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Adoptive transfer of aminobisphosphate-expanded V γ 9V δ 2⁺ T cells does not control HIV replication in a humanized mouse model

Aim: V γ 9V δ 2 $\gamma\delta$ T cells have effector potential against several cancers and infectious agents. Whether these cells control HIV replication was tested in humanized mouse model. **Methods:** NOD SCID gamma mice engrafted with human peripheral blood mononuclear cells (PBMCs) and infected with HIV_{BAL} were treated with zoledronate-expanded V γ 9V δ 2 T cells either alone or in combination with an anti-HIV envelope antibody b12. **Results:** Severe depletion of CD4+CCR5⁺ T cells was observed in PBMCs of all infected mice. HIV plasma p24 levels were comparable in all infected groups with no decrease in plasma viremia achieved by adoptive transfer of cells. **Conclusion:** Autologous transfer of *ex vivo* expanded V γ 9V δ 2 T cells may not be a successful treatment choice for controlling HIV replication *in vivo*.

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Keywords: gamma delta • HIV • humanized mouse • V γ 9V δ 2

The phenotype of majority of peripheral blood $\gamma\delta$ T cells is V γ 9V δ 2 and cytotoxicity of these cells is well demonstrated against solid tumors, melanoma, myeloma and lymphoma [1,2]. Among infectious diseases, expansion of this subset of T cells is observed in *Mycobacterium tuberculosis*, malaria and bacterial infections [3,4]. Adoptive transfer of *ex vivo* aminobisphosphate-expanded V γ 9V δ 2 T cells is being tested for therapeutic potential against metastatic solid tumors [5], where such treatment showed some additive effect when given alongside previously ineffective cancer therapy. Recently, a role for pamidronate expanded V γ 9V δ 2 T cells against influenza virus was shown [6]. These cells effectively lysed influenza virus infected cell lines through the Fas-FasL and perforin-granzyme B pathways. In a humanized mouse model, cells expanded by aminobisphosphate pamidronate reduced disease severity and mortality caused by seasonal and avian influenza virus [7].

While it is confirmed that there is significant depletion in both numbers and func-

tion of V γ 9V δ 2 T cells in HIV infected individuals [8,9], whether this subset has an anti-HIV effect remains to be established. Natural history studies have shown a correlation between V δ 2 T cell frequency and lower vRNA [10] and reconstituted V δ 2 T-cell numbers are observed in elite controllers of HIV [11]. Cytolytic capacity of these cells against HIV-infected cells has been shown using *in vitro* systems [12,13]. The mucosal $\gamma\delta$ T cells were expanded in rhesus macaques that were protected in a challenge study indicating their role in protection [14], however, these mucosal cells are primarily of V δ 1 subtype. In sooty mangabeys, the natural hosts of SIV, dysfunction of V γ 9V δ 2 T cells is not observed [15] suggesting that the dysfunction observed in pathogenic HIV infection may play role in disease progression.

Materials & methods

Mice

Use of humanized mouse model can be an efficient way to observe direct effects of

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such interventions like adoptive cell transfers on HIV pathogenesis. 4- to 6-week-old NOD SCID gamma (NSG) mice were used for engraftment of human peripheral blood mononuclear cells (PBMCs). These mice lack the IL-2 receptor γ chain and are defective in cytokine signaling, leading to successful engraftment of human cells. All protocols described here were approved by the University of Maryland Institutional Animal Care and Use Committee (IACUC). PBMCs were obtained from healthy individuals and a total of 5×10^6 cells were inoculated by intraperitoneal route in each mouse to generate humanized mice. After 4 weeks of engraftment, blood was collected from retro-orbital sinus and stained for analysis using flow cytometry. Successful engraftment was determined by positive staining of mice whole blood with human CD45 antibody. Mice were then divided into groups of five and assigned to various treatment groups.

HIV infection, treatments & assays

HIV_{BAL} with a p24 concentration of 160 ng was used to infect mice via intraperitoneal route. This concentration was previously found to result in successful infection of all animals resulting in massive loss of CD4 T cells. Groups of uninfected and infected but untreated mice were controls. In treatment groups, mice were either treated with zoledronate-expanded cells, a combination of expanded cells and anti-HIV envelope antibody b12 or with b12 antibody alone.

Autologous PBMC were expanded by using a combination of zoledronic acid (zoledronate; Novartis, Basel, Switzerland) and recombinant human IL-2 (Tecin Biological Resources Branch, NIH, MD, USA) [16]. During HIV infection, the numbers and function of V γ 9V δ 2 T cells are severely compromised and efforts to stimulate them with $\gamma\delta$ stimulant, phosphoantigen isopentenyl pyrophosphate (IPP), are not successful. Aminobisphosphonate zoledronate overcomes this defect and leads to expansion of functionally active V γ 9V δ 2 T cells [16]. Frequency of V δ 2 expressing cells was determined by flow cytometry and cultures with ~90% V δ 2 expressing cells were used for adoptive transfers.

The role of ADCC in HIV is increasingly being recognized [17]. Cells expanded with zoledronate have potent ADCC activity when used in combination with specific antibodies against tumors [16] and also against HIV envelope coated target cells in *in vitro* systems (data not shown). We attempted to use the expanded $\gamma\delta$ T cells along with 10 mg/kg of b12 antibody to test this anti-HIV ADCC effect *in vivo*. The b12 monoclonal antibody is a broadly HIV neutralizing antibody, and has potent ADCC capacity [18].

Animals were infected 4 weeks after engraftment. Cell and antibody transfers were carried out at days 3, 7 and 10 after infection in relevant groups. A total of 15×10^6 zoledronate-expanded cells were injected at each time point per animal via lateral tail vein. At the same time, 250 μ g of b12 antibody was injected in relevant groups by the same route. The dose of antibody and cell injections are based on published work in a mouse model using similar antibody/cell suspensions [19,20]. Blood was collected at various times after infection for flow cytometric evaluation of surface markers as well as for measurement of HIV core antigen p24 in plasma. Monoclonal antibodies were from BD biosciences unless otherwise specified. Whole blood was stained for cellular markers following lysis and fixing using a commercial kit (Beckman Coulter) and acquisition was done on an FACS Calibur. Data analysis was performed using FlowJo software (Tree Star, OR, USA). The p24 antigen was detected in plasma samples from individual mice at various times post-infection using a commercial ELISA kit.

Expanded cells were injected at days 3, 7 and 10 after infection with HIV. We are testing the potential of $\gamma\delta$ T cells as an immunotherapy in individuals whose infection is detected in very acute stages. Accordingly, this regimen was adopted. Flow cytometry showed significant frequency of V δ 2+ cells (average 4.5% of T cells) in inoculated animals tested 7 days after adoptive transfer. This represents tenfold higher frequency compared with circulating V δ 2+ T cells (average V δ 2+ frequency 0.4% of T cells) in our humanized mouse model.

Results

Severe depletion of HIV target cells expressing CD4 and CCR5 was observed in all infected groups by day 10 after infection (Figure 1) when compared with uninfected group. There were no improvements in frequency of this subset in animals inoculated with expanded cells and/or antibody compared with untreated groups by day 24, which was the last day of our experiments. Along with depletion of target cells, the CD4:CD8 ratio [21] was inverted in all infected groups irrespective of treatment (Figure 2). The levels of viral replication measured by detecting p24 antigen in plasma samples showed comparable p24 in all infected groups at most time points, with a peak replication observed in most animals at day 7. There was no decrease in viral load in any treated group compared with untreated infected group at any time tested (Figure 3).

Discussion

While there was no benefit of $\gamma\delta$ therapy in any infected animal in our system, we observed a significant spike

Figure 1. Effect of HIV replication on viral target cell frequencies in peripheral blood of mice. Percentages of CCR5+CD4+ T lymphocytes measured at various times after infection shows a severe depletion of this subset in all infected animals. HIV-infected mice are untreated (HIV), treated with Zol+IL-2 expanded cells (HIV+Cells), with Zol+IL-2 expanded cells and b12 antibody (HIV+Cells+b12), or with b12 antibody (HIV+b12). **(B)** By day 14 post-infection, significant decline in this subset in all infected groups was observed with no significant difference observed between the groups.

in viral replication in animals that were injected with expanded cells as well as the anti-HIV envelope antibody b12 at week 2. We have previously tested the antibody-dependent cytotoxicity of cells expanded in this manner against HIV envelope coated target cells (data not shown) and were expecting a similar beneficial effect of such treatment against HIV in this animal model. One explanation for the unexpected result could be the Fc receptor (CD16) mediated immune activation caused by such a treatment and resulting spike in HIV replication. Cross-linking of CD16 on immune cells results in release of cytokines such as TNF- α as well as upregulation of activation markers like CD25 (data not shown). Similar changes in

the microenvironment could possibly promote HIV replication. In an unexpected finding, recently it was shown that resting V δ 2+ cells act as reservoir for HIV [13] whereas HBMPP+IL-2 activated V δ 2+ cells could be readily infected with HIV JR-CSF in a CD4-dependent manner. Based on these findings, the study hypothesized that immune activation caused by HIV infection induces transient CD4 expression on this cell subset rendering them susceptible to HIV infection. Such a scenario is possible in our system where zoledronate +IL-2 expanded cells were injected during acute phase of infection.

Expanded V δ 2 cells have not been unambiguously shown to limit HIV in an *in vivo* model. Ami-

Figure 2. Inverted CD4:CD8 T lymphocyte ratios in HIV infected mice. The average ratio in uninfected animals was $3.26 (\pm 1.49)$ which is similar to that in infected animals at day 7 post HIV infection **(A)**. **(B)** By day 14 post-infection this ratio was significantly inverted in all infected groups with average values of $0.13 (\pm 0.04)$ for HIV, $0.3 (\pm 0.28)$ for HIV+cells, $0.31 (\pm 0.21)$ for HIV+cells+b12 and $0.29 (\pm 0.3)$ for HIV+b12 groups.

nobisphosphonates such as zoledronate along with cytokines IL-2 or IL-15 are used to expand these cells, which then have potential for immunotherapy against cancers and some infectious diseases via direct cytotoxicity or antibody-mediated cytotoxicity. Recent success using such expanded cells to control influenza virus replication in a humanized mouse model encouraged us to test the possibility against HIV. We showed here that adoptive transfer of expanded $\gamma\delta$ T cells either alone or in combination with anti-HIV envelope b12 antibody failed to suppress HIV replication in our system of acute HIV infection. It is possible that the concentration of HIV used for infection in our experiments is high and may have resulted in an uncontrollable infection. Previously, use of a lower dose of 120 ng/mouse resulted in a slower infection characterized by peak viral replication observed at week 2 instead of week 1 as well as significantly lower levels of p24 in

plasma (data not shown). However, in that case similar to here, no effect of adoptive transfer of expanded cell on viral p24 levels or depletion of viral target cell was observed. It is possible that during acute HIV infection, the immune activation caused by such treatment counteracts the possible beneficial effect by providing susceptible cells for virus replication. These expanded $\gamma\delta$ T cells are capable of producing pro-inflammatory cytokines that can potentially activate HIV replication. In a rhesus macaque SHIV model of HIV infection, injection of HMBPP+IL-2 during acute stage of infection resulted in an increase in virus replication whereas similar treatment during chronic stage had no effect on plasma viral loads [22]. Administration of adoptive transfers of expanded cells with or without anti-HIV antibodies during set point viremia phase of infection thus may have different outcomes and will be tested in the future.

Figure 3. HIV p24 levels in plasma of infected mice measured by ELISA. p24 levels for individual mice in each group are shown at various times after HIV infection. **(A)** Viral loads remained stable in untreated mice, with one animal showing a spontaneous reduction over time. **(B)** In mice treated with Zol+IL-2 expanded cells, viral loads were higher than in untreated animals at day 7 (not significant) but were reduced thereafter. **(C)** Cells+b12 group had significantly increased ($p=0.01$) viral loads at day 14 post-infection compared with all other groups. **(D)** Mice treated with b12 had detectable p24 values with averages slightly lower than untreated animals (not significant) at all time points. One animal injected with b12 antibody with significantly higher p24 levels compared to other animals in the group died before day 10 for unknown causes.

Conclusion

Adoptive transfer of zoledronate expanded $\gamma\delta$ T cells do not suppress HIV replication in acute model of HIV infection.

Financial & competing interests disclosure

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Executive summary

- This study tested the role of zoledronate-expanded V δ 2+ T cells in controlling HIV replication.
- NOD SCID gamma mice were engrafted with human peripheral blood mononuclear cells and infected with HIV_{BAL}.
- Respective groups of animals were adoptively transferred with zoledronate-expanded V γ 9V δ 2 T cells either alone or in combination with an anti-HIV envelope antibody b12 and outcome was compared with untreated animals.
- HIV target cell depletion and HIV replication was comparable in all infected groups suggesting no protective effect of these cells during acute infection.

Ethical conduct of research

The author has obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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