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Differential loss of invariant NKT cells and FoxP3+ regulatory T cells in HIV-1 subtype A and subtype D infections

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Abstract

HIV-1 subtype D is associated with faster disease progression as compared to subtype A. Immunological correlates of this difference remain undefined. We investigated invariant natural killer T cells and FoxP3+ regulatory T cells in Ugandans infected with either subtype. Loss of iNKT cells was pronounced in subtype D, whereas Tregs displayed more profound loss in subtype A infection. iNKT cell levels were associated with CD4 T cell IL-2 production in subtype A, but not D, infection. Thus, these viral subtypes are associated with differential loss of iNKT cells and Tregs that may influence the quality of the adaptive immune response.

Keywords

HIV-1; viral subtype; AIDS; iNKT cell; CD1d; T regulatory cell

INTRODUCTION

HIV-1 subtypes A and D are the predominant circulating viral subtypes in Uganda.¹ Several studies in East and West African cohorts indicate that clinical endpoints such as AIDS-free survival, rate of CD4 T cell decline, and death are delayed in infection with HIV-1 subtype A compared to subtype D infection.^{1–5} Despite efforts to understand the biological mechanisms behind this subtype difference in disease progression, the underlying mechanisms are incompletely understood. Bousheri et al. found that CD4 T cells in subtype D infected patients express more PD-1 and are more prone to apoptosis than CD4 T cells in

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subtype A infected patients.⁶ This suggests that immune activation, regulation and cell death may differ with subtype. Based on these results we speculated that immunoregulatory mechanisms might be involved in determining the difference in HIV-1 A and D pathogenesis.

Invariant Natural Killer T (iNKT) cells are innate-like T cells with an invariant T cell receptor (TCR) that recognize CD1d-presented antigens.⁷ iNKT cells respond rapidly with secretion of immunoregulatory and activating cytokines that direct responses by other innate and adaptive lymphocytes. HIV-1 infection is associated with reduced number and function of iNKT cells.^{8–12} Furthermore, IFN γ produced by iNKT cells have anti-HIV activity *in vitro*¹³, and HIV-1 has evolved Vpu- and Nef-dependent mechanisms to interfere with CD1d-mediated antigen presentation and activation of iNKT cells.^{14–16} In SIV infection, recent findings indicate that iNKT cells and their anti-inflammatory functions are lost in pathogenic SIV infection in sooty mangabeys.¹⁷ In humans, recent results suggest the preferential loss of anti-inflammatory iNKT cells in the HIV-infected gut.¹⁸ These findings together suggest a role of iNKT cells in HIV-1 immunity and pathogenesis.^{19,20} FoxP3+ regulatory T cells (Tregs) down-regulate self-reactive T cell responses in humans to avoid autoimmunity, and control immune responses to pathogens to avoid excessive and sustained immune activation.²¹ A hallmark of HIV-1 infection is chronic T cell immune activation, which is detrimental to the host.²² Treg activity may mediate two counteracting effects in HIV infection.²³ Suppression of anti-viral T cell responses might impair immune control of virus, whereas suppression of generalized immune activation might be working to limit immune pathology.²⁴

Here, we hypothesized that subtype divergence in disease progression might be associated with differences in regulatory T cell subsets. We therefore investigated iNKT cells and Tregs in HIV-1 subtype A and subtype D infected Ugandans. The results identify the differential distribution of these two immune cell compartments as a potential immune correlate of the difference between these viral subtypes.

PATIENTS AND METHODS

Study Cohort and Samples

Study participants were from a prospective community-based cohort to characterize HIV-1 infection in Rakai District, Uganda, from 1998 until 2004 (Table 1).¹ The study was approved by institutional Review Boards of Uganda's National Council for Science and Technology and the Uganda Virus Research Institute's Science and Ethics Committee, as well as the Division of Human Subjects Protection at the Walter Reed Army Institute of Research. Written informed consent was provided by all participants. Previously cryopreserved peripheral blood mononuclear cells (PBMC) from 103 HIV-1 sero-positive individuals and 40 community-matched sero-negative controls were randomly selected. No patients were on antiretroviral therapy. HIV-1 testing was performed, and Amplicor HIV-1 Monitor test, version 1.5 (Roche Diagnostics, Indianapolis, IN) was used to quantify viremia. Viral subtype was determined previously.²⁵

Flow Cytometry

PBMC were thawed and washed in RPMI medium containing 10% fetal bovine serum, L-glutamine, penicillin/strep, and HEPES. Guava PCA and ViaCount were used to determine viability and cell concentration (Guava Technologies, Hayward, CA). To study iNKT cells, samples were stained at 4°C for 30 min in the dark. Aqua Live Dead Stain (Invitrogen, Carlsbad, CA) was used to discriminate viable cells. Commercially available mAbs included anti-CD3 PerCP-Cy5.5, anti-CD4 Pacific Blue, and anti-CD161 APC, all from BD

Biosciences (San Jose, CA). The anti-V α 24 FITC and anti-V β 11 PE were from Beckman Coulter (Brea, CA). To identify Tregs, the mAb cocktail substituted anti-CD25 PE, anti-CD8 PE-Cy7, and anti-CD127 Alexa Fluor® 647, all from BD Biosciences. After surface staining, samples were washed, permeabilized/fixed and stained with anti-FoxP3 Alexa Fluor® 488 (clone 206D, BioLegend, San Diego, CA). Samples were acquired on a BD FACSCanto II (BD Biosciences) and analyzed using FlowJo version 9.4.10 (Tree Star, Ashland, OR).

Functional Assays

To assess CD4 T cell responses, PBMC were thawed and cultured overnight with HIV-1 Gag PepMix (subtype B consensus pool) or CMVpp65 PepMix (both from JPT Peptide Technologies GmbH, Berlin, Germany). Staphylococcal Enterotoxin B (SEB) (Sigma, St. Louis, MO) was used at 10 ng/ml. Brefeldin A (Sigma) was present during the final 6 hrs of incubation. After incubation, samples were fixed, washed, and permeabilized using BD Perm/Wash (BD Biosciences), and stained with anti-IFN γ APC (eBiosciences), anti-TNF α PE (BD Biosciences), anti-IL-2 FITC (BD Biosciences) before acquisition on a BD FACSCanto II cytometer.

Statistical Analysis

Graph Pad Prism version 5.0a for Mac OSX was used for statistical analysis (GraphPad Software, La Jolla, CA). Comparisons between two groups were done using Mann-Whitney test, and between three groups using non-parametric one-way ANOVA. Associations were determined by Spearman's rank correlation. P values < 0.05 were considered statistically significant.

RESULTS

Lower CD4 counts in HIV-1 subtype D infection compared to subtype A infection

HIV-1 subtype A (n = 35) and subtype D (n = 68) infected subjects, as well as uninfected community-matched controls (n = 40), were randomly identified from a cohort designed to characterize incident HIV-1 infection in Rakai District, Uganda (Table 1).¹ Age and sex of the participants were similarly distributed between the groups. Viral load did not differ significantly between HIV-1 subtype A and D infection (p = 0.388), despite a higher median among subtype D infected subjects (50,225 vs. 22,521 copies/ml). Absolute CD4 T cell counts were significantly lower in people infected with HIV-1 subtype D compared to those infected with subtype A (p = 0.008). The median time from sero-conversion was, however, similar between the two groups (928 and 913 days, respectively, p = 0.413). These data are consistent with more rapid CD4 T cell decline in subtype D infection.¹⁻⁴

Differential patterns of iNKT cells and Tregs in HIV-1 subtype A and D infection

PBMC of HIV-1 subtype A and subtype D infected patients and negative control subjects were analyzed for levels of iNKT and Treg cells. iNKT cells positive for TCR chains V α 24 and V β 11 were identified by polychromatic flow cytometry (Fig. 1A). The levels of iNKT cells were significantly reduced in individuals infected with HIV-1 subtype D, when compared to HIV-1 negative controls (p = 0.006, Fig. 1B). In contrast, HIV-1 subtype A infection was not associated with significant loss of iNKT cells. Next, Tregs were identified as CD3+CD4+CD25+FoxP3+CD127- cells (Fig. 1C), and their frequency was reduced in patients infected with either subtype of HIV-1 as compared to uninfected controls (Fig. 1D). Interestingly, individuals infected with HIV-1 subtype A displayed a more pronounced decrease in Tregs than did subtype D infected subjects (p < 0.001, Fig. 1D).

Levels of iNKT cells are associated with CD4 T cell function

T cell responses to CMV, HIV-1 Gag, as well as responsiveness to SEB, were analyzed by intracellular flow cytometry. iNKT cell levels were positively associated with IL-2 production ($\rho = 0.472$, $p = 0.004$) and, to a lesser extent, TNF α production ($\rho = 0.382$, $p = 0.025$) in CD4 T cells after stimulation with SEB in subtype A infected, but not in D infected, subjects (Fig. 1E and F). CD4 T cells with triple-expression of TNF α , IFN γ and IL-2 were less common, but also correlated directly with levels of iNKT cells in subtype A infection ($\rho = 0.456$, $p = 0.006$; Fig. 1G). In contrast, after stimulation with CMV peptide pool, cells producing IL-2 or TNF α were inversely associated with the frequency of iNKT cells in subtype A infection ($\rho = -0.611$, $p = 0.02$ and $r = -0.696$, $p = 0.005$; Fig. 1H and I, respectively). CD4 T cells producing IL-2, TNF α and IFN γ concomitantly showed a modest inverse correlation with iNKT levels ($\rho = -0.520$, $p = 0.056$, Fig. 1J). No correlation was found when comparing the function of CD4 and CD8 T cells to iNKT cell levels in patients infected with HIV-1 subtype D, nor between iNKT cell levels and T cell responses to HIV-1 Gag in patients infected with either subtype (data not shown). There were also no significant correlations between iNKT or Treg cell levels and viral load or CD4 counts (data not shown). Likewise, no associations were present between levels of Tregs and measures of antigen-specific T cell responses (data not shown).

DISCUSSION

In the present study we found that the frequency of iNKT cells was significantly reduced in patients infected with HIV-1 subtype D, but not in those infected with subtype A, compared to uninfected subjects. In contrast, levels of Tregs were decreased in HIV-1 infection overall, but remained higher in patients infected with subtype D compared to A. In addition, iNKT cell levels in individuals infected with HIV-1 subtype A correlated positively with CD4 T cell responsiveness to oligoclonal SEB stimulus, and negatively with CMV-specific CD4 T cell responses. These associations were not observed in patients infected with HIV-1 subtype D. These results identify a possible association between the differential decline of immunoregulatory T cell subsets and differences in disease progression in patients infected with different HIV-1 subtypes.

Absolute CD4 counts were significantly lower in subtype D infected patients as compared to subtype A infected patients, despite similar viral load and time from sero-conversion. These findings confirm previous reports where subtype D infection displayed faster disease progression than subtype A infection, independently of viral load.¹⁻⁴ The mechanism behind this difference is not yet understood. Subtype A viruses may switch from R5 to X4 co-receptor usage at a slower rate.²⁶ To what extent such a difference in co-receptor usage might be linked to the differences in iNKT cells and Tregs we have observed here remains to be investigated. iNKT cells have relatively high levels of CCR5 suggesting that an early co-receptor switch might not hit these cells specifically, but other unknown subtype-specific viral characteristics could be involved. It is possible that iNKT cells become involved in responses against HIV-1 or opportunistic pathogens, that might lead to their activation-induced cell death or redistribution to peripheral or lymphoid tissues. The decrease in Tregs in peripheral blood during HIV-1 infection is due at least partly to sequestration in lymph-nodes as a part of the pathological immune activation during HIV disease.²⁷ The finding of higher levels of PD-1 expression and apoptosis in CD4 T cells in subtype D infection might suggest a difference between subtypes in the ability to cause persistent immune activation.⁶ However, the possibility that subtype differences in disease progression are due to differences in immune activation has not been thoroughly addressed.

We investigated possible relationships between CD4 T cell responses to antigens and the levels of iNKT cells and Tregs. Interestingly, the levels of iNKT cells in subtype A infection

were directly correlated with SEB-induced CD4 T cell responses, and in particular with the ability to produce IL-2. This suggests that maintenance of healthy iNKT cell levels in subtype A, but not in subtype D, infection is associated with the ability of the CD4 T cell compartment to respond to antigen with IL-2 production. SEB activates a broad range of naïve, memory and effector cells sharing certain V β 's and many of those cells will not be engaged in defense against HIV-1. It is interesting to note that maintenance of IL-2 production in CD4 T cells have previously been associated with control of HIV viremia.^{28–30} In contrast, iNKT cell levels correlated inversely with CD4 T cell responses to CMV. The magnitude of T cell responses to CMV might in this context be interpreted as a proxy marker of disease progression, as CMV reactivation may cause those responses to accumulate later in disease. In conclusion, the present findings identify associations between viral subtype, iNKT cells and adaptive T cell responses that may contribute to the differential pathogenicity of HIV-1 subtypes A and D in Uganda.

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FIGURE 1. iNKT cells and Treg cells in HIV-1 subtype A and D infection. Contour plots illustrating the gating strategy used to identify iNKT cells (A) and CD4+CD25+FoxP3+CD127– Treg cells (C). Box and Whisker plots showing the median and 10th–90th percentiles of iNKT cells (B) and Tregs (D) in HIV-1 negative and HIV-1 subtype A and D infected subjects. Statistically significant differences between groups were determined with non-parametric one-way ANOVA test. *** $p < 0.001$; ns = non-significant. Expression of IL-2 (E) and TNF α (F) alone, or triple-expression of IL-2, TNF α and IFN γ (G) by CD4 T cells correlates directly with the levels of iNKT cells in HIV-1 subtype A infected participants after stimulation of cells with SEB. An inverse correlation is seen between the same parameters after stimulation with CMV peptide pool (H–J). Correlation analysis between groups was performed with Spearman's rank correlation.

Table 1

Descriptive Statistics for Study Population

	HIV-1 negative (n = 40)	HIV-1 subtype A (n = 35)	HIV-1 subtype D (n = 68)
Age, median years (range)	29 (20 – 42)	31 (18 – 54)	31 (19 – 53)
Sex, no. (percent)			
Female	20 (50%)	20 (57%)	45 (66%)
Male	20 (50%)	15 (43%)	23 (34%)
Estimated time from seroconversion, median days	NA	928 (233 – 2103)	913 (22 – 2284)
Viral load, median copies/ml (range) ^a	NA	22,521 (1,071 – 1,685,638)	50,225 (528 – 1,310,492)
CD4 T cell absolute count, median cells/ μ l (range)	NA	670 (88 – 1,385)	489 (1 – 1,092)

NA indicates not applicable

^aViral load measured by Roche Amplicor Monitor v1.5, limit of detection 400 copies/ml