1.3. Fourth and fifth terms to present (1995–2009)

Monkey infected with BV and SVV were completely eliminated from TPRC in the late 90s. Three viruses, simian cytomegalovirus (CMV), simian Epstein-Barr virus (EBV, simian lymphocryptoviruses (LCV)) and simian foamy virus (SFV), were added as target viruses in a new plan in 1995 to establish SPF monkey colonies. Simian CMV infections have been reported in various species of monkeys, including macaques [15]. This virus is readily transmitted in oral secretions, breast milk and urine [16], and 3% of adult monkeys in TPRC were infected with the virus. CMV infection was detected by IFA or an ELISA system using CMV Ag. Simian EBV has also been detected in several species of Old World and New World primates [17]. This virus is also readily transmitted, and serological surveys indicated that about 90% of adult cynomolgus monkeys in TPRC were infected. Detection of EBV infection was usually done by using commercial available human IFA kit. Infection with these two viruses, CMV and EBV, in macaques are opportunistic infections. Infection with the other virus, SFV, also does not seem to cause disease in nonhuman primates as natural hosts [18]. Humans can be infected with SFV, although the number of known SFV infection cases in humans is small [19]. SFV infection was detected by IFA using SFV Ag. Monkeys infected with SFV are fraught with hazards to workers in a primate center. The rate of infection with SFV in adult monkeys in TPRC was 80%. Detection of SFV was done by Ab response in sera using ELISA. Prevention of the spread of these three viruses, CMV, LCV and SFV, was performed by artificial nursing with feeding formula for baby monkeys that had been removed from their mothers immediately after birth. CMV infection in monkeys has not been detected in TPRC since 2005.

2. Conclusions

SPF nonhuman primate colonies are required for biomedical research with several beneficial effects such as animal health and occupational safety. High quality of laboratory animals is also required for advanced biomedical studies including vaccine research and development. Infectious agents frequently affect the results of animal experiments. The history of establishment of SPF cynomolgus monkeys in TPRC in Japan for evaluation of state-of-the-art medical technology, evaluation of the efficacy of new drugs and new vaccines, and safety assessments has been described in this review.

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The author would like to thank all workers in TPRC since 1978 for establishing SPF macaque colonies in Japan.

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Conflict of interest statement

The author states that they have no conflict of interest.

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Induction of CD56$^+$ T cells after prolonged activation of T cells *in vitro:* A possible mechanism for CD4$^+$ T-cell depletion in acquired immune deficiency syndrome patients

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ABSTRACT

The pathogenic mechanisms responsible for depletion of CD4$^+$ T cells in aquired immune deficiency syndrome (AIDS) are not fully understood. Systemic immune activation mediated by persistent infection of human immunodeficiency virus (HIV) seems to be one of the predictors of disease progression. We predicted that certain lymphocytes responsible for CD4$^+$ T-cell depletion could be induced in patients during prolonged activation of lymphocytes. Therefore, we have established an *in vitro* long-term culture system for peripheral blood mononuclear cells with PHA-P stimulation and Herpesvirus saimiri infection, and examined what types of cells having strong cytotoxic activity to be emerged under the activated conditions. We observed that percentage of CD56$^+$ T cells was gradually increased in cultures from 30 days after stimulation and exhibited a cytotoxic activity against both autologous and allogeneic targets. Interestingly, HIV-1 infection enhanced the susceptibility of CD4$^+$ T cells to their cytotoxic effectors, and CD4$^+$ T cells from HIV-1–infected individuals showed decreased survival rate in the presence of autologous CD56$^+$ T cells. These findings raised the possibility that induction of autoreactive CD56$^+$ T cells in consequence of immune activation might be contributed to the depletion of CD4$^+$ T cells in HIV-1–infected patients.

1. Introduction

The progressive depletion of CD4$^+$ T cells in human immunodeficiency virus (HIV) –infected patients is still a major fundamental and controversial question in acquired immunde deficiency syndrome (AIDS) research. HIV selectively infects and kills cells expressing the CD4 molecule on their surface, particularly T lymphocytes and cells of the monocyte/macrophage lineage. Direct killing of infected cells appears to contribute to the loss of CD4$^+$ T cells in primary HIV-1 infection [1,2]. However, the percentage of HIV-1–infected CD4$^+$ T cells is too small to account for the extensive depletion of T-helper cells in patients who are progressing to AIDS through chronic infection with HIV-1 [3,4]. Thus, several other mechanisms have been proposed to explain the deletion of CD4$^+$ T cells [5,6].

HIV infection leads to sustained immune activation and causes profound alterations in T-cell homeostasis [7]. HIV-infected patients display elevated expression of activation markers, such as CD38, CD25, and CD69, on CD4$^+$ and CD8$^+$ T cells. High levels of proinflammatory cytokines and chemokines, such as tumor necrosis factor–α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and macrophage inflammatory protein (MIP-1) are also observed in both plasma and lymph nodes in these patients [8,9]. Although the actual mechanisms underlying such an immune activation remain unclear, higher T-cell activation levels are associated with CD4$^+$ T-cell depression in untreated patients independent of plasma HIV RNA [10,11].

Previously, it has been reported that HIV-1–infected individuals had circulating cytotoxic T cells that were cytotoxic for noninfected CD4$^+$ T cells, and it was suggested that autoimmune phenomena contributed to the depletion of CD4$^+$ T cells [12,13]. It is interesting which types of cells are induced in consequence of immune activation and exhibit cytotoxicity against autologous CD4$^+$ T cells. In most *in vitro* studies, effector cells were prepared from peripheral blood mononuclear cells (PBMC) stimulated with mitogen followed by short-time culture in the presence of cytokines. We wanted to find effector cells in activated PBMC without adding any specific cytokines as well as antigens after long-term culture, because the presence of cytokines and antigens result in cytokine dependent expansions or antigen specific T-cell responses, and chronic immune activation persist for long time after HIV-1 infection *in vivo*. Therefore, we have established an *in vitro* long-term culture system for PBMC in the absence of cytokines and examined what types of cells are induced under the activated culture condition.

We found that CD56$^+$ T cells were gradually increased from long-cultured PBMC at approximately 30 days after stimulation, and we observed their cytotoxic activities against various tumor

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cells in a non–major histocompatibility complex (MHC)–restricted manner as well as autologous cells. It should be noted that HIV-1 infection enhanced the susceptibility of $CD4^+$ T cells to be killed by $CD56^+$ T cells. Moreover, $CD56^+$ T cells from HIV-1–infected patients exhibited a potent cytotoxicity against autologous $CD4^+$ T cells by *in vitro* activation. Therefore, if such autoreactive $CD56^+$ T cells will be expanded in HIV-1–infected patients, both non–HIV-1–infected and infected $CD4^+$ T cells might be strongly killed and depleted. Thus, we presented here a possible involvement of $CD56^+$ T cells in the depletion of $CD4^+$ T cells in HIV-1 infection.

2. Subjects and methods

2.1. Preparation of cells

Blood samples were obtained from healthy donors and highly active antiretroviral therapy (HAART)–treated or untreated asymptomatic HIV-1–infected individuals with peripheral $CD4^+$ T lymphocytes counts of $>300/\mu l$. All subjects gave informed consent under a protocol approved by the Institutional Review Board of the Nippon Medical School. PBMC were purified over a Ficoll density gradient and stimulated with 10 μg/ml of phytohemagglutinin-P (PHA-P: Sigma-Aldrich, St. Louis, MO) for 24 hours and subsequently inoculated with the Herpesvirus saimiri (HVS) strain C-488; kindly provided by M. Yasukawa at moi 10. Stimulated PBMC were cultured with T-cell culture medium (CTM) [14], composed of RPMI 1640 medium supplemented with 2 mmol/l of L-glutamine, 1 mmol/l of sodium pyruvate, 0.1 mmol/l of nonessential amino acids, a mixture of vitamins, 1 mmol/l of HEPES, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 50 μmol/l of 2-mercaptoethanol, and heat-inactivated 10% fetal calf serum (FCS). Approximately 50 days later, stimulated PBMC, termed long-cultured PBMC (LC-PBMC), were used as effector cells in cell-mediated cytotoxicity assays. MOLT-4, MOLT-4/HIV-1_{IIIB} (kindly provided by S. Harada), Jurkat, K562 cells were cultured in CTM. Human glioblastoma cells, A172 and A172-448 [15], were grown in Eagle's minimum essential medium (MEM) supplemented with 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. To establish the HVS transformed T-cell line, PBMC were stimulated with PHA-P and infected with HVS at moi 0.1. Stimulated cells were cultured in CTM with 100 U/ml of IL-2 for more than 3 months.

2.2. Antibodies and flow-cytometric analysis

Cells were pelleted and resuspended at a concentration of $<5 \times 10^5$ cells in 50 μl of PBS with 0.1% NaN_3 and 1% human serum containing each monoclonal antibody (mAb). FITC- or PE-labeled mouse anti-human CD3, CD4, CD8, CD56, CD1d, $\gamma\delta$, and Fas mAb were purchased from BD Biosciences (San Diego, CA). APC-labeled mouse anti-human CD3 and PE-labeled mouse anti-human MICA/B mAb were purchased from Biolegend (San Diego, CA). FITC-labeled mouse anti-human Vα24 mAb was purchased from Beckman Coulter, Inc. (La Brea, CA). PE-labeled mouse anti-human ULBP-2 mAb was purchased from R&D Systems, Inc. (Minneapolis, MN). After 30 minutes' incubation with each antibody at 4°C, cells were washed and resuspended in PBS for analysis by FACScan (BD Biosciences). Neutralizing antibodies against Fas ligand and NKG2D were purchased from R&D Systems, and CD1d were obtained from BD Biosciences.

2.3. Cytotoxicity assay

Cytotoxicity was assessed in a standard 4-hour ^{51}Cr-release assay with ^{51}Cr-labeled targets at various E:T ratios in 96well, U-bottomed culture plates. After incubation, the plates were centrifuged, and 100 μl of cell-free supernatants was collected to measure the radioactivity using a γ-counter. The percentage of specific ^{51}Cr release was calculated as follows: (experimental release- − spontaneous release)/(maximum release- − spontaneous release) × 100. In our laboratory, we cannot perform the ^{51}Cr-release assay using HIV-1–infected cells because of the constraint of Infectious Diseases Control Law in Japan. Therefore, we have established a method to measure the cytotoxic activity by flow-cytometric analysis using carboxyfluorescein succinimidyl ester (CFSE; Life Technology Corporation, Carlsbad, CA) staining [16]. Target cells were labeled with 0.8 nmol/l CFSE to differentiate from effector cells. Effectors and targets were mixed at a ratio of 1:1 and incubated at 37°C for 24 hours. After incubation, cells were harvested and resuspended in 4% paraformaldehyde in PBS and then stored at 4°C in the dark before cytometry. Figure 1A shows the cell size and granularity profiles of target cell alone (upper) and co-culture of targets and effectors (lower). High fluorescence intensity of CFSE in target cells was indicated by R1 region (R1 gate), and viable cells were gated from nonviable cells by their distinct forward scatter (FSC) versus side scatter (SSC) (R2 gate). R1 and R2 gates were used for estimation of survival target cell number. The percentage of survival target cells was calculated as follows: (survival target cells in co-culture of targets and effectors)/(survival targets in target cell alone) × 100.

2.4. Cell purification or depletion

Cells were incubated with biotinylated monoclonal antibody for 5 minutes at room temperature and washed three times with PBS. The labeled cells were then further incubated with streptoavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by magnetic column selection or depletion, respectively. Biotynylated mouse anti-human CD4 and CD8 were purchased from BD Biosciences, and CD56 were obtained from Biolegend.

2.5. Granzyme B activity

Granzyme B activity was determined by granzyme B (GrB)–specific hydrolysis of substrates. A 180-μl volume of the reaction mixture (200 mmol/l acetyl-Ile-Glu–Pro–Asp-paranitroanilide in reaction buffer containing 50 mmol/l HEPES, pH 7.5, 10% sucrose, 0.05% 3-(3-cholamidepropyl)dimethylammonio-1-propanesulphonate (CHAPS), and 5 mmol/l dithiothreitol (DTT)) was added to 20 μl of cell lysates. The absorbance at 405 nm was measured after incubation for 24 hours at 37°C. Concentration of samples were determined using a standard curve with recombinant mouse GrB (Sigma-Aldrich, St. Louis, MO).

2.6. Detection of HVS DNA

DNA was isolated from LC-PBMC and HVS transformed T-cell line using Blood & Tissue Genome Mini (Viogene, Sunnyvale, CA). HVS genome was detected by polymerase chain reaction (PCR) using the following primers for the STP gene from HVS subgroup C strain 488 to generate a 783-bp fragment: 5′-CTCTAAGCA-CAGGGGCACA-3′ and 5′-CTACGCAGAAGTCGGAAGCC-3′.

2.7. Statistical analysis

Student's *t* test was used to determine the significance of differences in means. A *p* value of <0.05 was considered significant.

3. Results

3.1. Expression of cytotoxic activity in LC-PBMC

To examine which types of cells to be emerged *in vitro* cultures of PBMC under the activated conditions, we have established a unique *in vitro* long-term culture system for PBMC without adding any exogenous cytokines as well as specific antigens, as the treatments lead to initiation of cytokine production or antigen-specific T-cell responses. To carry out the experiments, we cultured PBMC stimulated with PHA-P and subsequently infected with HVS. HVS, an oncogenic tumor virus of New World monkeys, has been reported to infect and to immortalize human CD4 and CD8 T cells

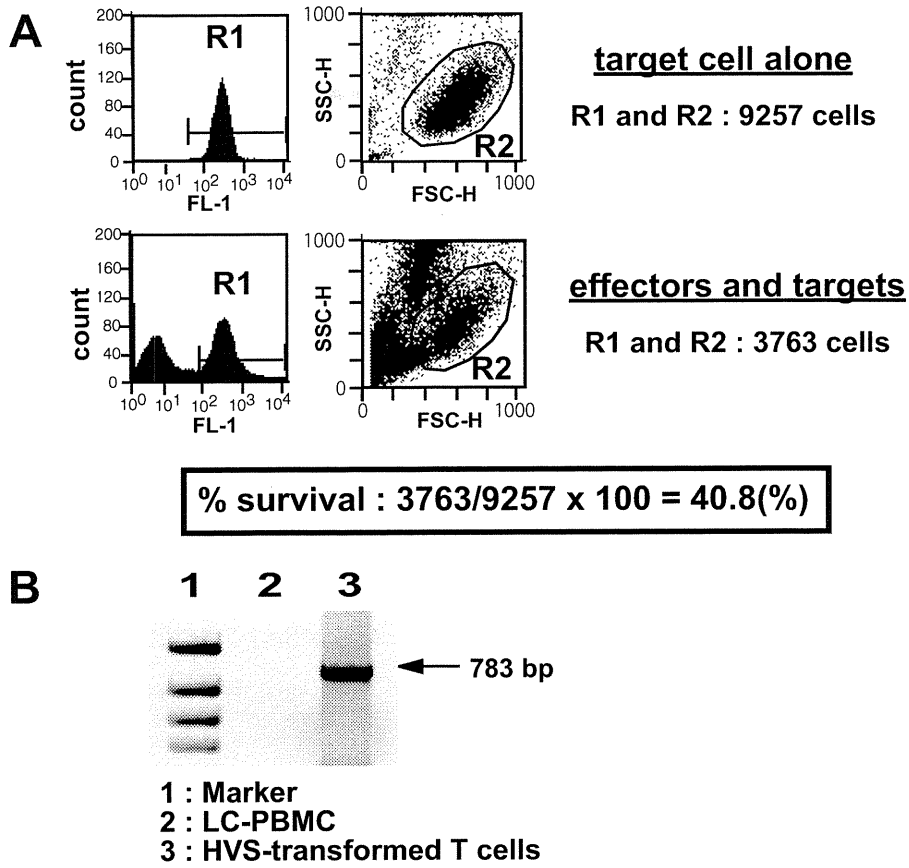

Fig. 1. Induction of long-cultured PBMC (LC-PBMC) and detection of their cytotoxic activity. (A) Method for measurement of cytotoxic activity by flow-cytometric analysis using carboxyfluorescein succinimidyl ester (CFSE) staining. R1, high fluorescence intensity of CFSE in target cells. R2, viable cells gated from nonviable cells by their distinct FSC versus SSC. A total of 10,000 cells gated in R1 were acquired, and R1 and R2 gates were used for estimation of survival target cell number. (B) PCR amplification of the Herpesvirus saimiri (HVS) genome. PCR products of the expected size were detected in HVS-infected owl monkey kidney cells and no PCR product was detected in LC-PBMC.

expressing $\alpha\beta$ and $\gamma\delta$ T cells [17,18]. We found that when PHA-stimulated PBMC were infected with HVS at high moi and then cultured in the absence of IL-2, T cells could maintain an activated state more than 30 days after stimulation. However, these treatments scarcely yielded HVS-transformed T-cells line. Indeed, as shown in Fig. 1B, we could not detect the PCR amplification of HVS gene in 60 days of cultured PBMC. We defined here such stimulated T cells as LC-PBMC, which could actively replicate more than 30 days after stimulation and maintain the cultures for more than 50 days without adding of any specific antigens and cytokines, but were not immortalized.

When we examined the cytotoxic potential of LC-PBMC after culturing them for more than 50 days in a ^{51}Cr -release cytotoxity assay, they showed significant killing activity against various T-cell lines, K562 cells, and A172 cells in a non–MHC-restricted manner. Moreover, LC-PBMC showed weak cytotoxicity against autologous PBMC (Fig. 2A). Next, we examined whether virus infection would affect the susceptibility to cytotoxic activity of LC-PBMC, by using persistently infected cell lines. Although MOLT-4 were efficiently killed by LC-PBMC, HIV-1-producing counterpart cells MOLT-4/HIV-1 were more sensitive to LC-PBMC. However, measles virus (MV)–infected A172-448 or HVS-transformed T cells were not susceptible compared with noninfected cells (Fig. 2B). These findings suggest that HIV-1 infection might enhance the susceptibility of infected cells to LC-PBMC.

3.2. $CD3^+CD56^+$ cells might be major effectors in LC-PBMC

We wanted to address the question of which types of cells among LC-PBMC are responsible for producing such cytotoxic activities. Therefore, aliquots of the cultures were removed on days 0, 3, 10, 20, 30, 40, and 50 of cultures for phenotypic analysis and measurement of cytotoxic activity against both MOLT-4 and MOLT-4/HIV-1. Time course analysis showed that until 30 days after, LC-PBMC exhibited little cytotoxic activity against both MOLT-4 and MOLT-4/HIV-1. However, after 40 days of stimulation, LC-PBMC mediated approximately 75% and 50% survival of MOLT-4 and MOLT-4/HIV-1, respectively, and another 10 days later LC-PBMC mediated less than 50% and 20% survival (Fig. 3A). The percentage of $CD3^-CD56^+$ cells was decreased by 20 days after *in vitro* stimulation, whereas that of $CD3^+CD56^+$ cells was increased gradually from 30 days after stimulation. Changes in the relative percentage of $\gamma\delta$T cells were minimal (Fig. 3B). These results suggested that $CD3^+CD56^+$ cells might be the major effectors in LC-PBMC, and natural killer (NK) cell and $\gamma\delta$ T-cell contribution was limited.

3.3. Characterization of cytotoxic cells in LC-PBMC

When the effector and target cells were separated by permeable filter, LC-PBMC showed minimal killing (data not shown). Therefore, cell–cell contact between effectors and targets are required for efficient killing by LC-PBMC. Figure 3 demonstrates that cytotoxic activity was elevated in parallel with increase of $CD3^+CD56^+$ cells. Thus, to examine the possible engagement of $CD3^+CD56^+$ cells in this cytotoxicity, $CD56^+$ cells were depleted from LC-PBMC by magnetic bead selection. An apparent reduction in cytotoxic activity was observed against MOLT-4/HIV-1 (Fig. 4A). In addition, depletion of $CD8^+$ cells had a significant effect on the cytotoxicity of LC-PBMC, as two-thirds of $CD3^+CD56^+$ cells were CD8 positive (Fig. 4B). Taken together, these results suggested that $CD3^+CD8^+CD56^+$

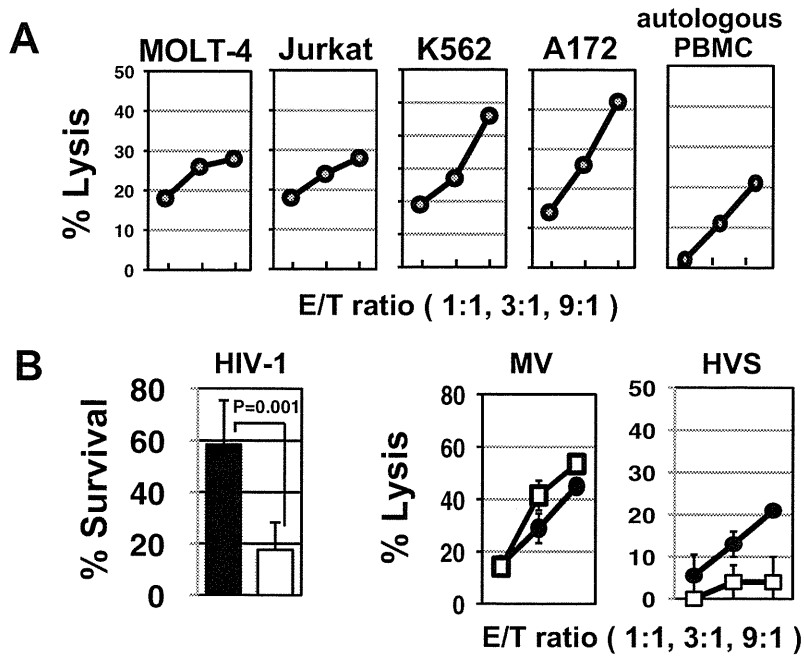

Fig. 2. Cytotoxic activity of long-cultured PBMC (LC-PBMC) against MOLT-4, Jurkat, K562, A172, and autologous PBMC (A). Cytotoxicity was assessed in ^{51}Cr-release assay. (B) Effect of virus infection on the susceptibility of target cells to cytotoxic activity of LC-PBMC. Assessment of cytotoxic activity was evaluated as survival rate in HIV-1 infection (closed bar; MOLT-4, open bar; MOLT-4/HIV-1) and was done as killing rate in measles virus (MV) (filled circle, A172; open square, A172-448) or Herpesvirus saimiri (HVS) infection (filled circle, PHA-P–stimulated T cells; open squares, HVS-transformed T cells).

cells might be the predominant effectors in LC-PBMC. We then assessed the contribution of FasL and NKG2D to LC-PBMC–mediated cytotoxicity using the neutralizing antibodies to FasL and NKG2D. Figure 4C demonstrates that anti-NKG2D antibody significantly affected the cytotoxic activity of LC-PBMC against MOLT-4/HIV-1, whereas anti-FasL antibody blocked the cytotoxicity only slightly. Moreover, increased granzyme B activity was observed when LC-PBMC were incubated with target cells (Fig. 4D).

Type 1 NKT cells is characterized by a semi-invariant T-cell receptor (TCR) using a unique TCR Vα24Jα18 chain in human and by its recognition of the glycolipid α-galactosylceramide (α-GalCer) loaded onto CD1d molecules. We tested the expression of Vα24 on CD3$^+$CD56$^+$ cells in LC-PBMC, but they did not express this molecule (Fig. 4E, left panel). We also examined whether cytotoxicity of target cells by LC-PBMC was in a CD1d-restricted manner, target cells were incubated with anti-CD1d neutralizing antibody [19] before co-culturing with effector cells. As shown in Fig. 4E, cytotoxic activity of LC-PBMC was not affected, suggesting that LC-PBMC-mediated cytotoxicity was not CD1d restricted.

3.4. HIV-1–infected CD4$^+$ T cells are more susceptible to cytotoxic activity of LC-PBMC

We then asked why HIV-1–infected cells are more susceptible to LC-PBMC–mediated killing than are noninfected cells. Because the killing of target cells was mostly NKG2D mediated, we examined the cell surface expression of ligands for NKG2D on both MOLT-4 and MOLT-4/HIV-1. Phenotypic analysis showed that the level of CD1d and MICA/B expression on MOLT-4/HIV-1 were lower than MOLT-4, whereas that of ULBP-2 was higher (Fig. 5A). These findings agree with previous report that HIV-1-Vpr up-regulated the expression of ligands for the activating NKG2D receptor, including ULBP-1, −2, and −3 but not MICA/B in infected cells [20]. However, as treatment of targets cells with anti–ULBP-2 antibody slightly blocked the cytotoxiciy of LC-PBMC (data not shown), all other ULBPs might be involved in enhancing susceptibility of HIV-1–infected cells to LC-PBMC–mediated killing.

Finally, to confirm the susceptibility of HIV-1–infected CD4$^+$ T cells to the cytotoxic activity of LC-PBMC, we infected primary CD4$^+$ T cells from healthy or HIV-1–infected donors with HIV-1 IIIB at moi of 0.1 for 4 days, followed by co-culturing with autologous LC-PBMC for 1 day. As shown in Fig. 5B, LC-PBMC were cytotoxic against autologous CD4$^+$ T cells and became more cytotoxic against HIV-1–infected CD4$^+$ T cells. Of note, although the percentages of CD3$^+$CD56$^+$ cells in LC-PBMC from HIV-1–infected individuals were little less than from healthy donors (data not show), CD4$^+$ T cells from HIV-1–infected patients showed a decreased survival rate in the presence of autologous LC-PBMC compared with healthy donors.

4. Discussion

Chronic immune activation is one of the characteristic features in HIV infection [21]. Polyclonal B-cell activation, increased T-cell turnover, increased frequencies of T cells with an activated phenotype, and increased serum levels of proinflammatory cytokines and chemokines were observed in HIV-1–infected patients [22]. In the absence of antiretroviral treatment, markers of T-cell activation and T-cell turnover predicted the rate of disease progression and CD4$^+$ T-cell depletion [10,23]. Chronic simian immunodeficiency virus (SIV) infection in macaques provides a relevant and useful model to explore the mechanisms for progressive CD4$^+$ T-cell decline in AIDS pathogenesis. Rhesus macaques, which develop progressive CD4$^+$ T-cell depletion and progression to AIDS upon SIV infection, are characterized by strong T-cell activation. In contrast, SIV-infected sooty mangabeys and African green monkeys, the natural hosts of SIV, which do not develop any immunodeficiency, exhibit minimal T-cell activation despite evident viral replication [24]. Although the underlying causes of immune activation have remained elusive, several mechanisms have been proposed. For example, CD4$^+$ T-cell depletion during the acute stage of HIV-1 infection occurs rapidly within the first few weeks of infection and is predominantly localized to the gastrointestinal tract. Therefore, in the context of a compromised gastrointestinal mucosal surface,

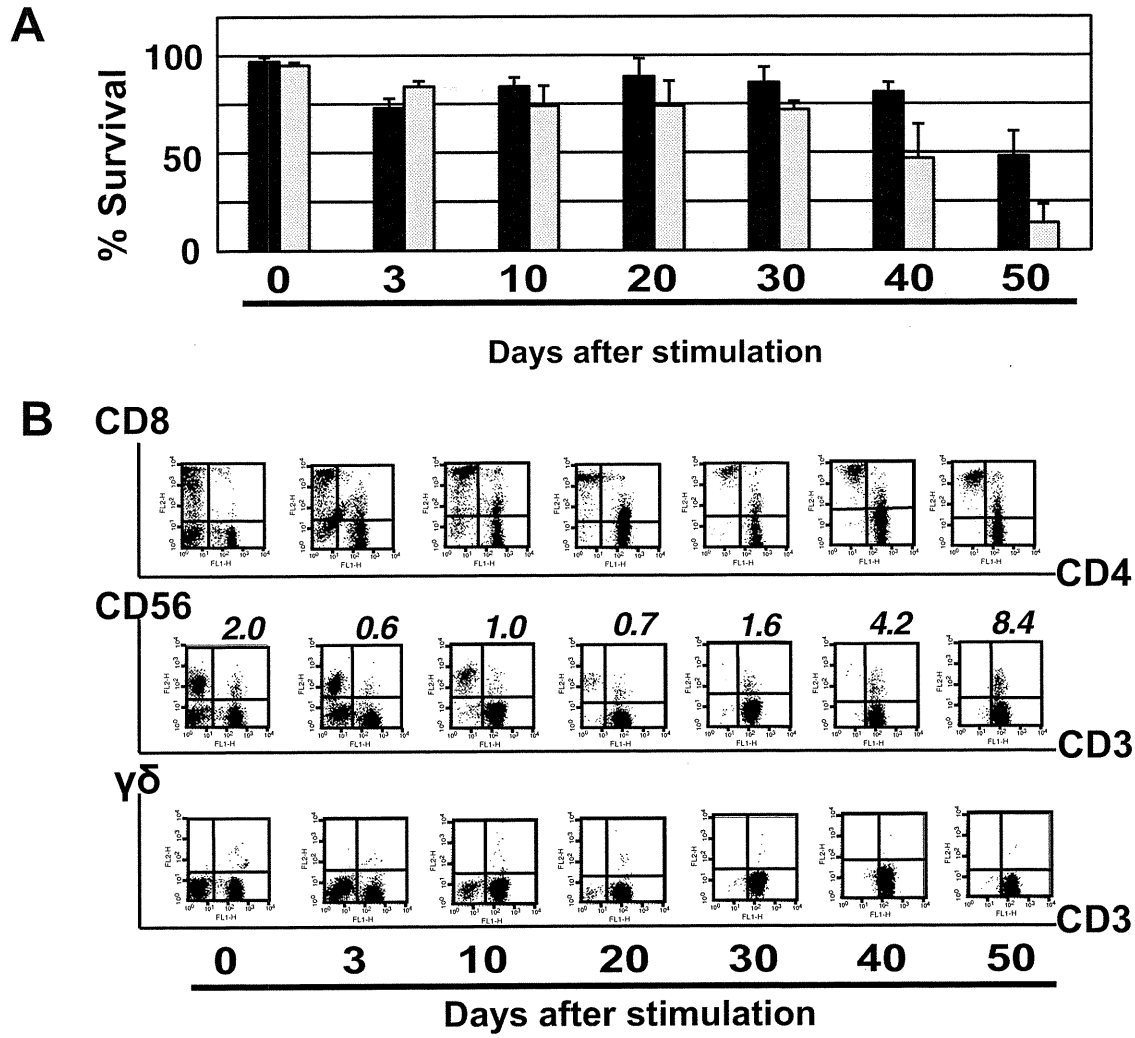

Fig. 3. $CD3^+CD56^+$ cells might be the major effectors in long-cultured PBMC (LC-PBMC). (A) Time course analysis of cytotoxic activity in LC-PBMC. PBMC were stimulated with PHA-P and subsequently infected with Herpesvirus saimiri (HVS). Aliquots of the cultures were removed on days 0, 3, 10, 20, 30, 40, and 50 of culture for measurement of cytotoxic activity against both MOLT-4 (filled bar) and MOLT-4/HIV-1 (open bar). Assessment of cytotoxic activity was evaluated as survival rate of target cells in triplicates (mean ± SD). (B) Time course analysis of phenotype in LC-PBMC. PBMC were stimulated with PHA-P and subsequently infected with HVS. Aliquots of the cultures were removed on days 0, 3, 10, 20, 30, 40, and 50 of culture for phenotypic analysis. The indicated numbers represent the percentages of $CD3^+CD56^+$ cells.

circulating microbial products (*e.g.*, lipopolysaccharide [LPS]) might be a possible cause of HIV-related systemic immune activation [25]. Furthermore, HIV-1 gene products such as gp120 or Nef are able to activate lymphocytes or to enhance their responsiveness to activation [26].

Activation of T cells includes their turnover, differentiation from naive to memory cells, and various type of cytokine production. These events might cause the alterations of constitution in immune cell populations. In this study, we applied an alternative approach using PHA-P and HVS infection to maintain an activated condition of T cells and examined which types of cells would emerge *in vitro* in cultures. We observed that $CD56^+$ T cells were gradually increased in the long-term culture conditions from 30 days after stimulation and exhibited a potent cytotoxic activity against both autologous and allogeneic targets. In human beings, innate and acquired immune responses are thought to be mediated in part by NK cells, γδ T cells, and T cells that express both NK cell-associated markers and TCR. Among NK-like T cells, $CD56^+$ T cells are well characterized for their phenotype and function [27–30]. $CD56^+$ T cells are a heterogeneous population that include $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ cells expressing αβ or γδ TCR and various combinations of NK cell receptors. They account for a small percentage (~5%) of PBMC, but are present in remarkably high numbers in the liver and bone marrow of healthy adults, accounting for 15–55% of all T cells in these organs [31]. $CD56^+$ T cells possess dual innate and adaptive immune functions displaying properties of both T and NK cells capable of both MHC-restricted and non-MHC-restricted cytotoxicity and secretion of cytokines, including IFN-γ, TNF-α, and IL-4 [32]. These properties provide a role for $CD56^+$ T cells to regulate the immune responses against microorganisms and tumors. Indeed, decreased numbers of $CD56^+$ T cells have been reported to occur in the livers of chronically hepatitis C virus (HCV) -infected patients, and failure to eliminate HCV is thought to result from a deficiency of such innate lymphocytes [33,34].

Previous studies reported that invariant NKT (iNKT) cells, which comprise a small portion of peripheral $CD56^+$ T cells, expressing an invariant Vα24Vβ11 and recognizing glycolipid antigens presented by CD1d, were highly susceptible to HIV-1 infection, and that selective depletion of these cells might contribute to developments in HIV pathogenesis [35]. Because $CD56^+$ T cells observed in this study did not show the expression of Vα24 and CD1d-mediated cytotoxicity, they were not iNKT cells. There are few studies on the expres-

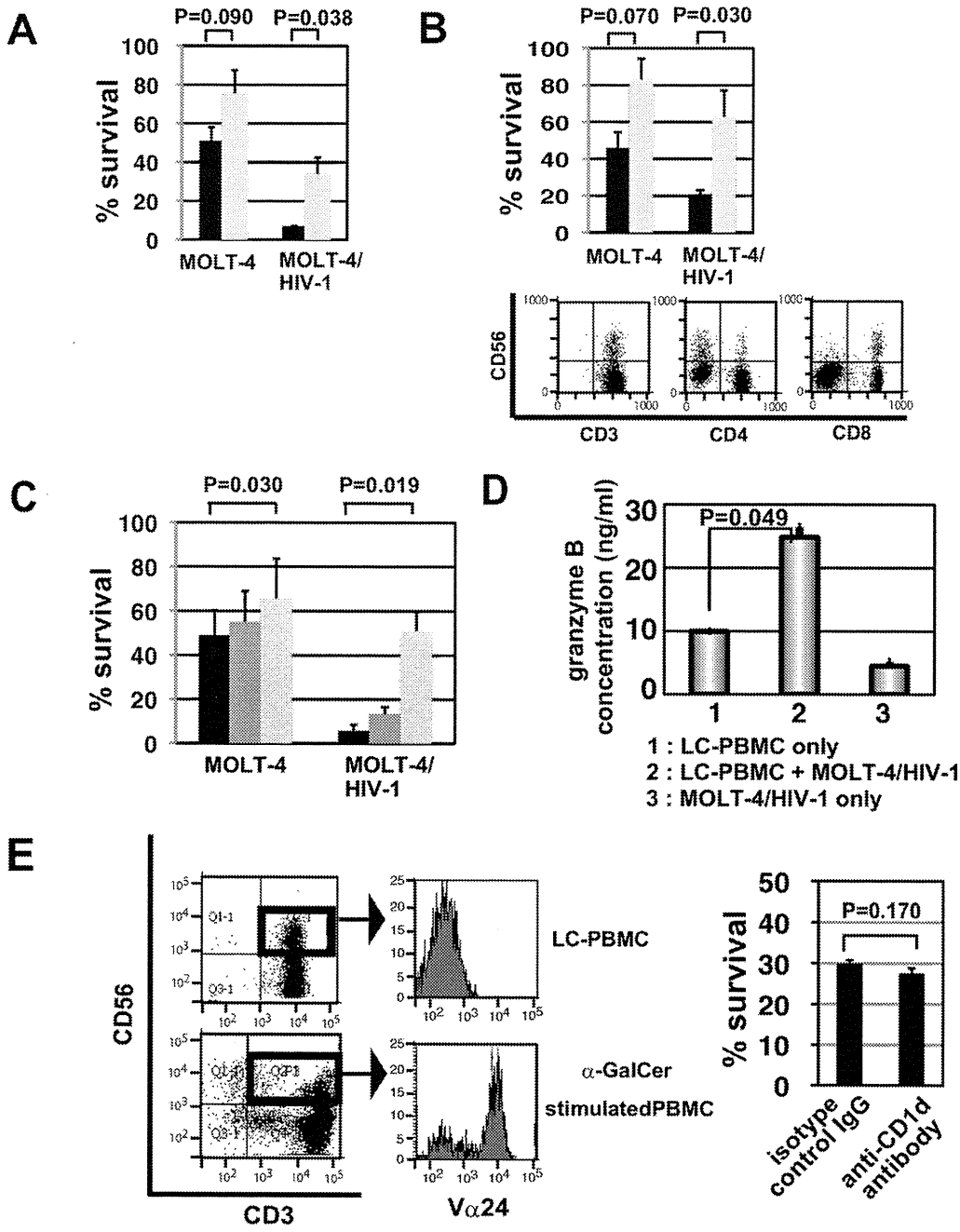

Fig. 4. Characterization of effector cells in long-cultured PBMC (LC-PBMC). Identification of effector cells in LC-PBMC. $CD56^+$ cells (A) or $CD8^+$ cells (B) were depleted from LC-PBMC by magnetic beads selection and subjected to cytotoxicity assay. (C) Effect of anti-FasL or NKG2D neutralizing antibody on cytotoxic activity of LC-PBMC. Effector cells and target cells were co-cultured in the presence of neutralizing antibodies and subjected to cytotoxicity assay. (A–C); Assessment of cytotoxic activity was evaluated as survival rate of target cells in triplicate (mean ± SD). (D) Granzyme B activity of LC-PBMC. LC-PBMC were cultured with or without MOLT-4/HIV-1. After 1 day, cells were harvested and subjected to measurement of granzyme B activity. (E) Examination of Vα24 expression on $CD3^+CD56^+$ cells. LC-PBMC and α-galactodemide stimulated PBMC were stained with APC (anti-CD3), PE, (anti-D56) and FITC (anti-Vα24) labeled antibodies (left panel). Target cells were incubated with anti-CD1d neutralizing antibody before coculturing with effector cells (right panel).

sion and function of $CD56^+$ T cells in HIV infection besides iNKT cells. Tarazona et al. observed a decreased number of $CD8^{bright}$ T cells expressing CD56 in non–HAART-treated HIV-1 patients [36]. Parsons et al. reported that the phenotypic changes of CD56 from positive to negative on $CD8^+$ T cells with non–MHC-restricted cytotoxicity occurred in progressive HIV infection, and suggested that these changes might reflect autoreactive and pathogenic diversion of the $CD8^+$ T-cell repertoire [37].

Induction of $CD56^+$ T cells by PHA-P stimulation and HVS infection do not necessarily reflect cytotoxic T cells *in vivo*. However, when we examined the cytotoxic activity of $CD56^+$ T cells from HIV-1–infected or noninfected donors, $CD56^+$ T cells were cytotoxic against autologous $CD4^+$ T cells and became more cytotoxic against HIV-1–infected $CD4^+$ T cells. It should be noted that $CD4^+$ T cells from HIV-1–infected patients showed significant susceptibility against autologous $CD56^+$ T cells. The percentages of $CD3^+CD56^+$ cells in LC-PBMC from HIV-1–infected patients were little less than from healthy donors. These findings suggest that $CD56^+$ T cells from HIV-1–infected patients are more likely to acquire a potent cytotoxicity under the activated culture condition.

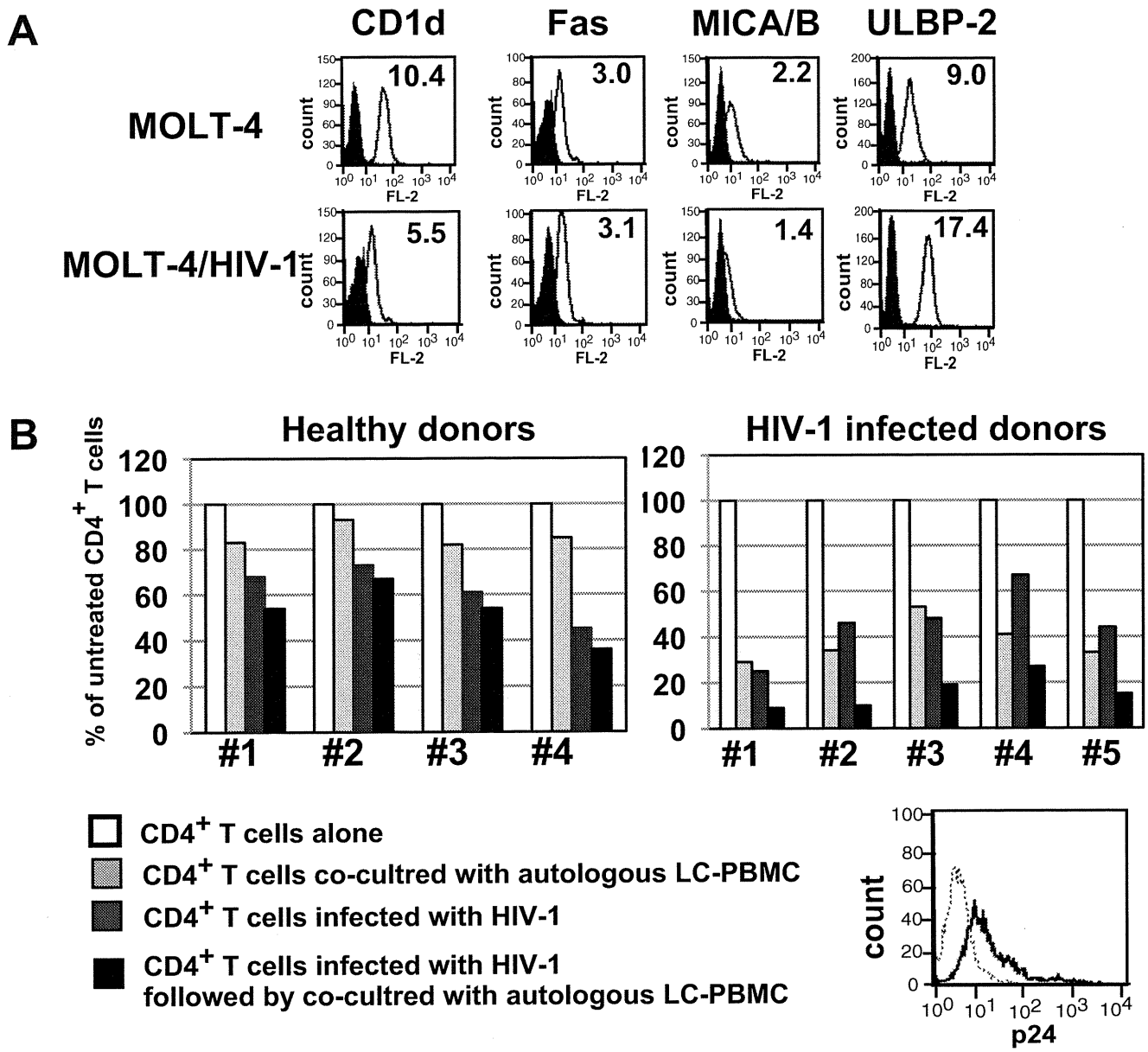

Fig. 5. $CD4^+$ T cells from HIV-1–infected individuals are more susceptible to cytotoxic activity of autologous long-cultured PBMC (LC-PBMC). (A) Cell surface expression of ligands for NKG2D on both MOLT-4 and MOLT-4/HIV-1. Both cells were stained with anti-CD1d, Fas, MICA/B, or ULBP-2 antidody. MFI values are indicated in the upper right corner of histogram. (B) Enhancement of susceptibility of $CD4^+$ T cells against autologous LC-PBMC by HIV-1 infection. PBMC from healthy and HIV-1–infected donors were stimulated with PHA-P followed by HVS infection. After ~50 days' incubation, cells were harvested and used as LC-PBMC. $CD4^+$T cells were purified from PHA-P–stimulated PBMC by magnetic beads selection and infected with HIV-1 IIIB at 0.1 moi for 4 days, and then co-cultured with autologous LC-PBMC for 1 day. Assessment of cytotoxic activity was evaluated as survival rate of target cells. HIV-1 infection was confirmed by intracellular staining of HIV-1 gag antigen p24. Results were normalized and are expressed as percentages of intact $CD4^+$ T cells.

We assumed that if the level of immune activation was highly elevated, autoreactive $CD56^+$ T cells might be up-regulated and expanded in HIV-1–infected patients. Therefore, we present here our findings on the possible involvement of $CD56^+$ T cells in the depletion of $CD4^+$ T cells.

Some HIV-1–infected individuals have autoreactive antibodies directed CD4 molecules, human leukocyte antigen (HLA) class II, and myelin basic protein [38,39]. Recently Kuwata et al. demonstrated that increased autoreactive antibodies correlated with the extent of $CD4^+$ T-cell depletion in an SIV/macaque model [40]. Induction and development of autoreactive antibodies is also likely to be caused by immune activation in HIV-1–infected patients. Therefore, it is necessary to block or to minimize immune activation and inflammation to prevent such unfavorable responses. Antiretroviral therapy exerts a suppressive effect on T-cell activation and apoptosis through its potent and prolonged inhibition of HIV replication. However, there are several limitations to application of HIV therapy for HIV-1–infected individuals, such as drug resistance and dose-limiting side effects. Use of immunosuppressive agents or inhibitors of proinflammatory cytokines might also be effective for suppression of immune activation. However, because the immune system also has to cope with other persisting or exogenous pathogens, casual use of these agents may cause progression toward severe infectious disease. Therefore, a better understanding of the mechanisms by which HIV-1 infection causes immune activation is required for the development of strategies for control of immune activation, that is, what types of agents we can use, or under what conditions these agents can be used.

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Species-specific CD1-restricted innate immunity for the development of HIV vaccine

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ABSTRACT

The human immune defense system is composed of two distinct elements: innate immunity located primarily at body surfaces restricted by species-specific CD1 molecules and acquired immunity found mainly in internal compartments associated with individually restricted MHC molecules. Historically, effective vaccines have focused on eliciting pathogen epitope-specific acquired immune responses to protect against infectious diseases; however, such traditional approaches to developing HIV vaccines have been unsuccessful. This review addresses the importance of activating host species-restricted innate immunity to enhance the virus epitope-specific acquired immunity that is needed for HIV vaccines.

1. Introduction

Our internal defense system is composed of two distinct elements. One is local innate immunity principally arranged on surface areas, such as skin or mucous membrane to establish barriers against various pathogens, and the other is systemic acquired immunity, mainly found in systemic components, for example, circulating blood or lymphoid organs, such as lymph nodes and spleen, to survey and control internal damage and disorders. The former innate arm is chiefly regulated via species-restricted CD1 antigen-presenting molecules and the latter acquired arm is orchestrated by individually restricted MHC molecules (Fig. 1).

In vaccine development for both the prevention of pathogen intrusion and suppression of its expansion as well as tumor growth, we have been focusing on the induction of acquired immune responses composed of MHC molecule-restricted peptide epitope-specific T cells and antibodies that bind specifically to the particular epitopes on pathogens or tumors through their definite receptors created by gene re-arrangements. Thus, the main work to advance vaccine development has been focusing on the identification of epitopes and the establishment of a powerful and non-toxic adjuvant for the induction of epitope-specific immunity. However, because pathogen- or tumor-derived epitopes vary among diverse MHC molecules, the analysis and discovery of cross-reactive immuno-dominant epitope(s) should be considered to overcome MHC diversity [1,2].

Under these conditions, a lack of correlation between acquired virus-specific immunity and resistance to infection with simian immunodeficiency virus (SIV) in rhesus monkeys has been reported recently [3]. Also, most exposed, uninfected commercial sex workers eventually became infected after quitting their jobs to limit mucosal human immunodeficiency virus type-1 (HIV-1) exposure, although virus-specific cell-mediated immunity and immunoglobulin A (IgA) antibody responses had been confirmed [4,5], suggesting that continuous mucosal virus stimulation may be required to maintain protective acquired immunity against persistently infected pathogens. Moreover, the reservoir for HIV-1 in persistently infected patients with no free virus particles in the circulating blood after highly active anti-retroviral treatment (HAART) has been identified as innate CD4-positive dendritic cells (DC) or natural killer T (NKT) cells in the small intestine (J.M. and H.T.; unpublished observation). In the present review, based on our recent progress, the importance of activating species-restricted local innate immunity to develop and HIV-1 vaccine rather than individually restricted systemic acquired immunity will be addressed.

2. Species-specific antigen-presenting molecule CD1s

Species-specific CD1 molecules are further divided into two sub-classes, group I CD1 (CD1a–CD1c) and group II CD1 (CD1d) [6]. These CD1s have been found to present lipid/glycolipid antigens to the appropriate T cells bearing relatively invariant

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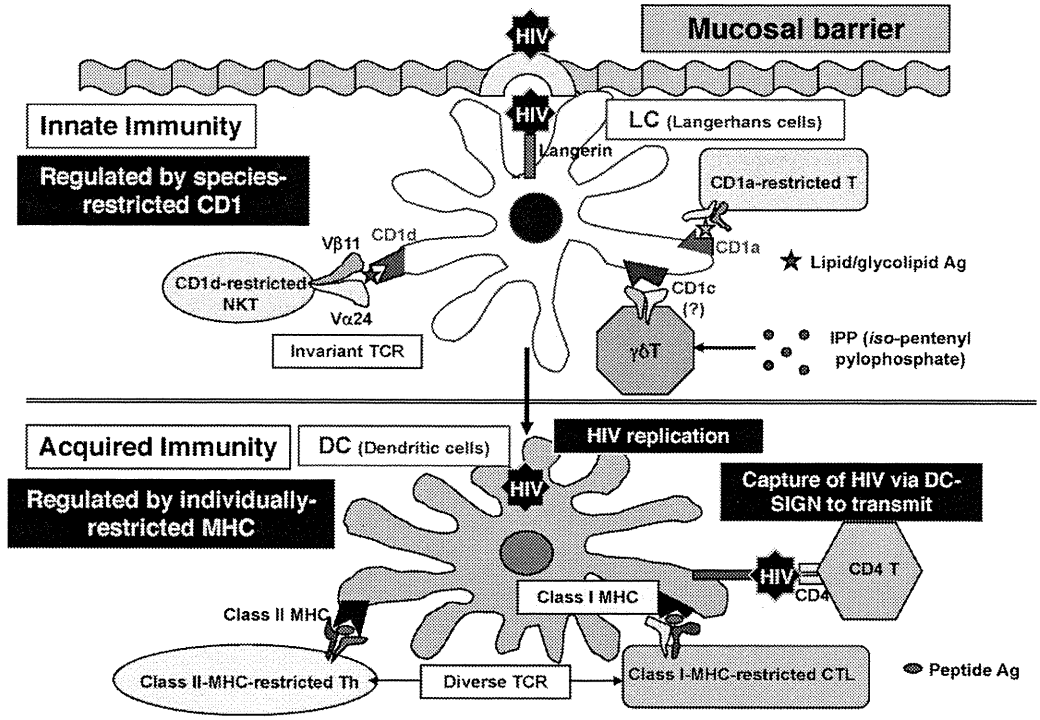

Fig. 1. Innate immunity and acquired immunity. Our internal defense system is composed of two distinct elements. One is innate immunity composed of γδT cells and NKT cells as effectors expressing fixed invariant receptors controlled mainly by species-restricted CD1 molecules on Langerhans cells and (LC) dendritic cells (DC), and the other is systemic acquired immunity composed of helper T cells (Th), cytotoxic T lymphocytes (CTL), and antibodies bearing diverse receptors from re-arranged genes orchestrated by individually restricted MHC molecules.

αβT-cell receptors (TCR), most of which are conserved among species; for example, highly conserved CD1d molecules present α-galactosyl ceramide (α-GalCer) to natural killer T (NKT) cells of their own species. Indeed, human NKT cells generally express unique combinations of TCRs that consist of an invariant Vα24 chain preferentially paired with a Vβ11 [7], while murine α-GalCer-reactive CD1d-restricted NKT cells express invariant Vα14 paired with various Vβ combinations [8].

The structures of CD1 molecules are similar to those of class I MHCs bearing non-covalently bound β2-microglobulin that may regulate the antigen-binding capacity of the presenting molecules; however, CD1s show limited polymorphism and do not map to MHC genes [9]. Also, CD1-encoding genes are highly conserved and their structures are shared among species [10]. We have confirmed recently that the genetic structure of CD1d molecules is very tightly conserved among species, such as rhesus macaques, African green monkeys (AGMs), and chimpanzees, and would not be affected by long-term environmental stimulation [11]. It is important to note that, unlike rhesus macaques and AGMs, both α1 and α2 domains of the CD1d of chimpanzees were identical to those of humans, although 4 amino acids in α3 domain differed [11]. Since the α2 domain of CD1d molecules with a hydrophobic ligand-binding pocket critical for antigen presentation [12], changes of amino acids in the α2 domain may alter the capacity of the presented glycolipid/lipid antigens to effector NKT cells. It is widely known that both humans and chimpanzees are susceptible to HIV-1 [13,14] but very weakly to SIV, to which rhesus macaques and AGMs are susceptible. These findings suggest an evolutionary relationship between species-specific CD1d molecules and retrovirus susceptibility through the activation of innate effector NKT cells.

3. Individually restricted antigen-presenting molecule MHC

In contrast, both class I and class II MHC molecules are extremely diverse among species with self-restricted elements that can present internally processed peptide antigens only to the same MHC molecule-bearing cells. Such individually restricted peptide epitopes will be recognized by highly diverse αβTCRs established via suitable intracellular gene re-arrangements that create antigen-specificity. In general, CD8αβ-positive T cells recognize the processed epitope peptide presented by class I MHC molecules, whereas CD4-positive T cells recognize epitope peptide in association with class II MHC [15]. Both class I and class II MHC molecule-restricted T cells can be elicited by individual class I and class II MHC molecule-expressing DC that capture antigenic proteins and select to present specific epitopes with their MHC molecules; therefore, the epitope-specific rearranged αβTCR-expressing T cells in the acquired arm seem to be controlled by individual antigen-captured DC *in vivo*.

In viral infection, various viral proteins and genetic components are disseminated throughout the body. The former viral proteins may be captured by immature DC (iDC) and the latter genetic components may stimulate antigen-loading iDC to mature via Toll-like receptors (TLR), inducing virus-specific cellular immunity, in particular, cytotoxic T lymphocytes (CTL) that eliminate virus-infected cells. Double-strand RNA, polyriboinosinic polyribocytidylic acid (poly(I:C)), which reflects a natural genetic product from a variety of viruses during replication, has recently been identified as one of the critical stimuli of TLR3 [16]. We and others have shown that iDC could present processed antigen from captured purified protein in association with class I MHC molecules via a cross-presentation mechanism when iDC were stimulated with poly(I:C) [17,18]. Also, such cross-presentation of externally added purified proteins can be achieved by a saponin-associated adjuvant like ISCOMs [19] or cholera toxin (CT) [20]. Taken together, virus-specific acquired immunity restricted by individual MHCs can be spontaneously elicited by the appropriate activation of innate iDC that capture viral antigenic molecules during the course of infection.

4. Interaction between HIV-1 and DC

DC at the mucosal site appear to be a natural reservoir for HIV-1, whose Nef protein is known as a key factor in disease progression. Indeed, nef-deficient HIV-1 as well as SIV markedly slowed the clinical manifestation of AIDS [21]. We and others have recently reported that the surface expression of CD1a and CD1d was selectively down-regulated among CD1 molecules as well as class I MHC on HIV-1 infected iDC by Nef [22,23], indicating that iDC may lose their function to present virus-associated antigens to both innate and acquired effectors, which may cause disease progression. Thus, stimulation of iDC or deletion of the Nef effect in HIV-1 infected DC may alter the immunological state of HIV-1-infected individuals.

Two distinct types of innate DC are observed at the local mucosal site. One is sentinel Langerhans cells (LC) that are present in the surface epithelial compartment to survey antigens, including viruses, via the LC-specific sampling receptor, Langerin; langerin-bound antigens are internalized into Birbeck granules and degraded. Recently de Witte et al. [24] proposed that LCs provide a barrier to HIV-1 infection by demonstrating that HIV-exposed skin-derived LCs captured HIV via Langerin and degraded the virus. However, evidence contradicting the capacity of LCs to protect against HIV was obtained using human vaginal explant cultures, a more direct, biologically relevant model of HIV-sexual transmission [25]. Hladik et al. showed that after HIV-1 exposure of vaginal explants, LCs were rapidly virus-penetrated primarily by endocytosis via multiple receptors and that endocytosed virions could persist in the cytoplasm. Also, DC-SIGN (CD209) and langerin (CD207) apparently had a negligible role in mediating endocytotic HIV infection by vaginal LCs. Thus, depending on the tissue source of LCs and the precise experimental conditions, human LCs are capable of degrading HIV-1 or being infected and harboring HIV-1. It is important to note that Haldik et al.'s results support prior observations of rapid vaginal LC infection in macaques after experimental SIV vaginal inoculation [26]. We have reported that DC-SIGN-positive macrophages in the early colostrum (breast milk macrophages: BrMMØ) and their DC-SIGN expression were markedly enhanced by externally added interleukin (IL)-4 [27]. IL-4-treated BrMMØ showed strong capacity to transmit HIV-1 to $CD4^+$ cells via DC-SIGN [28]. Therefore, evidence from different human tissue culture model systems indicates that both LCs and DC-sign-positive DCs can be reservoirs for HIV-1.

5. Selective activation of innate DC lineage cells for the induction of HIV-1-specific acquired immunity

The two major distinct subsets of DC are arranged to regulate immune responses *in vivo*; 33D1-positive and DEC-205-positive DC. Using anti-33D1-specific monoclonal antibody (mAb), 33D1-positive DC were successfully depleted from C57BL/6 mice *in vivo*. When the remaining DEC-205^+ DC in 33D1$^+$ DC-depleted mice were stimulated with LPS, serum IL-12 but not IL-10 secretion was markedly enhanced, which may induce Th1 dominancy upon TLR signaling. After implanting various syngeneic tumor cells into the dermis of 33D1$^+$ DC-depleted mice, subcutaneous injection with LPS resulted in significant suppression of tumor growth *in vivo*. Moreover, apparent proliferation of class I MHC molecule-restricted epitope-specific $CD8^+$ CTL among tumor infiltrating lymphocytes (TIL) against already established syngeneic tumors was observed in the LPS-stimulated 33D1$^+$ DC-deleted mice administered intraperitoneally with very small unaffected amount of melphalan (L-phenylalanine mustard; L-PAM) (K.M., A.W., and H.T.; unpublished observation).

These findings indicate the importance and effectiveness of selective targeting of a specific subset of innate DC, such as DEC-205^+ DC alone or with very small amount of anti-cancer drugs to activate functional acquired epitope-specific $CD8^+$ CTL without externally added antigen stimulation *in vivo*. This may be true for HIV-1 intrusion in the local mucosal area, in which selective activation of suitable DC with or without small amount of anti-HIV-1 drugs will induce effective acquired immunity specific for the pathogen.

6. Concluding remarks

Most vaccine work to block pathogens has focused on how to artificially elicit acquired individual MHC molecule-restricted effectors specific for pathogens or pathogen-infected cells before pathogen invasion by using attenuated pathogens or pathogen-derived immunogenic molecules containing epitopes with a strong adjuvant. However, to establish the most suitable pathogen-specific acquired immunity before intrusion in individuals may be very difficult and sometimes harmful since some deteriorate products, such as CTL and antibodies, may spread in the blood before pathogen intrusion. Thus, the acquired products circulating throughout the body may attack or bind the pathogens, their destructive products, or pathogen-infected cells, having negative effects on the body.

As indicated above, it should be considered that pathogens will attack species but not individuals like HIV-1, in humans and chimps, while SIV in monkeys. Humans have survived battled against various pathogens for a long period probably as a result of species-specific CD1-restricted innate immunity rather than individually restricted acquired immunity. Species-restricted innate cells are mainly arranged on the surface area where pathogens invade from outside. Here, an innovative vaccination strategy against various pathogens or tumors is suggested. Namely, similar to tumors, selective activation of innate immunity with slight damage of the pathogens using a potent drug or an antibody will spontaneously achieve to establish most favorable acquired immunity in each individual.

However, innate immune cells, such as DC, do not usually keep long, persistent memories, and thus have to be stimulated constantly to maintain their activities. Under the conditions in which selective and constant activation of DC is performed, attenuated or killed pathogens or their components, or even live infectious pathogens themselves, should be addressed to establish immunity. Such a procedure may help to generate the most suitable acquired immunity to control pathogens spontaneously. Also, as suggested in exposed, uninfected commercial sex workers, constant activation of innate DC is required to maintain the most favorable acquired immunity [29].

Intravesical bacillus Calmette-Guerin (BCG) therapy against human bladder carcinoma is considered the most successful immunotherapy against solid tumors [30]. During the course of determining the actual effector cells activated by intravesical BCG therapy to inhibit the growth of bladder carcinoma, we found that innate alert cells, such as $V\gamma2V\delta2$ T cells and NKT cells derived from peripheral mononuclear cells (PBMC) activated by live BCG-pretreated DC, appear to inhibit the proliferation of T24 tumor cells as well as eliminate them [31]. These findings strongly suggest that some products in live BCG or live BCG itself must stimulate suitable DC for tumor surveillance and such DC will help to induce and maintain most effective acquired effectors against the tumor. We are currently searching for the substances from live BCG.

Collectively, to develop an ideal vaccine, the activation of species-restricted innate immunity located at the surface compartment should be the focus to establish more favorable individual-restricted acquired immunity against external pathogens, such as HIV-1, or internal tumors. The fact that one can

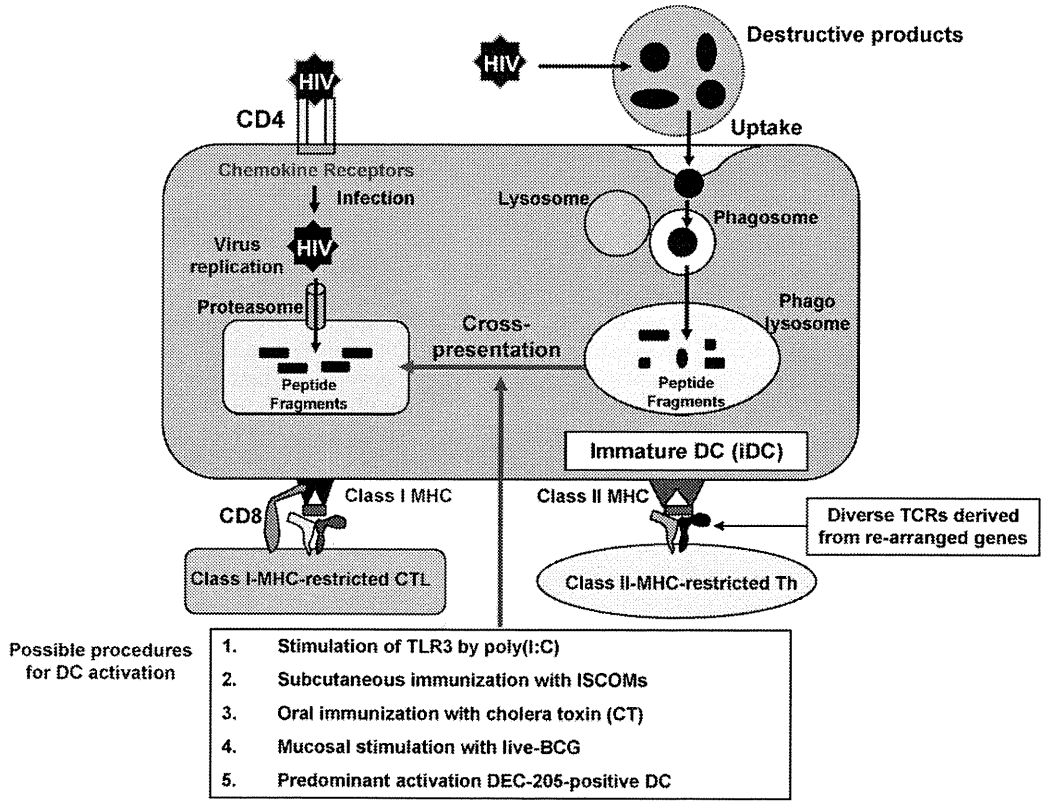

Fig. 2. Antigen-presentation by class I and class II MHC molecules. Intracellular antigens are degraded by proteasomes into peptides that are loaded into class I MHC and displayed on the cell surface to CD8-positive CTL. In contrast, extracellular antigens are taken up by phagosomes fused with lysosome containing various enzymes and processed into peptides that bind to class II MHC molecules to activate CD4-positive Th. Such extracellular peptide antigens can be displayed in association with class I MHC molecules to prime CD8-CTL when antigen-presenting DC are treated with various stimuli.

usually obtain a suitable protective acquired immunity including MHC molecule-restricted CTL after a pathogen infection indicates that some factors that activate innate DC to generate suitable acquired immunity via cross-presentation seem to be hidden in the pathogen itself (Fig. 2).

Conflict of interest statement

The author states that they have no conflict of interest.

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IMMUNOLOGY ORIGINAL ARTICLE

Inhibition of DC-SIGN-mediated transmission of human immunodeficiency virus type 1 by Toll-like receptor 3 signalling in breast milk macrophages

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Summary

The majority of cells in early/colostrum milk are breast milk macrophages (BrMMø) expressing dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN), and the expression level of DC-SIGN on BrMMø will determine cell-to-cell human immunodeficiency virus type 1 (HIV-1) transmissibility. Thus, one of the strategies to prevent vertical transmission of HIV-1 through breast-feeding is to find a way to suppress DC-SIGN expression on BrMMø. As for the expression of Toll-like receptors (TLRs) in BrMMø, TLR3 was always seen in BrMMø but not in peripheral blood monocytes (PBMo). Also, the expression of TLR3 was slightly enhanced in BrMMø when the cells were treated with interleukin (IL)-4. Moreover, when TLR3 was stimulated with its specific ligand, the double-stranded RNA (dsRNA) poly(I:C), DC-SIGN expression on BrMMø was reduced even in the IL-4-mediated enhanced state. Some reduction may be caused by type I interferons (IFNs), such as IFN-α/β, secreted from BrMMø. Indeed, both IFNs, particularly IFN-β, showed a strong capacity to suppress the enhancement of DC-SIGN expression on IL-4-treated BrMMø and such TLR3-mediated DC-SIGN suppression was partially abrogated by the addition of anti-IFN-α/β-receptor-specific antibodies. As expected, DC-SIGN-mediated HIV-1 transmission to CD4-positive cells by BrMMø was inhibited by either poly(I:C) stimulation or by treatment with type I IFNs. These findings suggest a possible strategy for preventing mother-to-child transmission (MTCT) of HIV-1 via breast-feeding through TLR3 signalling.

Keywords: breast milk macrophages; colostrum/early breast milk; dendritic cell-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN); human immunodeficiency virus type 1 mother-to-child transmission; Toll-like receptor 3; type I interferons

Introduction

Although mother-to-child transmission (MTCT) of human immunodeficiency virus type 1 (HIV-1) has been markedly reduced by antiretroviral treatment and avoidance of breast-feeding,[1] around 400 000 newly infected children have been born, particularly in resource-limited countries (AIDS epidemic update. UNAIDS, http://www.UNAIDS.org accessed 29 July 2008), via vertical transmission during pregnancy, delivery and breast-feeding. Among these three distinct routes, breast-feeding is still a major public health concern in developing countries. The risk of HIV-1 infection of infants via breast-feeding has been found to be influenced by breast milk virus load, which is significantly higher in early/colostrum milk than in mature breast milk.[2]

The majority of cells in colostrum milk have been identified as unique large cells, termed breast milk macrophages (BrMMø), expressing both CD4 and CD14.[3] Importantly, BrMMø also express chemokine receptors such as chemokine (C-X-C motif) receptor 4 (CXCR4) and chemokine (C-C motif) receptor 5 (CCR5), which permit HIV-1 entrance, as well as CD83, a maturation marker of dendritic cells (DCs).[4] Thus, BrMMø have been

identified as DC-lineage HIV-1-vulnerable cells and also express C-type lectin DC-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN),[5] which will tightly capture free HIV-1 virions and transmit them to HIV-1-susceptible infant CD4-positive cells.[3] Moreover, after co-culture with interleukin (IL)-4, BrMMø were found to have enhanced DC-SIGN expression,[4] and became resistant to HIV-1 infection. Therefore, IL-4-treated BrMMø will not be infected by HIV-1 but will rather capture free virus particles via DC-SIGN, and such cell-associated virions would more readily be transmitted to HIV-1-susceptible cells via breast-feeding.

Local production of IL-4 in mastitis may up-regulate the expression of DC-SIGN in BrMMø, which may explain why mastitis is linked to higher HIV load in breast milk and a higher risk of mother-to-infant vertical transmission of the virus.[6] Indeed, it has recently been reported that increased cell-associated HIV-1 but not cell-free virion shedding in breast milk could mediate the association between mastitis and MTCT.[7] In addition, we reported previously that high transmissibility was mediated through HIV-1 virions captured by DC-SIGN but not through cell-free virus particles released from HIV-1-infected cells,[3] although some reports indicate that cell-free HIV-1 in breast milk may contribute to vertical transmission.[8] Therefore, in order to prevent vertical transmission of HIV-1 through breast-feeding, it is necessary to find a way to inhibit the acquisition of free HIV-1 virions via DC-SIGN by suppressing its expression on BrMMø.

In the present study, careful examination of BrMMø revealed the apparent expression of Toll-like receptor 3 (TLR3) in freshly isolated BrMMø, although we could not detect TLR3 in peripheral blood monocytes (PBMo). However, TLR3 was detected in PBMo when they were stimulated with granulocyte–macrophage colony-stimulating factor (GM-CSF), which is spontaneously produced in BrMMø.[4] Moreover, freshly isolated TLR3-positive BrMMø also expressed DC-SIGN and the expression of TLR3 was slightly enhanced in IL-4-treated BrMMø, in which DC-SIGN expression is significantly enhanced. Thus, we attempted to stimulate TLR3 with one of its ligands, poly(I:C), which is a double-stranded RNA (dsRNA),[9,10] to investigate its effect on DC-SIGN expression, and found a reduction in DC-SIGN expression in both freshly isolated BrMMø and IL-4-treated BrMMø. Also, poly(I:C)-stimulated BrMMø secreted considerable amounts of type I interferons (IFNs), such as IFN-α and IFN-β. As expected, DC-SIGN-mediated HIV-1 transmission to susceptible CD4-positive cells by BrMMø was inhibited by TLR3 signalling with poly(I:C) or treatment of BrMMø with their products, type I IFNs. We discuss our findings in terms of this unique feature of BrMMø and propose a possible strategy for preventing MTCT of HIV-1 via breast-feeding through TLR3 signalling.

Materials and methods

Isolation and culture of BrMMø and PBMo

Breast milk was collected from healthy women within 2–6 days of delivery after informed consent had been obtained under a protocol approved by the Institutional Review Board of Nippon Medical School. Breast milk cells were isolated from freshly obtained breast milk by Ficoll-Hypaque (Amersham-Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation methods as described previously.[4] BrMMø were isolated from freshly collected breast milk cells, and allowed to adhere to polystyrene tissue culture dishes (Corning, New York, NY) for 1–2 hr at 37°. After non-adhering cells had been gently removed, adherent cells were washed with warm RPMI-1640 medium containing 2% fetal calf serum (FCS) (HyClone Laboratories, Logan, UT). The remaining adherent cells were then removed by incubation with 5 mM ethylenediaminetetraacetic acid (EDTA) for 30 min at 4° and confirmed to express homogeneous $CD14^+$ cells at approximately 95% using a FACScan (BD Biosciences, Mountain View, CA). To obtain PBMo, $CD14^+$ monocytes were isolated from peripheral blood of healthy volunteers by magnetic depletion using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) containing hapten-conjugated antibodies to CD3, CD7, CD16, CD19, CD56, CD123 and Glycopholin A and a magnetic antibody cell sorter (MACS; Miltenyi Biotec) according to the manufacturer's instructions, routinely resulting in > 90% purity of $CD14^+$ cells. The isolated monocytes were cultured in 24-well culture plates (Corning) for 6 days in RPMI-1640-based complete culture medium (CCM)[11] supplemented with 10% FCS (HyClone Laboratories), 20 mM HEPES (Invitrogen, Carlsbad, CA), 50 mM 2-mercaptoethanol (2-ME) (Sigma-Aldrich, St Louis, MO), 2 mM L-glutamine (Sigma-Aldrich) and 100 units of penicillin-streptomycin (Sigma-Aldrich), together with 100 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) and 20 ng/ml IL-4 (Biosource Intl., Camarillo, CA) to obtain immature DCs (iDCs).

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted from 3×10^5 cells of each cell preparation using the commercial RNeasy Kit (Qiagen, Hilden, Germany), and first-strand DNA was synthesized as described previously.[12] Transcripts of TLRs as well as the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified by PCR reaction. The primer sets were: GAPDH sense, 5′-GCC TCA AGA TCA TCA GCA ATG C-3′; GAPDH anti-sense, 5′-ATG CCA GTG AGC TTC CCG TTC-3′; TLR1 sense, 5′-CGC ATG GTC CAC ATG CTT T-3′; TLR1 anti-sense, 5′-GCC

ACA TCC AGG AAG GTC AGT-3′; TLR2 sense, 5′-CCC TGG GCA GTC TTG AAC ATT-3′; TLR2 anti-sense, 5′-GCC TCC GGA TTG TTA ACG TTT-3′; TLR3 sense, 5′-GGG TCC CAG CCT TAC AGA GAA-3′; TLR3 anti-sense, 5′-CTA GGT GGC CCA ACC AAG AG-3′; TLR4 sense, 5′-TGG TGT CCC AGC ACT TCA TC-3′; TLR4 anti-sense, 5′-CTG CAT ATC TAG TGC ACC ATG G-3′; TLR5 sense, 5′-TCC ACG GAA GGT TGT GAT GA-3′; TLR5 anti-sense, 5′-GAC CCA ACC ACC ACC ATG A-3′; TLR6 sense, 5′-CCT CAT GCA CCA AGC ACA TT-3′; TLR6 anti-sense, 5′-TCT GGC AGC TCT GGA AGA AA-3′; TLR7 sense, 5′-CGA ACC TCA CCC TCA CCA TTA-3′; TLR7 anti-sense, 5′-GGG ACG GCT GTG ACA TTG TTA-3′; TLR8 sense, 5′-GCC AGC GAG TCT CAC TGA ACT-3′; TLR8 anti-sense, 5′-GCC AGG GCA GCC AAC ATA-3′; TLR9 sense, 5′-TGG ACA CTC CCA GCT CTG AAG-3′; TLR9 anti-sense, 5′-TGG GAC ACT TGG CTG TGG ATG-3′. After 35 cycles of PCR reaction, the PCR products were resolved by electrophoresis in agarose gels and visualized by ethidium bromide staining using an ultraviolet (UV) light source.

BrMMø treatment with various reagents

To treat BrMMø with IL-4, cells were plated at 1×10^6 cells/ml in 10% FCS containing CCM, and cultured at 37° for 5 days in the presence of IL-4 (20 ng/ml) (Biosource Intl.). To treat BrMMø with poly(I:C) (Amersham-Pharmacia Biotech), cells were placed at 1×10^6 cells/ml on a 24-well flat-bottom tissue culture plate in CCM with or without 50 μg/ml poly(I:C). After incubating for 24 hr at 37°, poly(I:C) was washed out three times with RPMI-1640 medium. Cells were further cultured for 4 days with or without IL-4. In some experiments, after culture of BrMMø with CCM for 24 hr at 37°, IL-4 was added and the cells were further cultured for 3 days, and then poly(I:C) was added and the cells were incubated for an additional 24 hr. To treat BrMMø with IFNs, freshly isolated BrMMø (1×10^6) placed in a 24-well plate were stimulated with 100 IU/ml of either IFN-α (INTRON® A; Schering-Plough, Kenilworth, NJ) or IFN-β (Toray Medical Co. Ltd, Tokyo, Japan) together with IL-4 for 5 days.

Measurement of IFN by enzyme-linked immunosorbent assay (ELISA)

Freshly isolated BrMMø (1×10^6 cells/ml) were stimulated in a 24-well flat-bottomed tissue culture plate with either 50 μg/ml poly(I:C) or 50 μg/ml poly(I:C) plus 20 ng/ml IL-4 in 1 ml of CCM for 24 hr at 37°, and the levels of IFN-α and IFN-β in culture supernatants were measured by ELISA using human an IFN-α and IFN-β ELISA kit (PBL Interferon Source, Piscataway, NJ).

Antibodies and flow cytometry

Phycoerythrin (PE)-conjugated and unlabelled anti-human monoclonal antibodies (mAbs) to DC-SIGN (120507) for blocking experiments were purchased from R & D Systems (Minneapolis, MN). PE-conjugated isotype-matched control antibody (MOPC-21), PE-conjugated anti-human monoclonal antibody (mAb) to CD4 (RPA-T4) and fluorescein isothiocyanate (FITC)-conjugated anti-human mAb to CD14 (M5E2) were purchased from BD Biosciences.

Cells were stained with the relevant antibody on ice for 30 min in phosphate-buffered saline (PBS) with 2% FCS and 0·01 M sodium azide (PBS-based medium), washed twice, and re-suspended in PBS-based medium. Labelled cells were then analysed with a FACScan (BD Biosciences) using CELLQUEST software (BD Biosciences). Live cells were gated based on propidium iodide gating.

For intracellular staining of TLR3, cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 20 min on ice. After washing twice with Perm/Wash solution (BD Biosciences), cells were incubated with AB serum to prevent non-specific binding for 30 min and further incubated with anti-human mAb to TLR3 (TLR3.7) (eBioscience, San Diego, CA) for 30 min on ice in the dark. After washing twice, cells were incubated with PE-conjugated secondary anti-mouse immunoglobulin G (IgG) (Beckman Coulter, Fullerton, CA) for 30 min on ice in the dark and re-suspended in PBS-based medium for analysis by FACScan.

Infection of cultured BrMMø with NL(AD8) isolate

After treatment with either poly(I:C) or IFNs, BrMMø were harvested and added to a 1·5-ml micro-tube (Greiner Bio-one, Frickenhausen, Germany). Spanned cells were then incubated with the macrophage-tropic NL(AD8)[13] HIV-1 isolate, which has a multiplicity of infection (MOI) of 0·2, at $1–2 \times 10^5$ cells for 2 hr at 37°. Cells were washed three times with RPMI-1640 containing 2% FCS and used as HIV-1-loaded cells.

HIV-1 transmission assay

An an indicator cell line, Ghost X4/R5 cells,[14] kindly provided by the National Institutes of Health (NIH) AIDS Reagent Repository, were used to examine the capacity of NL(AD8)-sensitized cells to transmit that isolate. Ghost X4/R5 cells (1×10^4 cells/well) were plated in a flat-bottomed 96-well micro-plate (Corning) with Dulbecco's modified Eagle's minimal essential medium (DMEM) (Sigma-Aldrich) supplemented with 10% FCS (D-10) the day before co-culturing with target cells. After removal of the medium from each well, 5×10^3 thoroughly washed NL(AD8)-infected BrMMø were added. After incubation

for an additional 16 hr, the loaded BrMMø were removed by gentle washing with warmed D-10. After 24 hr, NL(AD8)-infected Ghost X4/R5 cells expressing green fluorescent protein (GFP) were analysed by flow cytometry to estimate transmissibility.

Results

TLR3 expression in freshly isolated BrMMø

It has previously been shown that freshly isolated BrMMø are DC-lineage cells expressing DC-SIGN, which will capture free HIV virions and transmit them to HIV-susceptible CD4-positive cells.[3] Such DC-SIGN-mediated spread of HIV appears to be the principal route of MTCT via breast milk. The magnitude of DC-SIGN expression on BrMMø is greatly enhanced when BrMMø are treated with IL-4.[4] In order to find a way to prevent MTCT of HIV via BrMMø through down-modulation of DC-SIGN, we first examined the TLR expression of BrMMø, through which they might be regulated. As demonstrated in Fig. 1a, although TLR3 expression was not detected in freshly isolated PBMo by RT-PCR analysis, TLR3 was clearly observed in both DCs and BrMMø. Also, as shown in Fig. 1b, the expression of TLR3 was slightly enhanced in BrMMø when they were treated with IL-4 at both the transcriptional level, as determined by RT-PCR [Fig. 1b, left panel; the mean fold increase (MFI) ± standard deviation (SD) was 1·5 ± 0·3], and the protein level, as determined by flow cytometry (Fig. 1b, middle panel; MFI ± SD was 1·4 ± 0·1). As in a previous study,[4] we found marked enhancement of DC-SIGN expression on IL-4-treated BrMMø (Fig. 1b, right panel; MFI ± SD was 21·9 ± 7·6). TLR3 expression among a series of TLRs

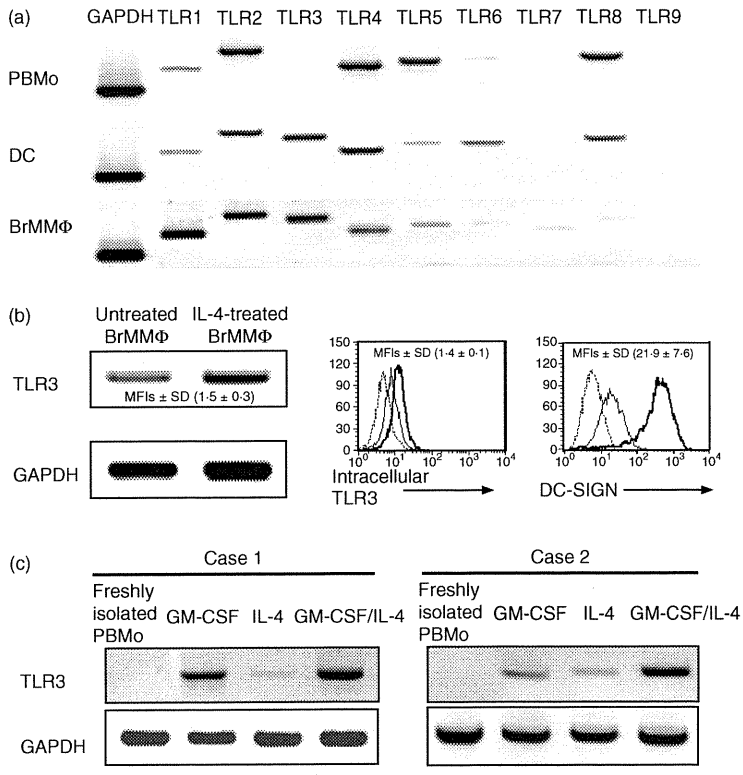


Figure 1. Analysis of Toll-like receptors (TLRs) in breast milk macrophages (BrMMø). (a) Expression of all TLRs in freshly isolated peripheral blood monocytes (PBMo), dendritic cells (DCs) generated from isolated PBMo with 20 ng/ml interleukin (IL)-4 plus 100 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF), and freshly isolated BrMMø was analysed by reverse transcription–polymerase chain reaction (RT-PCR). (b) The expression level of TLR3 in freshly isolated BrMMø was slightly increased in IL-4-treated BrMMø both at the transcriptional level, as determined by RT-PCR [the mean fold increase (MFI) ± standard deviation (SD), determined using a densitometer and the bio-imaging software ImageJ (widely provided by NIH, USA) in four separate experiments, was 1·5 ± 0·3; left panel), and at the protein level, as determined by flow cytometry (the MFI ± SD for four separate experiments was 1·4 ± 0·1; middle panel). The expression level of DC-SIGN was markedly increased in the IL-4-treated BrMMø (the MFI ± SD for four separate experiments was 21·9 ± 7·6; right panel). The dotted lines indicate isotype control, the thin line freshly isolated BrMMø, and the thick line IL-4-treated BrMMø in these panels. (c) Effect of externally added GM-CSF on TLR3 detection in PBMo. TLR3 was detected in PBMo when cells were co-cultured with 100 ng/ml GM-CSF. We used samples from four donors, and two representative cases are shown (cases 1 and 2). In case 2, it should be noted that weak expression of TLR3 was found when cells were incubated with IL-4 alone. Each experiment shown was performed for at least four donors and representative results are shown. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

seems to be a critical difference between PBMo and BrMMø.

In the light of our previous finding[4] that freshly isolated BrMMø produced GM-CSF spontaneously, but PBMo did not, and that TLR3-expressing DCs could be obtained from PBMo by GM-CSF plus IL-4 stimulation, we stimulated freshly isolated PBMo with GM-CSF to see whether they would express TLR3. As expected, TLR3 was detected in PBMo by RT-PCR analysis when cells were co-cultured with GM-CSF (Fig. 1c; cases 1 and 2). It should be noted that low levels of expression of TLR3 was sometimes found in PBMo from individual donors (e.g. case 2) when they were incubated with IL-4 alone. This could be because PBMo may produce a small amount of GM-CSF during co-culture with IL-4.[15] These results suggest that TLR3 expression in freshly isolated BrMMø may be regulated by their internal production of GM-CSF, which was not blocked by the external addition of anti-GM-CSF-specific antibodies (data not shown).

Effect of stimulation with the TLR3 ligand poly(I:C) on DC-SIGN expression on BrMMø

As shown in Fig. 1b (middle panel), the slight enhancement of TLR3 expression in IL-4-treated BrMMø with augmented DC-SIGN (right panel) was confirmed by flow cytometry. This finding may suggest a relationship between DC-SIGN and TLR3. Thus, we investigated the effect of TLR3 stimulation with a known dsRNA ligand, poly(I:C), on DC-SIGN expression on BrMMø. Because a number of freshly isolated BrMMø died after co-culture with 200 μg/ml poly(I:C) for more than 24 hr, we incubated cells with 50 μg/ml poly(I:C) for 24 hr after confirming that there was no toxic effect associated with this procedure, and used them in the following experiments after extensive washing to remove free poly(I:C).

Using two-colour staining, dot-plot analyses were performed by FACScan (Fig. 2a). In the upper left panel of Fig. 2a, the expression of both DC-SIGN and CD14 on freshly isolated BrMMø was confirmed. Dot-plot data to evaluate the pattern of CD14 and DC-SIGN on IL-4- and/or poly(I:C)-treated-BrMMø are shown in the other panels of Fig. 2a. The total incubation period for BrMMø was 5 days. The CD14 expression (indicating a macrophage lineage) observed on the freshly isolated BrMMø completely disappeared after treatment with 20 ng/ml IL-4 (upper right, lower middle and lower right panels) and decreased on cultured BrMMø. The up-regulation of DC-SIGN expression on BrMMø produced by IL-4 treatment (upper right) was significantly inhibited when freshly isolated BrMMø pretreated with 50 μg/ml poly(I:C) for 24 hr were further stimulated for an additional 4 days with IL-4 (lower middle), and was slightly inhibited when BrMMø treated with IL-4 for 4 days were incubated with 50 μg/ml poly(I:C) for the final 24 hr (lower right). Moreover, DC-SIGN expression on untreated BrMMø (upper middle) was decreased by pretreatment with 50 μg/ml poly(I:C) for 24 hr (lower left).

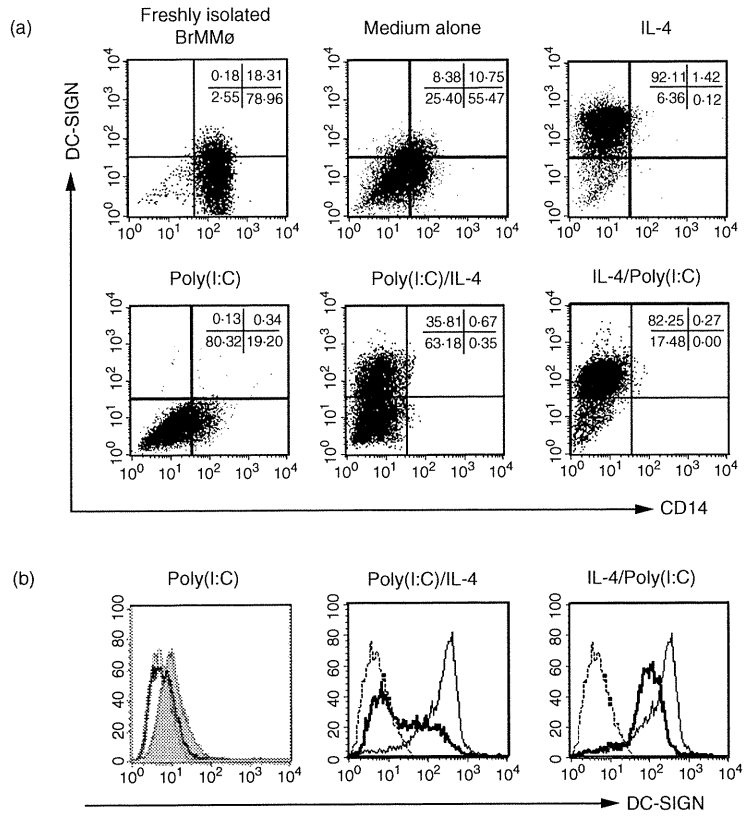
The above results were confirmed by flow cytometry analysis (Fig. 2b). It should be noted that DC-SIGN expression on BrMMø in culture medium alone [Fig. 2a, upper middle panel and Fig. 2b, left panel (shaded line)] was decreased by treatment with 50 μg/ml poly(I:C) for 24 hr [Fig. 2a, lower left panel and Fig. 2b, left panel (thick line)]. Moreover, up-regulation of DC-SIGN by IL-4 treatement was also strongly inhibited when freshly isolated BrMMø were pretreated with 50 μg/ml poly(I:C) for 24 hr and stimulated for an additional 4 days with 20 ng/ml IL-4 [Fig. 2a, lower middle panel and Fig. 2b, middle panel (thick line)]. In addition, the already enhanced DC-SIGN expression induced by IL-4 co-culture for 4 days was partially suppressed when IL-4-treated BrMMø were incubated with 50 μg/ml poly(I:C) for the final 24 hr [Fig. 2a, lower right panel and Fig. 2b, right panel (thick line)]. Thus, DC-SIGN expression on BrMMø can be down-regulated by brief TLR3 stimulation with the dsRNA poly(I:C).

Effect of poly(I:C) on the production of type I IFNs (IFN-α and IFN-β) by BrMMø

Upon dsRNA stimulation via TLR3, cells will produce and secrete type I IFNs, such as IFN-α and IFN-β. We thus investigated whether BrMMø were able to produce and secrete type I IFNs after brief exposure to the dsRNA poly(I:C). As expected, not only freshly isolated but also IL-4-treated BrMMø produced and secreted both IFN-α and IFN-β after stimulation with poly(I:C) (Fig. 3a,b). These results suggest that freshly isolated BrMMø as well as IL-4-stimulated BrMMø with high HIV-1 transmissibility will gain the capacity to produce and secrete antiviral cytokines, such as type I IFNs, upon stimulation of TLR3 with viral substances such as poly(I:C).

Effect of type I IFNs on DC-SIGN expression on BrMMø

We further examined whether type I IFNs can affect the DC-SIGN expression of IL-4-treated BrMMø with high HIV-1 transmissibility. As shown in Fig. 4a, both IFNs at non-toxic concentrations, particularly IFN-β, showed a strong capacity to suppress the enhancement of DC-SIGN expression on IL-4-treated BrMMø in a dose-dependent manner when IFNs were added together with IL-4. However, once the enhanced DC-SIGN expression on BrMMø had been established by co-culture with IL-4 for 4–5 days, IFN treatment did not affect the expression of DC-SIGN (data not shown). The finding that treatment of BrMMø with type I IFN, particularly IFN-β, seemed to reduce the enhancement of DC-SIGN expression mediated by



Cell treatment	DC-SIGN expression (MFI) (mean ± SD)	Percentage of positive cells (%) (mean ± SD)	Viability of cells after 5 days of culture (%) (mean ± SD)
Medium alone	2·9 ± 1·5	15·2 ± 4·2	87·5 ± 4·1
IL-4	47·4 ± 3·9	93·7 ± 4·7	85·5 ± 2·3
Poly(I:C)	1·7 ± 0·4	4·8 ± 4·4	85·9 ± 2·4
Poly(I:C)/IL-4	13·6 ± 1·6	30·8 ± 5·9	84·3 ± 2·6
IL-4/poly(I:C)	28·1 ± 4·6	80·8 ± 2·6	82·8 ± 0·7

Figure 2. Effect of stimulation with a known Toll-like receptor 3 (TLR3) ligand, poly(I:C), on dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN) expression of breast milk macrophages (BrMMø). (a) Dot-plot data from two-colour FACScan analysis are shown for evaluation of the patterns of DC-SIGN and CD14 expression on freshly isolated BrMMø (upper left panel), as well as on interleukin (IL)-4- and/or poly(I:C)-treated BrMMø (other panels). The total incubation period for BrMMø was 5 days. CD14 expression, indicating a macrophage lineage, observed on the freshly isolated BrMMø completely disappeared after treatment with 20 ng/ml IL-4 (upper right, lower middle and lower right panels) and that on cultured BrMMø was reduced. Up-regulation of DC-SIGN expression on BrMMø by 4 days of IL-4 treatment (upper right panel) was strongly inhibited when freshly isolated BrMMø pretreated with 50 μg/ml poly(I:C) for 24 hr were stimulated for an additional 4 days with IL-4 (lower middle panel) and slightly inhibited when BrMMø treated with IL-4 for 4 days were incubated with 50 μg/ml poly(I:C) for the final 24 hr (lower right panel). Moreover, DC-SIGN expression on untreated BrMMø was decreased by pretreatment with 50 μg/ml poly(I:C) for 24 hr (lower left panel). (b) The results presented in (a) were confirmed by flow cytometry analysis. The effect of poly(I:C) on DC-SIGN expression in BrMMø is shown in the table, which presents the mean fluorescence intensity (MFI), the percentage of positive cells, and their viability (mean ± standard deviation). Dotted lines show isotype controls and thick lines indicate poly(I:C)-treated BrMMø. The shaded line in the left panel indicates DC-SIGN expression of BrMMø cultured for 5 days with medium alone, and thin lines in the middle and right panels indicate DC-SIGN expression of BrMMø stimulated for 4 days with IL-4. Each experiment shown was carried out on samples from at least four different donors, and representative results for the same donor are shown in (a) and (b) for comparison.