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NK cells and CD1d-restricted NKT cells respond in different ways with divergent kinetics to IL-2 treatment in primary HIV-1 infection

Carlotta Kuylensstierna^{*}, Jennifer E. Snyder-Cappione[†], Christopher P. Loo[†], Brian R. Long[†], Veronica D. Gonzalez^{*}, Jakob Michaëlsson^{*}, Markus Moll^{*}, Gerald Spotts[†], Frederick M. Hecht[‡], Douglas F. Nixon^{†,1}, and Johan K. Sandberg^{*,1}

^{*}Center for Infectious Medicine, Department of Medicine, Karolinska Institutet, Karolinska University Hospital Huddinge, 14186 Stockholm, Sweden.

[†]Division of Experimental Medicine, Department of Medicine, University of California, San Francisco, CA, USA.

[‡]Positive Health Program, San Francisco General Hospital, San Francisco, California, USA.

Abstract

Cytokine immunotherapy is being evaluated as adjunct treatment in infectious diseases. The effects on innate and adaptive immunity *in vivo* are insufficiently known. Here, we investigate whether combination treatment with antiretroviral therapy (ART) and IL-2 of patients with primary HIV-1 infection induces sustained increases in circulating NKT cell and NK cell numbers and effector functions, and investigate how changes are coordinated in the two compartments. Patients with primary HIV-1 infection starting ART were analyzed for numbers, phenotype, and function of NKT cells, NK cells and dendritic cells (DCs) in peripheral blood before, during and after IL-2 treatment. NKT cells expanded during IL-2 treatment, as expected from previous studies. However, their response to α -galactosyl ceramide antigen were retained but not boosted. Myeloid DCs did not change their numbers or CD1d-expression during treatment. In contrast, the NK cell compartment responded with rapid expansion of the CD56^{dim} effector subset, and enhanced IFN γ production. Expansions of NKT cells and NK cells retracted back towards baseline values at 12 months after IL-2 treatment ended. In summary, NKT cells and NK cells respond to IL-2 treatment with different kinetics. Effects on cellular function are distinct between the cell types, and the effects appear to not be sustained after IL-2 treatment ends. These results improve our understanding of the effects of cytokine immunotherapy on innate cellular immunity in early HIV-1 infection.

Introduction

The innate immune system provides the first line of defense against invading pathogens. Invariant CD1d-restricted NKT cells are unconventional T cells, operating on the border between the innate and adaptive immune systems [1,2]. They are believed to be important immunoregulatory cells, rapidly secreting cytokines upon activation and modulating the

Correspondence: Dr. Johan K. Sandberg, CIM, Department of Medicine, F59, Karolinska Institutet, Karolinska University Hospital Huddinge, 14186 Stockholm, Sweden. Tel: +46-8-58589687; Fax: +46-8-7467637; johan.sandberg@ki.se.

¹These authors contributed equally and should both be considered last and senior authors.

Disclosures

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activity of other immune cells [3,4]. NKT cells express the HIV-1 co-receptor CCR5 and are susceptible to infection [5,6]. In particular the CD4⁺ NKT cell subset is rapidly lost in the majority of infected subjects, and the recovery of these cells in response to antiretroviral therapy (ART) is slow at best [5,7,8]. Some patients, however, maintain relatively healthy numbers of NKT cells even in chronic HIV-1 infection, but NKT cells retained under these circumstances are functionally impaired [9,10]. HIV-1 uses several mechanisms to inhibit CD1d-mediated antigen presentation, suggesting that CD1d-restricted NKT cells play a role in immune control of the virus [11]. NK cells fight HIV-1 infection via direct lysis of infected cells or antibody-dependent cytotoxicity [12–14]. They also secrete chemokines that inhibit HIV-1 target cell entry [12–14]. NK cells suffer changes in subset distribution and receptor expression during chronic HIV-1 infection [15–18], and the main cytolytic CD56^{dim} NK cell subset is numerically reduced [19].

Interleukin-2 (IL-2) treatment in combination with ART supports an increase in CD4⁺ T cells, NKT cells and CD56^{dim} NK cells in HIV-1 infected subjects [20,21]. The clinical benefits of these effects, if any, are at present unclear [22,23], and merit further investigation. In this study we therefore aimed to investigate whether treatment of HIV-1 infected subjects with ART+IL-2 can induce sustained increases in circulating NKT cell and NK cell numbers and effector functions, and determine how changes are coordinated in the two immune compartments.

Materials and methods

Patients and cells

Study subjects were from the University of California, San Francisco (UCSF) OPTIONS cohort of acute and early HIV-1 infections [24]. All subjects were ART-naïve when entering the study. Those who decided to start ART were offered the opportunity to participate in a randomized, controlled trial adding IL-2 to ART. This study included eight patients starting ART+IL-2. The IL-2 treatment required subjects to have reached HIV-1 RNA levels below 500 copies/ml prior to initiation. HIV-1 RNA levels were well ART controlled during the course of the study. IL-2 was given within the first month after starting ART as subcutaneous injections (7.5 million units, twice daily) for 5 days in 8-week intervals. Samples were taken at month 0 (ART-naïve), 1 (before initiation of IL-2 therapy), 6 (2 cycles of IL-2), 12 (5 cycles of IL-2) and at month 18 and 24 (post IL-2). PBMCs were isolated from whole blood and frozen in heat-inactivated FBS containing 10% DMSO in liquid nitrogen and stored at the UCSF AIDS Specimen Bank. Thawed samples were washed twice in RPMI 1640 supplemented with 2 mM L-glutamine, 1% streptomycin, penicillin (Invitrogen, Carlsbad, CA), 10 mM HEPES (Hyclone, Logan UT), and 10% heat-inactivated FCS (Invitrogen).

Flow cytometry

The following mAbs were used in flow cytometry: Anti-CD16 Pacific Blue (PB), anti-CD56 PE-Cy7, anti-CD11c APC, anti-CD14 APC-Cy7, anti-CD19 APC-Cy7, anti-HLA-DR PerCp, anti-CD1d PE, anti-CD4 PB, anti-CD8 PerCp, anti-CD38 PE-Cy7, anti-CD3 PerCp, anti-IFN γ FITC, anti-MIP1 β PE, anti-IgG1 PE, anti-IgG1 PE-Cy7 and anti-IgG2a APC-Cy7 were all from BD Biosciences (San Diego, CA). Anti-CD4 Qdot605, anti-CD3 Qdot655 and Aqua Live/dead marker were from Invitrogen (Carlsbad, CA). Anti-V β 11 FITC and anti-V α 24 APC were from Beckman Coulter (Fullerton, CA). Anti-6-sulfoLacNAc (Slan) FITC and anti-CD161 biotin were from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-PD1 PE was from Biosite (San Diego, CA), and anti-CD3 Cascade Yellow from Dako (Carpinteria, CA). For phenotypic analyses, PBMCs were stained in 96-well V-bottomed plates for 20–30 min at 4°C and subsequently fixed (CellFix, BD Biosciences). For

measurement of cytokines, cells were permeabilized (FACS Permeabilizing Solution 2, BD Biosciences) prior to incubation with the respective cytokine mAbs. Flow cytometry data were acquired on a BD LSR II instrument and analyzed using FlowJo software (Tree Star, OR, USA).

Functional assays

Cytokine expression in NK and NKT cells was detected after *ex vivo* stimulation. NK cell function was assessed by incubating 5×10^5 PBMCs in U-bottomed plates in complete medium supplemented with 50 ng/ml IL-12 and 50 ng/ml IL-18 (R&D Systems) at 37°C for 18 h, with 1 μ g/ml brefeldin A (Golgi Plug, BD Biosciences) present for the final 6 h. Similarly, to assess NKT cell function, 5×10^5 PBMCs were incubated in medium supplemented with 100 ng/ml α -galactosyl ceramide (α GalCer) (Biomol, Hamburg, Germany) for 2 h, and after addition of Golgi Plug, for another 6 h.

Statistical analysis

Changes in cell numbers and other measures over time were analyzed with repeated measures ANOVA and paired t test as indicated, using Sigma Stat software (SPSS, Chicago, IL).

Results

NKT cells and NK cell subsets expand with different kinetics during IL-2 treatment

Previous studies have shown that addition of IL-2 treatment to effective ART leads to significant increases in mean NKT cell and NK cell counts in patients with primary HIV-1 infection [20,21]. Both the kinetics and sustainability of the effects of IL-2 on these cells are, however, unknown and therefore we studied the changes in absolute numbers of NKT cells and NK cells and their subsets longitudinally before, during, and post IL-2 treatment. Invariant NKT cells were identified by flow cytometry using double staining for their invariant TCR composed of V α 24 and V β 11 (Fig. 1A). NKT cells expressed CD161 and CCR5 as expected, with frequent expression of PD-1 but with low levels of CD38. NK cells were identified by excluding cells expressing CD3, CD4, CD14, CD19 and Slan from the lymphocyte population, and subsets of NK cells were defined as CD56^{bright}, CD56^{dim} and CD56^{neg}CD16⁺ (CD56^{neg}) (Fig. 1B). We observed a continuous increase in NKT cell numbers over the time of IL-2 treatment (month 1 to 12) (Fig. 1C). Interestingly, that trend appear to continue for an additional six months after discontinuation of IL-2 treatment, but at one year after IL-2 discontinuation NKT cell numbers had declined to levels seen before initiation of treatment. Also, the expansion of NKT cells upon ART+IL-2 treatment occurred in both the CD4⁺ and CD4⁻ NKT cell subsets as the relationship between these subsets were unchanged in patients (data not shown). The expression levels of CD161, CCR5, PD-1 and CD38 did not change significantly over time, although there was a slight downward trend in CCR5 and CD38 expression over the course of IL-2 treatment (data not shown).

NK cells increased in response to initiation of IL-2 treatment from month 1 to month 6, but then gradually declined to pre-ART levels (Fig. 1D). It was predominantly the CD56^{dim} subset which expanded, although CD56^{neg} NK cells displayed a similar trend. CD56^{bright} NK cells appeared to be somewhat boosted by the initiation of ART, but IL-2 had no measurable numerical effect on this subset (Fig. 1E). Thus, NKT cells and NK cells both expanded in response to IL-2 treatment but with different kinetics, with NK cells responding more rapidly (Fig. 1F). However, these initial increases in numbers of NKT cells and NK cells were not sustained for long periods after discontinuation of IL-2 therapy. CD4 T cells

followed a similar trend as the NKT cells in that they gradually increased (Fig. 1F). CD4 T cell numbers, however, appeared better sustained post IL-2 treatment.

NKT cells retain their responsiveness, whereas NK cells enhance their IFN γ production with IL-2 treatment

The ability of NKT cells and NK cells to rapidly produce cytokines and chemokines to activate and regulate other immune cells is important for their ability to help control infections. Therefore, the capacity of these cells in blood from HIV-1 infected subjects treated with IL-2 to produce IFN γ and MIP1 β was measured. To determine the functional capacity of NKT cells, PBMCs were stimulated with α GalCer for 8 h (Fig. 2A). Commencing ART initially appeared to boost IFN γ and MIP1 β production slightly, but there was no further increase after initiation of IL-2 treatment, and responses gradually returned to pre-ART levels over the two year's time of study (Fig. 2C). To assess NK cell activity, PBMCs were stimulated with a combination of IL-12 and IL-18 for a total of 18 h (Fig. 2B). There was a clear increase in IFN γ expression in response to stimulation during the first months of IL-2 treatment but there was little change in the MIP1 β -production over time. Though IFN γ production increased in the CD56^{dim} NK cell subset, there was no clear change in the CD56^{bright} cells, and a tendency towards a decrease in CD56^{neg} cells (Fig. 2E). Taken together, these data suggest that the two innate immune cell compartments respond functionally in different ways to IL-2 treatment. The NKT cell response to α GalCer is maintained during IL-2 treatment, whereas NK cells increase their responsiveness to IL-12 and IL-18 as determined by IFN γ production (Fig. 2F). As IFN γ expression is increased in NK cells and MIP1 β is unchanged, this also leads to a change in the functional profile in the NK cell compartment, which may influence their anti-viral activity.

Dendritic cell numbers and CD1d expression are not significantly affected by IL-2 treatment

We next investigated if the increase in NKT cells might be accompanied by an expansion of myeloid DCs expressing CD1d. Myeloid DCs were identified in PBMCs by gating on size, followed by a gate on cells negative for lineage markers (CD3, CD8, CD14, CD16, CD19, and CD56). CD11c+ HLA-DR+ DCs were subsequently identified among the lineage-negative cells. Both the number of myeloid DCs (Fig. 3A), and the expression of CD1d on these cells were generally unchanged during IL-2 treatment (Fig. 3B). These data suggest that IL-2 treatment does not affect myeloid blood DC numbers and CD1d expression, and such effects are thus unlikely to underlie the changes in numbers and function of NKT cells and NK cells.

Discussion

Many aspects of innate cellular immunity in HIV-1 infection remains to be discovered. It is likely that the role of NK cells and NKT cells is both multifaceted and important. Combination therapy with ART and cytokines adds a new level of complexity to the role of innate immunity in this infection, and studies of NK and NKT cells during ART+IL-2 therapy has so far been limited [20,21]. Here, we simultaneously assessed NK cells, NKT cells, and myeloid DCs longitudinally in patients with primary HIV-1 infection initiating first ART and, one month later, IL-2 treatment. Importantly, we also followed patients and evaluated these cell types after discontinuation of IL-2 treatment. We found that these innate cellular compartments respond with different kinetics and in different ways to IL-2 administration. NK cells respond rapidly with an expansion of CD56^{dim} effector type NK cells. This expansion is not sustained over time or post IL-2 treatment. In addition to the numerical changes, IFN γ production in this NK cell subset increases with IL-2 treatment. As their MIP1 β production is unchanged, this results in a skewing of the functional profile of

CD56^{dim} NK cells that may have implications for their ability to contain HIV-1. Nevertheless, the rapid expansion of CD56^{dim} effector NK cells may open the possibility of using pulsed IL-2 treatment to specifically expand and activate this innate immune compartment.

The NKT cells responded to IL-2 treatment with a gradual numerical increase, but it appeared that the NKT cell expansion retracted back after cessation of IL-2 treatment. There were no measurable functional changes in the NKT cells during the course of the study. The functional deficiencies of NKT cells we have previously described in HIV-1 infection [9,10], may therefore not be alleviated by IL-2 treatment. The increase in NKT cell numbers was kinetically different from that of NK cells, and instead largely tracked the increase in CD4⁺ T cells. We hypothesized that the increase in NKT cells could potentially be paired with an increase in CD1d-expressing DCs in blood. However, neither the number of DCs nor their CD1d expression showed any clear change in response to IL-2 treatment. The changes in NKT cells can thus probably not be explained at that level. It remains possible that functional changes in the DC compartment during IL-2 therapy may play a role. Overall, the results presented here help the understanding of innate immune cells in the context of HIV-1 infection, as well as their response to IL-2 treatment in combination with ART. We speculate that the inability of IL-2 to induce sustainable increases in NK cells and NKT cells may contribute to the apparent failure of this treatment to achieve clinical benefits in HIV-1 infected patients [22,23].

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Figure 1.

IL-2 treatment is associated with rapid expansion of the CD56^{dim} NK cells, whereas the invariant NKT cells expand with delayed kinetics. **(A)** Identification of NKT cells in PBMCs from HIV-1 infected subjects by co-expression of V α 24 and V β 11, and their phenotypical characterization (one representative donor is shown). **(B)** Identification of NK cells by excluding CD3, CD4, CD14, CD19 and Slan expression and definition of the CD56^{bright}, CD56^{dim} and CD56^{neg} NK cell subsets (one representative donor is shown). **(C and D)** Longitudinal measurement of the NKT and NK cell counts in HIV-1 patients (n = 8) following ART and IL-2 treatment. **(E)** The relative changes in the proportion of the CD56^{bright}, CD56^{dim} and CD56^{neg} NK cell subsets. * p < 0.05 with repeated measures ANOVA; (*) p < 0.05 with paired t-test. **(F)** The relative changes in the NKT cell, NK cell and CD4 T cell compartments. Mean and standard error is shown.

Figure 2.

NKT cells retain their response to stimuli, while NK cells enhance their IFN γ production with IL-2 treatment. **(A and B)** Representative contour plots showing IFN γ and MIP1 β production in NKT cells stimulated with α GalCer and NK cells stimulated with IL-12 and IL-18, respectively. **(C and D)** Longitudinal measurement of IFN γ and MIP1 β responses in NKT and NK cells in patients treated with ART+IL-2 ($n = 8$). **(E)** Effect of IL-2 treatment on NK cell subsets. **(F)** Differences between NK and NKT cells in the kinetics of their functional response. Mean and standard error is shown. * $p < 0.05$ with repeated measures ANOVA.

Figure 3.

Myeloid DC numbers and their CD1d expression during the course of the study. **(A)** Enumeration of myeloid DCs in PBMC by flow cytometry before, during and after IL-2 treatment. Myeloid DCs were identified in PBMCs by gating on size, followed by a gate on cells negative for lineage markers (CD3, CD8, CD14, CD16, CD19, and CD56). CD11c+ HLA-DR+ DCs were subsequently identified among the lineage-negative cells. **(B)** CD1d expression levels on myeloid DCs as measured by flow cytometry.