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Phenotypic Analysis of Peripheral Blood γδ T Lymphocytes and Their Targeting by Human Immunodeficiency Virus Type 1 in Vivo

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INTRODUCTION

Infection with human immunodeficiency virus type 1 (HIV-1) invariably leads to the destruction of CD4+ T helper lymphocytes whose loss contributes to the profound immunodeficiency underlying the development of clinical AIDS. The tropism of HIV for this cell type results from its expression of CD4, and the necessary coreceptors CXCR4 and/or CCR5 (Klatzmann et al., 1984; Dalgleish et al., 1984; Maddon et al., 1986; Feng et al., 1996; Alkhattib et al., 1996; Dragic et al., 1996), which act as receptors for the attachment and entry of HIV-1. In principal, expression of CD4 and either chemokine receptor could make other lymphoid and nonlymphoid cell types susceptible to HIV-1 infection in vivo. For example, combined CD4 and CCR5 expression likely accounts for the infection of macrophages and macrophage-derived cell types in vivo. Documenting the extent of infection of nonlymphoid cell types by HIV-1 is important as their destruction or functional impairment potentially further harms the immune system and contributes to the pathogenesis of AIDS.

We have previously demonstrated infection of both CD4 and CD8 lymphocytes in peripheral blood mononuclear cells (PBMCs) by PCR analysis of highly purified subsets of lymphocytes isolated by magnetic bead separation methods (Livingstone et al., 1996; McBreen et al., 2001). Recently we have used a pan-T negative isolation method to purify T lymphocytes from PBMCs, followed by positive selection for CD8 and CD4. Quantitation of proviral sequences by limiting PCR indicated substantial infection of both lymphocyte subsets in the majority of individuals, although we also detected a significant reservoir of infection in the remaining lymphocytes (γδ T lymphocytes and CD3 “nul” cells), in agreement with a previous study describing infection of null (CD4−, CD8−) T lymphocytes with HIV in vivo (Mardon et al., 1999).

Although it possible that the infected cells represent CD4 lymphocytes with Nef-induced down-regulation of CD4, it is also possible that HIV infects other classes of T lymphocytes. Among these, numerically the most prominent are those expressing the γδ T cell receptor (TCR) that differentiate from intrathymic T cell precursors following rearrangement of the T cell receptor to express γ and δ gene segments (reviewed in MacDonald et al., 2001). These lymphocytes are predominantly distributed in the gut, lung, and genitourinary tracts and are believed to play an role in mucosal immunity, in both coordination of immune reactions through release of cytokines, primarily of the Th1 spectrum, and direct cytolytic effects particularly against cells infected with intracellular pathogens such as mycobacteria and parasites. Recognition of pathogens by γδ T lymphocytes is not MHC restricted nor limited to antigenic peptides and they do not require antigen-presenting cells to become activated (Pfeffer et al., 1990). γδ lymphocytes account for between 0.5 and 15% of peripheral blood T lymphocytes, although
whether they play any functional role in immunity outside of mucosal tissues is unknown. Frequencies of circulating γδ lymphocytes show a modest increase in PBMCs from HIV-infected individuals (Moreau et al., 1996; Roederer, 2000), and it has been suggested that this represents a homeostatic mechanism that maintains lymphocyte numbers to compensate for destruction of CD4 lymphocytes (Roederer, 2000).

There is limited information on the effect of HIV infection on γδ T lymphocyte function, and the contribution of such damage to the immunodeficiency observed in AIDS. Increased γδ T lymphocyte numbers in the gastrointestinal epithelium is associated with increasing disease severity, which may represent increased immune activation (Nilssen et al., 1996; Norazmi et al., 1995). A repeated observation is the numeric decline and functional impairment of the γδ lymphocyte subset expressing the Vγ9/Vδ2 receptors (Wallace et al., 1997; Poccia et al., 1996). These cells have demonstrated cytolytic activity against cells infected with a range of viruses including HIV (Sciammas and Bluestone, 1999; Poccia et al., 1999), although whether infection of γδ lymphocytes by HIV underlies the functional impairment and repertoire changes observed in HIV-infected individuals remains difficult to assess. The majority of peripheral γδ T lymphocytes are double negative for CD4 and CD8, with CD4 expression less frequent than CD8 (Hayday, 2000). CD8 expression has to be reported on activated γδ T lymphocytes, although they express the αα CD8 homodimer rather than the αβ CD8 heterodimer found on αβ TCR+ CD8 lymphocytes (Hayday, 2000; Taupin et al., 1999). Of relevance to HIV pathogenesis is the recent observation of high frequencies of expression of the principal coreceptor for HIV, CCR5, on the Vδ2 subset of γδ lymphocytes in the peripheral circulation (Glatzel et al., 2002). Whether the numerical decline of this subset in HIV infection is associated with reduction in the frequency of CCR5-positive γδ lymphocytes on the development of AIDS is currently unknown.

In this study, we investigated the expression of CD4, CD8, and the chemokine receptors CCR5 and CXCR4 on γδ lymphocytes in the peripheral circulation, as part of a preliminary assessment of their infectibility by HIV. We also modified our CD4 and CD8 lymphocyte separation method to enable the isolation of highly purified γδ lymphocytes from PBMCs to enable their contribution of proviral load to be quantified. We also investigated possible genetic differences between variants of HIV-1 isolated from the CD3+ CD4+ lymphocytes and γδ lymphocytes from four HIV-seropositive individuals that might determine differences in cellular tropism. Finally, an in vitro infectivity assay was used to determine if HIV-1 can replicate in γδ T lymphocytes and investigate the entry requirements.

RESULTS

Expression of CD4, CD8, CCR5, and CXCR4 on peripheral γδ T lymphocytes

To investigate the surface phenotype of γδ lymphocytes in vivo, and thus their potential susceptibility to HIV infection, PBMCs from HIV-seronegative and -seropositive individuals were analysed for expression of CD4, CD8, CCR5, and CXCR4 by three-colour FACS analysis with different combinations of monoclonal antibodies (Mabs). Using Mabs to CD4, CD8, and the γδ receptor, CD4 expression was detected on distinct populations of γδ lymphocytes at frequencies ranging from 6.97 to 9.57% (mean, 8.14%, n = 5) for the seronegative individuals and between 2.06 and 9.25% (mean, 4.44%, n = 7) of γδ lymphocytes from the seropositive individuals. Irrespective of HIV infection, levels of expression of CD4 on positive γδ lymphocytes were approximately fivefold lower than found on CD4 T lymphocytes [mean fluorescence intensity for γδ lymphocytes: 28 (range, 12–49), compared with 140 (range, 99–170) for CD4+ αβ TCR+ lymphocytes]. The numeric decline in the frequency of CD4 expression on γδ lymphocytes in HIV infection contrasted with the observation of more frequent CD8 expression in this subset in samples from HIV-infected individuals (mean, 53.41%; range, 28.8–87.47%, n = 7), compared with a mean value of 10.63% for HIV-uninfected individuals (range, 0.26–17.46%, n = 5; Figs. 1A and 1B). Dual expression of CD4 and CD8 on γδ lymphocytes was detected on a small population of cells, with 0.57% (range, 0.4–1.24%, n = 5) from seronegative individuals and 0.95% (range, 0.52–2.16%, n = 7) from seropositive individuals (Figs. 1A and 1D).

Expression of the chemokine receptors expression on γδ lymphocytes was determined by a FACS analysis of PBMC using a second set of monoclonal antibodies to CXCR4, CCR5, and the γδ receptor. The majority (mean, 62.70%, n = 5) of γδ lymphocytes from seronegative individuals expressed high levels of CCR5 (Figs. 1A and 1C), while lower frequencies of expression were detected in those from seropositive subjects (mean, 31.70%, n = 7; Figs. 1A and 1C). In contrast, while expression of CXCR4 occurred at a generally low level on a minority of the γδ population (mean, 34.75%, n = 5; Figs. 1A and 1C) from seronegative individuals, higher CXCR4 expression levels were found on the γδ lymphocytes from infected individuals (mean, 44.53%, n = 7, Figs. 1A and 1C). Combining the results of the two FACS analyses, the expression of CD4, CCR5, and CXCR4 indicates the potential infectibility of at least a proportion of γδ lymphocytes by HIV.

HIV-1 proviral burden of γδ lymphocytes in vivo

To investigate the extent of HIV infection of γδ lymphocytes in vivo, γδ, CD4, CD8, and non-T lymphocytes were
were contaminating CD4 lymphocytes.

500 copies/10^6 cells

frequencies in

sponded to complete proviruses. Comparison of proviral
indicating that the majority of or all sequences corre-
and C-LTR primers were comparable (Table 1, column 3),
the assay, proviral loads measured using the pan-LTR
using the pan-LTR primers. Within the accuracy limits of
scripts only (C-LTR; Table 1). Frequencies of proviral
sequences detected in

tRNA binding site, designed to detect completed tran-

tectors (pan-LTR) and using those that span the
tially transcribed region of the LTR that detects all pro-

retectable proviral sequences using primers from the ini-

HIV seropositive individuals ranked from low CD4 count (P8) to high CD4 count (P14).

<table>
<thead>
<tr>
<th>Study subject</th>
<th>Percentage in PBMC sample</th>
<th>Frequency/10^6 cells</th>
<th>Proportion proviral load</th>
<th>Contribution CD4+ αβ TCR cells</th>
<th>Proportion CD4 in γδ subset</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pan-LTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P8</td>
<td>9.4%</td>
<td>200</td>
<td>13%</td>
<td>3.1</td>
<td>1.89%</td>
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<td>P10</td>
<td>4.9%</td>
<td>500</td>
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<td>P11</td>
<td>1.4%</td>
<td>100</td>
<td>5%</td>
<td>0.99</td>
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<td>300</td>
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<td>1.43</td>
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<tr>
<td>P13</td>
<td>14.7%</td>
<td>200</td>
<td>14%</td>
<td>2.68</td>
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<tr>
<td>P14</td>
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<td>10</td>
<td>5%</td>
<td>0.13</td>
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<tr>
<td>C-LTR</td>
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<td></td>
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</tr>
<tr>
<td>P8</td>
<td>9.4%</td>
<td>200</td>
<td>45%</td>
<td>0.24</td>
<td>0.15%</td>
</tr>
<tr>
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<td>26%</td>
<td>1.25</td>
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<td>4%</td>
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<td>2.38</td>
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<tr>
<td>P14</td>
<td>7.8%</td>
<td>20</td>
<td>10%</td>
<td>0.29</td>
<td>1.25%</td>
</tr>
</tbody>
</table>

HIV seropositive individuals ranked from low CD4 count (P8) to high CD4 count (P14).

a Proviral copies per 10^6 γδ lymphocytes.

b Proportion to overall proviral load in PBMCs contributed by γδ lymphocytes.

c Contribution of CD4 lymphocytes to proviral load in γδ lymphocyte subset based on the mean contamination level of 0.21% (range 0.03%-0.46%, n = 3).

d Percentage of proviral load in γδ lymphocyte subset attributable to CD4 lymphocyte contamination.

separated from PBMCs from seven HIV-seropositive indi-

ciduals, and proviral sequences quantified by limiting
dilution PCR (Simmonds et al., 1990b) using LTR primers
as previously described (Imlach et al., 2001). The purity of
each immunomagnetically isolated subset of lympho-
cytes apart from γδ lymphocytes was previously as-

sessed (Imlach et al., 2001). The isolation method for γδ
lymphocytes achieved very high purities of γδ lympho-
cytes, with between 98.8 and 99.9% (mean, 98.9%, n = 3)
of this isolated subset expressing CD3 and the γδ TCR.
Frequencies of non-γδ lymphocytes were extremely low;

between 0.03 and 0.46% (mean, 0.21%, n = 3) of cells
were contaminating CD4 lymphocytes.

All samples of purified γδ lymphocytes contained de-
tectable proviral sequences using primers from the ini-
tially transcribed region of the LTR that detects all pro-

viral transcripts (pan-LTR) and using those that span the
tRNA binding site, designed to detect completed tran-
scripts only (C-LTR; Table 1). Frequencies of proviral
sequences detected in γδ lymphocytes ranged from 10 to 500 copies/10^6 cells, as estimated by limiting dilution
using the pan-LTR primers. Within the accuracy limits of
the assay, proviral loads measured using the pan-LTR
and C-LTR primers were comparable (Table 1, column 3),
indicating that the majority of or all sequences corre-
sponded to complete proviruses. Comparison of proviral
frequencies in γδ lymphocytes, CD4, CD8, and residual
null cells allowed the contribution of each lymphocyte
population to overall proviral load in PBMCs to be cal-
culated, taking into account their relative frequencies in

the total lymphocyte population (Table 1, Fig. 2). γδ lympho-
cytes contributed between 3 and 45% of proviral load
in PBMCs as assessed with the C-LTR primers. No
obvious associations between disease progression (as
indicated by CD4 absolute count) with frequency of in-
fection of any of the lymphocyte subsets was apparent.

As described for our previous investigation of CD8
lymphocyte infection (Imlach et al., 2001), we needed to
calculate the frequency of contaminating CD4 lympho-
cytes in the purified γδ lymphocyte subset to exclude the
former as the source of the proviral sequences. Based
on the purity estimations of the separated γδ lymphocytes,
“worst case” frequencies of infection ranging from only
0.24 to 4.1 copies/10^6 cells, or 0.57–2.9% of the proviral
load detected in the γδ lymphocytes were contributed by
CD4 lymphocytes, based on 0.46% contamination (Table
1). CD4 lymphocyte contamination was therefore not the
source of proviral sequences detected in γδ lympho-
cytes.

To investigate the genetic relationship between HIV-1
variants from immunomagnetically isolated γδ lympho-
cytes and CD3^+ CD4^- lymphocytes, phylogenetic analy-
thesis was carried out on the V3 hypervariable region of
gp120 amplified from proviral sequences detected in the
two lymphocyte subsets (Fig. 3). From the four HIV se-
ropositive individuals, V3 sequences from γδ lympho-
cytes and CD3^+ CD4^- lymphocytes were found to be
generally interspersed, indicating that HIV-1 variants in-
festing the two cell types were genetically similar. To
formally demonstrate the degree of population segrega-
tion, we applied a newly developed method to score phylogenetic trees, by calculation of an association index (AI) (Wang et al., 2001). AI scores ranging from 1.1086 to 0.5339 in three of the individuals indicated a complete absence of cell type-specific segregation of V3 sequences (Fig. 3), while the value of 0.4932 for P10 showed only marginal separation, quite different from previous comparisons of HIV recovered from different autopsy tissues (Wang et al., 2001).

**In vitro infection of γδ lymphocytes**

To directly investigate the susceptibility of γδ lymphocytes to be infected with HIV, *in vitro* infectivity assays of activated PBMCs were carried out using a laboratory isolate of HIV-1 that expresses GFP, and therefore, allows infection of each of the constituent lymphocyte subsets to be monitored by flow cytometry (Figs. 4A–4E). Following PHA activation, PBMCs isolated from healthy donors were infected with stock virus derived from the pNL4.3ΔGFP clone at a multiplicity of infection of 0.1. Following 5 days of culture, lymphocytes were stained with different combinations of Mabs (CD4, CD8α chain, CD8β chain, and γδ TCR) and analysed by flow cytometry using the FL1/green channel to detect GFP expression. From this we were able to determine the frequencies of infection of γδ lymphocytes expressing CD4 or CD8, CD4 lymphocyte, and both CD4⁺ and CD4⁻ subsets of CD8β⁺ lymphocytes (typical results for two combinations of Mabs are shown in Figs. 4A and 4B).

Reproducible infection of each of the target lymphocyte subsets was found in the four PBMC samples subjected to the infectivity assay (Figs. 4C–4E). Both CD8β⁻ and γδ lymphocytes were susceptible to HIV infection and showed frequencies of infection comparable or frequently greater than found in CD4 lymphocytes. For example, CD8 lymphocytes coexpressing CD4 [representing an activated phenotype (Imlach et al., 2001; Flamand et al., 1998; Kitchen et al., 1998; Yang et al., 1998)] showed a mean frequency of GFP-expression of 1.3-fold higher that measured in CD4 lymphocytes. Remarkably, γδ lymphocytes, particularly those expressing CD8 or CD4, were even more susceptible to HIV infection, with frequencies of GFP expression approximately 3- and 10-fold greater than found in CD4 lymphocytes.

To investigate the relationship between cell-surface CD4 expression and susceptibility of γδ lymphocytes and other subsets to infection, the effect of a blocking anti-CD4 monoclonal antibody on the infectivity of each lymphocyte subset was determined (Fig. 4D). As controls, each experiment was carried out in parallel using the reverse transcriptase inhibitor, AZT. At a concentration of 2.5 μg/ml, frequencies of infection of each lymphocyte subset was reduced to generally less than 20% of the untreated control level (Fig. 4E).

Each lymphocyte subset showed evidence for CD4-
FIG. 1. (A) Three-colour, dual-parameter FACS plots on PBMC samples from a representative HIV-seronegative and a representative HIV-seropositive individual analysing the expression of CD4, CD8, CXCR4, and CCR5 on peripheral γδ lymphocytes. Percentage values of expression refer to expression on γδ lymphocytes only. (B) Expression of CD4 and CD8 on peripheral γδ T lymphocytes from HIV-seronegative (S1–S5) and HIV-seropositive individuals (P1–P7). (C) CCR5 and CXCR4 on peripheral γδ T lymphocytes from HIV-seronegative (S1–S5) and HIV-seropositive individuals (P1–P7). (D) Dual expression of CD4 and CD8 on peripheral γδ T lymphocytes from HIV seronegative (S1–S5) and HIV seropositive individuals (P1–P7). Seronegative and seropositive groups are indicated below the histograms. A minimum of 10,000 γδ T lymphocytes were collected and analysed from each individual. HIV-seropositive group ranked from low CD4 count (P1) to high CD4 count (P7).
dependence for virus entry. For example, pretreatment of PBMCs with anti-CD4 antibody before infection reduced the frequency of GFP-expressing T lymphocytes approximately 12-fold in the CD4-expressing subset and by 8.5-fold in those expressing CD8. CD4-dependence on entry of HIV into lymphocytes was comparable to that observed for the infection of CD4 and CD8 lymphocytes (including those in the latter cell type without detectable surface expression of CD4).

**DISCUSSION**

**Surface phenotype of peripheral γδ lymphocytes**

This study is the first to determine the effect of HIV infection on the in vivo expression of the principal receptors for HIV-1 CD4, CXCR4, and CCR5 on the surface of peripheral γδ lymphocytes. In this lymphocyte subset, expression of CCR5 was detected at substantial frequencies in samples from HIV-uninfected individuals (mean, 62.7%) consistent with previous estimates (Glatzel et al., 2002). The frequency of CCR5-expressing γδ lymphocyte was reduced in the HIV-seropositive group (mean frequency, 31.7%), possibly reflecting the previously observed numerical decline in the frequency of the Vγ9/ Vδ2-expressing subset (Wallace et al., 1997; Poccia et al., 1996), which expresses CCR5 (Glatzel et al., 2002). The observed reduction in CCR5 expression may alternatively or additionally be the result of the greater activation of γδ lymphocytes in HIV infection (as manifested by up-regulation of CD8); it has been previously observed that physiological stimuli, such as extracts of Mycobacterium tuberculosis, down-regulates CCR5 expression in γδ lymphocytes in vitro (Glatzel et al., 2002). These frequencies of CCR5 expression contrast markedly to other lymphocyte subsets, notably CD4 and CD8 lymphocytes in which CCR5 expression in vivo is generally associated with cellular activation by antigenic stimuli (Wu et al., 1997).

The information gained on CD4 and chemokine receptor expression on γδ lymphocytes provides additional insights into the potential susceptibility of this to HIV infection, as it is generally accepted from both in vitro and in vivo studies that CD4 in association with either CCR5 or CXCR4 is essential for HIV-1 to infect cells (Dalgleish et al., 1984; Cloyd and Moore, 1990; Deng et al., 1996; Dragic et al., 1996; Alkhatib et al., 1996; Berson et al., 1996; Kozak et al., 1997; Feng et al., 1996). Clearly, the expression of CD4+ , CCR5/CXCR4+ and the phenotypically activated γδ lymphocytes renders this subset potentially susceptible to productive HIV infection in vivo by both R5 and X4-using variants of HIV-1, particularly with the observed association between CD4 expression and cellular activation (0.95% of CD4+ γδ lymphocyte coexpressed CD8; Fig. 1H). Indeed, it is possible that the observed reduction in frequency of CD4 and CCR5 expression in the γδ lymphocyte subset in HIV-seropositive individuals (Fig. 2) reflects the specific targeting and

![FIG. 2. Contribution to overall proviral load of CD4+ lymphocytes (CD4+), CD8+ lymphocytes (CD8+), γδ T lymphocytes (GD T cells) CD3+ 'Null' cells, and non-T cells (expressed as a percentage contribution to the overall measured viral load) in peripheral blood samples from seven HIV seropositive individuals. Seropositive individuals were ranked from low CD4 count (P8) to high CD4 count (P14). VL: circulating viral load (RNA copies per millilitre of plasma).](image-url)
destruction of the CCR5+ γδ/Vδ2 subset by HIV. Our observations of HIV-infected γδ lymphocytes in the peripheral circulation, and their susceptibility to infection with a laboratory isolate of HIV in vitro, clearly supports this hypothesis (see below).

Susceptibility of peripheral γδ lymphocytes to HIV infection

This study demonstrates for the first time HIV infection of peripheral γδ lymphocytes in vivo. Although γδ lymphocytes represented between 1.4 and 14.7% of the cells within the PBMC population, they represented between 3 and 45% of the proviral load detected when using C-LTR primers (Table 1 and Fig. 2). An inherent problem of immunomagnetic isolations is achieving a pure population of cells, as contamination with another cell type would alter proviral load measured by limiting dilution PCR. Within this study, as in previous studies carried out by our group (McBreen et al., 2001; Imlach et al., 2001), calculation of the mean level of αβ TCR+ CD4+ lympho-

FIG. 3. Phylogenetic analysis of V3 region from different lymphocyte subsets from four seropositive individuals ranked from low CD4 count (P8) to high CD4 count (P14). Symbols: □ = CD3+ CD4+ lymphocytes, ○ = γδ T lymphocytes. Trees were rooted using HIVSF162 sequence as an outgroup; a scale bar is indicated at the bottom of each tree. Numbers at branches indicate bootstrap values greater than 70. AI: association index value between CD3+ CD4+ T lymphocyte V3 sequences and γδ T lymphocyte V3 sequences.
cyte contamination within our isolated γδ lymphocyte fractions combined with measurements of proviral loads within this cell type indicated that CD4 lymphocyte contamination (0.07–0.70% of the proviral load detecting using C-LTR primers; Table 1) could not account for the proviral loads measured in γδ lymphocytes.

To provide more direct evidence for the susceptibility and mechanism of γδ lymphocyte infection, we developed...
oped an in vitro model of the entry and replication stages of HIV infection using the NL4.3 HIV-1 isolate that expresses GFP. γδ lymphocytes expressing CD4 were found to be the phenotype most frequently infected by HIV of all the lymphocyte subsets, with those expressing CD8 the next highest (Fig. 3). Separate analysis of CD8 lymphocytes further indicated the substantial in vitro susceptibility of cell with the CD8⁺ CD4⁺ double-positive phenotype as previously reported (Imlach et al., 2001). Observations of the exquisite susceptibility of activated γδ lymphocytes to infection confirm and extend the findings of SIV infection of this subset in vivo (Veazey et al., 2000), and the previous report of in vitro productive infection of cloned γδ T lymphocytes with the CXCR4-utilising isolate, HIV-1_{NL4.3} (Wallace et al., 1997). Pretreatment with anti-CD4 antibody before addition of pNL4.3ΔGFP dramatically reduced the levels of GFP levels detected in γδ, CD4, and CD8 lymphocytes, suggesting the major route of HIV-1 entry into γδ lymphocytes is mediated by CD4, and in the case of the isolate used in the current study, by CXCR4. Commonality in the mechanism of infection of γδ and CD4 lymphocytes in vivo is provided by the evidence for genetic similarity between variants infecting these two cell types in peripheral blood (Fig. 4). Indeed, the detection of HIV variants with signature sequences characteristic of both X4- and R5-utilizing variants of HIV (data not shown) in different study subjects suggests some breadth in possible coreceptor usage of HIV infecting this cell type in vivo.

Interestingly, lower frequencies of in vitro infection were also found in subsets of γδ and CD8 lymphocytes that lacked detectable surface expression of CD4 and/or
CD8. Although it is possible that such infections may result from non-CD4-dependent mechanisms of entry, the observation that infection of these subsets was also inhibited by the anti-CD4 Mab (Fig. 3U) suggests alternative explanations. For example, it is possible that productive infection led to the down-modulation of CD4 by the action of Nef (Garcia and Miller, 1991).

**Clinical significance of γδ lymphocytes infection**

The significance of infection of γδ lymphocytes arises first through the possibility that infection of this subset contributes substantially to the reservoir of HIV-infected cells in vivo, and second, that such infection leads to measurable immune cell depletion and functional impairment of mucosal defenses.

At this stage it is not possible to determine the contribution of γδ lymphocyte infection to the reservoir of HIV-infected cells. First, this study was dependent on samples of peripheral blood as a source of γδ lymphocytes, and therefore, does not provide direct information on the cell number, frequency of infection, or surface phenotype of the likely much larger population resident in mucosal tissues. A second unknown factor is how labile the virus population is within γδ lymphocytes. Without information on the turnover of infected cells of this subset, it is difficult to predict the extent to which this population would persist during antiretroviral therapy and therefore contribute to the reactivation of virus replication during treatment cessation. Numerically, γδ lymphocytes represent a major cell type in adult gut tissue, but there is evidence that their representation increases in HIV infection, possibly by the homeostatic mechanisms that increases their frequency in peripheral blood (Nilssen et al., 1996). If the frequency of infection in gut and lung-associated γδ lymphocytes was comparable to that observed in peripheral blood, this cell type could therefore harbour a substantial virus reservoir of infection.

The other potentially clinical relevant outcome of this study is the possibility that infection of γδ lymphocytes contributes to immunodeficiency, and in particular, the host response to respiratory and gastrointestinal pathogens and neoplasia in which γδ lymphocytes are involved. The mucosal element of the immune system fails during infection with HIV-1, as highlighted by the development of opportunistic diseases of the mucosal surfaces upon progression to AIDS. Whether γδ lymphocyte infection and possible functional impairment or depletion contributes to mucosal disease observed in AIDS is controversial. The issue of whether γδ lymphocytes are a significant contributor to mucosal immunity either directly through cytolytic activity and release of cytokines or by suppressing the response of αβ TCR+ lymphocytes has yet to be properly address in humans (Hayday, 2000). For example, there is evidence that γδ lymphocytes may only play a significant role early in neonatal development before the development of effective adaptive immune responses mediated by the αβ TCR+ lymphocytes and are redundant in the adult immune system (Hayday, 2000). On the other hand, γδ lymphocytes have been shown to have antiviral activity through direct lysis of infected cells and release of HIV inhibitory chemokines such as stromal cell derived factor 1α (Valenzuela-Fernandez et al., 2001). Resolving this issue will be important in understanding the effect of HIV-induced destruction of this subset in HIV-infected individuals.

In the future, it will be necessary to determine whether the extensive infection of γδ lymphocytes in the peripheral circulation is paralleled by infection of γδ lymphocytes in mucosal tissues lining the GI and respiratory tracts. Similarly, to understand more about the potential contribution of γδ lymphocytes to the virus reservoir, investigation of sequential samples from individuals receiving HAART treatment will provide information on the lability of this subset and its ability to persist during periods of virus suppression. The findings obtained to date do however indicate the possibility that HIV infection plays a direct aetiological role in impaired immune defenses at mucosal surfaces, which should be considered in future investigations of HIV immunopathology and in the development of mucosal vaccination strategies.

**MATERIALS AND METHODS**

**Samples and clinical details**

Peripheral blood samples (20–30 ml) anticoagulated with heparin sulphate were obtained by venipuncture from 5 HIV-seronegative individuals and from 14 HIV-seropositive individuals attending the Regional Infectious Disease Unit, Western General Hospital, Edinburgh, U.K. Information about CD4 count, viral load, duration of anti-viral therapy, and risk group for the HIV-seropositive individuals is presented in Table 2.

**Immunomagnetic T cell separation**

Peripheral blood mononuclear cells were isolated from whole blood by density centrifugation over Lymphoprep medium (Nycomed Pharma AS). T lymphocytes were purified from PBMC samples via immunomagnetic negative selection on an AutoMACS system using a Pan-T cell isolation kit (Miltenyl Biotec) according to the manufacturer’s instructions, with all solutions kept on ice throughout the isolation procedure. Those cells retained on the column (i.e., those cells expressing either CD11b, CD16, CD19, CD36, or CD56) after the Pan-T cell isolation had been carried out were eluted and referred to as non-T cells. γδ T lymphocytes were isolated from purified T lymphocytes by positive selection using a TCR γδ-negative microbead kit (Miltenyl Biotec). To the TCR γδ-negative
fraction, CD8⁺ lymphocytes were isolated by positive selection using CD8 microbeads (Miltenyi Biotec) and CD4⁺ lymphocytes were isolated from the TCR γδ negative fraction, CD8 negative fraction by positive selection using CD4 microbeads (Miltenyi Biotec). Those cells that remained (i.e., TCR γδ negative, CD8 negative, and CD4 negative) were retained and referred to as CD3⁺ “Null” cells.

Detection and quantitation of HIV sequences

DNA was extracted from selected cell subsets using QIAamp DNA Mini kit (Qiagen Ltd., Crawley, U.K.). HIV proviral sequences were quantified by limiting dilution nested PCR using a nested set of highly conserved PCR primers from the complete long terminal repeat (C-LTR) and pan-LTR (P-LTR) amplified using previously described primers and amplification conditions (Imlach et al., 2001). Between 8 and 10 replicates at limiting dilution were used for quantitation, providing an accuracy of approximately 0.5 log₁₀ in the estimation of proviral load (Donaldson et al., 1994). Nucleotide sequences from the V3 region of the HIV-1 genome were amplified from single molecule DNA templates from P8, P9, P10, and P14 study subjects. Single molecule V3 amplicons were isolated by limiting dilution nested PCR and sequencing directly using ABI Prism BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Briefly, V3 amplicons were purified before sequencing using QiAquick PCR purification kit (Qiagen). The sequencing reaction consisted of approximately 10 ng of secondary V3 amplicon, 3.2 pmol of V3 inner sense primer, 8 µl terminator ready reaction mix, and deionized nuclease-free water up to a total volume of 20 µl. Sequencing reactions were carried out using the following parameters: 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min for 25 cycles at a thermal ramp rate of approximately 1°C/s, followed by a thermal ramp to 4°C and held until the products were ready to be purified. Amplicons were purified before sequencing using QiAquick PCR purification kit (Qiagen). The sequencing reaction consisted of approximately 10 ng of secondary V3 amplicon, 3.2 pmol of V3 inner sense primer, 8 µl terminator ready reaction mix, and deionized nuclease-free water up to a total volume of 20 µl. Sequencing reactions were carried out using the following parameters: 96°C for 30 s, 50°C for 15 s, and 60°C for 4 min for 25 cycles at a thermal ramp rate of approximately 1°C/s, followed by a thermal ramp to 4°C and held until the products were ready to be purified. Amplicons were purified before sequencing using QiAquick PCR purification kit (Qiagen) and the products run on an ABI Prism 3100 genetic analyser. Returned sequence files were checked using Chromas 1.45 and imported into and aligned using the Simmonic 2000 sequence editor package. Phylogenetic trees were constructed by neighbour-joining using Jukes–Cantor corrected sequence distances in the MEGA 2.1 package. The degree of genetic segregation of V3 sequences recovered from different cell types within the same patient was scored by an association index as previously described (Wang et al., 2001). Nucleotide sequences obtained during the study

### TABLE 2
Clinical Profile of HIV-Seropositive Study Subjects

<table>
<thead>
<tr>
<th>Study subject</th>
<th>Sex</th>
<th>Age</th>
<th>Risk group*</th>
<th>CD4 count/µl</th>
<th>Viral load/µl</th>
<th>Duration of therapy (months)</th>
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<tbody>
<tr>
<td>P1</td>
<td>F</td>
<td>41</td>
<td>IVDU</td>
<td>25</td>
<td>109,000</td>
<td>NT*</td>
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<tr>
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<tr>
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<td>MSM</td>
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<tr>
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<td>334</td>
<td>139,000</td>
<td>NT</td>
</tr>
<tr>
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<td>334</td>
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<td>430</td>
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<td>&lt;50</td>
<td>12</td>
</tr>
</tbody>
</table>

* F, female; M, male.
* Risk groups, IVDU = intravenous drug abuser, MSM = men who have sex with men, WSM = women who have sex with men, NI = needlestick injury.
* No therapy.
have been submitted to GenBank and have been assigned the accession numbers AY 171414–AY 171559.

Peripheral blood mononuclear cell isolation and culture

PBMCs were isolated from approximately 40–50 ml whole blood from HIV-seronegative individuals by density centrifugation over Lymphoprep medium (Nycomed Pharma AS). Isolated PBMCs were counted using a haemocytometer and the cell concentration adjusted to 1 × 10^6 PBMCs/ml in RPMI 1640 medium containing 10% v/v foetal calf serum, 2 mM l-glutamine, 100 IU/ml penicillin with 100 μg streptomycin (RF10 medium) and activated using phytohemagglutinin-L (PHA-L) at 5 μg/ml (Sigma, Poole, U.K.). Cells were placed into culture for 72 h at 37°C and 5% CO₂ atmosphere before infection with HIV-1.

Infection of PHA-L-activated PBMC cultures with HIV-1

Infection assays of different lymphocyte subsets in PBMCs were carried out using a green fluorescent protein (GFP)-expressing HIV isolate derived from the pNL4.3ΔGFP molecular clone of a CXCR4-utilising subtype B laboratory strain of HIV-1 (Welker et al., 1996). PHA-L-activated PBMC cultures were pelleted by centrifugation and resuspended in 500 μl of RF10 medium and 2 ml of cell-free culture supernatant of pNL4.3ΔGFP HIV-1 isolate and incubated for 2 h at 37°C. PBMCs were pelleted by centrifugation and resuspended in RF10 medium at a concentration of 1 × 10^6 PBMC/ml containing 20 IU recombinant interleukin 2/ml (rIL-2, ARP901, ADP, NIBSC, Potters Bar, U.K.) and cultured for 4 days at 37°C. Virus replication and entry was inhibited in control cultures through the use of anti-CD4 antibodies or zidovudine (AZT). Briefly, before infection with HIV-1, 1 × 10^7 PBMC were centrifuged and resuspended in 500 μl of RF10 and treated with either 100 μg anti-CD4 antibody (ARP318, ADP, NIBSC) or 50 μg AZT (ARP952, ADP, NIBSC) for 30 min at 4°C. After incubation, HIV-1 was added to the treated cells as above and incubated for 2 h at 37°C. The virus was removed by centrifugation and the PBMCs were resuspended in RF10 medium containing 20 IU rIL-2/ml and either 2 μg anti-CD4 antibody/ml or 2.5 μg AZT/ml and analysed by flow cytometry after 5 days in culture.

Flow cytometric analysis of GFP expression in pNL4.3ΔGFP HIV-1-infected PBMC

Flow cytometric analysis was carried out on a FACS-Calibur flow cytometer (Becton–Dickinson, Crawley, U.K.) by gating on lymphocytes based on their forward- and side-scatter characteristics, with a minimum of 100,000 events collected. Monoclonal antibodies and isotype-matched mouse immunoglobulin controls [labelled with phycoerythrin (PE), PE-Cy5 (Cy5), and fluorescein isothiocyanate isomer 1 (FITC)] to the following cell-surface markers were obtained: CD4-Cy5 (clone MT310, Dako, Ely, U.K.), CD8 β-chain-PE (clone 2ST8.5H7, Coulter Immunotech, High Wycombe, U.K.), and γδ TCR-PE (clone 11F2, BD Biosciences, Cowly, U.K.). Approximately 2 × 10^6 infected PBMCs were resuspended in 200 μl PBS containing 1% w/v bovine serum albumin (BSA) + 2 mM ethylenediaminetetraacetic acid (EDTA). The appropriate antibodies were added at the manufacturers’ recommended concentration and incubated at 4°C for 30 min. Cells were washed twice with PBS containing 1% w/v BSA + 2 mM EDTA before fixation in PBS containing 2% w/v paraformaldehyde for 1 h at 4°C prior to flow cytometric analysis.

To accommodate day-to-day variation in the infectivity assays associated with the use of different PBMC preparations and virus stocks, frequencies of infection of different lymphocytes were expressed as ratios to those recorded for CD4 lymphocytes in each culture. Typically between 0.35 and 0.99% (mean, 0.55%, n = 4) of the CD4 lymphocytes were productively infected over the 5-day culture, as measured by levels of GFP by flow cytometry.

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REFERENCES


