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Impairment of *in vitro* generation of monocyte-derived human dendritic cells by inactivated HIV-1: involvement of type-I interferon produced from plasmacytoid dendritc cells

Akira KODAMA[a], Reiko TANAKA[a], Li Feng Zhang[a], Tetsuya Adachi[a], Mineki Saito[a]
Aftab A. Ansari[b] and Yuetsu TANAKA[a, c]

Running title: Impairment of DC generation by HIV-1

[a] Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Okinawa , Japan,
[b] Department of Pathology and Laboratory Medicine and Department of Microbiology and Immunology, Emory University School of Medicine, Georgia , U.S.A.

Keywords: AIDS, HIV-1, Vaccination, Dendritic cells, Apoptosis

[c] The corresponding author
Dr. Yuetsu TANAKA, Ph.D. Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Uehara 207, Nishihara-cho, Nakagami-gun, Okinawa 903-0215, Japan
Phone: +81-98-895-1202 Fax: +81-98-895-1437
E-mail address: yuetsu@s4.dion. ne.jp

Abstract

In an attempt to simplify the protocol of DC generation *in vitro*, studies conducted herein show that functional DCs could be generated from bulk peripheral blood mononuclear cells (PBMCs) in media containing GM-CSF and IL-4. Interestingly, when PBMCs, but not purified monocytes, were exposed to either CCR5- or CXCR4-tropic inactivated HIV-1 isolates (iHIV-1) at the initiation of the culture, DC yields were significantly reduced in a dose-dependent manner due to monocyte apoptosis. Similar impairment of DC generation was noted with the use of type-I IFNs and poly I:C not only in cultures of PBMCs but also using highly enriched monocytes. This effect was reversed by anti-human type-I IFN receptor, but not by anti-FasL, anti-TRAIL, anti-TNF or a mixture of these antibodies. iHIV-1-exposed PBMCs, but not monocytes, produced high levels of IFN-α but not IFN-β. PBMCs depleted of $CD123^+$ plasmacytoid DCs produced low levels of IFN-α and were resistant to iHIV-1-mediated DC impairment. Interestingly, exogenously added TNF reversed the impairment by iHIV-1 in the PBMC cultures. In conclusion, the present results indicate that iHIV-1 impairs the *in vitro* generation of functional DCs from PBMCs through the induction of IFN-α from plasmacytoid DCs in a CD4-dependent fashion in the absence of TNF.

1. Introduction

The high potency of dendritic cells (DCs) in the processing and presentation of antigens to T cells *in vivo* has prompted their use as potential therapeutic antigen specific delivery vehicles to promote tumor- and virus-specific T-cell responses in patients with cancer and a variety of infectious diseases including HIV-1 [1-3]. These strategies were based on the successful employment of such techniques not only in murine but also in simian models [4]. Several laboratories including ours have also successfully utilized DC based HIV specific immunization protocols utilizing the humanized mouse model (hu-PBL-SCID) [5, 6]. Of importance was the finding that such DC-based immunizations were found to be safe in a number of vaccine trials using HIV-1 CD8-epitope peptides in humans [7]. However, implementing such DC based immunization protocols requires the isolation of significant numbers of DC's which in the case of untreated HIV-1 infection provides a challenge. Thus, decreased numbers and function of circulating myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) has been recorded as one of the hallmarks of untreated HIV infection [8-11]. In addition, for reasons unclear at present, there is poor reconstitution of pDC during ART [12, 13], which has been reasoned to contribute to the poor immune responses in these patients in trials that involved immunization with inactivated autologous HIV-1. The remarkable potency of peptide based DC vaccination thus holds great promise if methodologies can be identified to procure the required number of DCs for such immunization [14-16].

DCs for such clinical studies are obtained either directly from blood or generated *in vitro* from circulating $CD34^+$ hematopoietic stem cells [17]. More recently, however, it has become common to generate DCs from peripheral blood monocytes by *ex vivo* culture. The specific methods used for cell purification, culture and maturation vary widely. Commonly, monocytes are isolated from blood by adherence [18], elutriation [19] or positive or negative selection using immunomagnetic beads [20]. These enriched population of monocytes are then induced to differentiate into DCs in vitro using medium supplemented with granulocyte-macrophage–colony-stimulating factor (GM-CSF) and interleukin (IL)-4 [21]. However, since the dose, quality of reagents, additional use of other cytokines, culture conditions, the cocktail of reagents utilized for maturation and the methods of antigen loading vary considerably, it is reasonable that the quality and biological activity of each DC preparation have been shown to differ [3, 18, 22-26].

In an attempt to simplify the methods currently being used for DC generation, we found that enrichment of monocytes as an initial step was not essential since functional DCs were successfully generated from unfractionated PBMCs. During the process of optimizing such a protocol for the eventual use for immunization of HIV-1 infected patients, we found that an exposure of PBMCs to inactivated HIV-1 (iHIV-1) at the initiation of culture resulted in a marked reduction in the yield of DCs due to cell death. Studies designed to elucidate the mechanisms for such cell death revealed that IFN-α produced from the iHIV-1-exposed $CD123^+$ plasmacytoid DCs in PBMCs impairs DC generation *in vitro*. Results of these studies constitute the basis of this report.

2. Subject and Methods

2.1. Reagents

Medium used throughout this study consisted of RPMI-1640 supplemented with 5% heat-inactivated fetal calf serum (FCS) (Sigma chemical co, St. Louis, MO), (heretofore referred to as RPMI medium). The recombinant human cytokines used included IL-4, GM-CSF, TNF-α, TNF-β, IL-1β (PeproTech, London, United Kingdom), IFN-α A (α 2a) (R&D System, Minneapolis, MN), IFN-β (Torey, Tokyo Japan), and IL-2 (provided by the U.S. National Institutes of Health Acquired Immune Deficiency Syndrome (AIDS) Research and Reference Reagent Program). Poly I:C was purchased from Alexis Biochemicals (San Diego, CA). Aldrithiol-2 (AT-2) and low-endotoxin bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). The monoclonal antibodies against human cell surface molecules used included anti-CD4 blocking antibody (SIM-2) (from the AIDS Research and Reference Reagent Program), anti-TRAIL (CD253), anti-Fas-L (CD178), anti-CD279 (PD-1) (BioLegend, San Diego, CA), anti-type-I IFN receptor 2 blocking antibody (IFNAR2) (LIFESPAN biosciences, Seattle, WA), anti-TNF-α, anti-TNF-β and anti-TNF receptor 1 (R&D systems, Minneapolis, MN). Fluorescent-dye labeled mouse mAbs used included anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD80, anti-HLA-DR, and isotype-matched control mAbs (Beckman Coulter, Fullerton CA), and anti-CD11c, anti-CD86 and anti-CD83 (BioLegend, San Diego, CA).
ELISA kits for the quantitation of human IFN-α were purchased from MABTECH (Mariemont, OH), human IFN-β from Invitrogen (Carlsbad, CA), human IFN-γ, human IL-10 and IL-4 from Biolegend (San Diego, CA). The human monocyte negative isolation kits, the human T cell isolation kits, and the human naïve $CD4^+$ T cell isolation kits were purchased from Invitrogen (Carlsbad, CA) and Miltenyi Biotec (Gladbach, Germany), respectively. The Vybrant™ CFSE cell tracer kit was purchased from Invitrogen.

2.2. Generation of DC

PBMCs were isolated from heparinized peripheral blood obtained from normal healthy adult volunteer donors by standard density gradient centrifugation. Cells at the interface were collected and washed three times in cold phosphate-buffered saline (PBS) containing 0.1% low-endotoxin BSA and 2 mM Na_2-EDTA. For select experiments, monocytes were purified from these PBMCs by using the $CD14^+$ monocyte negative isolation kit (Invitrogen, Carlsbad, CA). An aliquot of cells from each monocyte preparation was examined by flow cytometry and found to contain >90% $CD14^+$ cells. PBMC (2.5 x 10^6 cells/ml) or the purified monocytes (5×10^5 cells/ml) were cultured in RPMI medium containing human GM-CSF (500 ng/ml) and human IL-4 (200 ng/ml) at 37° C in 24-well plates in a 5% CO_2 humidified incubator for 5 days. In some experiments, these DCs were further matured by incubation with poly I:C (20 ug/ml) and IL-1β (10 ng/ml) for an additional 2 days. The viability of myeloid DCs in each PBMC culture on day 5 was calculated as follows: (the number of viable cells counted by using a Bilker-Chulk hemocytometer) x (percent $CD11c^+$ cells within the viable cell gate as determined by flow cytometry).

2.3. HIV-1 preparation and inactivation

$HIV\text{-}1_{JR\text{-}CSF}$ viral stock was produced by transfection of the 293T cells with the appropriate HIV-1 infectious plasmid DNA utilizing the calcium phosphate method followed by *in vitro* culture of the transfectd cells in RPMI medium for 2 or 3 days [5]. $HIV\text{-}1_{IIIB}$ was cultured and harvested from Molt-4/IIIB cell cultures. Batches of each HIV-1 preparation were inactivated with AT-2 as described previously [5]. These AT-2-inactivated HIV-1 (iHIV) were then purified by pelleting down the virus at 20,000 x g for 2 hr 3 times in 0.1% BSA-PBS. The virus pellet was then resuspended in 0.1% BSA-PBS, aliquoted and stored at -80°C until use. The concentration of HIV-1 was estimated by measuring levels of p24 antigen utilizing an ELISA kit (ZeptoMetrix, Buffalo, NY). An aliquot of 1 μg/ml of the AT-2-treated HIV-1 preparation when incubated with previously activated human PBMCs as previously described [5] failed to demonstrate the presence of any detectable infectious virions.

2.4. Flow cytometry

Aliquots of the cells to be analyzed were incubated in PBS containing 0.1% BSA and 0.1% NaN_3 (FACS buffer) supplemented with 2 mg/ml normal human IgG on ice for 15 min to block Fc receptors. The cell suspension was then incubated with a pre-determined optimal concentration of the appropriate fluorescent dye-labeled mAbs against human cell surface molecules on ice for 30 min. After washing with FACS buffer, cells were fixed in 1%

paraformaldehyde (PFA)-containing FACS buffer. The cells were analyzed by standard flow cytometry using a FACS-Calibur and the data obtained analyzed using the Cell Quest software (BD Pharmingen, San Diego, CA).

2.5. Stimulation of T cells

Enriched populations of naïve $CD4^+$ T cells and bulk T cells with > 90% purity were isolated from normal human PBMCs by using appropriate negative cell isolation kits. These cells were labeled with CFSE (carboxy-fluoresecein diacetate, succinimidyl ester) according to the manufacture's protocol (Invitrogen, Carlsbad, CA). These T cells (4×10^4 cells/well) were co-cultured with allogeneic DCs at a T:DC ratio of 50:1 in 100 ul of RPMI medium supplemented with 20 U/ml human IL-2 in 96-well U-bottom plates. Cell proliferation and cytokine production were determined on day 4.

2.6. Statistical analysis

Data were tested for significance using the Student's *t* test by using Prism software (GraphPad Software).

Results

3.1. Myeloid DCs are generated from bulk PBMC cultures in the presence of IL-4 and GM-CSF.

According to the most commonly used current protocols to generate myeloid DCs *in vitro*, the precursor monocytes are first commonly enriched from PBMCs with varying degrees of efficiency by a variety of methods prior to cultivation. In an attempt to reduce the cost, the labor and any loss of potential precursors from PBMCs, we first attempted to determine whether functional DCs could be generated by culturing bulk unfractionated PBMC's in the presence of IL-4 and GM-CSF for 5 days followed by a 2-day maturation by incubation in media containing poly I:C and IL-1β. As shown in Fig.1a, cells expressing high levels of CD11c, CD83, HLA-DR and CD86, highly reminiscent of mature myeloid DCs, represented 10% of the cultured cells. The other smaller cells (90% in the viable fraction) consisted of 90% $CD3^+$ T and 10% $CD20^+$ B cells. The average yield of DCs from 2.5×10^6 PBMC cultures was $2\sim2.5 \times 10^5$ cells depending on the donor.

The PBMC-derived myeloid DCs (PB-DCs) showed potent allo-T cell stimulating activity at levels comparable to those generated from purified monocytes (Mo-DCs) (Fig.1b). In separate experiments, similarly generated ovalbumin (OVA)-pulsed PB-DCs were also able to induce OVA-specific T cell and OVA-specific antibody responses using the hu-PBL-SCID mouse model (Kodama et al. manuscript in preparation). Thus, it is clear that functional immuno-stimulating DCs can be generated from bulk PBMCs without the prior requirement for using enriched population of monocytes *in vitro*.

3.2. AT-2-inactivated HIV-1 (iHIV) impairs generation of DCs from PBMCs.

Previously, our laboratory reported that conventionally prepared DCs sensitized with iHIV-1 induce HIV-1 specific Th1 immune responses in the hu-PBL-SCID mice [6]. In efforts to test whether the bulk PB-DCs could similarly induce such anti-HIV-1 T cell responses in the hu-PBL-SCID mice, attempts were made to first determine the time of culture and dose of iHIV-1 which was optimal for the derivation of sensitized PB-DCs. To our surprise, incubation of the PBMC cultures with such iHIV-1 at the initiation of the culture, resulted in impairment of DC development by day 5. However, such impairment of DC development was not observed in the iHIV-1 pulsed purified monocyte cultures set up in parallel (Fig.2a). In addition, if the iHIV-1 was added to the cultures on day 3, such impairment was not detectable on day 5 (data not shown).

As shown in Fig. 2b, flow cytometric analysis of the cells cultured for 5 days in the presence of iHIV-1 showed a marked dose-dependent impairment in the generation of DCs as determined by examining the high forward and side scatter profile of an aliquot of such cultured cells (typically used for the analysis of DCs). Such impairment by iHIV-1 exposure appeared to be induced as early as day 3 post culture as determined by flow cytometry (Fig.2c). It was reasoned that apoptosis of the cells could account for such impairment and thus aliquots of similar cultures were analyzed for the frequency of Annexin-V binding cells. As seen in Fig.2d, there were more Annexin-V-binding apoptotic cells in the iHIV-1 exposed PBMCs as early as 24 hrs post culture as compared to the control non-iHIV-1 exposed cultured cells ($P<0.01$). Taken together, these results show that iHIV-1 impaired the generation of DCs from unfractionated PBMCs in a dose-dependent fashion, which is secondary to apoptosis. The impaired generation of

DCs was also not secondary to residual levels of AT-2 since the use of mock virus preparation treated with AT-2 had no detectable effect on such cultures (data not shown).

3.3. Both CCR5- and CXCR4-tropic using HIV-1 isolates impair DC generation from PBMCs and modify CD86 expression.

The role of co-receptor tropism of HIV-1 isolates in the impairment of DC generation was examined next. As shown in Fig.3a, both the CCR5-tropic HIV-$1_{JR\text{-}CSF}$ and CXCR4-tropic HIV-1_{IIIB} isolates that had been inactivated with AT-2 similarly reduced the numbers of viable myeloid DCs generated from unfractionated PBMCs on day 5. Again, no impairment was observed in the similarly iHIV-1-exposed purified monocyte cultures by either of the iHIV-1 isolates. It was of interest to note that the DCs that remained in the iHIV-1-exposed PBMC cultures expressed about 6-8-fold higher levels of CD86 (note the MFI scale) on their cell surface than the DCs from the control non-HIV exposed ones even though they were not stimulated for maturation (Fig.3b). However, these high density $CD86^+$ HIV-1-exposed DCs led to reduced levels (approx. 50%, $p< 0.05$) of IFN-γ production when co-cultured with allogeneic T cells as compared with the DCs from the control cultures (Fig.4). In contrast, iHIV-1 treated and untreated Mo-DCs induced similar levels of IFN- γ when co-cultured with allogeneic T cells (Fig.4). The decreased levels of IFN-γ were not secondary to increase in the levels of IL-4 or IL-10 production. These data indicate that the *in vitro* impairment of DC generation from unfractionated PBMCs is independent of HIV-1 co-receptor usage and the residual DCs show markedly reduced capacity to induce IFN-γ although they expressed high levels of CD86.

3.4. IFN-α is involved in the impairment of DC generation

The previous published reports that type-I IFNs inhibit DC generation *in vitro* [27, 28] prompted us to determine whether the synthesis of type-I IFNs was the basis of iHIV-1-mediated impairment of DC generation. As shown in Fig.5a, the addition of either recombinant IFN-α or the type-I IFN-inducing agent poly I:C could induce impairment of DC generation similar to that induced by iHIV-1 in DC from not only unfractionated PBMCs but also from highly enriched preparation of monocytes. Again, the DCs that remain in these IFN-α or poly I:C-treated cultures expressed high levels of CD86 (Fig.5a). These IFN-α or Poly I:C exposed MO-DCs showed diminished function as measured by their ability to induce allogeneic Th1 responses (data not shown) similar to that noted for iHIV-1 exposed PB-DCs. Importantly, the impaired generation of DCs induced by iHIV-1, IFN-α and poly I:C were specifically reversed by the prior addition of anti-type-I IFN-R antibody to the cultures (Fig.5b). Altogether, these data suggest that type-I IFNs produced by unfractionated PBMC in response to iHIV-1 is the basis for the impaired generation of DC *in vitro*.

The levels of both IFN-α and β in the supernatant fluid of unfractionated PBMCs cultured in the presence of iHIV-1, poly I:C stimulated unfractionated PBMCs and monocytes were also quantitated by ELISA. As seen in Fig.6a, high levels of IFN-α but not IFN-β were measured in such supernatant fluids, suggesting that it is IFN-α not β that is the cytokine that is likely involved. Since pDCs are known to be a major IFN-α producing cell lineage, we next tested whether pDCs were involved in our culture system. Freshly obtained PBMCs were thus depleted of pDCs by incubation with anti-CD123 antibody-conjugated magnetic beads and the remaining PBMCs were cultured in the presence of iHIV-1 using our standard culture system. As shown in Fig.6b, prior depletion of CD123-bearing pDCs from PBMCs markedly decreased the ability of iHIV-1 to impair the generation of DC's *in vitro*. Taken together, these data suggest that IFN-α produced by pDCs in response to iHIV-1 is involved in the diminished generation of DCs from unfractionated PBMCs *in vitro*.

3.5. HIV-1 impairs DC generation from PBMCs in a CD4 dependent fashion.

Since an interaction between HIV-1 gp120 and CD4 has been shown to be requisite for IFN-α production by pDCs, we next examined the effect of blocking anti-CD4 antibody on DC generation from PBMCs. As shown in Fig.7, the prior addition of anti-CD4 antibody ameliorated the inhibitory effect of iHIV-1 on DC generation (Fig.7a) and CD86 up-regulation (Fig.7b), and inhibited the production of IFN-α (Fig.7c) in the iHIV-1-exposed PBMCs, confirming that IFN-α is produced by pDCs in a CD4 dependent fashion.

3.6. TNF blocks the DC impairment by AT-2 inactivated iHIV-1.

Finally, we examined whether the common apoptosis inducing cytokines TNF-α, TNF-β and the apoptosis-related cell surface antigens including FasL, TRAIL and TNF were involved in the impairment of DC generation from iHIV-1-exposed PBMCs. As shown in Fig.8a, the addition of blocking antibodies against FasL, TRAIL, TNF-α and TNF-β and TNF-R, failed to show any detectable effects on the impaired generation of DCs by iHIV-1. This failure was not secondary to the amount of antisera utilized. To our surprise, however, the addition of soluble recombinant TNF-α (or TNF-β) to the iHIV-1-exposed PBMC cultures at the time of initiation of the cultures reversed the iHIV-1-mediated inhibition of DC generation from PBMCs (Fig.8b). Consistent with these findings was the observation that the addition of TNF-α to these cultures led to a marked reduction in the level of IFN-α production from these PBMCs (Fig.8c). Therefore, these data suggest that iHIV-1 induced IFN-α directly impairs DC generation, and that TNF counteracts the iHIV-1 effect by reducing IFN-α production by pDCs in PBMCs.

4. Discussion

Data presented herein show that myeloid DCs can be generated *in vitro* by culturing unfractionated human PBMCs in media containing IL-4 and GM-CSF for 5 days followed by 2 days of further maturation in media containing poly I:C and IL-1β. These PB-DCs expressed high levels of CD86 and HLA-DR, and were capable of inducing not only alloreactive T cell responses *in vitro* but also antigen-specific T and B cell responses *in vivo*. This finding is important to reduce the cost, the labor and any loss of precursors from PBMCs in generating immuno-stimulating DCs *in vitro*. The superiority of using unfractionated PBMCs over enriched population of monocytes for the generation of immature DCs with high rates of recovery has also been reported by Goxe et al. [29]. They showed that unfractionated PBMCs cultured in a serum-free medium containing IL-13 and GM-CSF led to 38~54% higher recovery than the use of enriched population of monocytes. However, in the studies reported herein, we observed little or no difference in the yields of DCs between PBMCs and highly purified monocyte cultures. This discrepancy is likely due to either the higher efficiency of the kit we utilized for the isolation of monocytes or differences in the cytokines used for the derivation of the DCs which included IL-4 instead of IL-13 in our studies. The fact that one can utilize unfractionated PBMCs for the generation of highly functional DCs *in vitro* reduces not only the time involved in setting up cultures but also limits the number of manipulations and facilitates the generation of such cultures for clinical studies. Thus, the present data may provide an alternative simple and low-cost protocol for the *in vitro* generation of conventional human DCs with immuno-stimulating function. The role of the residual population of lymphoid cells in such cultures remains to be elucidated. The fact that there was no detectable expression of CD69 on such residual lymphoid cells (data not shown) supports the view that there is minimal if any effect of such quiescent cells on the generation of DC's. Further studies on this issue are in progress.

The finding that both CCR5- and CXCR4-tropic iHIV-1 interfered with DC generation from unfractionated PBMCs when added at the initiation of culture is important because of its potential *in vivo* relevance. It is known that 60-70% of monocytes are prone to die of apoptosis and necrosis by 7 days when left unstimulated *in vitro*, with the main loss occurring within the first 24 hrs [30, 31]. Such spontaneous monocyte apoptosis can be ameliorated to varying degrees by the addition of a variety of pro-inflammatory cytokines [32] or agents such as LPS [33]. It has also been reported that even in the presence of both IL-4 and GM-CSF about 40% of monocytes spontaneously undergo apoptosis during maturation into DCs *in vitro* [34]. The results of the studies reported herein show that approximately 50% of the monocytes (or DCs) from the PBMCs die following exposure for 5 days to iHIV-1. Since IFN-α alone could induce a similar loss of DCs in both unfractionated PBMCs and monocyte cultures, and that anti-type-I IFN receptor antibody reversed the effects of both iHIV-1 and IFN-α, it is clear that IFN-α is likely the major effector molecule responsible for the impairment of DC generation induced by iHIV-1. Similar impairing effects of type-I IFNs on monocyte-derived DC has been previously reported by McRae [28]. In contrast, Lehner et al. [27] reported that IFN-α alone is not capable of inducing impairment of DC generation unless an additional stimulus like LPS or LTA was included in such cultures. Since the media and cytokines that we utilized for the studies reported herein were free of endotoxin and bacterial contamination, the reason for this discrepancy remains undefined. Based on the results of our studies, the major mechanism for the decrease in the yield of viable DCs by iHIV-1 appear to be cell death, and was not likely due to static suppression of differentiation. Recently, it has been demonstrated that IFN-β induces apoptosis of immature murine DCs thorough caspase activation [35]. Therefore, it is possible that human IFN-α similarly induces apoptosis of immature DCs directly through type-I IFN receptors. Since soluble gp120 has been previously shown to induce $CD4^+$ T cell apoptosis, studies were conducted to determine whether soluble gp120 (commercially available) could inhibit conventional DC generation from PBMCs and monocytes in our culture system. Results of these studies showed that the addition of soluble gp120 to such cultures did not have

any detectable inhibitory effect (data not shown), indicating that whole HIV-1 virion is required for the induction of monocyte apoptosis.

Results from a large number of studies have documented the role of type-I IFNs in the modulation of DC biology at different levels. In addition to its well-known antiviral effect, type-I IFNs induce the maturation of DCs [5, 36-38]. However, it has been shown that when fresh monocytes are cultured in the presence of IFN-α or IFN-β at culture initiation, their maturation into DCs and IL-12 producing activity is severely diminished [28, 39]. It should be noted that in the culture system utilized in the studies reported herein some (about 50%) DCs survived in the iHIV-1 or IFN-α treated PBMCs. It is possible that these remaining DCs are resistant to the effects of IFN-α because they are a fraction of the terminally differentiated DCs that are known not to express receptors for IFN-α [40]. On the other hand, these cells could have developed resistance to undergo apoptosis. The fact that these remaining DCs as compared with control DCs were poor inducers of Th1 responses despite their expression of high levels of CD86 similar to previous observations [28, 39] suggests that either these cells are immune exhausted or that such culture conditions lead to the down regulation of some other co-stimulatory molecules required for optimum Th1 cell activation.

Based on the present data, it is clear that IFN-α secreted from $CD123^+$ pDCs as a result of interaction between HIV-1 gp120 and CD4 affects DC maturation, which is in support of previous findings [41, 42]. The reasons why iHIV-1 can induce large amounts of IFN-α has been thought to be due to the high efficiency by which CD4 expressing pDCs pick up HIV-1 particles through the interaction with envelope gp120 followed by pinocytosis of HIV-1 into the endosome compartments where viral RNA stimulates TLR-7, which results in the production of IFN-α [43-45]. The HIV-1-induced IFN-α has also been reported to induce bystander maturation of myeloid DCs [46]. Whereas the impairment of DC generation by iHIV-1 was found to be CD4 dependent, the addition of anti-CD4 to such cultures did not completely block IFN-α production (Fig.7c). While the reasons for the failure of anti-CD4 to completely block IFN-α production remains to be defined, we reason that some whole virion or degraded components of HIV-1 can be incorporated into pDCs in a CD4-independent manner and stimulate IFN-α production.

The present data also showed that FasL, TRAIL, TNF-α and TNF-β have limited if any relevance to the impairment of DC generation from PBMCs induced by iHIV-1, IFN-α or poly I:C. Although the major mechanism responsible for cell death still remains to be elucidated, it is of interest that TNF-α or TNF-β reversed the iHIV-1-, IFN-α-, or poly I:C-mediated DC impairment. TNF has been known to act not only in maturation [47] and killing of DCs [48], but also in the prevention of apoptosis of monocytes [32]. Recently, it has been reported that TNF-α blockade impairs DC survival and function in rheumatoid arthritis [49], suggesting that TNF may play a role in determining the longevity of DCs. Our present data suggest that the blocking activity of TNF against iHIV-1 or type-I IFN-mediated impairment of DC generation may be related to the down-modulation of IFN-α production from pDCs, since TNF did not act to down-modulate type-I IFN receptors expressed on monocytes (data not shown). Alternatively, there may be additional mechanisms by which TNF rescues DC generation in the presence of iHIV-1. Further studies are in progress aimed at analysis of the biological role of TNF in DC generation under inflammatory conditions.

In conclusion, data from the present study suggests that unfractionated PBMCs are a good source for the generation of conventional DCs *in vitro* in media supplemented with IL-4 and GM-CSF, and that HIV-1 impairs DC differentiation from PBMCs due to apoptosis via pDC-produced IFN-**α**. Therefore, strategies need to be devised to inhibit and/or limit the production of type-I IFNs in such cultures in order to maintain a good yield of functional DCs, especially from PBMC samples from individuals infected with HIV-1.

Acknowledgement

This work was supported by grants from a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; Research on HIV/AIDS and Health Sciences focusing on Drug Innovation from the Ministry of Health, Labor and Welfare of Japan; and Japan Human Science Foundation.

Legends for Figures

Fig 1.
Unfractionated PBMCs can serve as a source of functional myeloid DCs *in vitro*.
Purified monocytes or PBMCs were cultured at 5 x 10^5 and 2.5 x 10^6 cells/ml, respectively, in media containing GM-CSF and IL-4 for 5 days followed by maturation by incubation with poly I:C and IL-1β for an additional 2 days. (a) Representative phenotypic profile of the matured cells as determined by flow cytometry is shown. Mo-DC from purified monocytes, and PB-DC from bulk PBMCs. (b)The T cell stimulating activity of the matured DCs was analyzed by co-culture with CFSE-labeled purified allogeneic $CD4^+$ T cells at a DC to T cell ratio of 1:50 in RPMI medium containing 20 U/ml IL-2 in U-bottom 96-well plates for 4 days. Proliferation of the CFSE-labeled $CD4^+$ T cells was assayed by flow cytometry. Data shown are representative of three independent experiments utilizing blood from 3 different donors.

Fig 2.
Exposure of PBMCs to iHIV-1 impairs DC generation.
Highly enriched populations of monocytes and bulk PBMCs were cultured as described in Fig.1 in the presence or absence of iHIV-1.
(a) Cultures of DCs generated from monocytes or PBMCs either in the presence or absence of iHIV-$1_{JR\text{-}CSF}$ (50 ng/ml p24 level) were visualized on day 5 using an inverted microscope (200x).
(b) The impairment of DC generation from PBMCs in the presence of various concentrations of iHIV-$1_{JR\text{-}CSF}$ added at the initiation of cultures. On day 5, cells were analyzed by flow cytometry. Note that normal DCs have a high FSC and high SSC profile. The profiles of CD11c and CD86 expression by un-gated PBMCs and statistical analysis of %$CD11c^+$ cells was shown.
(c) Time course of HIV-1-mediated impairment of DC generation from PBMCs. PBMCs cultured in the presence or absence of iHIV-$1_{JR\text{-}CSF}$ (at 50 ng/ml p24 level) as in Fig.1, and the FSC and high SSC profile and %CD11c+ cells in un-gated PBMCs were examined on days 3, 4 and 5.
(d) PBMCs cultured in the presence or absence of iHIV-$1_{JR\text{-}CSF}$ (at 50 ng/ml p24 level) were examined for $CD14^+$ monocyte apoptosis on day 1 by CD14 and Annexin-V staining. Gates were set based on background profile of cells incubated with Annexin-V on day 0.
Data shown are representatives of three independent experiments utilizing blood from 3 different donors.

Fig 3.
Both CCR5- and CXCR4-tropic iHIV-1 isolates impair DC generation from PBMCs and modify CD86 expression.
Aliquots of highly enriched preparation of monocytes and unfractionated PBMCs were cultured as in Fig.1 in the presence or absence of AT-2 inactivated CCR5-tropic HIV-$1_{JR\text{-}CSF}$ or CXCR4-tropic HIV-1_{IIIB} each at 50 ng/ml p24 level for 5 days.
(a) The total number of viable $CD11c^+$DCs were determined as described in the "Material and Methods" section and data expressed as percent viable cells.
(b) The levels of CD86 expression on DCs was determined by flow cytometry and are shown as the mean fluorescence intensity (MFI).
Representative data from 3 different donors are shown.

Fig 4.
DCs generated from iHIV-1-exposed PBMC cultures are less potent in IFN-γ production than those from control cultures.
Unfractionated PBMCs (PB-DC) or monocyte derived DC's (Mo-DC's) were cultured in the presence or absence of iHIV-$1_{JR\text{-}CSF}$ (50 ng/ml of p24) for 5 days as described under Fig.1. The resulting DCs were then co-cultured with allogeneic bulk T cells at a DC:T ratio of 1:50 in RPMI medium containing 20 U/ml IL-2 in U-bottom 96 well-plates for 4 days. The supernatants were assayed for IFN-γ, IL-10 and IL-4 production by ELISA. All results are expressed as the mean ± standard deviation from three independent experiments. Representative data from 3 different donors are shown.

Fig 5.
Type-I IFNs impair DC generation in both unfractionated PBMCs and monocytes.
Highly enriched preparation of monocytes and unfractionated PBMCs were pretreated with either (a) medium alone

or (b) 10 ug/ml anti IFN-receptor 2 (IFNR2) antibody for 30 min at 37°C, and then, without washing, cultured as in the Fig.1 in the presence or absence of type-1 IFNs (5 ng/ml IFN-α) or IFN-β, 5 ng/ml each), 20 ug/ml poly I:C or iHIV-1_{JR-CSF} (at 50 ng/ml p24) for 5 days, and the total number of viable $CD11c^+$ DC and the relative level (MFI) of CD86 expression determined. As IFN-α, 5 ng/ml IFN-β showed similar results (data not shown).
Representative data from 3 different donors are shown.

Fig 6.
HIV-1 induces IFN-α but not IFN-β from $CD123^+$ plasmacytoid DCs in unfractionated PBMCs.
(a) Unfractionated PBMCs and highly enriched preparation of monocytes were cultured as in the Fig.1 either in the presence or absence of iHIV-1_{JR-CSF} (50 ng/ml of p24) or poly I:C for 3 days, and the level of IFN-α production in the culture supernatants was determined by ELISA.
(b) Unfractionated PBMCs or PBMCs depleted of $CD123^+$ cells were cultured either in the presence or absence of iHIV-1_{JR-CSF} (50 ng/ml of p24) for 5 days, and the total number of viable $CD11c^+$DC determined.
All results are expressed as the mean ± standard deviation from three independent experiments. Representative data from 3 different donors are shown.

Fig 7.
iHIV-1 impairs DC generation from PBMCs in a CD4 dependent fashion.
Aliquots of unfractionated PBMCs were pretreated with either 20 ug/ml anti-CD4 mAb or control IgG for 30 min at 37°C, and subsequently, without washing, cultured as in the Fig.1 either in the absence or presence of inactivated HIV-1_{JR-CSF} (at 50 ng/ml p24) for 5 days, and aliquots evaluated for the total number of viable $CD11c^+$DCs (a), elative levels of CD86 expression (b). The levels of IFN-α production in the culture supernatants collected on day 3 were determined by ELISA.
All results are expressed as the mean ± standard deviation from three independent experiments. Representative data from 3 different donors are shown.

Fig 8. iHIV-1-mediated impairment of DC generation is reversed by TNF but not by antibodies against TRAIL, Fas ligand, TNF-α, TNF-β or TNF receptor.
(a) Aliquots of unfractionated PBMCs were either untreated or pretreated with 5 ug/ml of anti-TRAIL mAb, anti-FasL, anti-TNF-α, anti-TNF-β or anti-TNF receptor-I at 37°C for 30 min, and subsequently, without washing, cultured as in the Fig.1 either in the absence or presence of iHIV-1_{JR-CSF} (at 50 ng/ml p24) for 5 days. The total numbers of viable $CD11c^+$ DC were then determined.
(b) Unfractionated PBMCs were cultured as in Fig.1 either in the presence or absence of 5 ng/ml IFN-α, 20 ug/ml poly I:C or iHIV-1_{JR-CSF} (50 ng/ml of p24) for 5 days either with 20 ng/ml TNF (α or β), and then the total number of viable $CD11c^+$ DC determined.
(c) IFN-α production in the culture supernatants (except from the IFN-α added cultures) from Fig.8(b) was determined on day 3 by ELISA
All results are expressed as the mean ± standard deviation from three independent experiments.

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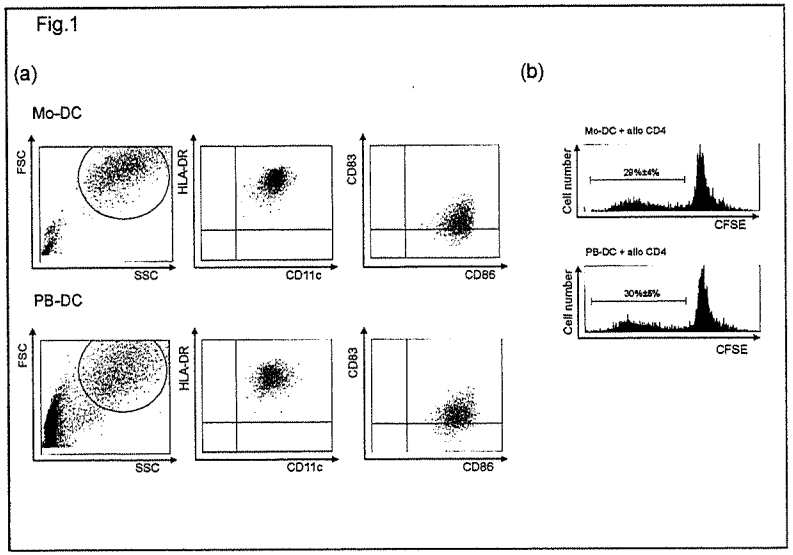


Fig.1
(a)
Mo-DC
FSC
SSC
HLA-DR
CD11c
CD83
CD86
PB-DC
(b)
Mo-DC + allo CD4
Cell number
CFSE
PB-DC + allo CD4

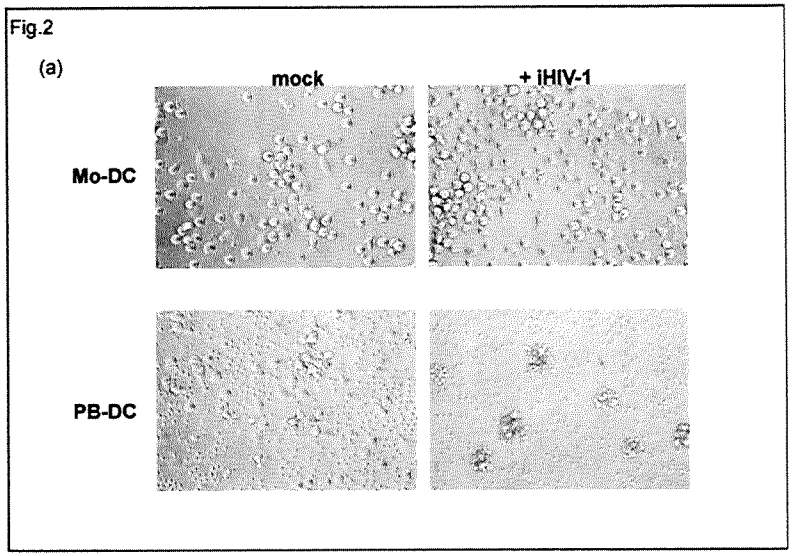


Fig.2
(a)
mock
+ iHIV-1
Mo-DC
PB-DC

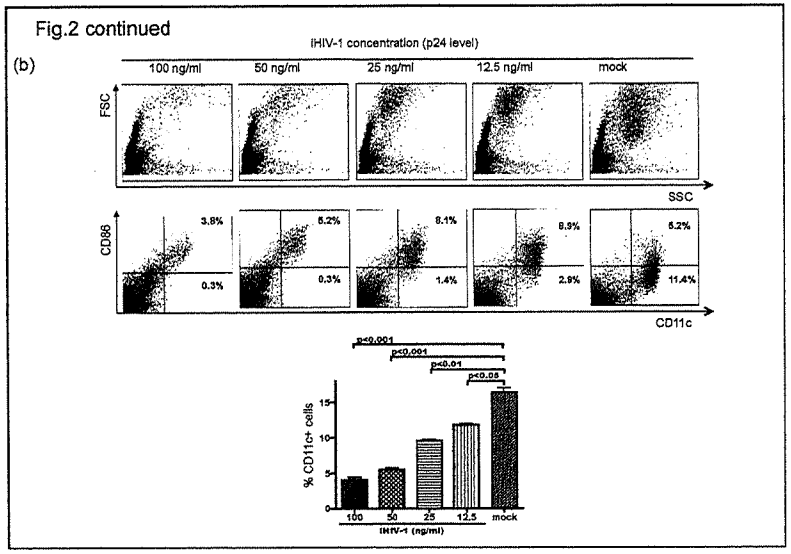


Fig.2 continued
(b)
iHIV-1 concentration (p24 level)
100 ng/ml
50 ng/ml
25 ng/ml
12.5 ng/ml
mock
FSC
SSC
CD86
CD11c
% CD11c+ cells
iHIV-1 (ng/ml)

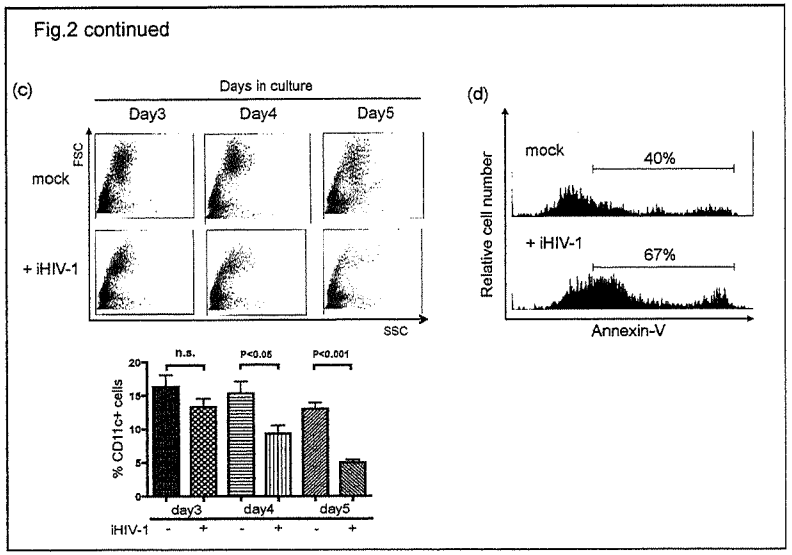


Fig.2 continued
(c)
Days in culture
Day3
Day4
Day5
mock
+ iHIV-1
FSC
SSC
% CD11c+ cells
day3
day4
day5
iHIV-1
(d)
Relative cell number
40%
67%
Annexin-V

Fig.3

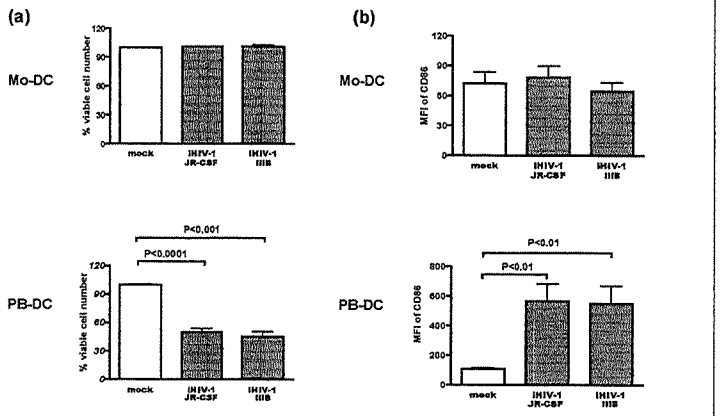


Fig.4

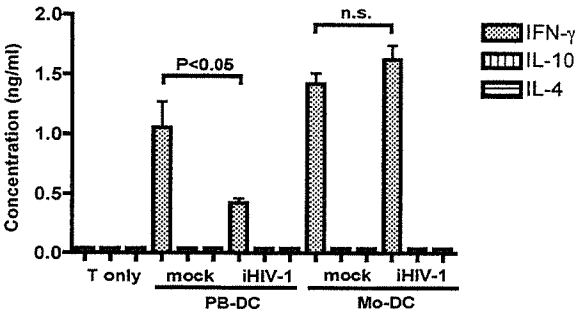


Fig.5

(a)

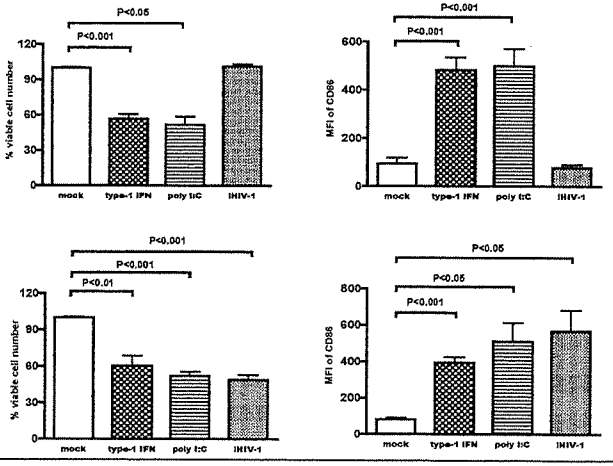


Fig.5 continued

(b)

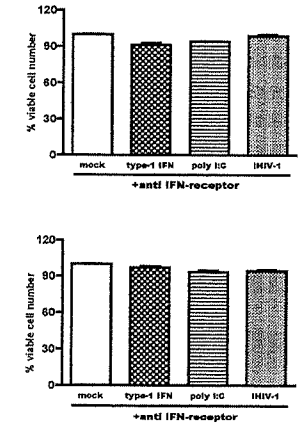


Fig.6

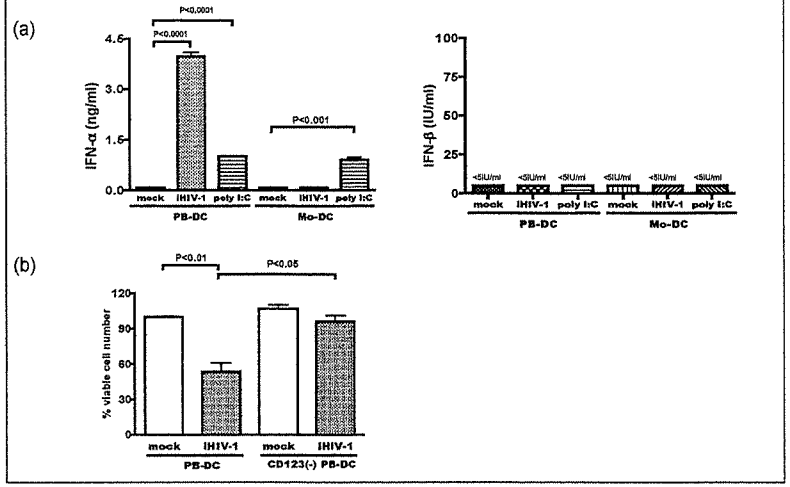


Fig.7

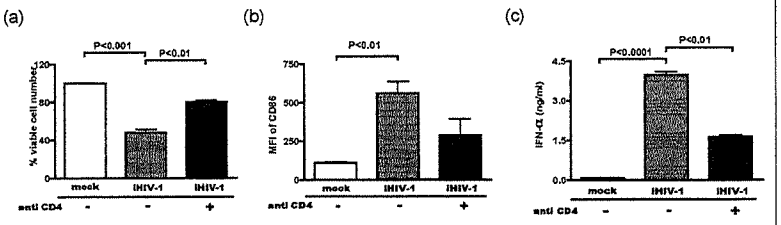
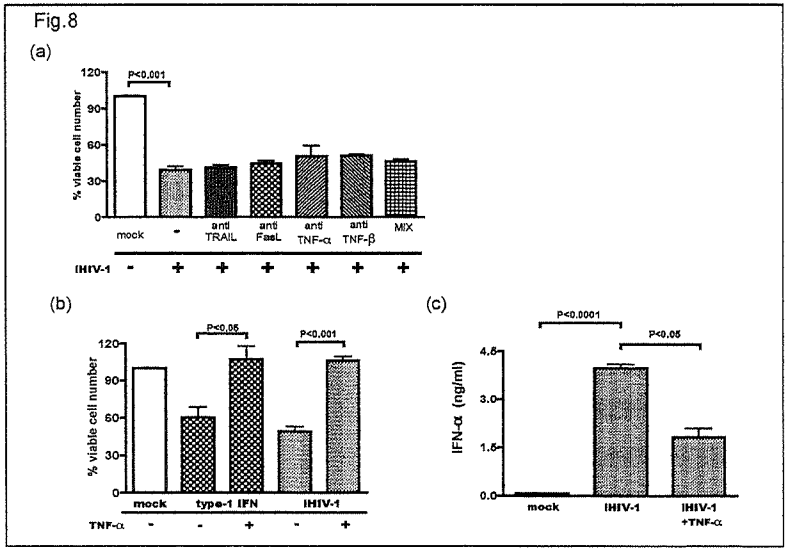


Fig.8



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Vaccine xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Dynamics of memory and naïve CD8$^+$ T lymphocytes in humanized NOD/SCID/IL-2Rγ^{null} mice infected with CCR5-tropic HIV-1

Kei Sato[a,1], Chuanyi Nie[a,1], Naoko Misawa[a,1], Yuetsu Tanaka[b], Mamoru Ito[c], Yoshio Koyanagi[a,*]

[a] Laboratory of Viral Pathogenesis, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan
[b] Department of Immunology, Graduate School of Medicine, University of the Ryukyus, Nishihara, Okinawa 903-0125, Japan
[c] Central Institute for Experimental Animals, Kawasaki, Kanagawa 216-0001, Japan

ARTICLE INFO

Article history:
Received 15 May 2009
Received in revised form 3 August 2009
Accepted 29 October 2009
Available online xxx

Keywords:
HIV-1
Humanized mice
Immunopathogenesis

ABSTRACT

Creating a novel small animal model of HIV-1 infection that can support long-term systemic HIV-1 infection and produce HIV-1-specific immune response has a great benefit for studying HIV-1 pathogenesis *in vivo*. In the present study, we have generated a humanized mouse, NOG-hCD34 mouse, by transplanting newborn NOD/SCID/IL-2Rγ^{null} mice with human hematopoietic stem cells through hepatic injection. These mice were infected with a CCR5-tropic HIV-1 and were analyzed for plasma viral load, changes in peripheral blood T lymphocytes, and HIV-1-specific antibody production. High level of viral replication, increase in effector/memory CD8$^+$ T lymphocytes, class-switching to IgG, and production of HIV-1-specific IgGs were observed. Our findings suggest that NOG-hCD34 mice may have a wide variety of application in HIV-1 research.

1. Introduction

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that causes immunodeficiency exclusively in human [1]. The inability of HIV-1 to cause immunodeficiency in other animals has made authentic investigations in live animals extremely difficult, thus preventing the advancement of our understanding on the pathogenesis of HIV-1 *in vivo*.

Tremendous efforts have been made to generate a cost-efficient and readily accessible small animal model that can be used to investigate the pathogenesis of HIV-1, to test the effectiveness of anti-HIV-1 drug candidates, and to test HIV-1 vaccines *in vivo* [2–4]. Initial successful attempts included C.B17-severe combined immunodeficient (SCID) mice transplanted under the kidney capsule with fragments of human fetal thymus, lymph node, and human hematopoietic cells-containing human fetal liver (Thy/Liv SCID-hu mice) [5,6] or in the peritoneal space with human peripheral blood lymphocytes (hu-PBL-SCID mice) [7]. Thy/Liv SCID-hu mice are able to support *de novo* generation of human T lymphocytes and thus are useful for the studies of HIV-1 intrathymic infection and hematopoiesis suppression [8]. However, HIV-1 infection in Thy/Liv SCID-hu mice is restricted to the transplanted organ, and Thy/Liv SCID-hu mice are not able to support multilineage differentiation of human hematopoietic cells [9]. On the other hand, hu-PBL-SCID mice are able to reproduce high level of systemic HIV-1 infection, but the lack of on-going *de novo* human hematopoiesis and the short life span limited their application [10].

In order to improve a small animal model for the testing of anti-HIV-1 therapy and HIV-1 vaccines, HIV-1 infection must be sustained and immune response against HIV-1 must be generated within the animal. We have previously created a novel non-obese diabetic/severe combined immunodeficient (NOD/SCID) interleukin-2 receptor gamma chain (IL-2Rγ) knockout (NOD/SCID/IL-2Rγ^{null}; NOG) mouse strain, which lacks intrinsic T and B lymphocytes and functional natural killer cells [11]. NOG mice have been shown to be feasible for transplantation of human CD34$^+$ hematopoietic stem cells (hHSCs) and for supporting the differentiation of human T lymphocytes [11–13]. In adult NOG mice transplanted with hHSCs, it has been found that the CD34$^+$ cells successfully differentiated into human T and B lymphocytes, monocytes/macrophages, natural killer (NK) cells, as well as plasmacytoid and myeloid dendritic cells (DCs), and that these human cells were maintained for more than 150 days [12,14]. It was also reported that adult NOG mice transplanted with hHSCs supported HIV-1 infection for more than 3 months and produced HIV-1-specific antibodies [12]. These findings suggested that hHSCs-transplanted NOG mouse would be a useful animal model to study HIV-1 infection. However, whether hHSCs-transplanted NOG mice can mount a CD8$^+$ T lymphocyte-mediated immune response against HIV-1 has not been investigated yet.

In this paper, we generated humanized mice by transplanting newborn NOG mice with hHSCs via hepatic injection (NOG-hCD34 mice), because it has been suggested that transplantation of cord

* Corresponding author. Tel.: +81 75 751 4811.
E-mail address: ykoyanag@virus.kyoto-u.ac.jp (Y. Koyanagi).
[1] These authors contributed equally to this work.

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doi:10.1016/j.vaccine.2009.10.154

Please cite this article in press as: Sato K, et al. Dynamics of memory and naïve CD8$^+$ T lymphocytes in humanized NOD/SCID/IL-2Rγ^{null} mice infected with CCR5-tropic HIV-1. Vaccine (2009), doi:10.1016/j.vaccine.2009.10.154

blood (CB)-derived hHSCs into newborn mice results in better immune reconstitution [15,16]. Our humanized mice supported high level of HIV-1 replication. We observed that *de novo* generated $CD8^+$ T lymphocytes gained effector/memory phenotype in response to HIV-1 infection and massively proliferated. Based on our findings, we discuss the possibility of the application of NOG-CD34 mice, particularly those transplanted during the neonatal period, for HIV-1 research.

2. Material and methods

2.1. Mice

NOD/SCID/IL-2Rγ^{null} mice (NOG mice [11]) were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). The mice were maintained under specific pathogen-free conditions and were handled in accordance with the Regulation on Animal Experimentation at Kyoto University.

2.2. Purification and transplantation of CB-derived hHSCs

The purification and transplantation of CB-derived hHSCs was conducted as described previously with some modification [14–16]. Fresh human CB was obtained with parent written informed consent from healthy full-term newborns and CD34 MicroBead Kit (Miltenyi Biotec, Auburn, CA) was used according to the manufacturer's instructions. $CD34^+$ cells ($5–12 \times 10^4$) were intrahepatically injected into newborn mice of ages between 0 and 2 days after total radiation of 10 cGy per mouse in MBR-1520 X-ray irradiator (Hitachi Medico, Tokyo, Japan).

2.3. HIV-1 infection

~~NOG~~ mice were injected intraperitoneally with RPMI1640 or 1×10^5 50% tissue culture infective doses ($TCID_{50}$) of HIV-$1_{JR\text{-}CSF}$ [17] between 12 and 13 weeks post-transplantation. HIV-$1_{JR\text{-}CSF}$ solution was prepared and titrated previously described [18,19].

2.4. Quantification of HIV-1 RNA in plasma

The quantification of HIV-1 RNA in the plasma of the infected mice was routinely carried out using Amplicor HIV-1 monitor v1.5 according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

2.5. Peripheral blood collection and isolation of nucleated cells from organs

Peripheral blood (PB) was routinely collected from NOG-hCD34 mice as described previously [16]. Red blood cells in the PB were lysed with 1× BD Lysis Buffer (BD Pharmingen, San Diego, CA). Mononuclear cells from bone marrow (BM), thymus, spleen, and lymph nodes were collected as described previously [16], and single cell suspensions were used for flow cytometric analysis or stored at −80 °C until use.

2.6. Flow cytometry

Flow cytometric analysis was performed with some modifications to the protocol previously described [15,19]. Following mouse monoclonal antibodies (mMAbs) and reagents were used: FITC-conjugated anti-CD19 (Dako, Tokyo, Japan), anti-CD8 (Dako), anti-CD4 (eBioscience, San Diego, CA), and anti-CD14 (Miltenyi Biotec) mMAbs, PE-conjugated anti-CD3 (Dako), anti-CD4 (Dako), anti-CCR5 (BD Pharmingen), and anti-CD34 (Miltenyi Biotec) mMAbs, biotinylated anti-CD45 (eBioscience) and anti-CD45RA (BD Pharmingen) mMAbs, PerCP-conjugated streptavidin (BD Immunocytometry systems, San Jose, CA), and FcR blocker (Myltenyi Biotec). Each antibody was controlled with appropriate isotype IgGs (Dako). Following first incubation, the cells were washed and further incubated with PerCP-conjugated streptavidin if needed. For p24 staining, the cells were permeabilized and fixed by treatment with BD Cytoperm/Cytofix solution (BD Pharmingen) and were stained with FITC-conjugated rat anti-HIV-1 p24 MAb (clone 2C2) [20] for 30 min at 4 °C in 1× BD PermWash buffer (BD Pharmingen). Data collection was performed on BD FACSCan (BD Biosciences, San Jose, CA), and the obtained data was analyzed with CellQuest software (BD Biosciences).

2.7. Detection of reactive human IgG against HIV-1 antigens

Plasma was collected from 1 mock-infected and 7 ~~HIV-1JR-CSF-infected~~ NOG-hCD34 mice upon the time of sacrifice. The presence of human IgGs against HIV-1 antigens in the collected plasma was examined by using a New Lav Blot 1 kit (Bio-Rad, Hercules, CA) according to the manufacturer's protocol.

2.8. Statistic analysis

Significant differences were determined by Student's t test or paired t test. P value less than 0.05 was considered significantly different.

3. Results

3.1. Reconstruction of human leukocytes in NOG-hCD34 mice

We first investigated the ability of newborn NOG mice transplanted with hHSCs to support human hematopoiesis. These mice are referred to as NOG-hCD34 mice. The flow cytometric analyses revealed that substantial percentages of human $CD45^+$ leukocytes including $CD3^+$ T lymphocytes and $CD19^+$ B lymphocytes already presented in the PB of 13-week-old mice and were stably maintained for at least 31 weeks (Table 1). $CD3^+$ T lymphocytes in the PB of these mice were singly positive for either CD4 or CD8 (Table 1). In the thymi of NOG-hCD34 mice, $CD45^+$ cells were detected in great abundance, which were predominantly CD4CD8 double positive cells (Table 1). In the BM sampled from femurs of NOG-hCD34 mice, we found large number of human $CD45^+$ leukocytes including $CD34^+$ hematopoietic cells, $CD3^+$ T lymphocytes, $CD19^+$ B lymphocytes, and $CD14^+$ monocytes (Table 1). In the secondary lymphoid organs of the mice, large populations of human leukocytes including $CD3^+$ and $CD19^+$ lymphocytes were detected (Table 1). In addition, we also found significant number of $CCR5^+CD4^+$ T lymphocytes, which are the target cells for CCR5-tropic HIV-1, in the spleen of NOG-hCD34 mice (7.5 ± 2.6% in splenic mononuclear cells).

3.2. Significant and persistent viremia in PB of NOG-hCD34 mice

NOG-hCD34 mice between 12 and 13-week old were infected with HIV-$1_{JR\text{-}CSF}$ (1×10^5 $TCID_{50}$/mouse). HIV-$1_{JR\text{-}CSF}$ uses the chemokine receptor CCR5 as the co-receptor for its infection [21]. In the plasma of HIV-$1_{JR\text{-}CSF}$-infected NOG-hCD34 mice, high levels of HIV-1 were detected starting 2–3 weeks post-infection (wpi) ($1.28 \pm 0.65 \times 10^5$ copies/ml) and were maintained throughout the period of investigation (Fig. 1A). Of note, the plasma RNA levels were comparable to that found in patients with acute HIV-1 infection [22]. We also confirmed that HIV-1 RNA was undetectable in the plasma of mock-infected mice (<1600 copies/ml). In addition to the detection of viral RNA in plasma (Fig. 1A), we detected the cells positive for an HIV-1 antigen, p24, in the spleen of the infected mice

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