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Citation for published version:

Imlach, S, Leen, C, Bell, JE & Simmonds, P 2003, 'Phenotypic analysis of peripheral blood gammadelta T lymphocytes and their targeting by human immunodeficiency virus type 1 in vivo', *Virology*, vol. 305, no. 2, pp. 415-27. <https://doi.org/10.1006/viro.2002.1759>

Digital Object Identifier (DOI):

[10.1006/viro.2002.1759](https://doi.org/10.1006/viro.2002.1759)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Virology

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Phenotypic Analysis of Peripheral Blood $\gamma\delta$ T Lymphocytes and Their Targeting by Human Immunodeficiency Virus Type 1 *in Vivo*

S. Imlach,* C. Leen,† J. E. Bell,‡ and P. Simmonds*¹

*Laboratory for Clinical and Molecular Virology, University of Edinburgh, Summerhall, Edinburgh, EH9 1QH, United Kingdom; and †Regional Infectious Diseases Unit and ‡Department of Neuropathology, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, United Kingdom

Received July 1, 2002; returned to author for revision August 8, 2002; accepted August 27, 2002

There is increasing evidence that a wider range of lymphoid cell types other than CD4⁺ T helper lymphocytes are infected with HIV-1 *in vivo*, including CD8 lymphocytes, natural killer cells, and reticulodendritic cells. Each potentially contributes to the reservoir of infected cells that resist antiviral treatment and to the impairment of immune responses in AIDS. By quantitative PCR for HIV proviral sequences we have now obtained evidence for substantial infection of $\gamma\delta$ lymphocytes, contributing 3–45% of the proviral load in peripheral blood. A large proportion of $\gamma\delta$ lymphocytes constitutively expressed the chemokine receptors CCR5 and CXCR4, with evidence for marked up-regulation of CD8 in samples from HIV-infected individuals, corresponding to an activated phenotype. That $\gamma\delta$ lymphocytes might be susceptible to HIV infection was investigated using *in vitro* infectivity assays of recombinant HIV-expressing green fluorescent protein, followed by flow cytometry. $\gamma\delta$, CD4, and CD8 lymphocytes were each productively infected, with $\gamma\delta$ lymphocytes showing the greatest susceptibility. For each cell type, blocking assays with an anti-CD4 monoclonal antibody indicated that entry was CD4-dependent. © 2003 Elsevier Science (USA)

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; Dalglish *et al.*, 1984; Maddon *et al.*, 1986; Feng *et al.*, 1996; Alkhatib *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 ($\gamma\delta$ 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 $\gamma\delta$ 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 a role in mucosal immunity, in both coordination of immune reactions through release of cytokines, primarily of the T_H1 spectrum, and direct cytolytic effects particularly against cells infected with intracellular pathogens such as mycobacteria and parasites. Recognition of pathogens by $\gamma\delta$ 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). $\gamma\delta$ lymphocytes account for between 0.5 and 15% of peripheral blood T lymphocytes, although

¹ To whom correspondence and reprint requests should be addressed. Fax: +44 131 650 6511. E-mail: Peter.Simmonds@ed.ac.uk.

whether they play any functional role in immunity outside of mucosal tissues is unknown. Frequencies of circulating $\gamma\delta$ 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 $\gamma\delta$ T lymphocyte function, and the contribution of such damage to the immunodeficiency observed in AIDS. Increased $\gamma\delta$ 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 $\gamma\delta$ lymphocyte subset expressing the V γ 9/V δ 2 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 $\gamma\delta$ lymphocytes by HIV underlies the functional impairment and repertoire changes observed in HIV-infected individuals remains difficult to assess. The majority of peripheral $\gamma\delta$ 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 $\gamma\delta$ T lymphocytes, although they express the $\alpha\alpha$ CD8 homodimer rather than the $\alpha\beta$ CD8 heterodimer found on $\alpha\beta$ 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 V δ 2 subset of $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ T lymphocytes and investigate the entry requirements.

RESULTS

Expression of CD4, CD8, CCR5, and CXCR4 on peripheral $\gamma\delta$ T lymphocytes

To investigate the surface phenotype of $\gamma\delta$ 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 $\gamma\delta$ receptor, CD4 expression was detected on distinct populations of $\gamma\delta$ 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 $\gamma\delta$ lymphocytes from the seropositive individuals. Irrespective of HIV infection, levels of expression of CD4 on positive $\gamma\delta$ lymphocytes were approximately fivefold lower than found on CD4 T lymphocytes [mean fluorescent intensity for $\gamma\delta$ lymphocytes: 28 (range, 12–49), compared with 140 (range, 99–170) for CD4⁺ $\alpha\beta$ TCR+ lymphocytes].

The numeric decline in the frequency of CD4 expression on $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ lymphocytes was determined by a FACS analysis of PBMC using a second set of monoclonal antibodies to CXCR4, CCR5, and the $\gamma\delta$ receptor. The majority (mean, 62.70%, $n = 5$) of $\gamma\delta$ 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 $\gamma\delta$ population (mean, 34.75%, $n = 5$; Figs. 1A and 1C) from seronegative individuals, higher CXCR4 expression levels were found on the $\gamma\delta$ 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 $\gamma\delta$ lymphocytes by HIV.

HIV-1 proviral burden of $\gamma\delta$ lymphocytes *in vivo*

To investigate the extent of HIV infection of $\gamma\delta$ lymphocytes *in vivo*, $\gamma\delta$, CD4, CD8, and non-T lymphocytes were

TABLE 1
Frequency of Infection and Contribution to Overall Proviral Load of Immunomagnetically Isolated $\gamma\delta$ Lymphocytes from HIV Seropositive Individuals

Study subject	Percentage in PBMC sample	Frequency/ 10^6 cells ^a	Proportion proviral load ^b	Contribution CD4+ $\alpha\beta$ TCR cells ^c	Proportion CD4 in $\gamma\delta$ subset ^d
Pan-LTR					
P8	9.4%	200	13%	3.1	1.89%
P10	4.9%	100	8%	2.60	2.88%
P11	1.4%	200	5%	0.99	0.57%
P12	2.4%	500	26%	1.43	0.29%
P13	14.7%	100	14%	2.68	2.66%
P14	7.8%	10	5%	0.13	2.31%
C-LTR					
P8	9.4%	200	45%	0.24	0.15%
P10	4.9%	200	25%	1.25	0.77%
P11	1.4%	300	4%	0.99	0.35%
P12	2.4%	300	3%	2.38	0.97%
P13	14.7%	300	26%	4.16	1.54%
P14	7.8%	20	10%	0.29	1.25%

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

^a Proviral copies per $10^6/\gamma\delta$ lymphocytes.

^b Proportion to overall proviral load in PBMCs contributed by $\gamma\delta$ lymphocytes.

^c Contribution of CD4 lymphocytes to proviral load in $\gamma\delta$ lymphocyte subset based on the mean contamination level of 0.21% (range 0.03%–0.46%, $n = 3$).

^d Percentage of proviral load in $\gamma\delta$ lymphocyte subset attributable to CD4 lymphocyte contamination.

separated from PBMCs from seven HIV-seropositive individuals, 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 lymphocytes apart from $\gamma\delta$ lymphocytes was previously assessed (Imlach *et al.*, 2001). The isolation method for $\gamma\delta$ lymphocytes achieved very high purities of $\gamma\delta$ lymphocytes, with between 98.8 and 99.9% (mean, 98.9%, $n = 3$) of this isolated subset expressing CD3 and the $\gamma\delta$ TCR. Frequencies of non- $\gamma\delta$ 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 $\gamma\delta$ lymphocytes contained detectable proviral sequences using primers from the initially transcribed region of the LTR that detects all proviral transcripts (pan-LTR) and using those that span the tRNA binding site, designed to detect completed transcripts only (C-LTR; Table 1). Frequencies of proviral sequences detected in $\gamma\delta$ 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 corresponded to complete proviruses. Comparison of proviral frequencies in $\gamma\delta$ lymphocytes, CD4, CD8, and residual null cells allowed the contribution of each lymphocyte population to overall proviral load in PBMCs to be calculated, taking into account their relative frequencies in

the total lymphocyte population (Table 1, Fig. 2). $\gamma\delta$ lymphocytes 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 infection 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 lymphocytes in the purified $\gamma\delta$ lymphocyte subset to exclude the former as the source of the proviral sequences. Based on the purity estimations of the separated $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ lymphocytes.

To investigate the genetic relationship between HIV-1 variants from immunomagnetically isolated $\gamma\delta$ lymphocytes and CD3⁺ CD4⁺ lymphocytes, phylogenetic analysis 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 seropositive individuals, V3 sequences from $\gamma\delta$ lymphocytes and CD3⁺ CD4⁺ lymphocytes were found to be generally interspersed, indicating that HIV-1 variants infecting 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).

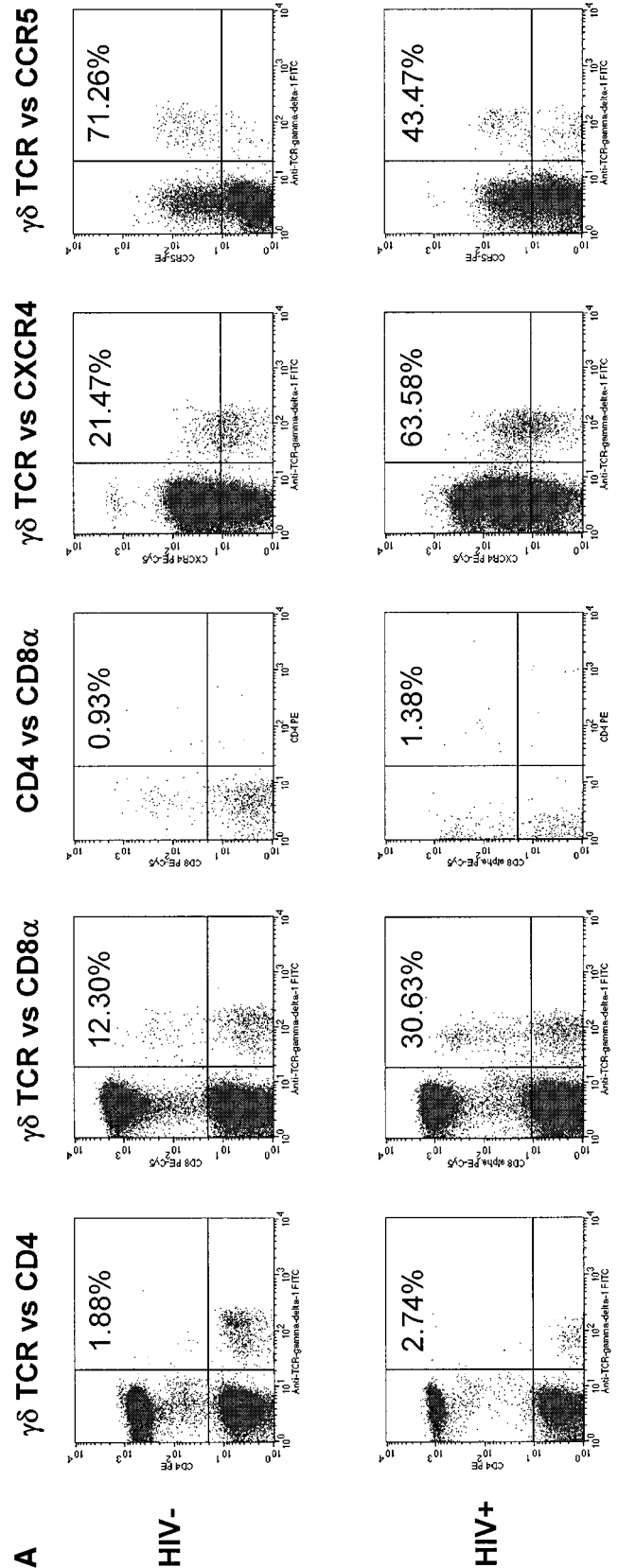
In vitro infection of $\gamma\delta$ lymphocytes

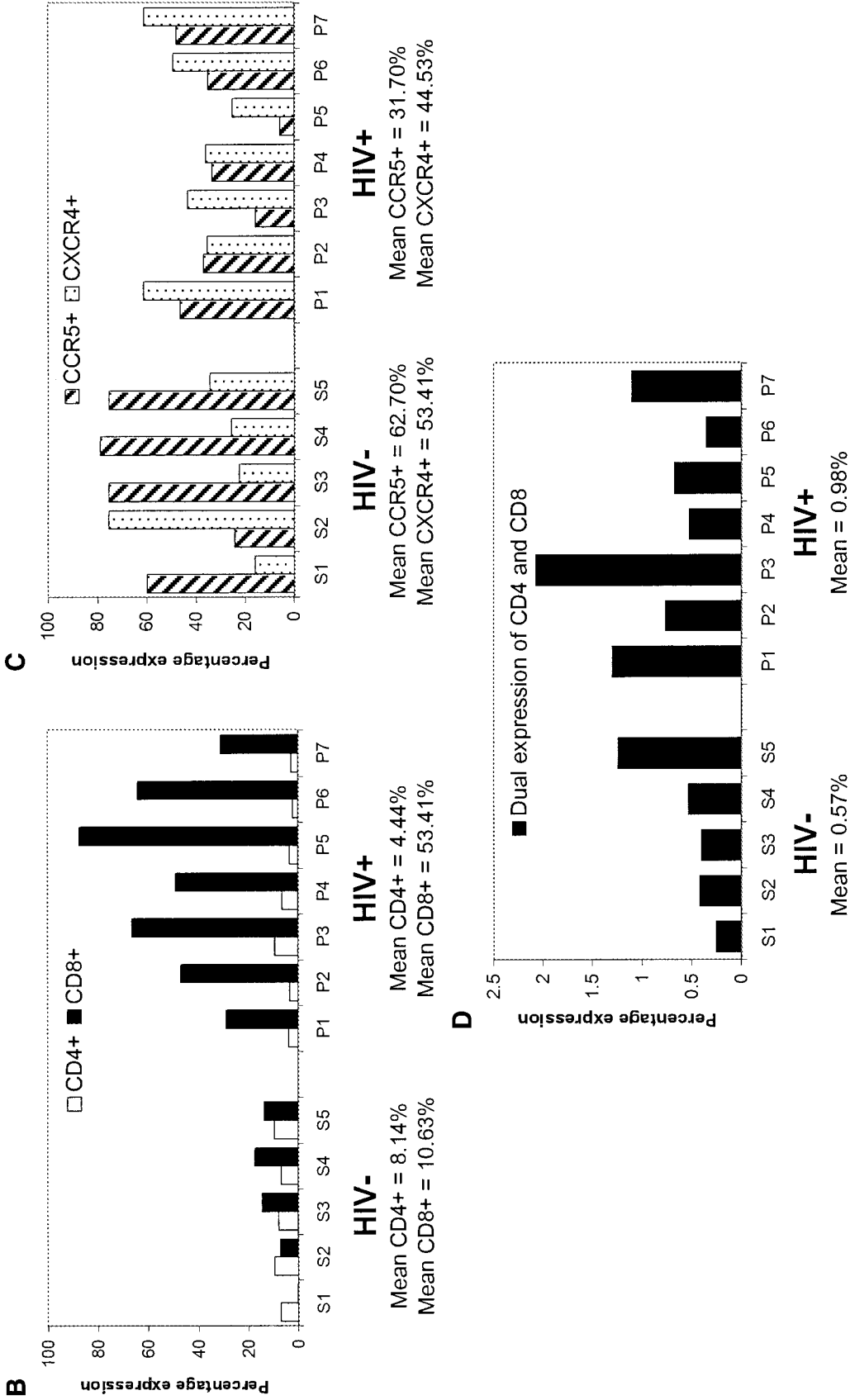
To directly investigate the susceptibility of $\gamma\delta$ lymphocytes to be infected with HIV, *in vitro* infectivity assays of activated PBMCs were carried out using a laboratory isolate of HIV-1 that expresses GFP, and therefore, allows infection of each of the constituent lymphocyte subsets to be monitored by flow cytometry (Figs. 4A–4E). Following PHA activation, PBMCs isolated from healthy donors were infected with stock virus derived from the pNL4.3 Δ GFP clone at a multiplicity of infection of 0.1. Following 5 days of culture, lymphocytes were stained with different combinations of Mabs (CD4, CD8 α chain, CD8 β chain, and $\gamma\delta$ 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 $\gamma\delta$ lymphocytes expressing CD4 or CD8, CD4 lymphocyte, and both CD4⁺ and CD4⁻ subsets of CD8 β + 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 CD8 β ⁺ and $\gamma\delta$ 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 than that measured in CD4 lymphocytes. Remarkably, $\gamma\delta$ 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 $\gamma\delta$ 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 μ g/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-





Study subject

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 $\gamma\delta$ lymphocytes. Percentage values of expression refer to expression on $\gamma\delta$ lymphocytes only. (B) Expression of CD4 and CD8 on peripheral $\gamma\delta$ T lymphocytes from HIV-seronegative (S1–S5) and HIV-seropositive individuals (P1–P7). (C) CCR5 and CXCR4 on peripheral $\gamma\delta$ T lymphocytes from HIV-seronegative (S1–S5) and HIV-seropositive individuals (P1–P7). (D) Dual expression of CD4 and CD8 α on peripheral $\gamma\delta$ 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 $\gamma\delta$ T lymphocytes were collected and analysed from each individual. HIV-seropositive group ranked from low CD4 count (P1) to high CD4 count (P7).

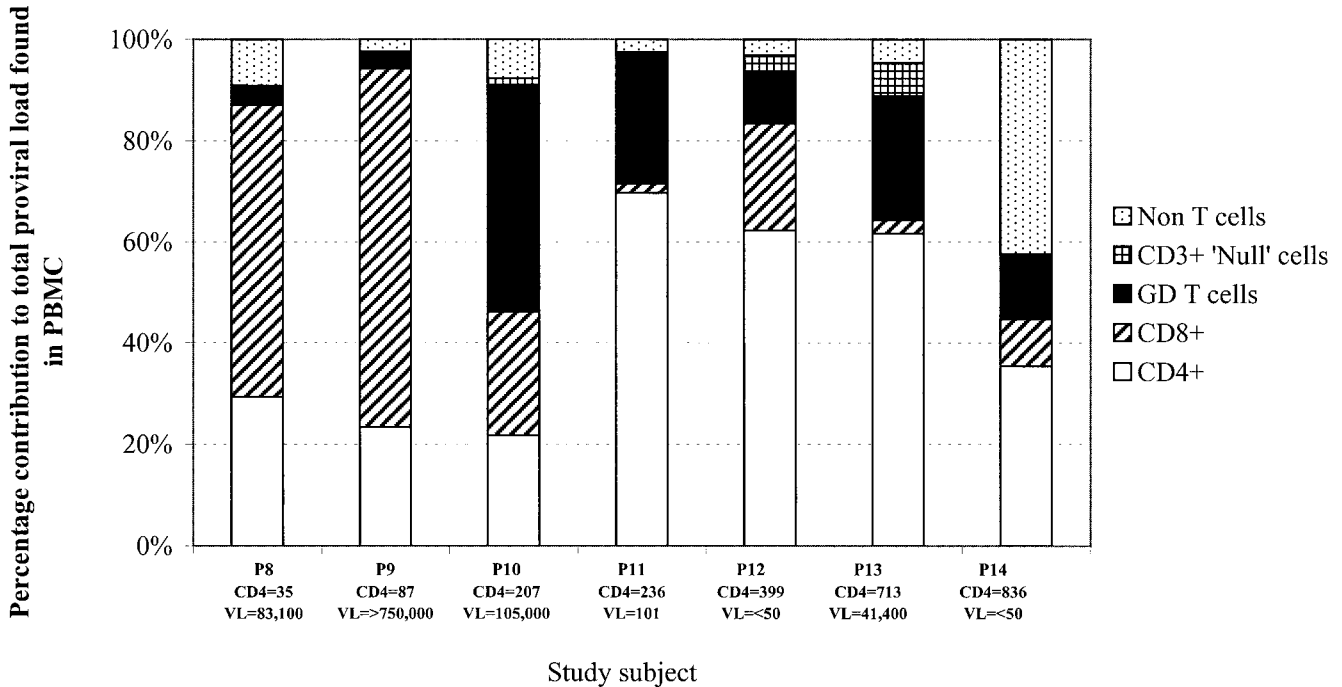


FIG. 2. Contribution to overall proviral load of CD4⁺ lymphocytes (CD4⁺), CD8⁺ lymphocytes (CD8⁺), $\gamma\delta$ T lymphocytes (GD T cells) CD3⁺ "Null" cells, and non-T cells (expressed as a percentage contribution to the overall measured viral load) in peripheral blood samples from seven HIV seropositive 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).

dependence for virus entry. For example, pretreatment of PBMCs with anti-CD4 antibody before infection reduced the frequency of GFP-expressing $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ lymphocyte was reduced in the HIV-seropositive group (mean frequency, 31.7%), possibly reflecting the previously observed numerical decline in the frequency of the V γ 9/V δ 2-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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 (Dagleish *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 $\gamma\delta$ 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⁺ $\gamma\delta$ lymphocyte coexpressed CD8; Fig. 1H). Indeed, it is possible that the observed reduction in frequency of CD4 and CCR5 expression in the $\gamma\delta$ lymphocyte subset in HIV-seropositive individuals (Fig. 2) reflects the specific targeting and



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: \square = CD3⁺ CD4⁺ lymphocytes, \bullet = $\gamma\delta$ T lymphocytes. Trees were rooted using HIV_{SF162} 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⁺ lymphocyte V3 sequences and $\gamma\delta$ T lymphocyte V3 sequences.

destruction of the CCR5⁺ V γ 9/V δ 2 subset by HIV. Our observations of HIV-infected $\gamma\delta$ 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 $\gamma\delta$ lymphocytes to HIV infection

This study demonstrates for the first time HIV infection of peripheral $\gamma\delta$ lymphocytes *in vivo*. Although $\gamma\delta$ 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 $\alpha\beta$ TCR⁺ CD4⁺ lympho-

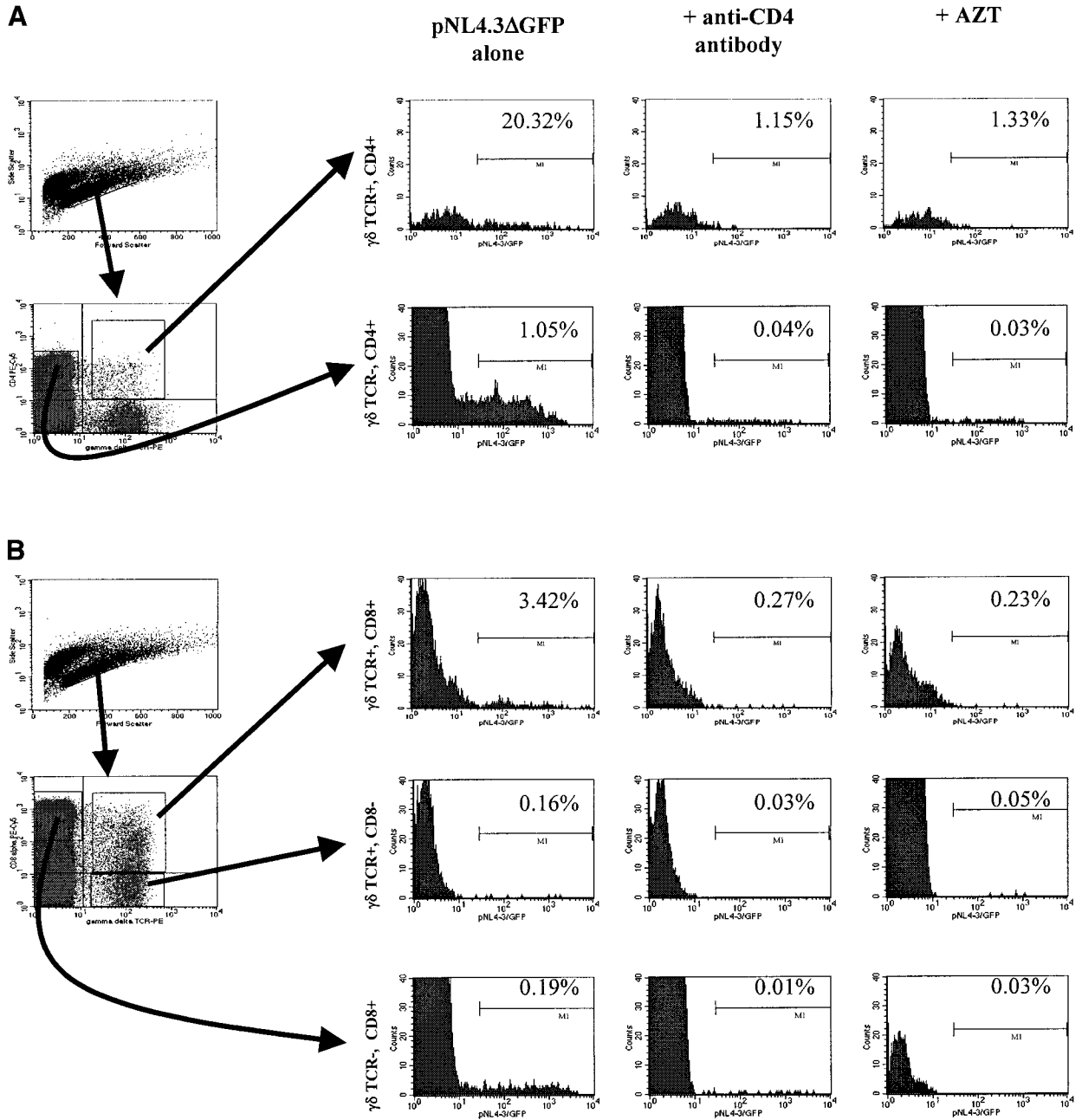


FIG. 4. Flow cytometric analysis of GFP expression in PHA-L-activated PBMC showing $\gamma\delta$ T lymphocytes expressing CD4 (A) or CD8 (B). Forward- and side-scatter plots highlighting the lymphocyte gate set on the PBMC sampled and dual parameter plot of CD4 and $\gamma\delta$ TCR expression from the R1 lymphocyte gate highlighting the $\gamma\delta$ TCR⁺ CD4⁺ gate (R2) and $\gamma\delta$ TCR⁻ CD4⁺ gate (R3) are shown in A. In B, the dual parameter plot of CD8 and $\gamma\delta$ TCR expression from the R1 lymphocyte gate highlighting the $\gamma\delta$ TCR⁺ CD8⁺ gate (R2), $\gamma\delta$ TCR⁺ CD8⁻ gate (R3), and $\gamma\delta$ TCR⁻ CD8⁺ gate (R4) is shown. The CD8 gate used to select CD8⁺, $\gamma\delta$ ⁻ cells was placed at 100 to exclude natural killer and activated CD4 lymphocytes (Sullivan *et al.*, 2001) from the analysis. Single-parameter histograms showing GFP expression in the gated lymphocytes from incubation with pNL4.3ΔGFP alone, treated with anti-CD4 antibody, and treated with AZT. A minimum of 100,000 cells in the R1 gate was collected. C–E, comparison of the levels of GFP found in CD4⁺ lymphocytes, CD8β⁺ CD4⁺ lymphocytes, CD8β⁺ CD4⁻ lymphocytes, $\gamma\delta$ TCR+CD4⁺ lymphocytes, $\gamma\delta$ TCR+CD4⁻ lymphocytes, $\gamma\delta$ TCR+CD8⁺ lymphocytes, and $\gamma\delta$ TCR+CD8⁻ lymphocytes in PHA-L-activated PBMC cultures from HIV-seronegative individuals infected with pNL4.3ΔGFP HIV-1 for 5 days by flow cytometry (C) treated with anti-CD4 antibody (D) or treated with AZT (E). Results (mean ± SD) were calculated from four separate experiments.

cyte contamination within our isolated $\gamma\delta$ 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 us-

ing C-LTR primers; Table 1) could not account for the proviral loads measured in $\gamma\delta$ lymphocytes.

To provide more direct evidence for the susceptibility and mechanism of $\gamma\delta$ lymphocyte infection, we devel-

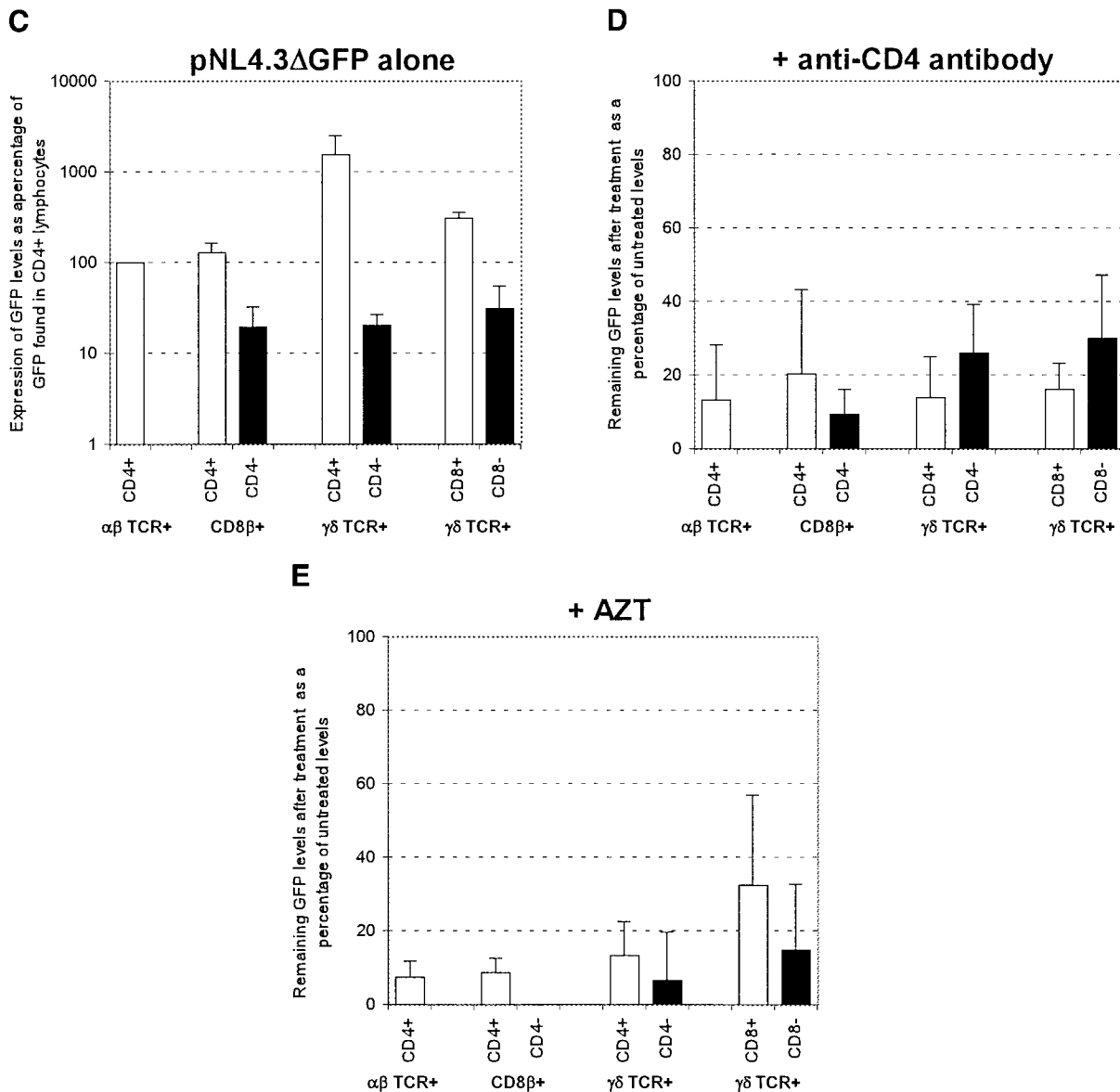


FIG. 4.—Continued

oped an *in vitro* model of the entry and replication stages of HIV infection using the NL4.3 HIV-1 isolate that expresses GFP. $\gamma\delta$ 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 β^+ CD4 $^+$ double-positive phenotype as previously reported (Imlach *et al.*, 2001). Our observations of the exquisite susceptibility of activated $\gamma\delta$ 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 $\gamma\delta$ T lymphocytes with the CXCR4-utilizing isolate, HIV-1_{LAI} (Wallace *et al.*, 1997). Pretreatment with anti-CD4 antibody before addition of

pNL4.3 Δ GFP dramatically reduced the levels of GFP levels detected in $\gamma\delta$, CD4, and CD8 lymphocytes, suggesting the major route of HIV-1 entry into $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ lymphocytes infection

The significance of infection of $\gamma\delta$ 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 $\gamma\delta$ lymphocyte infection to the reservoir of HIV-infected cells. First, this study was dependent on samples of peripheral blood as a source of $\gamma\delta$ 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 $\gamma\delta$ 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, $\gamma\delta$ 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 $\gamma\delta$ 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 $\gamma\delta$ lymphocytes contributes to immunodeficiency, and in particular, the host response to respiratory and gastrointestinal pathogens and neoplasia in which $\gamma\delta$ 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 $\gamma\delta$ lymphocyte infection and possible functional impairment or depletion contributes to mucosal disease observed in AIDS is controversial. The issue of whether $\gamma\delta$ lymphocytes are a significant contributor to mucosal immunity either directly through cytolytic activity and release of cytokines or by suppressing the response of $\alpha\beta$ TCR+ lymphocytes has yet to be properly address in humans (Hayday, 2000). For example, there is evidence that $\gamma\delta$ lympho-

cytes may only play a significant role early in neonatal development before the development of effective adaptive immune responses mediated by the $\alpha\beta$ TCR+ lymphocytes and are redundant in the adult immune system (Hayday, 2000). On the other hand, $\gamma\delta$ 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 1 α (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 $\gamma\delta$ lymphocytes in the peripheral circulation is paralleled by infection of $\gamma\delta$ lymphocytes in mucosal tissues lining the GI and respiratory tracts. Similarly, to understand more about the potential contribution of $\gamma\delta$ 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. $\gamma\delta$ T lymphocytes were isolated from purified T lymphocytes by positive selection using a TCR $\gamma\delta$ microbead kit (Miltenyl Biotec). To the TCR $\gamma\delta$ -negative

TABLE 2
Clinical Profile of HIV-Seropositive Study Subjects

Study subject	Sex ^a	Age	Risk group ^b	CD4 count/ μ l	Viral load/ μ l	Duration of therapy (months)
P1	F	41	IVDU	25	109,000	NT ^c
P2	F	40	IVDU	104	14,800	NT
P3	M	37	MSM	252	1030	3
P4	F	35	IVDU	334	139,000	NT
P5	M	64	NI	334	<400	7
P6	M	37	MSM	430	20,500	NT
P7	F	41	WSM	546	<50	33
P8	M	53	IVDU	35	83,100	24
P9	F	35	WSM	87	>750,000	2
P10	F	43	WSM	207	105,000	12
P11	F	38	WSM	236	101	14
P12	F	33	WSM	399	<50	25
P13	M	44	IVDU	713	41,400	NT
P14	M	41	IVDU	826	<50	12

^a F, female; M, male.

^b Risk groups, IVDU = intravenous drug abuser, MSM = men who have sex with men, WSM = women who have sex with men, NI = needlestick injury.

^c No therapy.

fraction, CD8⁺ lymphocytes were isolated by positive selection using CD8 microbeads (Miltenyl Biotec) and CD4⁺ lymphocytes were isolated from the TCR $\gamma\delta$ negative fraction, CD8 negative fraction by positive selection using CD4 microbeads (Miltenyl Biotec). Those cells that remained (i.e., TCR $\gamma\delta$ 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 (from study subjects P8, P9, P10, and P14) were amplified using previously described primers and amplification conditions (Kaufmann *et al.*, 1998; Sanchez *et al.*, 1997; Simmonds *et al.*, 1990a). PCR amplicons were run at 150 V for 30 min on 2% w/v agarose gels containing 0.5 μ g ethidium bromide per milliliter and visualised under UV light. Quantification of the proviral load in each of the selected cell subsets was performed by limited dilution PCR as previously described (Simmonds *et al.*, 1990b).

Nucleotide sequencing and analysis

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 μ l terminator ready reaction mix, and deionized nuclease-free water up to a total volume of 20 μ l. 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 difference 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

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 μ g streptomycin (RF10 medium) and activated using phytohemagglutinin-L (PHA-L) at 5 μ g/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 μ l 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 μ l of RF10 and treated with either 100 μ g anti-CD4 antibody (ARP318, ADP, NIBSC) or 50 μ g 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 μ g anti-CD4 antibody/ml or 2.5 μ g 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 iso-

thiocyanate 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 $\gamma\delta$ TCR-PE (clone 11F2, BD Biosciences, Cowly, U.K.). Approximately 2×10^6 infected PBMCs were resuspended in 200 μ l 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.

ACKNOWLEDGMENTS

The authors are grateful to staff at the Regional Infectious Diseases Unit, Western General Hospital, and at the Rayne Laboratory for FACS services. This study was funded in part by a Medical Research Council Strategic Project grant (G9632414).

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