

Broadening of coreceptor usage by human immunodeficiency virus type 2 does not correlate with increased pathogenicity in an *in vivo* model

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The pathogenic properties of four primary human immunodeficiency virus type 2 (HIV-2) isolates and two primary HIV-2 biological clones were studied in an *in vivo* human-to-mouse chimeric model. The cell-associated viral load and the ability to reduce the severity of the induced graft-versus-host disease symptoms, the CD4/CD8 ratio and the level of repopulation of the mouse tissues by the graft, were determined. All HIV-2 strains, irrespective of their *in vitro* biological phenotype, replicated to high titres and significantly reduced graft-versus-host disease symptoms as well as the CD4/CD8 ratios. Reduction of graft repopulation caused by infection with the respective HIV-2 strains showed that the *in vitro* replication rate, syncytium-inducing capacity and ability to infect human macrophages did influence the *in vivo* pathogenic potential whereas broadening of coreceptor usage did not.

Introduction

Human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) are the causative agents of AIDS (Barre-Sinoussi *et al.*, 1983; Clavel *et al.*, 1986; Levy *et al.*, 1986). The most prominent feature of the pathogenesis of AIDS is the quantitative and qualitative deterioration of the CD4⁺ T cell subset. Although CD4⁺ T cells are already affected soon after infection, it may take from 1 to more than 15 years before immune failure leads to overt clinical symptoms (Gruters *et al.*, 1990, 1991). Both virological and host-delimited factors have been shown to determine the length of the asymptomatic period (Cheng-Mayer *et al.*, 1988; Dean *et al.*, 1996; Fenyo *et al.*, 1988; van Baalen *et al.*, 1997). Epidemiological studies have shown that, although progression to AIDS may in specific cases be relatively fast, disease progression in HIV-2-infected individuals is in general much slower than disease progression in HIV-1-infected individuals (Marlink *et al.*, 1994; Whittle *et*

al., 1994; van der Ende *et al.*, 1996). Virological factors which underlie this difference between HIV-1- and HIV-2-induced disease progression are presently not known. Studies in *in vitro* and *in vivo* systems, specifying viral characteristics which determine the pathogenicity of HIV strains, may aid in the understanding of HIV-induced pathogenesis.

The characteristics used to classify HIV strains *in vitro* include replication rate, ability to induce the formation of multinucleated cells [syncytium-inducing (SI) capacity], coreceptor requirement and ability to infect different target cells (T cell lines versus macrophages) (Asjö *et al.*, 1986; Berger *et al.*, 1998; Cheng-Mayer *et al.*, 1988; Schuitemaker *et al.*, 1992*b*; Tersmette *et al.*, 1989). For HIV-1 it has been shown that these *in vitro* characteristics exhibit a high degree of correlation. For example, SI strains in general have a high replication rate, infect target cells via α and β chemokine receptors, may infect immortalized T cell lines and only infect macrophages inefficiently. NSI strains, on the other hand, have a low replication rate, require expression of β chemokine receptors, do not infect T cell lines and replicate efficiently in macrophages (Berger *et al.*, 1998; Schuitemaker *et al.*, 1991). It

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should, however, be noted that individual virus strains may also display intermediate biological phenotypes (Groenink *et al.*, 1991; Sabri *et al.*, 1996; Schuitemaker *et al.*, 1992*a*). Several differences in *in vitro* characteristics of HIV-1 and HIV-2 have become apparent. The replication rate, as defined by the time required to detect virus after initiation of standard virus culture from donor peripheral blood mononuclear cells (PBMC), is generally lower for HIV-2 than for HIV-1 (Albert *et al.*, 1990; van der Ende *et al.*, 1996). Furthermore, we and others have recently shown that clear differences exist in coreceptor requirements (Guillon *et al.*, 1998; McKnight *et al.*, 1998). The linkage between HIV-1 SI phenotype and CXCR-4 coreceptor usage was not observed for HIV-2. Furthermore, HIV-2 strains in general have a broader coreceptor usage than HIV-1. Taken together, these observations suggest that *in vitro* usage of the CXCR-4 coreceptor and broadening of the coreceptor usage by HIV-2 do not result in enhanced *in vivo* pathogenicity.

To further address this issue we studied the *in vivo* pathogenic potential of HIV-2 isolates and biological clones in a chimeric human-to-mouse model for *in vivo* HIV infection [the xeno-GvHD (graft-versus-host disease) mouse model] (Hupples *et al.*, 1992, 1993; Schutten *et al.*, 1996). In this model high numbers of human PBMC are grafted into the peritoneal cavity of immune-deficient mice. In these mice an acute graft-versus-host reaction develops within 7 to 14 days. The human lymphocyte population that repopulates the mouse tissues is characterized by high CD4/CD8 ratios (Schutten *et al.*, 1996). Depletion of human CD4⁺ T cells from the graft results in a complete abrogation of the acute xeno-GvHD reaction. Depletion of antigen-presenting cells (APC; macrophages) from the human graft results in lower CD4/CD8 ratios and a concomitant delay in appearance of the xeno-GvHD symptoms (Hupples *et al.*, 1993). We therefore set out to study in this model the direct (killing of infected CD4⁺ T cells) and indirect (inhibition/modulation of antigen presentation) pathogenic effect of different HIV-2 strains and isolates on the development of acute xeno-GvHD symptoms, CD4/CD8 ratio and the ability of the graft to repopulate mouse tissues.

Methods

■ **Virus strains and xeno-GvHD mice.** Xeno-GvHD mice were prepared as described previously (Hupples *et al.*, 1992, 1993; Schutten *et al.*, 1996). Briefly, 3–4-week-old XID mice (CBA/HNOlaHsd, Harlan Netherlands) were given total body irradiation (9 Gy) with syngeneic bone marrow support (5×10^5 cells per mouse, intravenously). Subsequently, Ficoll-gradient-isolated human PBMC from HIV-seronegative individuals (blood type A, Rhesus+) were given at 2×10^7 per gram mouse body weight intraperitoneally (i.p.). These xeno-GvHD mice were challenged i.p. with HIV-2 within 1 h of being grafted with human PBMC. Animals grafted with human PBMC from one and the same HIV-seronegative blood donor were equally distributed over groups of five mice receiving different HIV-2 strains. The primary HIV-2 strains used in this study had been isolated in mitogen-stimulated PBMC only and the *in vitro* characteristics have been described previously (van der Ende *et al.*,

1996; Guillon *et al.*, 1998). Relevant virus characteristics are given in Table 1. The doses of challenge virus were equilibrated on the basis of c.p.m. measured in an RT assay (van Baalen *et al.*, 1998) and equalled approximately 30 mouse infectious doses 50% per mouse.

■ **Parameters of acute xeno-GvHD reaction and HIV-2 infection.** Directly after the onset of acute xeno-GvHD symptoms (furry coat, breathing problems and wasting), mice were sacrificed and lymphocytes were isolated from the peritoneal cavity. Human-to-mouse chimerism was confirmed and the CD4/CD8 ratio of the human graft was calculated, using FACScan analysis with human CD3 and CD8 conjugates (Becton Dickinson) according to the manufacturer's instructions. The viral loads of the lymphocytes isolated from the peritoneal cavities were determined in an infectious centre assay (ICT) (Schutten *et al.*, 1996). Briefly, human lymphocytes isolated from the peritoneal cavity were counted and titrated in duplicate from 2×10^5 to 2 cells per well onward using fivefold dilution steps in 96-well (round bottomed) plates. PBMC, pre-stimulated with mitogen for 3 days, were added as feeder cells (5×10^4 per well). The cells were cultured for 7 days in RPMI 1640 (BioWhittaker) supplemented with 10% foetal calf serum (BioWhittaker), penicillin (100 U/ml), streptomycin (10 µg/ml) and 50 IU recombinant human IL-2/ml (Eurocetus), after which virus was detected by RT assay. The minimal number of cells isolated from the peritoneal cavity required for detection of virus was taken as a measure of the cell-associated viral load.

■ **HIV-2 *in situ* hybridization and immunohistochemistry.** Xeno-GvHD mouse tissues, including spleen, lung, bowel, heart, liver and bone, were prepared from groups of mice which had been grafted with PBMC isolated from the same individual. Tissues from two mice infected with the same HIV-2 isolate/strain were analysed pairwise. Tissues were fixed overnight with 4% formalin and subsequently embedded in paraffin. For routine immunohistochemistry, dewaxed 5 µm paraffin sections were heat-denatured with 0.01 M buffered sodium citrate (Norton *et al.*, 1994). The sections were incubated with CD45 (LCA) (Dakopatts) according to the manufacturer's instructions (Tenner-Racz *et al.*, 1998). Binding of antibodies was visualized by the alkaline phosphatase anti-alkaline phosphatase technique using New Fuchsin as red chromogen. We have previously shown that this conjugate does not stain mouse cells (Schutten *et al.*, 1996). After immunostaining the sections were either counter-stained with haematoxylin and mounted, or dehydrated and subjected to HIV RNA *in situ* hybridization. ³⁵S-labelled, single-stranded, anti-sense HIV-2 RNA probes (Lofstrand Laboratories), which contained 1.4–2.7 kb fragments, collectively representing approximately 90% of the HIV-2 genome, were used as described previously (Embretson *et al.*, 1993; Fox *et al.*, 1991). Briefly, paraffin sections were either treated with Proteinase K (0.01 mg/ml) for 8 min at room temperature or heat denatured. The sections were incubated with prehybridization mixture (50% formamide, 0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.02% Ficoll-polyvinylpyrrolidone-BSA and 7 mg of tRNA/ml) for 2 h at 37 °C and then covered with hybridization mixture (prehybridization mixture + 10% dextran sulfate and 2×10^6 d.p.m. of probe/ml) overnight at 45 °C. The sections were washed, RNase treated (Boehringer Mannheim) for 40 min at 37 °C and further developed, counter-stained with haemalaun and mounted as previously described. As a negative control, sections were hybridized with a radiolabelled sense probe. The sections were examined with a microscope equipped with epiluminescent illumination (Axiophot; Carl Zeiss). Cells were considered positive for viral gene expression if the number of grains overlying a cell was more than six times the background.

Table 1. Characteristics of the HIV-2 strains

HIV-2 strain	Replication rate*	Biological phenotypet	Coreceptor usage‡	Macrophage-tropism
RH2-1	42	Early NSI	R5	+
RH2-2	< 7	Late SI	R1,R3,R5,X4	-
RH2-6	< 7	Late SI	X4	+
PH2-1 E6	14	Intermediate NSI	R1,R3,R5,X4	+
PH2-1 D5	14	Intermediate SI	R5	+

* First day of a positive signal in p24 antigen ELISA after start of virus isolation (van der Ende *et al.*, 1996).

† Biological phenotype according to van der Ende *et al.* (1996).

‡ Coreceptor usage according to Guillon *et al.* (1998) and Berger *et al.* (1998).

Results

Acute xeno-GvHD symptoms and CD4/CD8 ratio

Grafting human PBMC from HIV-seronegative individuals at 2×10^7 per gram mouse body-weight i.p. has been shown to induce an acute xeno-GvHD reaction within 14 days in approximately 100% of the animals (Huppel *et al.*, 1992; Schutten *et al.*, 1996) (Table 2). In general, the development of acute xeno-GvHD symptoms is fast and little variation is observed between animals grafted with PBMC from donors with the same ABO/Rhesus factor. The acute xeno-GvHD symptoms observed in the HIV-2-infected xeno-GvHD mice were in general less severe than in the control mice (Table 2). No significant differences in absolute numbers of human lymphocytes isolated from the peritoneal cavity (data not

shown) or in the number of infected centres in the ICT (Table 2) were observed between the different groups of HIV-2-infected mice. Quite unexpectedly, no significant differences in symptoms or CD4/CD8 ratio were observed between xeno-GvHD mice infected with the early HIV-2 RH2-1 (R5) isolate or late isolate HIV-2 RH2-2 (R1,R3,R5,X4). Acute xeno-GvHD symptoms were not observed in the mice infected with the late HIV-2 subtype B isolate RH2-6 (X4) and CD4/CD8 ratios from these mice were lower compared to those in mice in the other groups (Table 2). Although the differences between mice infected with the SI and NSI biological clone from PH2-1 were minor, the xeno-GvHD mice infected with the SI biological clone PH2-1 D5 (R5) did show less severe xeno-GvHD symptoms as compared to the mice infected with the NSI biological clone PH2-1 E6 (R1,R3,R5,X4) (Table 2).

Table 2. Acute xeno-GvHD symptoms in HIV-2-infected mice

HIV-2 strain	Xeno-GvHD*	ICT†	CD4/CD8 ratio‡	Lung		Spleen	
				CD45 ⁺ cells§	HIV-2-infected cells	CD45 ⁺ cells§	HIV-2-infected cells
-	++	-	4-9	+++	-	NA	-
RH2-1	+	1.6×10^2	0.8-1.2	++	+++	+++	+++
RH2-2	+	4×10^2	0.6-1.1	++	+	+++	++
RH2-6	-	3×10^2	0.3-0.5	-	-	-	-
PH2-1 E6	+	2×10^3	0.7-1.2	++	+++	+++	+++
PH2-1 D5	+	1.6×10^2	0.6-0.8	-	-	+	+

* Severity of the xeno-GvHD symptoms: ++, severe; +, minor; -, no symptoms observed within the observation period (11 days).

† Number of infectious centres per 4×10^5 cells isolated from the peritoneal cavity.

‡ Range of the CD4/CD8 ratio within each group of mice.

§ Numbers of CD45⁺ cells in the organ: + + +, very high; ++, high; +, low; -, occasional positive cell; NA, not applicable due to xeno-GvHD induced necrosis of the organ.

|| Numbers of HIV-2-RNA-positive cells: + + +, very high; ++, high; +, low; -, occasional positive cell.

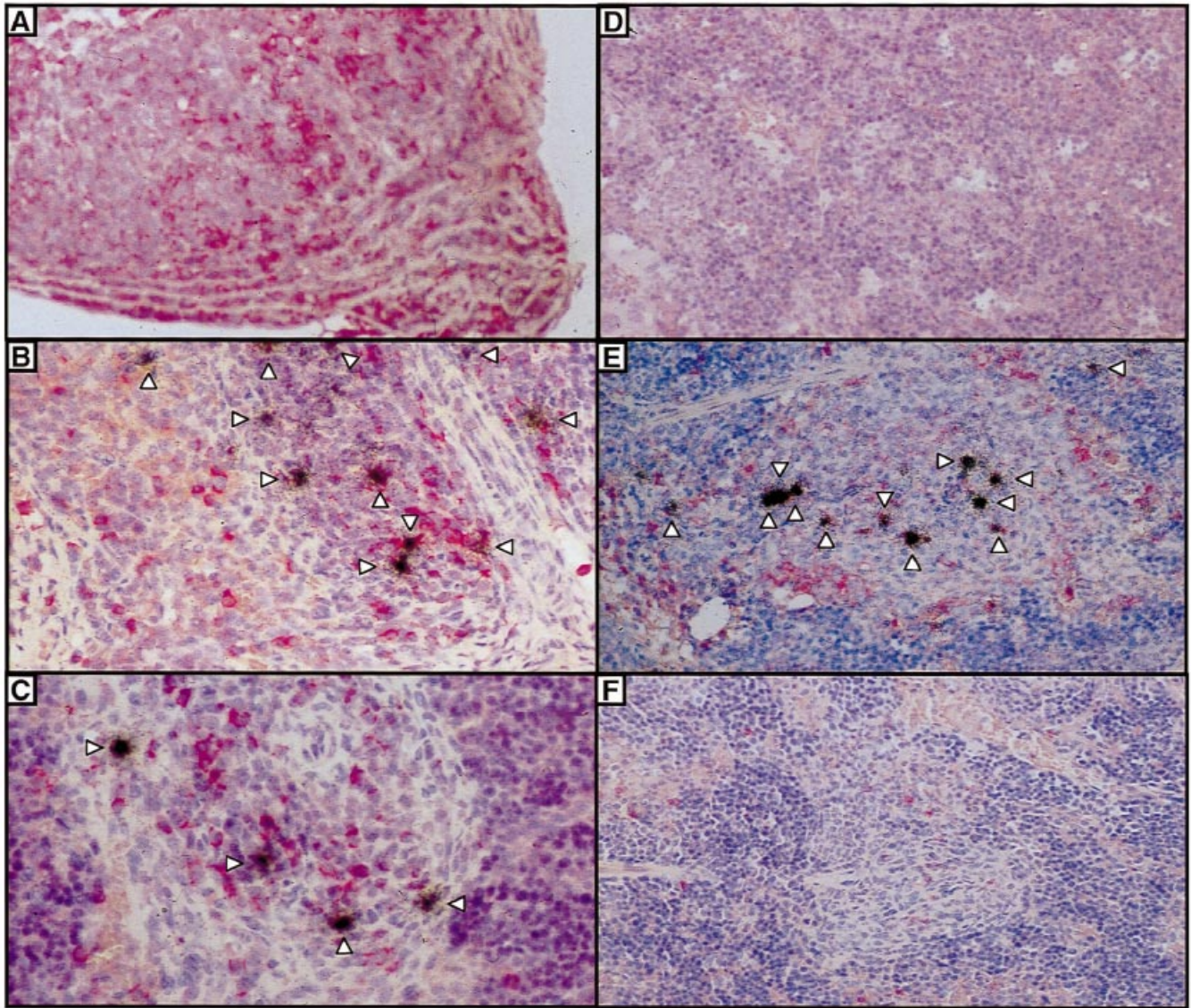


Fig. 1. Comparison of the numbers of human lymphoid cells and productively HIV-2-infected cells in xeno-GvHD mice as determined by CD45 staining (red alkaline phosphatase staining) and HIV-2 RNA *in situ* hybridization (black grains). (A) Uninfected control; (B) HIV-2 RH2-1 (R5); (C) HIV-2 RH2-2 (R1,R3,R5,X4); (D) HIV-2 RH2-6 (X4); (E) HIV-2 PH2-1 E6 (R1,R3,R5,X4); (F) HIV-2 PH2-1 D5 (R5).

Immunohistochemistry of tissues from xeno-GvHD mice

In order to study the ability of the human PBMC graft to repopulate the mouse tissues after infection with HIV-2, tissues from the xeno-GvHD mice (spleen, lung, heart, bone, bowel) were prepared and examined to determine the numbers human cells present as indicated by immunohistochemical staining for human CD45. Variation in the distribution of the human lymphocytes was low between mice grafted with the same donor PBMC (data not shown). High numbers of human cells, especially in highly vascularized tissues like the lung, were observed in xeno-GvHD mice which were uninfected (Table

2). Only small remnants of necrotized spleen could be obtained from these mice, in which the mouse tissue was almost completely replaced by human cells (Fig. 1A). Within these tissues only CD45⁺CD68⁻ cells (T cells) were found and CD45⁺CD68⁺ cells (macrophages) were exclusively found adhered to and migrating into tissues isolated from the peritoneal cavity (Schutten *et al.*, 1996). In all HIV-2-infected xeno-GvHD mice, relatively high numbers of human T cells and macrophages could be observed in the tissues isolated from the peritoneal cavity. Similar to what was found for uninfected mice, macrophages were exclusively found migrating into and adhered to the tissues isolated from the peritoneal cavity (data not shown). The numbers of human

cells in the lungs of the xeno-GvHD mice infected with HIV-2 RH2-1 (R5), RH2-2 (R1,R3,R5,X4) and PH2-1 E6 (R1,R3,R5,X4) was significantly lower than the number of human cells in the lungs of the control mice (Table 2). The structure of the spleen obtained from these mice was relatively intact and contained many CD45⁺ cells, mainly within the periarteriolar lymphocyte sheets (Fig. 1B, C, E; Table 2). Hardly any CD45⁺ cells were found in the lung and spleen of mice infected with HIV-2 RH2-6 (X4) (Fig. 1D; Table 2). Similarly, the lungs of HIV-2 PH2-1 D5 (R5)-infected mice was almost completely devoid of human cells and low numbers of human cells were observed in the spleen (Fig. 1F, Table 2).

HIV-2 infection of xeno-GvHD mice

The ability of the HIV-2 isolates and biological clones to productively infect cells expressing the macrophage marker CD68 was determined by double labelling (HIV-2 RNA *in situ* hybridization with CD68 immunohistochemistry) (Table 1). Productively infected macrophages were observed in all HIV-2-infected xeno-GvHD mice except for those infected with the late isolate RH2-2 (R1,R3,R5,X4) (Table 1). Also, the relative number of productively infected cells was determined in the tissues with high numbers of human lymphocytes (spleen and lung) (Table 2). HIV-2 RNA *in situ* hybridization signal above background was absent in the negative control mice. High numbers of HIV-2 RNA⁺ cells were observed in the peritoneal cavity of all mice infected with the different HIV-2 isolates and biological clones (data not shown). High numbers of productively infected cells could be observed in the lungs and spleens of xeno-GvHD mice infected with HIV-2 RH2-1 (R5) (Fig. 1C). Despite the fact that no significant differences in the numbers of CD45⁺ cells were observed between xeno-GvHD mice infected with HIV-2 RH2-1 (R5) (Fig. 1C) or HIV-2 RH2-2 (R1,R3,R5,X4) (Fig. 1D), a significantly lower number of productively infected cells was found in the tissues of xeno-GvHD mice infected with HIV-2 RH2-2 (R1,R3,R5,X4) (Table 2). Similar to HIV-2 RH2-1 (R5)-infected xeno-GvHD mice, many productively infected cells were found in the tissues of xeno-GvHD mice infected with HIV-2 PH2-1 E6 (R1,R3,R5,X4) (Fig. 1E, Table 2). Although the numbers of cells and therefore also the numbers of productively infected cells in the peripheral tissues of xeno-GvHD mice infected with HIV-2 PH2-1 D5 (R5) (Fig. 1F) were significantly lower as compared to HIV-2 RH2-1 (R5), RH2-2 (R1,R3,R5,X4) and PH2-1 E6 (R1,R3,R5,X4), a relatively high percentage of the cells observed in the peripheral tissues of these mice were still productively infected (Table 2).

Discussion

In the present paper we have shown that the SI capacity, replication rate and ability to infect macrophages influenced the pathogenic properties of HIV-2 strains in the xeno-GvHD mouse model for HIV infection. Moreover, an SI macrophage-

tropic HIV-2 biological clone that only uses CCR-5 as coreceptor proved to be more pathogenic than a highly homologous NSI macrophage-tropic biological clone with a potentially broader coreceptor usage (CCR-1, CCR-3, CCR-5 and CXCR-4). These data suggest that broadening of the potential cellular host-range does not necessarily increase the pathogenic potential of HIV-2 and probably HIV-1.

Both direct and indirect mechanisms have been suggested to contribute to the pronounced CD4⁺ T cell depletion observed in HIV-infected individuals (for review see Zinkernagel *et al.*, 1994). The direct mechanisms include acute single cell lysis of infected cells (Samson *et al.*, 1996) and depletion of uninfected CD4⁺ cells by fusion with infected cells (syncytium formation) (Tersmette *et al.*, 1993). It has also been shown that cells of the monocyte/macrophage lineage do not function properly, which may result in dysregulation of activation and/or maturation of CD4⁺ T cells due to functionally disturbed antigen presentation (Meyaard *et al.*, 1993). This may also indirectly contribute to the decline of CD4⁺ T cells. Two observations suggest that this last phenomenon contributed to the HIV-2-induced CD4⁺ T cell depletion in our xeno-GvHD mouse model. First, no significant differences were observed in the CD4/CD8 ratio and the number of CD45⁺ cells in spleens and lungs of mice infected with the early, macrophage-tropic NSI isolate RH2-1 (R5) and the late non-macrophage-tropic SI isolate RH2-2 (R1,R3,R5,X4). This is despite the fact that the late SI isolate is highly cytopathic for CD4⁺ T cells *in vitro* and the early NSI isolate does not induce single cell lysis to a significant extent (unpublished observations). The fact that the percentage of infected cells was high in the RH2-1 (R5)-infected mice and low in the RH2-2 (R1,R3,R5,X4)-infected mice also suggests that these virus strains cause CD4⁺ T cell depletion by two different mechanisms. It seems appropriate to assume that HIV-2 RH2-2 (R1,R3,R5,X4)-infected cells are directly killed and therefore do not reach the peripheral tissues studied, whereas RH2-1 (R5)-infected cells are not killed directly, but seem rather to be functionally affected. Second, RH2-2 (R1,R3,R5,X4) and RH2-6 (X4) are both highly cytopathic for CD4⁺ T cells *in vitro*. The macrophage-tropic isolate RH2-6 (X4) proved to be far more pathogenic *in vivo* with respect to abrogating GvHD symptoms and inhibiting migration of T cells to peripheral tissues than the non-macrophage-tropic isolate RH2-2 (R1,R3,R5,X4).

SI capacity, replication rate and coreceptor requirements have all been shown to positively correlate with rapid disease progression in HIV-1-infected individuals. Because, for HIV-1, these factors are generally linked, it has not been possible to distinguish which factors have a causal relation with disease progression. Since SI capacity and coreceptor usage are not linked for HIV-2, we could study these factors independently. The observation that *in vitro* coreceptor usage of HIV-2 strains is broader than that of HIV-1 has already suggested that broadening of coreceptor usage does not necessarily lead to increased *in vivo* pathogenicity. Indeed, in the xeno-GvHD

model, RH2-6 (X4) proved to be far more pathogenic with regard to all aspects studied than RH2-2 (R1,R3,R5,X4), suggesting that broadening of coreceptor usage does not significantly add to the *in vivo* pathogenicity. This is further supported by the observation that the NSI biological clone PH2-1 E6 (R1,R3,R5,X4) was less well able to inhibit migration of T cells and may therefore be regarded more pathogenic than its SI counterpart PH2-1 D5 (R5).

We have recently shown that the number of productively infected cells in lymphoid tissue from HIV-2-infected individuals is significantly lower than in HIV-1-infected individuals. Furthermore, it has been shown that the number of productively infected cells is positively correlated with the plasma viral load, which is in turn correlated with disease progression. Interestingly, we did not observe major differences in cell-associated viral loads or in pathogenicity of the HIV-2 strains tested when compared with previously studied HIV-1 strains (Schutten *et al.*, 1996). It therefore seems that in the absence of a substantial humoral and cellular antiviral immune response both *in vitro* and in our human-to-mouse chimeric model, HIV-1 and HIV-2 may have comparable replicative and pathogenic potential. We therefore hypothesize that the lower viral load and pathogenicity observed in HIV-2-infected individuals as compared to HIV-1-infected individuals is primarily related to a difference in interaction of the virus with the specific immune response.

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