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Resting CD4$^+$ T Cells with CD38$^+$CD62L$^+$ Produce Interleukin-4 Which Contributes to Enhanced Replication of T-Tropic Human Immunodeficiency Virus Type 1

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A significant increase in the CD38$^+$ population among T lymphocytes has been observed in human immunodeficiency virus type 1 (HIV-1)-infected carriers. We previously reported a higher replication rate of T-tropic HIV-1 in the CD4$^+$CD38$^+$CD62L$^+$ than CD38$^-$ subset under conditions of mitogen stimulation after infection. Here, we revealed a similarly high susceptibility in the CD38$^+$ subset on culture with conditioned medium containing Th2 cytokine, interleukin (IL)-4 that was produced endogenously from this subset on stimulation with mitogen or anti-CD3 antibody for 3 days. The contribution of IL-4 to the upregulated production of virus in the CD38$^+$ subset was confirmed by culture of this subset with recombinant human IL-4. In contrast, the rate of replication in the CD38$^-$ subset was not augmented in the conditioned medium from either subset or with IL-4. However, there were no differences in the surface expression of IL-4 receptor or HIV-1 receptors CD4 and CXCR4 between the two subsets. Thus, the CD4$^+$CD38$^+$CD62L$^+$ subset comprises a specific cell population secreting endogenous Th2 cytokine that contributes to the efficient production of T-tropic HIV-1 through upregulation at a certain stage of the viral life cycle, probably after the adsorption step.

INTRODUCTION

Disease progression after infection with human immunodeficiency virus type 1 (HIV-1) is well correlated with virus burden (Cao *et al.,* 1995; Michael *et al.,* 1995). Most of the HIV-1 in plasma is thought to be produced by newly infected CD4$^+$ T cells and there is a daily HIV-1-infected vs healthy CD4$^+$ T cell replacement war occurring in the patients, so that infected, dying cells continually are replaced (Ho *et al.,* 1995; Wei *et al.,* 1995; Coffin, 1996; Perelson *et al.,* 1996, 1997). This indicates that there may be a larger number of newly generating T cells in individuals at a more progressive stage of the disease.

The β chemokine receptor 5 (CCR5) and chemokine receptor 4 (CXCR4) are the two major coreceptors for infection by primary isolates, i.e., most of the primary isolates obtained from asymptomatic carriers are monocyte/macrophage (M)-tropic and use CCR5 (referred to as R5 virus), while most isolates from AIDS patients are T-tropic and use CXCR4 (referred to as X4 virus) (Feng *et al.,* 1996; Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Deng *et al.,* 1996; Doranz *et al.,* 1996; Dragic *et al.,* 1996; Bleul *et al.,* 1997).

Host factors have been known for some time to alter the rate of HIV-1 progression in individuals, including secretion of cytokines, e.g., proinflammatory cytokines such as tumor necrosis factor-α, interleukin (IL)-1β, and IL-6 upregulate HIV-1 replication, while transforming growth factor β and IL-10 downregulate HIV-1 replication (Poli *et al.,* 1990, 1994; Fauci, 1993; Vyakarnam *et al.,* 1990). In addition, recent studies have indicated that cytokines involved in the differentiation of immune cells also play a significant role in controlling HIV-1 replication, e.g., T-helper type 2 (Th2-type) cytokines enhance X4 HIV-1 production but also have an inhibitory effect on R5 HIV-1 production, while Th1 cytokine has completely the opposite effect on both types of HIV-1 (Salgame *et al.,* 1998; Galli *et al.,* 1998; Wang *et al.,* 1998; Suzuki *et al.,* 1999). This difference may partly correlate with differential regulation of CCR5 and CXCR4 by Th1 and Th2 cytokines in CD4$^+$ T cells, e.g., Th1 cytokines upregulate CCR5 (Patterson *et al.,* 1999) and downregulate CXCR4 (Galli *et al.,* 1998), while Th2 cytokines upregulate CXCR4 (Valentin *et al.,* 1998; Wang *et al.,* 1998; Patterson *et al.,* 1999; Suzuki *et al.,* 1999) and downregulate CCR5 (Patterson *et al.,* 1999). Furthermore, several reports have noted that *in vitro* stimulated peripheral blood mononuclear cells (PBMCs) and cloned T cells derived from HIV-1-infected individuals at early stages preferentially produce Th1-type cytokines, whereas cells derived from HIV-1-infected patients at late stages preferentially secrete Th2-type cytokines (Maggi *et al.,* 1987; Clerici *et al.,* 1993). Similarly, *in vitro* studies also showed differences

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in HIV-1 susceptibility between Th1 and Th2 subsets prepared after stimulation with Th1 and Th2 cytokines, respectively, i.e., a higher susceptibility of Th1 cells to R5 HIV-1 (Maggi *et al.,* 1994; Vyakarnam *et al.,* 1995; Suzuki *et al.,* 1999), and of Th2 cells to X4 HIV-1 (Suzuki *et al.,* 1999). Thus, Th1 and Th2 cytokines seem to play a key role in the susceptibility or productivity of R5 and X4 HIV-1, respectively.

In a previous report, we compared the replication rates of X4 and R5 HIV-1 in the resting $CD4^+$ T cells, i.e., $CD4^+$ CD38 subsets as well as $CD4^+$ naive/memory subsets (Horikoshi *et al.,* 2001). In the cultures, the cells were infected and then treated with phytohemagglutinin (PHA) for 3 days. The results showed that production of X4 HIV-1 was much higher in the $CD38^+$ than $CD38^-$ subset. On the other hand, there was no apparent difference in X4 HIV-1 production between naive and memory subsets. There were no apparent differences in CD4 and CXCR4 levels between $CD38^+$ and $CD38^-$ as well as naive and memory subsets, suggesting that the greater susceptibility of the $CD38^+$ subset to X4 HIV-1 is not related to the expression levels of HIV-1 receptor or coreceptor. In contrast, production of R5 HIV-1 was much higher in $CD38^-$ and memory than $CD38^+$ and naive subsets. This seems to be reasonable, because the $CCR5^+$ population was seen predominantly in $CD4^+CD38^-$ and memory subsets.

CD38 was reported to be expressed on T cells during the early and terminal stages of differentiation, but not during intermediate stages (Tedder *et al.,* 1984, 1985; Salazar-Gonzalez *et al.,* 1985; Dörken *et al.,* 1989), although it has been considered to be one of the T cell activation marker antigens along with CD25 and human leukocyte antigen (HLA)-DR (Janossy *et al.,* 1992; Kestens *et al.,* 1992, 1994; Mahalingam *et al.,* 1995; Yagi *et al.,* 1992). Therefore, this increase of $CD38^+$ T cell population may be due to the increase of newly generating cells in infected individuals. In our previous study, CD38 was also expressed on about half of the $CD4^+$ T cells from healthy donor-derived PBMCs, greatly different from the value of a few percentages obtained for activation marker antigens such as CD25 and HLA-DR (Horikoshi *et al.,* 2001). In addition, most of our $CD4^+CD38^+$ subset preparations also expressed CD62L and well overlapped with $CD4^+$ T cells in CD45RA expression (Horikoshi *et al.,* 2001).

In this report, we have focused on the mechanism of the increase in X4 HIV-1 productivity in $CD4^+CD38^+$ compared to $CD4^+CD38^-$ T cells. The secretion levels of Th2 cytokine after stimulation with PHA or anti-CD3 monoclonal antibody (MAb) were significantly higher in the $CD38^+$ than $CD38^-$ subset in all preparations from healthy donors. In fact, exogenously added IL-4 significantly increased the X4 HIV-1 productivity only in the $CD38^+$ subset. Thus, stimulation of the $CD4^+CD38^+$ subset with mitogen or anti-CD3 antibodies produces a specific cell population secreting Th2 cytokine that contributes to efficient replication of X4 HIV-1.

RESULTS

Higher susceptibility of the resting $CD4^+CD38^+$ than $CD4^+CD38^-$ subset to X4 HIV-1

We previously showed that the $CD38^+$ subset among $CD4^+$ T cells was more susceptible to a laboratory strain of X4 HIV-1 (LAI) than the $CD38^-$ subset (Horikoshi *et al.,* 2001). In the cultures, the CD38 subsets at resting stage were infected and then stimulated with PHA for 3 days. Therefore, we now compared the HIV-1 susceptibility between the CD38 subsets that were stimulated with PHA for 3 days before and after infection with the LAI strain.

The resting $CD4^+$ T cell fraction (99% in CD3; 95% in CD4; $<$0.5% in CD8; $<$0.1% in CD25; $<$0.5% in CD19; and $<$0.5% in CD14) prepared from the pooled blood of three healthy donors was separated into two subsets ($CD38^+$ and $CD38^-$). Both these subsets were certified to be negative ($<$0.1%) for CD25. The whole $CD4^+$ cell fraction and CD38 subsets were similarly infected with HIV-1 LAI strain. After adsorption for 1 h, the cells were incubated in the presence of PHA for 3 days. As shown in Fig. 1A, viable cell numbers for LAI infection were similar to those for mock infection. The viral production rates were significantly higher in the $CD38^+$ than $CD38^-$ subset on infection with LAI, as shown by p24 antigen-capture enzyme-linked immunosorbent assay (ELISA) of the culture media (Fig. 1A). Under the same culture conditions, similarly higher susceptibility of $CD38^+$ than $CD38^-$ subset was also confirmed by infection with X4-type primary isolates (17-3-6 and 0-4-26) (data not shown). On the other hand, there was no difference of the susceptibility to LAI infection between CD38 subsets when we infected after PHA treatment for 3 days (Fig. 1B). The same two CD38 subsets from healthy donors were pretreated with PHA for 3 days and then infected with LAI. In this case, the viable cell numbers as well as viral production rates in medium were almost the same between the $CD38^+$ and $CD38^-$ subsets.

Predetermination of the $CD38^+$ subset to secrete Th2 cytokine that contributes to efficient replication of X4 HIV-1

A clear difference in the susceptibility to X4 HIV-1 was observed in the CD38 subsets at the resting stage (Fig. 1A), but not in the cells already activated by stimulation with PHA (Fig. 1B). This may suggest that some soluble factor(s) responsible for X4 HIV-1 production was secreted from the $CD38^+$ subset during PHA treatment for 3 days. To test this hypothesis, we next examined the effect on HIV-1 production of the conditioned media from the two subsets that had been stimulated with PHA for 3

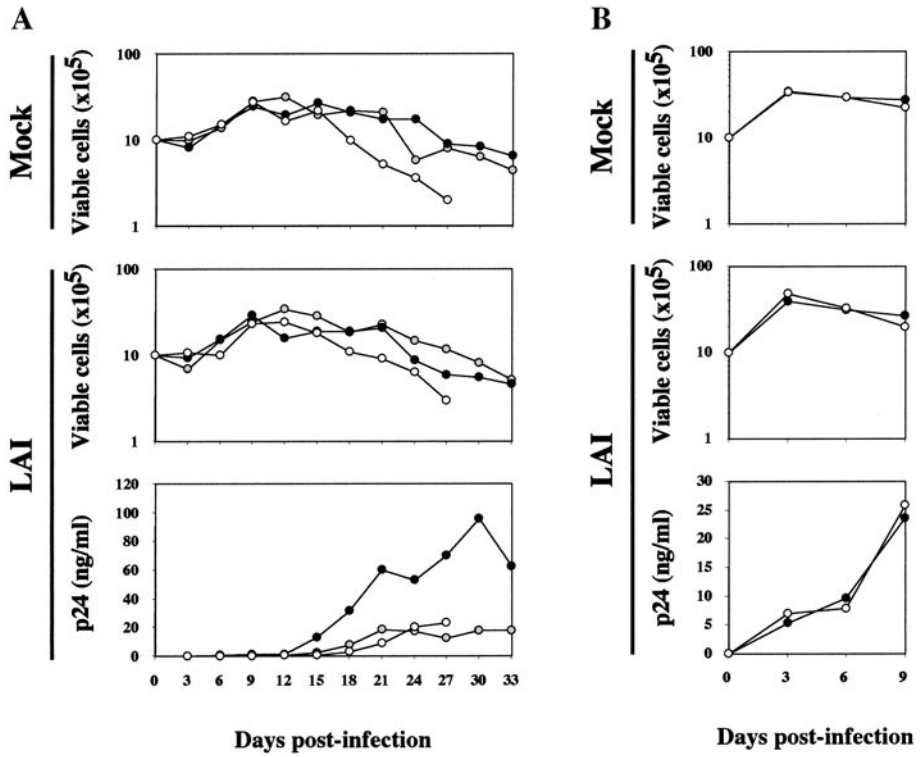

FIG. 1. Susceptibility of CD38 subsets to T-tropic HIV-1. (A) The CD38 subsets (●, $CD4^+CD38^+$; ○, $CD4^+CD38^-$) and whole $CD4^+$ T cells (◍) prepared as described under Materials and Methods were mock-infected or infected with LAI at 300 ng/ml for 1 h (corresponding to day 0 in figure) and then stimulated with PHA for 3 days. Subsequently, cultures were continued for 33 days after infection. The cells were replenished with fresh medium every 3 days. Cell density was adjusted to 1×10^6/ml at the time of the replenishment. (B) The same CD38 subsets (●, $CD4^+CD38^+$; ○, $CD4^+CD38^-$) were prestimulated with PHA for 3 days and then similarly infected with HIV-1 LAI (corresponding to day 0 in the figures). The cells were cultured for a further 9 days with replacement of the medium every 3 days. Kinetics on viable cell numbers and estimates of Gag p24 in individual culture medium are shown.

days. The subsets prepared as above were similarly adsorbed with LAI for 1 h and were then cultured as described in Fig. 1A in the presence of the conditioned media from the subsets in place of PHA. As shown in Fig. 2, an apparent increase in HIV-1 production was observed in the $CD38^+$ subset on treatment with the conditioned medium from the PHA-treated $CD38^+$ but not $CD38^-$ subset. The conditioned medium from PHA-treated $CD38^+$ cells also had only a slight effect to increase virus production in the $CD38^-$ subset. The viable cell count was almost the same between the subsets throughout the infection. Thus, some soluble factor(s) from the $CD38^+$ subset could contribute to the susceptibility of this subset to LAI infection.

There have been reports that the susceptibilities of $CD4^+$ T cells to X4 and R5 HIV-1 were significantly increased by treatment with Th2 and Th1 cytokines, respectively (Salgame *et al.,* 1998; Galli *et al.,* 1998; Wang *et al.,* 1998; Suzuki *et al.,* 1999). Therefore, we next estimated the amounts of such cytokines in the conditioned media used in Fig. 2 by ELISA. As representatives of Th1 and Th2 cytokines, we selected IFN-γ and IL-4, respectively. As summarized in Table 1, the conditioned media examined were obtained by the culture of $CD38^+$ and $CD38^-$ subsets derived from the blood of a single donor or pooled blood from two or three donors including the samples from Fig. 2. Unexceptionally, the $CD38^+$ subset secreted more IL-4 than the $CD38^-$ subset after stimulation with PHA or anti-CD3 MAb. Although the IL-4 levels were greatly variable among individual samples, there was no apparent difference between the stimulation with PHA and anti-CD3 MAb in the IL-4 secretion levels from $CD38^+$ subset. In addition, the whole $CD4^+$ T cell fraction showed intermediate values in most cases. In contrast, the $CD38^-$ subset showed a tendency to secrete larger amounts of IFN-γ in most cases (five of eight trials) than the $CD38^+$ subset, with some exceptions especially in the samples producing higher levels of this cytokine.

Further, when we examined the amounts of IL-4 in the conditioned media obtained from both subset cultures after LAI infection, which were the same as in Fig. 1A, it was found that this cytokine was secreted more efficiently from the $CD38^+$ than $CD38^-$ subset or whole $CD4^+$ T cell fraction (data not shown). In addition, it was found that IL-4 was secreted from the $CD38^+$ subset only in the first 3 days of incubation with PHA and not significantly in the conditioned media in subsequent cultures.

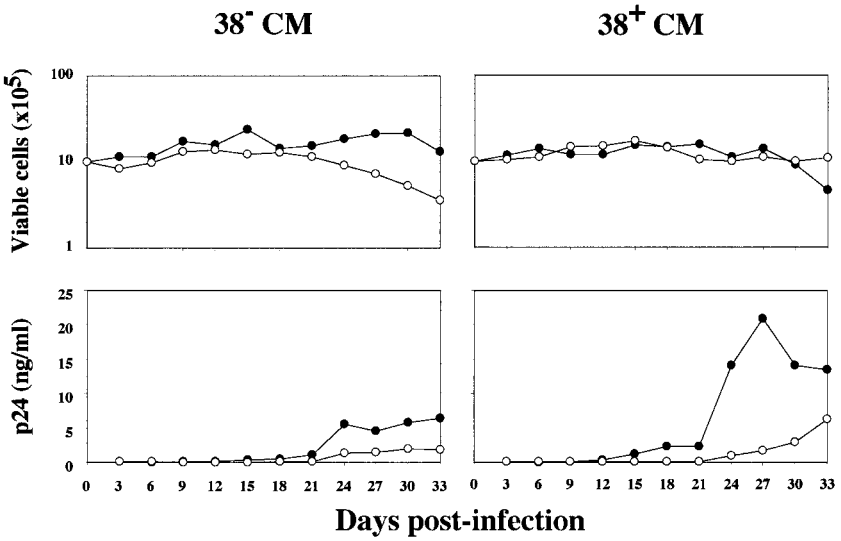

FIG. 2. Higher production of HIV-1 from the $CD4^+CD38^+$ than $CD4^+CD38^-$ subset on incubation with the conditioned medium from the PHA-stimulated $CD4^+CD38^+$ subset. CD38 subsets (●, $CD4^+CD38^+$; ○, $CD4^+CD38^-$) were infected with LAI at 300 ng/ml for 1 h and then cultured with the conditioned medium from $CD4^+CD38^+$ and $CD4^+CD38^-$ subsets (38^+ CM and 38^- CM, respectively). Subsequently, the cells were cultured until day 33 after infection with replacement of the medium every 3 days. Kinetics on viable cell numbers and estimates of Gag p24 in individual culture medium are shown.

Thus, the higher susceptibility of the $CD38^+$ subset to X4 HIV-1 could be due to the endogenous production of Th2 cytokine. To test this possibility, the HIV-1 production in this subset was further examined on addition of exogenous IL-4.

The CD38 subsets as well as whole $CD4^+$ T cell fraction prepared from the same donors were infected with LAI for 1 h. The cells were then washed, cultured at 2×10^6/ml in the presence or absence of 10 ng/ml of recombinant human IL-4 for 3 days, and cultured again without IL-4 for a further 30 days. The cell concentration was adjusted to 1×10^6/ml in fresh medium every 3 days. As shown in Fig. 3, the viable cell count was similar in all these populations until 9 days postinfection in both mock-infected and infected cultures in the absence or presence of IL-4. Thereafter, the number of viable cells gradually decreased in the $CD38^-$ subset, especially in the absence of IL-4. After LAI infection, the viable cell count gradually decreased in the $CD38^+$ subset and the whole $CD4^+$ T cell fraction even in the cultures with IL-4. HIV-1 production in the cultured medium as determined by p24 antigen-capture ELISA was not apparent in either

TABLE 1

Th1 and Th2 Cytokine Production Rates of $CD4^+CD38^+$ and $CD4^+CD38^-$ Subsets

		IL-4 production (pg/ml)			IFN-γ production (pg/ml)		
Donor	Stimulation	$CD4^+$	$CD4^+CD38^+$	$CD4^+CD38^-$	$CD4^+$	$CD4^+CD38^+$	$CD4^+CD38^-$
Single donor							
No. 1	PHA[a]	0.14	0.21	<0.1	189.03	96.37	174.15
No. 2	PHA[a]	2.05	3.27	<0.1	54.00	63.42	100.71
No. 3	PHA[a]	0.13	1.08	0.11	357.96	521.80	237.43
No. 4	anti-CD3[b]	ND[d]	16.97	1.62	ND	163.75	465.59
Two donors							
No. 1	anti-CD3[b]	0.47	4.33	2.13	2082.09	601.13	570.10
No. 2	anti-CD3[b]	ND	20.93	2.71	ND	1745.92	713.62
No. 3	anti-CD3[b]	ND	11.72	5.14	ND	242.31	943.65
Three donors							
No. 1	PHA[a,c]	0.48	1.03	0.33	103.89	22.15	66.69

[a] Cultures with IL-2 at 50 u/ml.
[b] Cultures with IL-2 at 100 u/ml.
[c] The same cell cultures used in Fig. 2.
[d] Not determined.

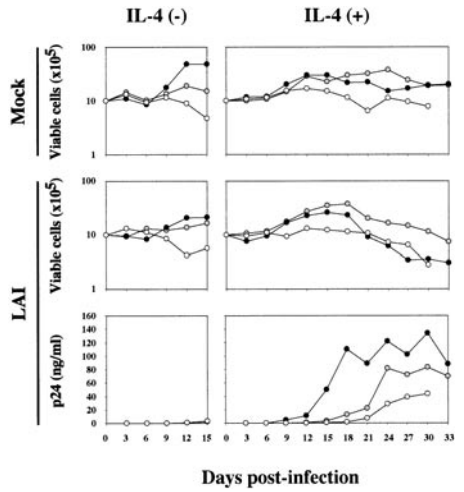

FIG. 3. Increase of HIV-1 production in the $CD4^+CD38^+$ subset on stimulation with IL-4. CD38 subsets (●, $CD4^+CD38^+$; ○, $CD4^+CD38^-$) and whole $CD4^+$ T cells (◍) were mock-infected or infected with LAI at 300 ng/ml for 1 h and then stimulated with IL-4 in place of PHA for 3 days. Subsequently, the cells were cultured until day 33 after infection with replacement of the medium every 3 days. Viable cell numbers and virus production rates are shown.

subset without IL-4, while production greatly increased in the $CD38^+$ compared to $CD38^-$ subset in the presence of IL-4. The $CD4^+$ T cell fraction produced HIV-1 at an intermediate rate. Flow cytometry of these infected subsets confirmed the higher susceptibility of $CD38^+$ than $CD38^-$ to LAI infection, as evidenced by expression of HIV-1 antigens on most of the cells from $CD38^+$ but not from $CD38^-$ which were cultured with IL-4 (Fig. 4).

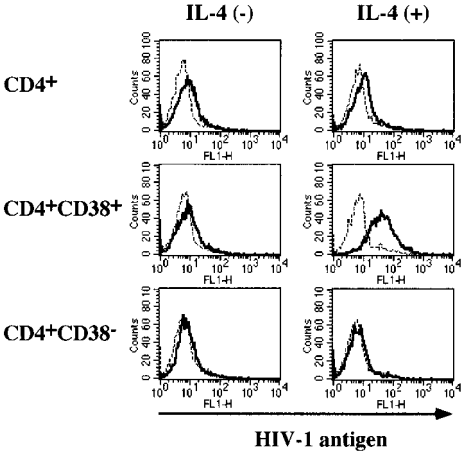

FIG. 4. Expression of HIV-1 antigens on most of the $CD4^+CD38^+$ cells after T-tropic HIV-1 infection followed by culture with IL-4. The CD38 subsets and whole $CD4^+$ T cells mock-infected (---) and infected with LAI (—) and cultured for 15 days, which were used in Fig. 3, were subjected to flow cytometry with the serum from a seropositive individual.

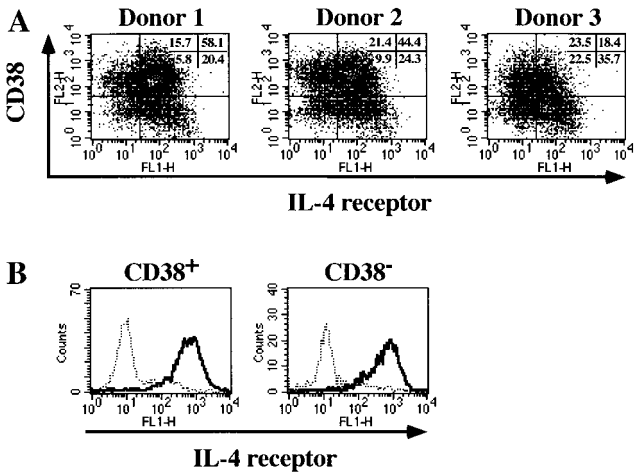

FIG. 5. No apparent difference of IL-4 receptor expression between $CD4^+CD38^+$ and $CD4^+CD38^-$ subsets. $CD4^+$ T cells from three donors were reacted with FITC-IL4 and then with PE-CD38 MAb (A). In (B), $CD4^+CD38^+$ and $CD4^+CD38^-$ subsets from a single donor were reacted with biotin-IL-4 followed by the FITC-avidine (—) and negative control reagent in the kit (---).

No apparent differences in the expression of IL-4 receptor as well as HIV-1 receptors CD4 and CXCR4 between CD38 subsets

Possible differences in the surface expression of the IL-4 receptor as well as HIV-1 receptors were examined between the two subsets to understand the mechanism behind the difference in susceptibility to X4 HIV-1. Whole $CD4^+$ T cell fractions from three donors were independently analyzed without culture for IL-4 receptor by two-color flow cytometry, i.e., fluorescein isothiocyanate (FITC)-IL-4 and phycoerythrin (PE)-CD38 MAb. The results showed that there was no apparent difference in the percentage of IL-4 receptor-positive cells between the CD38 subsets, i.e., positive for IL-4 receptor on 43–78% of $CD38^+$ and 61–78% of $CD38^-$ subset (Fig. 5A). Further, we analyzed this point by single staining of purified $CD4^+CD38^+$ and $CD4^+CD38^-$ subsets with biotin-IL-4 followed by the FITC-avidine. There was also no difference in their FITC intensity between the surfaces of purified CD38 subsets (Fig. 5B). In addition, the CD38 subsets after stimulation with PHA or IL-4 for 3 days as in Fig. 1A or Fig. 3, respectively, were subjected to flow cytometry to compare the cell surface expression levels of CD4 and CXCR4 (Fig. 6). Again, the results showed no apparent differences between the two subsets in receptor expression.

DISCUSSION

The susceptibility of the $CD4^+CD38^+$ subset to T-tropic laboratory strain LAI was shown to be much higher than that of the $CD4^+CD38^-$ subset in our previous study (Horikoshi *et al.,* 2001). This result was also confirmed by infection with primary isolates with T-tro-

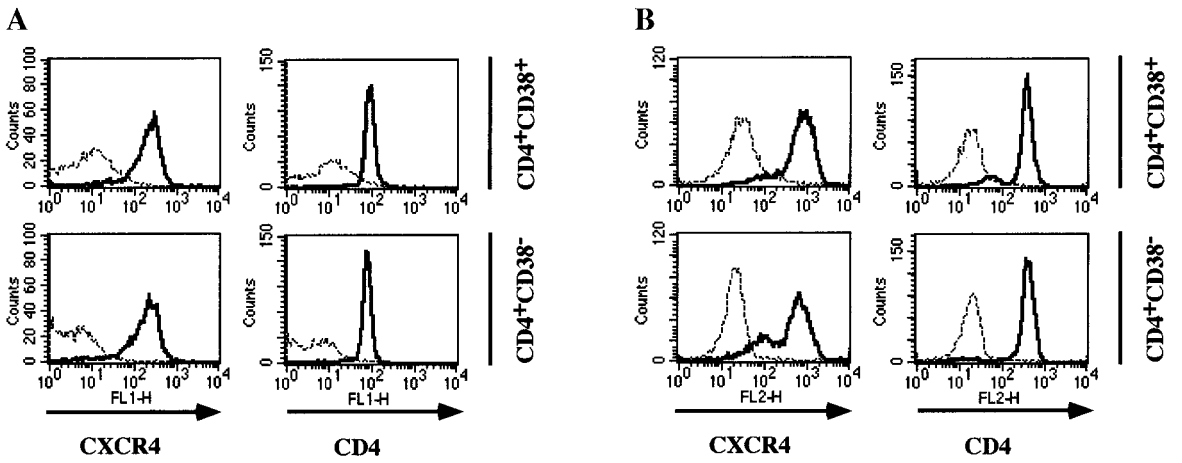

FIG. 6. No apparent difference in the surface expression levels of HIV-1 receptors CD4 and CXCR4 between CD38 subsets after PHA or IL-4 stimulation. $CD4^+CD38^+$ and $CD4^+CD38^-$ subsets after treatment with PHA (A) or IL-4 (B) for 3 days were subjected to flow cytometry with anti-CXCR4 or anti-CD4 MAb (—). The profiles with control IgG are shown by (---). Cells in A and B were stained by indirect and direct methods, respectively.

pism (data not shown). To understand the mechanism for this difference, we examined the effect of cytokines. The results clearly showed that (1) the $CD38^+$ subset is predestined to become the specific population with efficient secretion of Th2 cytokine, when the cells were stimulated with PHA or anti-CD3 MAb; and (2) Th2 cytokine endogenously secreted from the $CD38^+$ subset plays a significant role in the efficient production of X4 HIV-1 in this subset.

A difference between the two subsets in the susceptibility to X4 HIV-1 was observed in the cultures on infection followed by PHA stimulation for 3 days. However, we could not detect any difference in the susceptibility of the two subsets in such PHA-prestimulated PBMCs, where X4 HIV-1 showed more rapid replication kinetics. These results suggested the possible production of soluble factor(s) in the conditioned medium of PBMC cultures on stimulation with PHA. In fact, Th2 cytokine was apparently secreted from the $CD38^+$ subset on stimulation with PHA as well as anti-CD3 MAb. Time course analysis revealed that the production of Th2 cytokine was transient during the first 3 days of culture (data not shown). In contrast, the $CD38^-$ subset showed a tendency to produce higher levels of Th1 cytokine than the $CD38^+$ subset following stimulation in many preparations. Therefore, IL-4 in place of PHA was used as a stimulant and found to contribute to the efficient production of X4 HIV-1 in the $CD38^+$ subset. This cytokine did not significantly affect HIV-1 production in the $CD38^-$ subset. However, the slight effect of the conditioned medium from the $CD38^+$ subset on the production of X4 HIV-1 in the $CD38^-$ subset suggests the presence of an additional soluble factor(s) in the conditioned medium.

Flow cytometry showed similar expression levels of CD4 as well as CXCR4 on the surface of both subsets after PHA or IL-4 stimulation for 3 days. In fact, adsorption rates of LAI were also similar between the subsets before (Horikoshi *et al.,* 2001) and after stimulation with PHA or IL-4 (data not shown). In addition, the expression of IL-4 receptor did not differ significantly between the subsets. Thus, IL-4 cytokine may be responsible for the efficient production of HIV-1, which could be due to upregulation at a certain stage of the viral life cycle, probably after adsorption. For understanding the mechanism, we need further study to detect an IL-4-associated factor(s) responsible for this amplification only in $CD38^+$ subset.

There was a report of stronger expression of CXCR4 in the Th2 than Th1 subset (Suzuki *et al.,* 1999). In that study, the cells were used only after differentiation into Th1 or Th2 cells by stimulation with anti-CD3/IL-2/IL-12 or anti-CD3/IL-2/IL-4, respectively, long term (8 days) before HIV-1 infection. It was concluded that such Th2 and Th1 differentiated cells showed greater susceptibility to X4 and R5 HIV-1 due to a slight upregulation of CXCR4 and CCR5, respectively. On the other hand, we found a clear difference in the susceptibility to X4 HIV-1 between resting $CD38^+$ and $CD38^-$ subsets on infection before stimulation. Thus, the $CD38^+$ cells even in the resting stage comprise a specific cell population that can preferentially secrete the Th2 cytokine on stimulation with PHA or anti-CD3 MAb. Such an endogenously secreted Th2 cytokine could contribute to efficient replication of X4 HIV-1.

The presence of other factors contributing to the inhibition of HIV-1 replication, which may be secreted from the cells during PHA treatment, should also be considered. Cytokines such as IFN-γ could be candidates for the factor inhibiting HIV-1 replication, as reported (Galli *et al.,* 1998). More of IFN-γ was present in the culture of the $CD38^-$ than $CD38^+$ subset after infection followed by PHA stimulation. Since the replication rate of R5 HIV-1 was higher in the $CD38^-$ than $CD38^+$ subset (Horikoshi *et al.,* 2001), further study is necessary to clarify the role

of these cytokines in controlling the replication of X4 and R5 HIV-1s.

It has been documented that there is a daily infected vs healthy $CD4^+$ T cell replacement war occurring in the patients, so that infected, dying cells continually are replaced (Ho *et al.,* 1995; Wei *et al.,* 1995; Perelson *et al.,* 1996). Considering that the $CD4^+CD38^+$ subset showed greater susceptibility to X4 HIV-1 infection, this subset may represent newly generated $CD4^+$ T cells which can secrete Th2 cytokine and efficient infection of this subset by HIV-1 with X4 tropism can synergistically accelerate the course of HIV-1 disease.

MATERIALS AND METHODS

Viruses

Culture fluid of MOLT-4 cells persistently infected with LAI was used as the prototypic X4-type HIV-1. Two primary X4-type HIV-1 isolates were also used: 0-4-26 from a Japanese asymptomatic carrier (homosexual; $CD4^+$ cells, 322/mm^3) and 17-3-6 from a Japanese AIDS patient (hemophiliac; $CD4^+$ cells, 77/mm^3). HIV-1 particles in the conditioned media for primary isolates as well as LAI were pelleted by centrifugation at 100,000 *g* for 30 min and then suspended in RPMI 1640 supplemented with 10% fetal bovine serum, as previously described (Horikoshi *et al.,* 2001).

Preparation of normal peripheral blood-derived T cell subsets

Isolation of the whole $CD4^+$ T cell fraction from peripheral blood was performed by depletion of non-$CD4^+$ T cells. First, PBMCs were obtained by centrifugation over Ficoll–Paque (Pharmacia Biotech, Uppsala, Sweden). For depletion of B cells, monocytes, natural killer cells, $CD8^+$ T cells, dendritic cells, early erythroid cells, platelets, and basophils from PBMCs, such cells in the PBMCs were indirectly magnetically labeled using a cocktail of hapten-conjugated CD8, CD11b, CD16, CD19, CD36, and CD56 antibodies and MACS Microbeads coupled to an anti-hapten MAb (Miltenyi Biotec, Gladbach, Germany). The magnetically labeled cells are depleted by retaining them on a MACS column in the magnetic field of a MACS separator (Miltenyi Biotec). Subsequently, to obtain resting T cells, cells were labeled with CD25 MAb (anti-IL-2R; Becton-Dickinson Immunocytometry Systems) and magnetically labeled with goat anti-mouse IgG Microbeads (Miltenyi Biotec) and separated on a column, as above. The resting $CD4^+$ T cell fraction was further separated into two T cell subsets ($CD38^+$ and $CD38^-$) by panning with a MAb to CD38 (Leu-17; Becton-Dickinson Immunocytometry Systems) followed by addition of goat anti-mouse IgG Microbeads (Miltenyi Biotec). These cells were simulated with PHA at 2 μg/ml or IL-4 at 10 ng/ml before or after HIV-1 infection, as described below. Also, the cells were stimulated with PHA at 2 μg/ml or with anti-CD3 by culturing the cells in microplates for measuring cytokine production in individual subsets. For the stimulation with anti-CD3, microplates, coated with anti-CD3 MAb (PharMingen) at 10 μg/ml (Baroja *et al.,* 1989) for 12 h, followed by washing three times, were used.

HIV-1 infection

HIV-1 infection studies were performed by three protocols. First, HIV-1 infection of PBMC-derived resting $CD4^+$ T cell subsets prepared as above was performed by adsorption with HIV-1 for 1 h at 37°C. After extensive washing, cells at 2×10^6/ml were cultured in the presence of 2 μg/ml PHA and 50 u/ml IL-2 for 3 days. Second, the same PBMC-derived resting $CD4^+$ T cell subsets were pretreated with PHA at 2 μg/ml for 3 days. These stimulated T cell subsets were similarly infected with HIV-1 and cultured at 1×10^6/ml in medium containing 50 u/ml IL-2. Third, in place of PHA stimulation, the resting T cell subsets after HIV-1 infection as above were also treated with the conditioned medium of the T cell cultures stimulated with 2 μg/ml PHA for 3 days or 10 ng/ml IL-4 and cultured at 1×10^6/ml in medium containing 50 u/ml IL-2.

These cells were adjusted to 1×10^6/ml every 3 days and further cultured in medium containing 50 u/ml of IL-2. Viable cells were enumerated by the trypan blue exclusion assay.

Evaluation of T cell subsets for cell susceptibility to HIV-1 infection

HIV-1 susceptibility was determined from levels of HIV-1 production in conditioned media sequentially obtained from infected cells and from levels of HIV-1 antigen expression on the surface of infected cells at a late stage of infection. Virus production was measured by HIV-1 p24 antigen-capture ELISA (ZeptoMetrix Corp., Buffalo, NY). Viral antigen expression on the infected cells was measured by flow cytometry using the serum from an HIV-1-seropositive individual, as described below.

ELISA for cytokine production levels

The concentrations of IL-4 and IFN-γ in the culture supernatants from T cell subsets were measured with an ELISA kit (high-sensitivity interleukin-4 human, ELISA system and high-sensitivity interferon-gamma human, ELISA system, respectively; Amersham Pharmacia Biotech, Uppsala, Sweden).

Flow cytometry

For single cell staining by indirect flow cytometry, mock- or HIV-1-infected cells were incubated for 30 min

at 4°C with a 500-fold dilution (immunofluorescence titer, 1:4000) of serum from an HIV-1-seropositive individual as the polyclonal anti-HIV-1. In negative controls, the same amount of serum (500-fold dilution) from one seronegative healthy donor was used. Cells were washed with phosphate-buffered saline (PBS) and then further reacted with FITC-conjugated rabbit anti-human IgG (DAKO). Also, the cells were reacted with anti-CD4 MAb (Becton-Dickinson Immunocytometry Systems) or anti-CXCR4 MAb (PharMingen) and stained with FITC-conjugated rabbit anti-mouse IgG (DAKO). In addition, the following antibodies were used for direct flow cytometry: PE-conjugated MAbs to CD4 (Becton-Dickinson Immunocytometry Systems) and to CXCR4 (PharMingen). In negative controls for direct flow cytometry, the same amounts of PE-conjugated normal mouse IgG_1 (Becton-Dickinson Immunocytometry Systems) and IgG_{2a} (PharMingen) were used.

To analyze the IL-4 receptor expression, cells were reacted with biotin-IL-4, followed by the FITC-avidine in the IL-4 receptor detection kit (Biotinylated Human IL-4; Genzyme-Techne). In double staining, cells were first reacted with FITC-IL-4 as above and then with PE-conjugated anti-CD38 MAb (Becton-Dickinson Immunocytometry Systems).

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