCCAAGCTTCACCATGGACACCTACAGATACATAGATTATCAAGTGTCAAGAATCTATG and p3-CCR5-XbaI CCTCTAGACCTCACAAGCCCAC;AGATATTTC (for construction of AU1 tagged CCR5), p5-GPR15-AU1-HindIII CCAAGCTTCACCATGGACACCTACAGATACATAGACCCAGAAGAAACTTCAGTTTATTTGG and p3-GPR15-XbaI CCTCTAGACCTTAGAGTGACACAGACCTCTTCC (for construction of AU1 tagged GPR15) and p5-CXCR6-AU1-HindIII CC AAGCTTCACCATGGACACCTACAGATACATAGCAGAGCATGATTACCATGAAGACTATGG and p3-CXCR6-XbaI CCTCTAGACCCTATAACTGGAACATGCTGG (for construction of AU1 tagged CXCR6)

ccr5 genotyping

For the determination of the ccr5 genotype, mucosal epithelial cells were collected, washed with PBS, pelleted and treated with proteinase K for 60 min at 56 °C. Subsequently, the enzymatic activity was inactivated by incubation of the samples at 95 °C for 15 min. Finally, the ccr5 sequence was amplified employing primers 5-CCR5del32 CCC AGG AAT CAT CTT TAC CAG and 3-CCR5del32 CCC AGA AGA GAA AAT AAA CAA TCA T (Kantarci et al., 2005) and reaction products were analyzed by agarose gel electrophoresis.

FACS staining

For analysis of GPR15 expression on cell lines, a total number of 2×10^5 cells were washed with FACS buffer (1x PBS supplemented with 5% FCS), blocked for 30 min with 10 μl human IgG (Miltenyi Biotech) or 20 μl normal donkey serum (Sigma), and stained with mouse anti-GPR15 or anti-CXCR6 antibody (R&D Systems) or matched mIgG2b isotype control antibody diluted 1:50 in FACS buffer for 30 min at 4°C. The cells were washed again and stained with anti-mouse Cy5 (1:100) or anti-mouse Dylight649 (1:250) (Dianova) secondary antibody in FACS buffer for 30 min at 4°C, followed by washing and fixation in PBS/2%

paraformaldehyde. PBMCs were stained for GPR15 as described above and for CD4, CD8 and CD19 using directly labeled FITC-conjugated mouse monoclonal antibodies or the respective isotype matched controls (BD Biosciences). The cell staining was analyzed via FC500 cytometer (Beckman Coulter) and FCS Express (De Novo Software) or FlowJo (Tree Star) software. For subpopulation analysis GPR15 staining on $CD4^+$ cells was performed as described above, but in this case directly ex vivo without any blocking using anti-mouse Dylight 649 (Dianova) secondary antibody diluted 1:250 in FACS buffer and anti-CD3 V450, anti-CD4 PE, anti-CD45RA PE-Cy5.5, anti-CCR7 PE-Cy7 (BD Biosciences). The staining was analyzed via LSRII (BD Biosciences) and FlowJo (Tree Star) software.

Cell–cell fusion assay

293T cells were seeded at a density of 1.3×10^5 cells/ml in a 12-well plate. After 16 h cells were transfected with 1.5 μg of plasmid encoding SIVmac239 env and 1.5 μg of plasmid encoding the first exon of SIVmac239 tat using the calcium phosphate transfection method. At 24 h post transfection cells were mixed with 1×10^5 cells/ml CEMx174-SEAP or CEMx174R5 cells containing a secreted alkaline phosphatase (SEAP) or firefly luciferase expression cassette, respectively, under the control of a truncated SIV long terminal repeat (LTR) promoter. At 48 h post mixing of cells, SEAP activity in culture supernatants or luciferase activity in cell lysates were measured using the PhosphaLight assay system (Applied Biosystems) or the Beetle-Juice firefly luciferase assay system (PJK), respectively.

Virus production

Viruses were produced as described previously by us (Pöhlmann et al., 2007; Simmons et al., 2003). In brief, 293T cells were seeded in T-25 culture flasks at 7.5×10^5 cells per flask. After 16 h cells were transfected with either 12 μg of full-length SIVmac239 or SIVmac239/316Env proviral plasmid DNA or cotransfected with 6 μg of HIV-1 NL4–3 E^-R^-Luc plasmid DNA and 6 μg of envelope protein expression plasmids or empty vector (for production of pseudotypes) using the calcium phosphate transfection protocol. The transfection medium was replaced by fresh culture medium at 8–14 h post transfection. Culture supernatants were harvested at 48 h post transfection, separated from cell debris via filtration through a 0.445 μm pore size filter, aliquotted and stored at −80 °C. Virus stocks were normalized for comparable infectivity in TZM-bl cells.

Infection experiments

293T cells transfected to express CD4 and one of the coreceptors were seeded 24 h prior to infection at 1×10^4 cells/well and CEMx174-SEAP and CEMx174 R5 cells were seeded at 2×10^4 cells/well in 96-well plates and infected with HIV-1 NL4–3 E^-R^-Luc pseudotyped with MLV, HIV-1 NL4–3, SIVmac239 or SIVmac239 P321S envelope proteins which were normalized for comparable infectivity on TZM-bl cells. At 8 h post infection the medium was replaced by fresh culture medium. SEAP activity in culture supernatants or luciferase activity in cell lysates were measured 72 h post infection using the PhosphaLight assay system (Applied Biosystems) or the Beetle-Juice firefly luciferase assay system (PJK), respectively.

shRNA knock down of GPR15 and infection with pseudovirus

A total number of 1×10^7 cells/ml CEMx174 cells were electroporated with 20 μg of plasmids encoding GPR15 specific or control shRNA and a GFP expression cassette (pSiren RetroQ, Clontech). At 24 h post electroporation CEMx174 cells were sorted for GFP-positive (=shRNA-positive) cells using a FACSAria (BD Biosciences) or MoFlo (Beckman Coulter) cell sorter. Sorted cells were then seeded at 3×10^4 cells/well in 96 well plates and spinoculated (O'Doherty et al., 2000) with HIV-1 NL4–3 E^-R^-Luc pseudotyped with SIVmac239 or MLV Env proteins for 2 h at 2000 rpm at room temperature. At 8 h post infection the medium was replaced by fresh culture medium. Luciferase activities in cell lysates were measured at 72 h post infection using the Beetle-Juice firefly luciferase assay system (PJK).

PBMC infection with SIVmac239-GFP virus

Prior to infection PBMC were stimulated with 5 μg/ml phytohemagglutinin (PHA) and 100 units/ml interleukin-2 (IL-2) for 3–5 days and then seeded for infection at 5×10^5 cells/well in a 24-well plate. For infection the culture medium was removed and cells were resuspended in culture medium containing GFP-coding SIVmac239 or SIVmac239/316Env, respectively. Alternatively, cells were infected with GFP-encoding, env-defective HIV-1 bearing the Env protein of SIVmac239 or SIVmac239/316Env. After incubation at 37 °C for 8 h, the medium was replaced by fresh culture medium and 72 h post infection the cells were analyzed by flow cytometry for GPR15, CXCR6 and GFP expression. After gating, at least 100,000 events were counted.

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