Down-regulation of CXCR4 expression on human CD8$^+$ T cells during peripheral differentiation

Naoki Kobayashi[1,2], Hiroshi Takata[1], Shumpei Yokota[2] and Masafumi Takiguchi[1]

[1] Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, Japan
[2] Department of Pediatrics, Yokohama City University, Yokohama, Japan

Multi-color flow cytometric analysis on human CD8$^+$ T cell subsets revealed that CXCR4 is predominantly expressed on CD8$^+$ T cells with the naive CD27$^+$CD28$^+$CD45RA$^+$ phenotype, and is down-regulated during differentiation into those with an effector phenotype. The down-regulation of CXCR4 expression during peripheral differentiation was supported by the fact that the expression of CXCR4 on CD8$^+$ T cells was negatively correlated with that of perforin. The analysis of CCR5, CCR7, and CXCR4 co-expression further showed that CD8$^+$ T cells expressing a high level of CXCR4 are CCR7$^+$CCR5$^-$ naive or central memory subsets, and those expressing a low level of CXCR4 were included in the CCR7$^-$CCR5$^{+/-}$ memory/effector and effector subsets. Epstein Barr virus-specific CD8$^+$ T cells, which mostly express the memory phenotype, expressed CXCR4, while human cytomegalovirus-specific CD8$^+$ T cells, which mostly express the effector phenotype, partially expressed this receptor, showing that the expression of CXCR4 is also down-regulated during differentiation of viral antigen-specific CD8$^+$ T cells. The classification of human CD8$^+$ T cells based on the expression of these chemokine receptors should prove useful for studies that clarify the differentiation of human CD8$^+$ T cells.

Received	20/8/04
Accepted	9/10/04

Key words: CXCR4 / CD8$^+$ T cells / Naive T cells / Memory T cells

1 Introduction

CD8$^+$ T cells play an important role in viral eradication through their ability to produce various factors to suppress viral replication and to kill virus-infected cells [1–3]. Effector CD8$^+$ T cells have the ability to kill target cells through perforin, granzyme, and the Fas ligands [4, 5]. On the other hand, memory CD8$^+$ T cells can proliferate and produce cytokines such as IL-2 and IFN-γ in response to antigen stimulation, although they have no ability to directly kill target cells [6–8]. Phenotypic classification of memory and effector CD8$^+$ T cells has proven to be very useful in mouse and human immunological studies. In humans, the particular expression patterns of the costimulatory molecules CD27 and CD28 as well as CD45RA or CD45RO are associated with naive, memory and effector function of CD8$^+$ T cells [7, 9–15]. Indeed, effector and memory/effector CD8$^+$ T cells that were classified by the phenotypes of CD27$^-$CD28$^-$CD45RA$^{+/-}$ and CD27^{low}CD28$^-$CD45RA$^{+/-}$, respectively, possess cytotoxic activity and the ability to produce cytokines [6, 7, 14, 15].

[DOI 10.1002/eji.200425587]

Chemokine receptor signaling induces functional effects such as migration, rolling, sticking, the invasion and proliferation of granulocytes, monocytes and lymphocytes as well as increasing the intracellular calcium concentration in these cells [16–20]. Chemokine receptors are also useful as surface markers to discriminate naive, memory and effector subsets in human CD8$^+$ T cells. CCR7 is expressed on naive and memory CD8$^+$ T cells as a homing receptor to secondary lymphoid tissues [7, 15, 21, 22]. CCR5 is expressed on memory and memory/effector CD8$^+$ T cells and decreases during differentiation from memory to effector CD8$^+$ T cells; CD27$^+$CD28$^+$CD45RA$^-$ → CD27$^+$CD28$^-$CD45RA$^-$ → CD27$^-$CD28$^-$CD45RA$^-$ [7, 15, 23]. A recent study demonstrated that CXCR1 is expressed on effector and effector/memory CD8$^+$ T cells with phenotypes of CD27$^-$CD28$^-$CD45RA$^{+/-}$ and CD27^{low}CD28$^-$CD45RA$^{+/-}$, respectively, and that the expression of CXCR1 correlates with that of perforin [24].

CXCR4 is known to be a co-receptor for the entry of T-tropic HIV-1 into target cells [25]. Therefore, the expression of CXCR4 has been mostly studied in CD4$^+$ T cells. It is predominantly expressed on naive subsets of CD4$^+$ T cells [17, 26]. A previous study revealed that CXCR4 is expressed in both resting and activated human

CD8$^+$ T cells, and that the responsiveness for SDF-1 is diminished according to the following order: CD45RO$^-$CD62L$^+$ > CD45RO$^+$CD62L$^{+/-}$ > CD45RO$^-$CD62L$^-$ subsets [17], implying that CXCR4 is predominantly expressed on naive CD8$^+$ T cells. However, the expression of this receptor on human CD8$^+$ T cells has not yet been investigated sufficiently.

In the present study, the expression of CXCR4 on CD8$^+$ T cell subsets was analyzed using three surface markers, CD27, CD28 and CD45RA. In addition, we investigated the correlation of the expression of this receptor with that of perforin, CXCR1, CCR7, and CCR5, and finally analyzed the expression of CXCR4 on EBV-specific and human (H) CMV-specific CD8$^+$ T cells using HLA-class I-peptide tetrameric complexes (tetramers).

2 Results

2.1 Surface expression of CXCR4 on CD8$^+$ T cells

To analyze the expression of CXCR4 on total CD8$^+$ T cells, PBMC from healthy individuals were stained with anti-CD8, anti-CD3 and anti-CXCR4 mAb. The CXCR4 expression on CD3$^+$CD8$^+$ subsets was measured by flow cytometry. A representative result is shown in Fig. 1. Approximately 70% of CD8$^+$ T cells expressed CXCR4. The results from five healthy individuals showed that

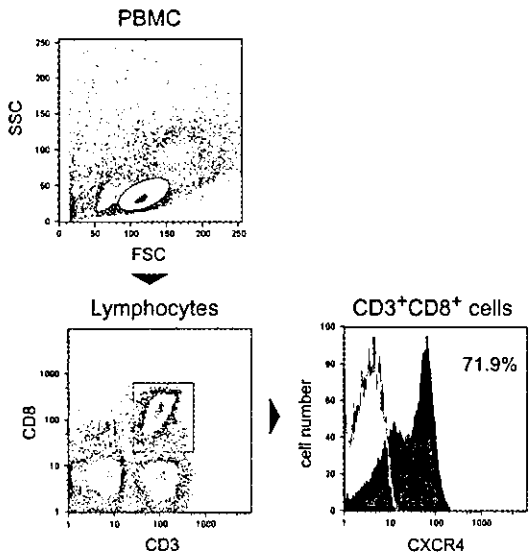

Fig. 1. Surface expression of CXCR4 on CD8$^+$ T cells. PBMC from a healthy donor, U-13, were stained with anti-CXCR4, anti-CD3 and anti-CD8 mAb. The CD3$^+$CD8$^+$ subset was gated, and then the surface expression of CXCR4 was analyzed by flow cytometry.

67–85% of CD8$^+$ T cells expressed CXCR4 (data not shown), suggesting that certain CD8$^+$ T cell populations may express CXCR4.

A recent study showed that CD8$^+$ T cells can be classified by the expression pattern of three cell surface markers, CD27, CD28 and CD45RA, as follows; naive cells: CD27$^+$CD28$^+$CD45RA$^+$, memory cells: CD27$^+$CD28$^+$-CD45RA$^-$, memory/effector cells: CD27^{low}CD28$^-$C-D45RA$^{+/-}$, and effector cells: CD27$^-$CD28$^-$CD45RA$^{+/-}$ [7]. To identify the populations expressing CXCR4, the surface CXCR4 expression on each CD27CD28CD45RA subset of CD8$^+$ T cells was investigated. CD8$^+$ T cells were isolated from eight healthy individuals, and then expression of CXCR4 was analyzed by four-color flow cytometric analysis with anti-CXCR4, anti-CD45RA, anti-CD27, and anti-CD28 mAb. A representative result is shown in Fig. 2A. The CXCR4$^+$ cells were predominantly found in the CD27$^+$CD28$^+$CD45RA$^+$ subset (>90%). The frequency of CXCR4$^+$ cells decreased according to the following order: CD27$^+$CD28$^+$CD45RA$^-$ → CD27^{low}-CD28$^-$CD45RA$^{+/-}$ → CD27$^-$CD28$^-$CD45RA$^{+/-}$ subsets. This result was confirmed by analysis of eight healthy individuals (Fig. 2B). These results suggest that CXCR4 is highly expressed on the naive subsets and that its surface expression is down-regulated during differentiation from memory to effector subsets.

2.2 Correlation between the expression of CXCR4 and perforin or CXCR1 in CD8$^+$ T cells

It is known that perforin is expressed in effector and memory/effector CD8$^+$ T cells [6, 14, 15]. Since CXCR4$^+$ cells were predominantly detected in CD8$^+$ T cells with naive and memory phenotypes, the assumption is that the expression of CXCR4 is negatively correlated with that of perforin. We investigated the correlation between the expression of CXCR4 and perforin on CD8$^+$ T cells from four healthy individuals. CD8$^+$ T cells were classified into three groups; CXCR4^{high}perforin$^-$, CXCR4^{low}perforin^{low}, and CXCR4$^-$perforin^{high} (Fig. 3A). These data indicate that the expression of CXCR4 is indeed negatively correlated with that of that of perforin.

Our recent study demonstrated that the surface expression of CXCR1 is positively correlated with that of perforin [24]. These findings suggest that the expression of CXCR4 correlates negatively with the expression of CXCR1. We therefore investigated the correlation between the CXCR4 and CXCR1 expressions on CD8$^+$ T cells from four healthy individuals (Fig. 3B). Most CXCR1$^-$CD8$^+$ T cells expressed a higher level of CXCR4, while a small population of CXCR1$^-$CD8$^+$ T cells expressed a lower level of or no CXCR4. In contrast,

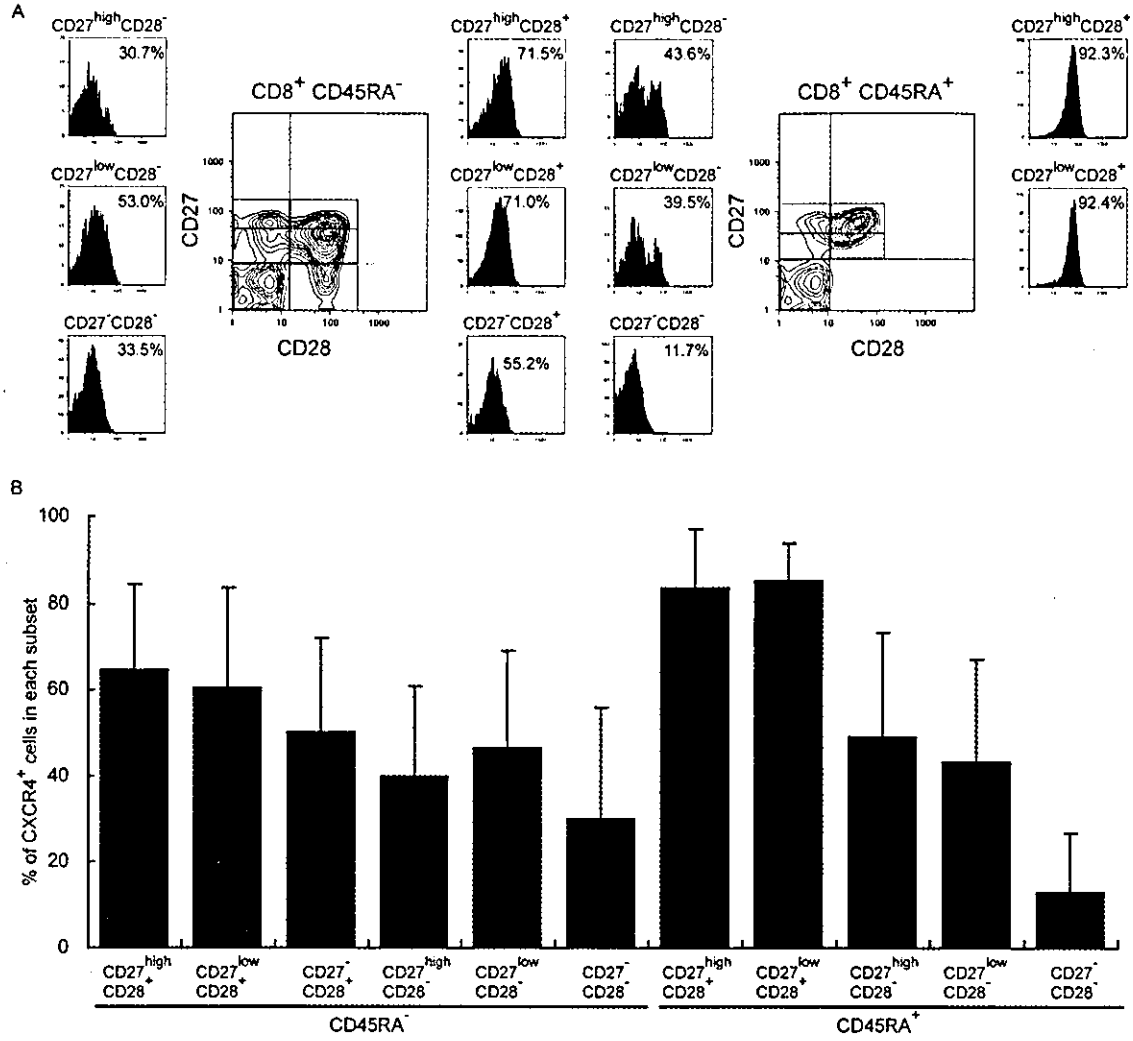

Fig. 2. Surface expression of CXCR4 on CD27CD28CD45RA subsets of $CD8^+$ T cells. (A) The frequency of $CXCR4^+$ cells in each CD27CD28CD45RA subset of $CD8^+$ T cells. $CD8^+$ T cells were isolated from one individual, U-13, and then stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CXCR4 mAb. CD27CD28CD45RA subsets were gated and then the expression of CXCR4 on each subset was analyzed. The percentage of $CXCR4^+$ cells in each subset is shown in each plot. (B) The frequency of $CXCR4^+$ cells in each CD27CD28CD45RA subset of $CD8^+$ T cells from eight individuals. $CD8^+$ T cells were isolated from eight individuals and then stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CXCR4 mAb. The mean percentage and SD of $CXCR4^+$ cells in each subset are shown.

$CXCR1^+CD8^+$ T cells expressed a low level of, or no, CXCR4. These findings indicate a reduced expression of CXCR4 on $CXCR1^+CD8^+$ T cells, but a considerable number of $CD8^+$ T cells lose both receptors.

2.3 The correlation between the expression of CXCR4 and CCR7 or CCR5 on $CD8^+$ T cells

CCR7 is expressed on naive $CD8^+$ T cells and a portion of memory $CD8^+$ T cells [7, 15, 21, 22], while CCR5 is predominantly expressed on memory $CD8^+$ T cells and a some effector $CD8^+$ T cells [15, 23, 27]. These observations suggest that the expression of CXCR4 is positively correlated with that of CCR7, but is not correlated with that of CCR5. To clarify the correlation of expression between CXCR4 and these two receptors, we investigated the co-expression of CXCR4 and CCR7 or CCR5 on $CD8^+$ T cells from healthy individuals. $CCR7^+CD8^+$ T cells expressed a high level of CXCR4 while $CCR7^-CD8^+$ T cells include both populations that express a low level of CXCR4 and no CXCR4 (Fig. 4A). These results show that CXCR4 is highly expressed on $CCR7^+CD8^+$ T cells, including naive and central memory $CD8^+$ T cells. On the other hand, analysis of $CD8^+$ T cells using anti-CXCR4 and CCR5 mAb showed that they

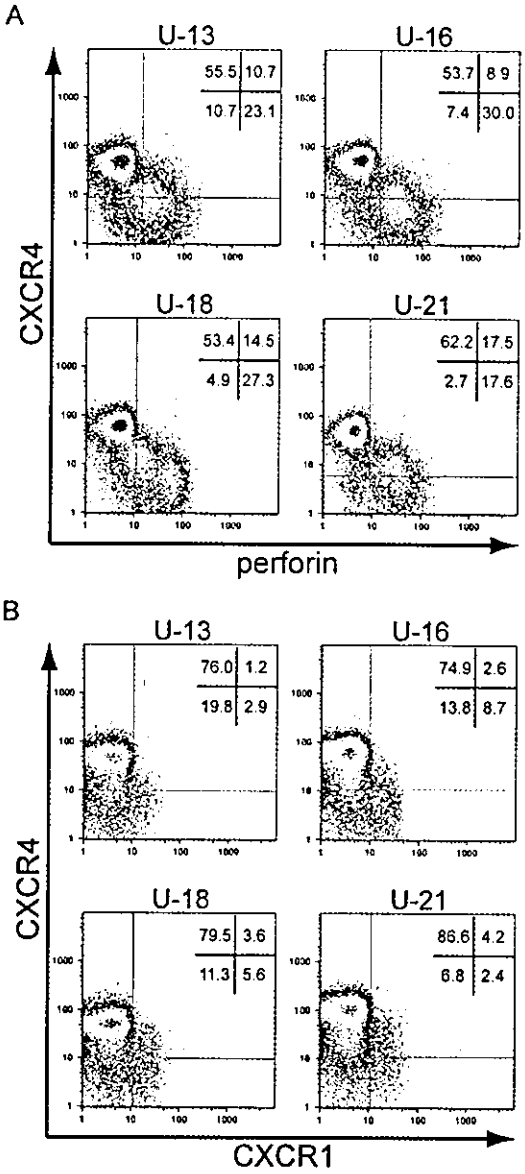

Fig. 3. Expression of perforin or CXCR1 on/in CXCR4$^{+/-}$ CD8$^+$ T cells. (A) PBMC from four individuals were stained with anti-CD8, anti-CD3, anti-perforin and anti-CXCR4 mAb, or mouse IgG mAb as an isotype control. The CD3$^+$CD8$^+$ subset was gated and then analyzed for the expression of CXCR4 and perforin. The percentage of each CXCR4 perforin subset in CD3$^+$CD8$^+$ cells is shown in each plot. (B) PBMC from four individuals were stained with anti-CD8, anti-CD3, anti-CXCR1 and anti-CXCR4 mAb, or mouse IgG mAb as isotype controls. The CD3$^+$CD8$^+$ subset was gated and then analyzed for the expression of CXCR4 and CXCR1. The percentage of each CXCR4CXCR1 subset in CD3$^+$CD8$^+$ cells is shown in each plot.

include three populations, CCR5$^-$CXCR4^{high}, CCR5$^+$-CXCR4$^{low/-}$, and CCR5$^{low/-}$CXCR4$^-$ (Fig. 4B). We hypothesized that the CCR5$^-$CXCR4^{high} population includes CCR7$^+$ naive and central memory CD8$^+$ T cells, because they express high level of CXCR4 but not CCR5 [7, 15, 27], and that other CCR5CXCR4 populations include both memory/effector and effector T cells. To investigate these CCR7/CXCR4 and CCR5/CXCR4 populations in detail, we directly analyzed the expression of these three receptors on CD8$^+$ T cells from the same individual using three mAb specific for CXCR4, CCR5, and CCR7. A representative result is shown in Fig. 4C. CD8$^+$ T cells were found to include five populations; CCR7$^+$CCR5$^-$CXCR4^{high}, CCR7$^-$CCR5$^+$CXCR4^{low}, CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$. CCR7$^+$CCR5$^-$CXCR4^{high} is the dominant population, and is found in half of total CD8$^+$ T cells, while each other population is found in approximately only 10% of total CD8$^+$ T cells. The same results were found in CD8$^+$ T cells from three other individuals (data not shown). The CCR7$^+$CCR5$^-$CXCR4^{high} population includes naive and a portion of memory cells, while the other populations were found to include the following populations; a CCR7$^-$CCR5$^+$CXCR4^{low} population that includes memory and memory/effector cells, and CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$ populations that include memory/effector and effector cells.

2.4 Surface expression of CXCR4 on EBV-specific and HCMV-specific CD8$^+$ T cells

The expression of CXCR4 on EBV- and HCMV-specific CD8$^+$ T cells was investigated. Most EBV-specific CD8$^+$ T cells express the CD27$^+$CD28$^+$CD45RA$^-$ memory phenotype [7, 28–30], while HCMV-specific CD8$^+$ T cells have CD27$^-$CD28$^-$CD45RA$^{+/-}$ effector phenotype or CD27^{low}CD28$^-$CD45RA$^{+/-}$ memory/effector phenotype [11, 15, 30]. Therefore, it is hypothesized that the former cells for the most part express CXCR4, while the latter do in part. We examined the CXCR4 expression on EBV-specific and HCMV-specific CD8$^+$ T cells using HLA-A*1101- and HLA-A*0206-tetramers, respectively. PBMC from HLA-A*0206$^+$ or HLA-A*1101$^+$ healthy individuals were stained with the combination of anti-CD8, anti-CXCR4 mAb and the tetramer, and with that of anti-CD8, anti-CD27, anti-CD28, anti-CD45RA mAb and the tetramer. All EBV-specific CD8$^+$ T cells, which mostly have the CD27$^+$CD28$^+$CD45RA$^-$ phenotype, expressed CXCR4 (Fig. 5A). On the other hand, approximately 35–43% of HCMV-specific CD8$^+$ T cells did not express CXCR4 (Fig. 5B). Approximately 40–50% of HCMV-specific CD8$^+$ T cells displayed a CD27$^-$CD28$^-$CD45RA$^{+/-}$ effector phenotype, which is in agreement with the percentage of CXCR4-negative cells in the HCMV-specific CD8$^+$ T cells.

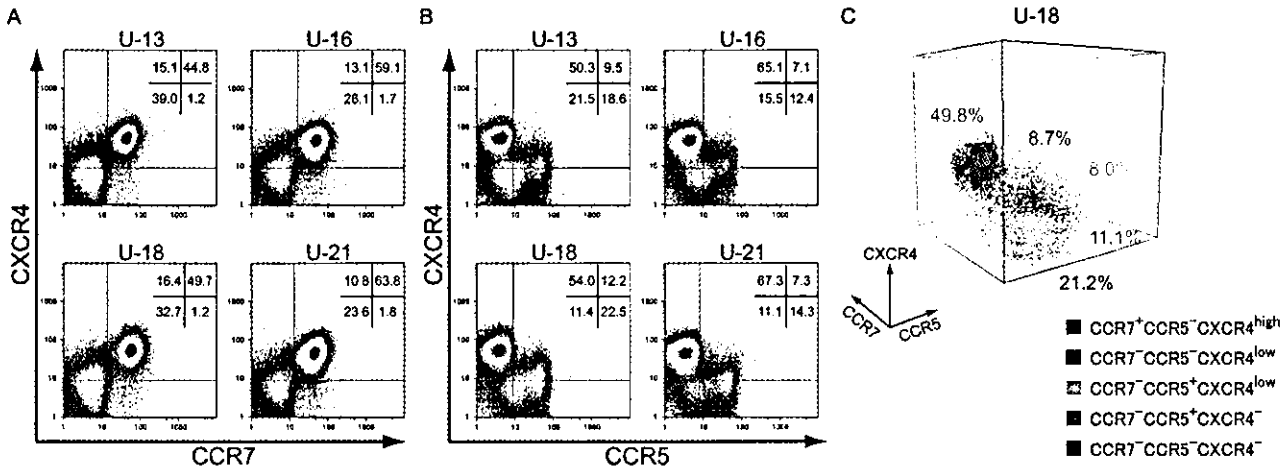

Fig. 4. Surface expression of CCR7 or CCR5 on CXCR4$^{+/-}$ CD8$^+$ T cells. (A) PBMC from four individuals (the same individuals analyzed in Fig. 3) were stained with anti-CD8, anti-CCR7 and anti-CXCR4 mAb, or mouse IgG mAb as negative control. The CD8$^+$ subset was gated and then analyzed for the expression of CXCR4 and CCR7. The percentage of each CXCR4CCR7 subset in CD8$^+$ cells is shown in each plot. (B) PBMC from the same four individuals were stained with anti-CD8, anti-CCR5 and anti-CXCR4 mAb, or mouse IgG mAb as negative control. The CD8$^+$ subset was gated and then analyzed for the expression of CXCR4 and CCR5. The percentage of each CXCR4CCR5 subset in CD8$^+$ cells is shown in each plot. (C) The co-expression of CXCR4, CCR5 and CCR7 on CD8$^+$ cells was examined using anti-CD8, anti-CCR5, anti-CCR7 and anti-CXCR4 mAb, or mouse IgG mAb as negative control. The CD8$^+$ subset was gated and then analyzed for the expression of CXCR4, CCR5 and CCR7 on CD8$^+$ cells using Paint-A-Gate PRO^{TM} software. The percentage of each subset in CD8$^+$ cells is shown.

3 Discussion

The present study demonstrated that CXCR4 is predominantly expressed on CD8$^+$ T cells of the naive and memory CD27CD28CD45RA phenotypes, while its expression is down-regulated during differentiation from CD8$^+$ T cells with the memory phenotype to those with the effector phenotype. In addition, the expression of CXCR4 was negatively correlated with that of perforin. These results indicate that the expression of CXCR4 is down-regulated during the differentiation to effector CD8$^+$ T cells. However, the precise functional role of this receptor on naive and memory CD8$^+$ T cells still remains unknown. A recent study demonstrated that central memory CD8$^+$ T cells in *plt/plt* mice that do not express CCR7 ligands in secondary lymphoid organs have the ability of rolling and sticking in the high endothelial venules (HEVs) of subiliac lymph nodes, and this sticking could be blocked by an anti-CXCL12 mAb, suggesting that CXCR4 on central memory CD8$^+$ T cells is involved in at least some of these functions [31]. CD45RO$^-$CD62L$^+$ naive and CD45RO$^+$CD62L$^{+/-}$ memory CD8$^+$ T cell subsets showed increased calcium flux in response to SDF-1 after stimulation with anti-CD3 antibodies, but unstiumlated CD8$^+$ T cells including these subsets did not respond to SDF-1 [17], suggesting that CXCR4 may effectively function on memory and memory/effector CD8$^+$ T cells expressing CXCR4 at the moment or just after these cells recognize antigens. Thus, these studies provide some evidence concerning the role of CXCR4 on CD8$^+$ T cells expressing this receptor. However, the precise functional role of CXCR4 on CD8$^+$ T cells still remains unclear.

The analysis using three chemokine receptors, CCR5, CCR7, and CXCR4, makes it evident that CD8$^+$ T cells consist of at least five populations: CCR7$^+$CCR5$^-$CXCR4^{high}, CCR7$^-$CCR5$^+$CXCR4^{low}, CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$. CCR7$^+$CCR5$^-$CD8$^+$ T cells, which are naive and central memory cells, express a high level of CXCR4. CD27$^+$CD28$^+$CD45RA$^+$ naive T cells are CCR7$^+$CCR5$^-$, while CD27$^+$CD28$^+$CD45RA$^-$ memory T cells include three CCR7CCR5 populations, CCR7$^+$CCR5$^-$, CCR7$^-$CCR5$^+$, and CCR7$^-$CCR5$^-$ (our unpublished observations). Since CD27$^+$CD28$^+$CD45RA$^-$ memory T cells with CCR7$^+$CCR5$^-$ are thought to be less mature cells than those with other CCR7CCR5 phenotypes, it is likely that CCR7$^+$CCR5$^-$CD8$^+$ T cells are naive and central memory cells. Thus, the finding that CCR7$^+$CCR5$^-$CD8$^+$ T cells express a high level of CXCR4 indicates that CXCR4 is highly expressed on naive and central memory CD8$^+$ T cells. CCR7$^-$CD8$^+$ T cells are mature memory (memory/effector) or effector cells. In addition, CCR5 is dominantly expressed on memory CD8$^+$ T cells with the phenotype CD27$^+$CD28$^+$CD45RA$^-$, and its expression is down-regulated during the maturation into effector cells [7, 15, 27]. These findings suggest

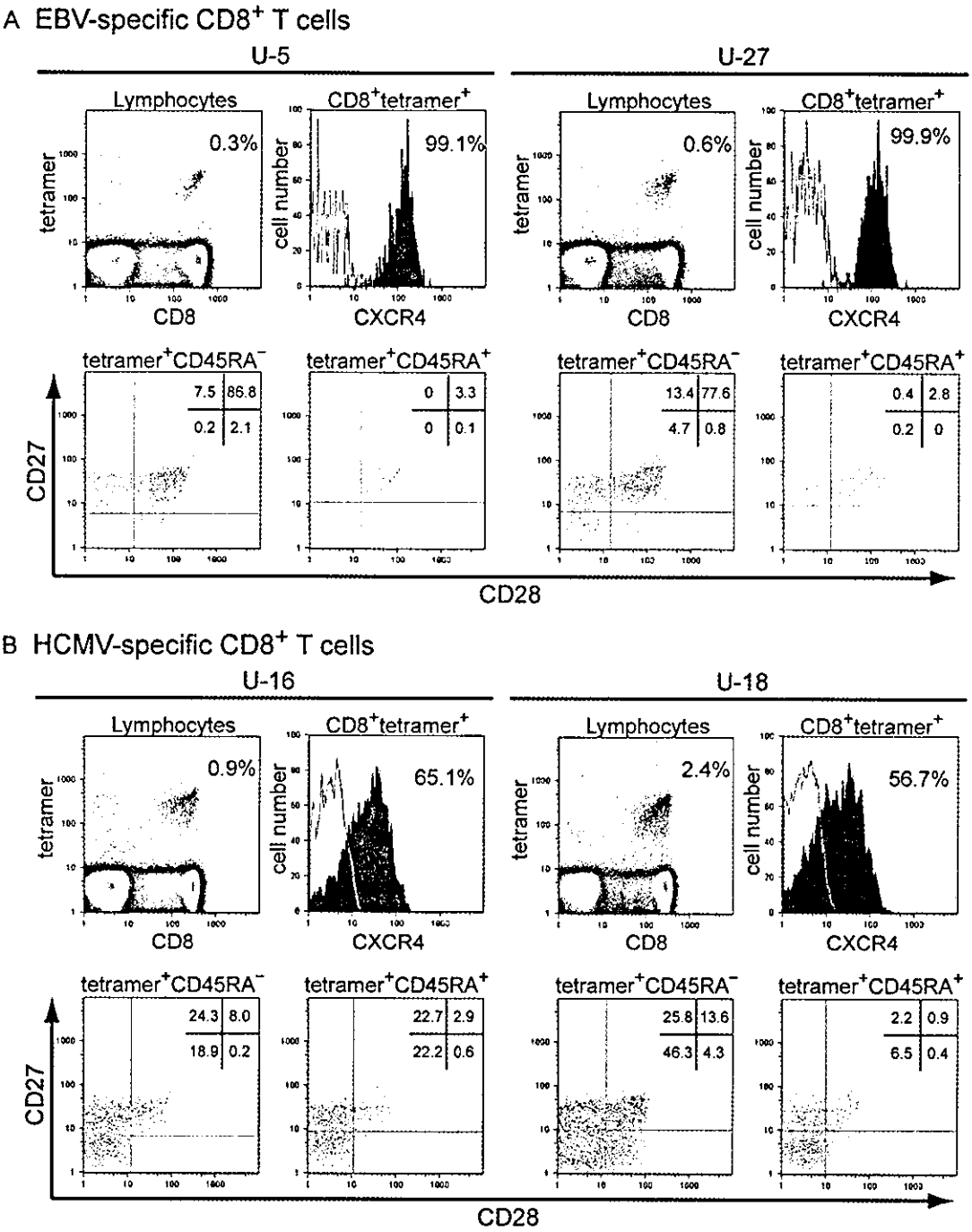

Fig. 5. Surface expression of CXCR4 on EBV-specific and HCMV-specific CD8+ T cells. (A) The surface expression of CXCR4 on EBV-specific CD8+ T cells. PBMC from two individuals (U-5 and U-27) with HLA-A*1101 were stained with anti-CD8 mAb, anti-CXCR4 mAb and the HLA-A*1101 tetramer. CD8+ tetramer+ cells were gated and then analyzed for the expression of CXCR4. The percentage of tetramer+ subsets in CD8+ T cells were shown. CD8+ T cells from the same individuals were also stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CXCR4 mAb, and with the HLA-A*1101 tetramer. The expression of CD27, CD28 and CD45RA on CD8+ tetramer+ cells from each individual is shown in each plot. (B) Surface expression of CXCR4 on HCMV-specific CD8+ T cells. PBMC from two individuals (U-16 and U-18) with HLA-A*0206 were stained with anti-CD8 mAb, anti-CXCR4 mAb and the HLA-A*0206 tetramer. CD8+ tetramer+ cells were gated and then analyzed for the expression of CXCR4. The percentage of tetramer+ subsets in CD8+ T cells is shown. CD8+ T cells from the same individuals were also stained with anti-CD27, anti-CD28, anti-CD45RA and anti-CXCR4 mAb, and with the HLA-A*0206 tetramer. The expression of CD27, CD28 and CD45RA on the CD8+ tetramer+ cells from each individual is shown in each plot.

that the CCR7$^-$CCR5$^+$CXCR4^{low} population may be more immature cells than the other three populations, CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$. However, the specific maturation/differentiation status of these three populations remains unclear. Further analysis of these three receptors on CD27CD28CD45RA subsets using multi-color flow cytometric analysis should enable a better characterization of the CCR7$^-$CCR5$^+$CXCR4$^-$, CCR7$^-$CCR5$^-$CXCR4^{low}, and CCR7$^-$CCR5$^-$CXCR4$^-$ subsets. The expression of the chemokine receptors on human CD8$^+$ T cells is summarized in Fig. 6.

Previous studies have reported that EBV-specific CD8$^+$ T cells, which are unable to kill target cells, have a memory phenotype (CD27$^+$CD28$^+$CD45RA$^-$) and that HCMV-specific CD8$^+$ T cells, which do have cytotoxic activity, have effector and memory/effector phenotypes (CD27$^-$CD28$^-$CD45RA$^{+/-}$ and CD27^{low}CD28$^-$CD45RA$^{+/-}$, respectively). The present study shows that EBV-specific CD8$^+$ T cells for the most part express CXCR4, while its expression decreased on HCMV-specific CD8$^+$ T cells (Fig. 5A, B). These findings indicate that surface CXCR4 is down-regulated during the maturation of virus-specific CD8$^+$ T cells.

In the present study, it has been shown that CXCR4 is highly expressed on naive and central memory CD8$^+$ T cells, and its expression is down-regulated during the maturation into effector cells. The expression pattern of this receptor is useful for functional classification of human CD8$^+$ T cells in conjunction with other chemokine receptors. The classification of human CD8$^+$ T cell subsets using the expression pattern of these chemokine receptors will contribute to the effort to clarify the steps in the differentiation pathway of human peripheral CD8$^+$ T cells.

4 Materials and methods

4.1 Blood samples

Blood samples were taken from healthy adult individuals. For analysis of HCMV-specific and EBV-specific CD8$^+$ T cells, samples were obtained from HCMV-seropositive adult individuals with HLA-A*0206 and EBV-seropositive adult individuals with HLA-A*1101, respectively.

4.2 Antibodies

Anti-CD27 FITC-labeled mAb, anti-CXCR4 PE-labeled mAb, anti-CD28 APC-labeled mAb, anti-CD8 PerCP-labeled mAb, anti-CD3 PerCP-labeled mAb, anti-CXCR1 APC-labeled mAb, anti-CD28 APC-labeled mAb, anti-CCR5 FITC-labeled mAb, anti-CCR7 PE-Cy7-labeled mAb, anti-mouse-IgG FITC- and PE-labeled mAb and anti-perforin FITC-labeled mAb were obtained from BD Biosciences (San Diego, CA). Anti-CD45RA ECD-labeled mAb was purchased from Immunotech (Marseille, France). Anti-CD8 Cascade Blue-labeled mAb was made by conjugating Cascade Blue (Molecular Probes, Eugene, OR) with anti-CD8 mAb OKT8.

4.3 HLA-class I tetramer

HLA-class I-peptide tetrameric complexes (tetramer) were synthesized as previously described [15]. The HCMV CTL epitope (HCMV-1 pp65 495-503: NLVPMVATV) and the EBV CTL epitope (EBNA3B 416-424: IVTDFSVIK) were used for

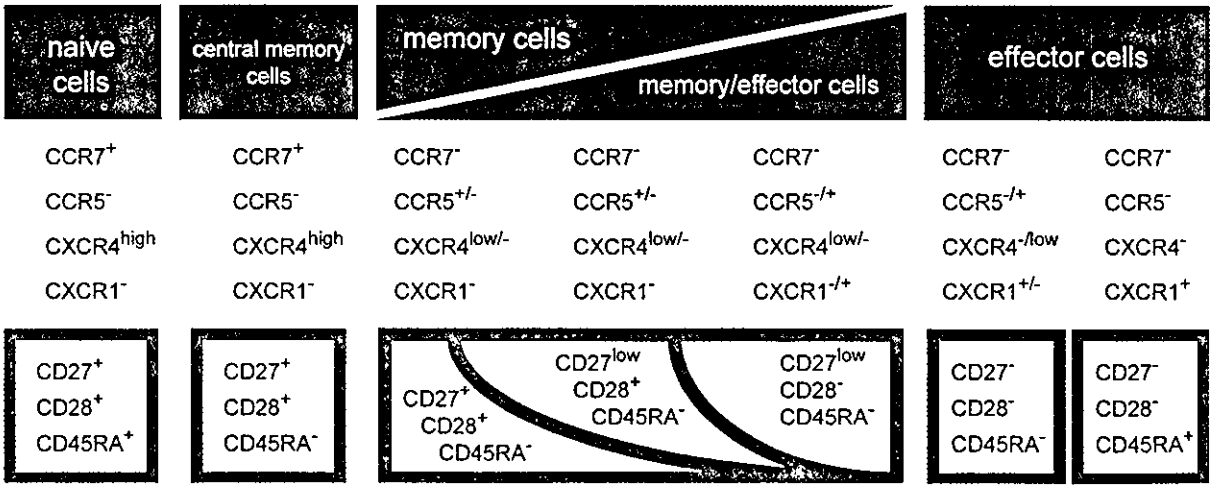

Fig. 6. Summary of the expression of chemokine receptors on human CD8$^+$ T cells.

the refolding of HLA-A*0206 and HLA-A*1101 molecules, respectively. PE-labeled streptavidin (Molecular Probes) was used for generation of the tetramers.

4.4 Flow cytometric analysis

PBMC from healthy individuals were stained with anti-CD3 and anti-CD8 mAb for 30 min at 4°C and were then washed twice with PBS containing 10% newborn calf serum (PBS/10% NCS). The cells were pre-treated with 1 μg human IgG per 1×10^5 cells for 15 min at room temperature, and then stained with anti-CXCR4 mAb for 20 min at room temperature. After washing twice with PBS/10% NCS, the percentage of $CXCR4^+CD8^+$ T cells in the total $CD8^+$ T cells was measured using a FACSCalibur (BD Biosciences).

To investigate the CXCR4 expression in each CD27CD28CD45RA subset of total $CD8^+$ T cells, we purified $CD8^+$ T cells from PBMC using anti-CD8-coated magnetic beads (Miltenyi Biotec, Gladbach, Germany). Purified $CD8^+$ T cells (>98%) were stained with anti-CD27, anti-CD28 and anti-CD45RA mAb at 4°C for 30 min, and were then washed twice with PBS/10% NCS. The blocking of the Fc receptor and staining of surface CXCR4 were carried out as described above. The percentage of $CXCR4^+$ cells in each subset was measured using a FACSCalibur.

To examine intracellular perforin expression in $CXCR4^+$ and $CXCR4^-$ subsets of total $CD8^+$ T cells, we stained PBMC with anti-CD8 and anti-CXCR4 mAb and then fixed them with 4% paraformaldehyde at 4°C for 20 min. The cells were permeabilized at 4°C for 10 min with PBS containing with 0.1% saponin and 20% NCS (permeabilizing buffer). The cells were washed with the permeabilizing buffer and then resuspended in 50 μl of the same buffer. After staining the cells with anti-perforin mAb at 4°C for 30 min, they were washed three times in the permeabilizing buffer at 4°C. FITC- and PE-labeled mouse IgG was used as a negative control.

To determine CXCR1 expression on $CXCR4^+$ and $CXCR4^-$ subsets of total $CD8^+$ T cells, we stained PBMC with anti-CXCR1 and anti-CD8 mAb and then blocked the Fc receptors as described above. The cells were then stained with anti-CXCR4 mAb for 20 min at room temperature.

To determine CCR7 and CCR5 expression on $CXCR4^+$ and $CXCR4^-$ subsets of total $CD8^+$ T cells, we stained PBMC with anti-CCR5 and anti-CD8 mAb and then blocked the Fc receptors as described above. The cells were then stained with anti-CCR7 mAb for 30 min at room temperature. After washing twice with PBS/10% NCS, the cells were stained with anti-CXCR4 mAb for 20 min at room temperature.

To clarify the expression of CXCR4 on HCMV-specific and EBV-specific $CD8^+$ T cells, PBMC were incubated with HCMV-A*0206 or EBV-A*1101 tetramers at 37°C for 30 min. The cells were washed twice with RPMI/10% NCS and then stained with anti-CD8 and anti-CXCR4 mAb following blocking of the Fc receptor, or anti-CD27, anti-CD28, anti-CD45RA and anti-CD8 mAb were added to the cell suspension. The cells were incubated at 4°C for 30 min, and were then washed twice with PBS/10% NCS.

Acknowledgements: The authors thank Sachiko Sakai for secretarial assistance. This research was supported by a Grant-in Aid for Scientific Research and a Grant for Child Health and Development (14-C2) from the Ministry of Health, Labor and Welfare, the government of Japan.

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Correspondence: Masafumi Takiguchi, Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan
Fax: +81-96-373-6532
e-mail: masafumi@kaiju.medic.kumamoto-u.ac.jp

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Epitope-Dependent Effect of Nef-Mediated HLA Class I Down-Regulation on Ability of HIV-1-Specific CTLs to Suppress HIV-1 Replication[1]

Hiroko Tomiyama, Mamoru Fujiwara,* Shinichi Oka,† and Masafumi Takiguchi[2]**

It is believed that Nef-mediated HLA class I down-regulation is one of the mechanisms that allow HIV-1-infected cells to escape from being killed by HIV-1-specific human CTLs. In this study, we show that the effect of Nef-mediated HLA class I down-regulation on the ability of HIV-1-specific CTLs to suppress HIV-1 replication is epitope dependent. The CTLs specific for two Pol epitopes presented by HLA-B*5101, one of the HLA alleles associated with slow progression to AIDS, effectively killed HIV-1-infected CD4$^+$ T cells and suppressed HIV-1 replication. In contrast, those specific for the other four epitopes failed to kill HIV-1-infected CD4$^+$ T cells and partially or hardly suppressed HIV-1 replication. The difference of the ability between these two types of CTLs may result from the difference of the number of HLA class I epitope complex on the surface of NL-432-infected CD4$^+$ T cells. *The Journal of Immunology*, 2005, 174: 36–40.

Human immunodeficiency virus-1 escape from HIV-1-specific CD8$^+$ T cells occurs during acute and chronic phases of HIV-1 infections, although the mechanisms of the HIV-1 escape still remain unclear. Previously, various investigators proposed several hypotheses concerning mechanisms of HIV-1 escape from the host immune system such as mutations of immunodominant epitopes (1), reduction in the number of HIV-1-specific CTLs by apoptosis of CD8$^+$ T cells via Fas and TNF (2), skewed maturation of HIV-1-specific CD8$^+$ T cells (3), and impaired cytolytic activity of HIV-1-specific CTL toward HIV-1-infected CD4$^+$ T cells by Nef-mediated down-regulation of HLA class I molecules (4).

Nef down-regulates the surface expression of both HLA-A and -B molecules in HIV-1-infected cells because of internalization of these molecules from the cell surface by endocytosis in the presence of Nef (5). A previous study showed that the expression of HLA-A2 molecules on Nef-positive (Nef$^+$) HIV-1-infected primary CD4$^+$ T cells was 200- to 300-fold lower than that on Nef-defective (Nef$^-$) HIV-1-infected ones (6). These observations suggested that the Nef-mediated HLA class I down-regulation may decrease the recognition of HIV-infected cells by HIV-1-specific CTLs. In fact, it was shown that HLA-A*0201-restricted HIV-1-specific CTLs failed to kill Nef$^+$ HIV-1-infected CD4$^+$ T cells but effectively killed Nef$^-$ HIV-1-infected ones (4). This was further confirmed by a study using two HLA-B*3501-restricted, HIV-1-specific CTL clones (7). The ability of HIV-1-specific CTLs to suppress HIV-1 replication was also impaired by Nef-mediated HLA class I down-regulation (7, 8). These studies strongly suggest that Nef-mediated HLA class I down-regulation is one of the major mechanisms by which HIV-1 escapes from HIV-1-specific CTLs. However, because only a very restricted number of HIV-1-specific CTLs has been tested for their abilities to kill Nef$^+$ and Nef$^-$ HIV-1-infected CD4$^+$ T cells and to suppress the HIV-1 replication, it still remains uncertain whether Nef-mediated HLA class I down-regulation affects the killing ability of all HIV-1-specific CTLs.

HLA-B57, -B51, and -B27 alleles are associated with slow progression to AIDS (9). It has been speculated that long-term nonprogressors (LTNPs)[3] and slow progressors carry CTLs specific for conserved and dominant epitopes whose recognition is not affected by Nef-mediated HLA class I down-regulation. However, no study has yet investigated this hypothesis. To clarify the effect of Nef-mediated HLA class I down-regulation on CTLs specific for HIV-1 epitopes presented by HLA alleles that are associated with or not associated with slow progression to AIDS, we investigated the ability of both HLA-B*5101-restricted and HLA-A*3303-restricted HIV-1-specific CTLs to recognize HIV-1-infected CD4$^+$ T cells. In this study, we show that HIV-1-specific CD8$^+$ T cells have various ranges of ability to kill HIV-1-infected CD4$^+$ T cells and to suppress the replication of HIV-1.

Materials and Methods

HIV-1-specific CTL clones and lines

HIV-1-specific CTL clones and CTL lines were generated previously (10–12). All CTLs were cultured in R10 medium supplemented with 200 U/ml recombinant human IL-2 and stimulated weekly with irradiated target cells prepulsed with the appropriate HIV-1-derived peptide.

*Division of Viral Immunology, Center for AIDS Research, Kumamoto University, Kumamoto, and †AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan

Received for publication August 18, 2004. Accepted for publication October 27, 2004.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This research was supported by a Grant-in Aid for Scientific Research from the Ministry of Education, Science, Sport and Culture; a Grant-in Aid for Scientific Research from the Ministry of Health, Labour and Welfare; the government of Japan; and a grant from Japan Health Science Foundation.

[2] Address correspondence and reprint requests to Dr. Masafumi Takiguchi, Division of Viral Immunology, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan. E-mail address: masafumi@kaiju.medic.kumamoto-u.ac.jp

[3] Abbreviation used in this paper: LTNP, long-term nonprogressor.

0022-1767/05/$02.00

HIV-1 clones

An infectious proviral clone of HIV-1, pNL-432, and its mutant, pNL-M20A (containing a substitution of Ala for Met at residue 20 of Nef), were reported previously (13).

Infection of CD4$^+$ T cells with HIV-1

CD4$^+$ T cells were purified from PBMCs of HIV-1-seronegative individuals with HLA-B*5101 or HLA-A*3303 by means of anti-human CD4 mAb-coated magnetic beads (MACS beads; Miltenyi Biotec). The purified CD4$^+$ T cells were cultured and infected with HIV-1 clones as previously shown (7).

CTL assay

The cytotoxicity of CTL clones for cultured CD4$^+$ T cells infected with HIV-1 (>40% p24 Ag-positive cells) was determined by a standard ^{51}Cr release assay as shown previously (7).

Flow cytometric analysis

To assess HLA class I expression in HIV-1-infected CD4$^+$ T cells, the cells were stained with anti-B5 mAb 4D12 following staining with allophycocyanin-labeled anti-mouse Ig (BD Pharmingen), and thereafter were fixed and permeabilized for intracellular HIV-1 p24 staining with FITC-labeled anti-p24 mAb KC-57. The expression of HLA class I molecules on HIV-1-infected CD4$^+$ T cells was analyzed by using a FACSCalibur with CellQuest software (BD Biosciences). For detection of intracellular cytokines, HIV-1-specific CTL clones were cocultured with peptide-prepulsed CD4$^+$ T cells or HIV-1-infected CD4$^+$ T cells for 6 h at a CTL:CD4$^+$ T cell ratio of 1:2. CTLs cocultured with CD4$^+$ T cells were used as a negative control. After a 2-h incubation, brefeldin A was added to each well (10 μg/ml). The cells were then stained as previously described with a FITC-labeled anti-human CD8 mAb, PE-labeled anti-human IFN-γ mAb, and allophycocyanin-labeled anti-human TNF-α mAb.

Suppression of HIV-1 replication by HIV-1-specific CTLs

The ability of HIV-1-specific CTLs to suppress HIV-1 replication was examined as previously described (7). After CD4$^+$ T cells had been incubated with the indicated HIV-1 clone following a 4-h incubation at 37°C with intermittent agitation, the cells were washed three times with R10 medium. HIV-1-infected CD4$^+$ T cells were cocultured with HIV-1-specific CTLs. From days 2 to 7 postinfection, 10 μl of culture supernatant was collected, and the concentration of p24 Ag in the supernatant was measured by enzyme immunoassay (HIV-1 p24 Ag ELISA kit; ZeptoMetrix). On days 3, 4, and 5 postinfection, cells were harvested and stained with a mixture of anti-CD4 and anti-CD8 mAbs and then with anti-p24 mAb. The percentage of intracellular p24 Ag-positive cells in the CD8$^-$ population was determined by flow cytometry.

Peptide binding assay

Binding of HIV-1 epitope peptides to HLA-B*5101 was examined by a peptide stabilization assay using RMA-S-B*5101 cells as previously described (10).

Results and Discussion

Ability of HIV-1-specific CTLs to suppress HIV-1 replication in HIV-1-infected CD4$^+$ T cells

To investigate the ability of HIV-1-specific CTLs to suppress HIV-1 replication, we selected the CTLs specific for four HLA-B*5101 epitopes and two HLA-A*3303 epitopes, whose sequences are found in the NL-432 clone. We measured the ability of seven CTL clones or lines specific for these epitopes to suppress HIV-1 replication in primary CD4$^+$ T cells infected with either HIV-1 clone NL-432 or its mutant NL-M20A, in which 1 aa of Nef has mutated and which has the ability to down-regulate cell surface expression of CD4 but not that of HLA class I molecules (13). The surface expression of HLA-B*5101 was indeed down-regulated in NL-432-infected CD4$^+$ T cells but not in NL-M20A-infected ones (Fig. 1*A*). CD4$^+$ T cells infected with the HIV-1 clones were cocultured with or without the HIV-1-specific CTLs. p24-positive CD4$^+$ T cells were not detected in the cultures of NL-M20A-infected CD4$^+$ T cells with the SF2-Pol283-8-specific CTL line, SF2-Pol743-9-51 CTL clone, or SF2-Gag327-9-249 CTL clone. They were also undetected in the cultures of NL-432-infected CD4$^+$ T cells with the SF2-Pol283-8-specific CTL line or SF2-Pol743-9-51 CTL clone, whereas the number of the p24-positive CD4$^+$ T cells was reduced by approximately one-half in the cultures with the SF2-Gag327-9-249 CTL clone. In contrast, the number of the p24-positive CD4$^+$ T cells was not reduced in the cultures of NL-432-infected and NL-M20A-infected CD4$^+$ T cells with HLA-mismatched HIV-1 Nef-specific CTL clones, SF2-6-218 and SF2-6-219 (Fig. 1*B*). These results suggest that SF2-Pol283-8-specific CTL line and SF2-Pol743-9-51 CTL clone completely suppressed Nef$^+$ HIV-1 replication and that SF2-Gag327-9-249 CTL clone only partially suppressed it. Two HLA-A*3303-restricted CTLs, the SF2-Gag144-152-10 clone and the SF2-Env697-706 line, gave the same results as the latter clones (data not shown).

The enzyme immunoassay analysis confirmed the results of the flow cytometric analysis (Fig. 1*C*). The SF2-Pol283-8 line and SF2-Pol743-9-51 clone completely suppressed replication of both NL-M20A and NL-432, whereas two CTL clones, SF2-Gag144-152-10 and SF2-Gag327-9-249, as well as one CTL line, SF2-Env697-706, partially suppressed NL-432 replication (21.7–44.0%) and effectively suppressed NL-M20A replication (82.4–89.9%). These results taken together suggest that the recognition by the latter CTLs was affected by Nef-mediated HLA class I down-regulation but that by the former ones was not.

To compare quantitatively the ability of these CTLs to suppress NL-432 replication, we tested the ability of the SF2-Pol283-8 or SF2-Pol743-9-51 at various E:T ratios to suppress NL-432 replication (Fig. 1*D*). Approximately 50% suppression of NL-432 replication was found when SF2-Gag144-152-10 and SF2-Gag327-9-249 CTL clones were tested at an E:T ratio of 1:1, whereas both SF2-Pol283-8 and SF2-Pol743-9-51 CTL clones showed ~50% suppression at an E:T ratio of 0.001:1, indicating that these CTLs have 1000-fold stronger ability to suppress NL-432 replication than SF2-Gag144-152-10 and SF2-Gag327-9-249 CTL clones.

The number of p24-positive CD4$^+$ T cells was not reduced in the culture of NL-432-infected CD4$^+$ T cells with the SF2-Rev71-11-55 clone, whereas it was partially reduced in that of NL-M20A-infected CD4$^+$ T cells with the same clone (data not shown). This clone also failed to suppress NL-432 replication but partially suppressed NL-M20A replication (Fig 1*C*). These results suggest that this CTL clone can weakly recognize NL-M20A-infected CD4$^+$ T cells but not NL-432-infected CD4$^+$ T cells.

Ability of HIV-1-specific CTLs to kill HIV-1-infected CD4$^+$ T cells and to produce cytokines by stimulation with HIV-1-infected CD4$^+$ T cells

To clarify the mechanism by which HIV-1-specific CTLs suppress HIV-1 replication, we investigated the activity of the HIV-1-specific CTL clones and lines to kill HIV-1-infected CD4$^+$ T cells and to produce cytokines when stimulated with HIV-1-infected CD4$^+$ T cells. SF2-Pol743-9-51 CTL clone and SF2-Pol283-8-specific CTL line, which showed strong suppression of NL-432 replication, effectively killed CD4$^+$ T cells infected with either NL-432 or NL-M20A. The result for the SF2-Pol743-9-51 clone was also confirmed by using the SF2-Pol743-9-specific CTL line (Fig. 2*A*). The cytolytic activity of these two CTLs for HLA-B*5101$^+$CD4$^+$ T cells infected with NL-432 was almost identical with that of those infected with NL-M20A at any E:T ratios (Fig. 2*B*). These results

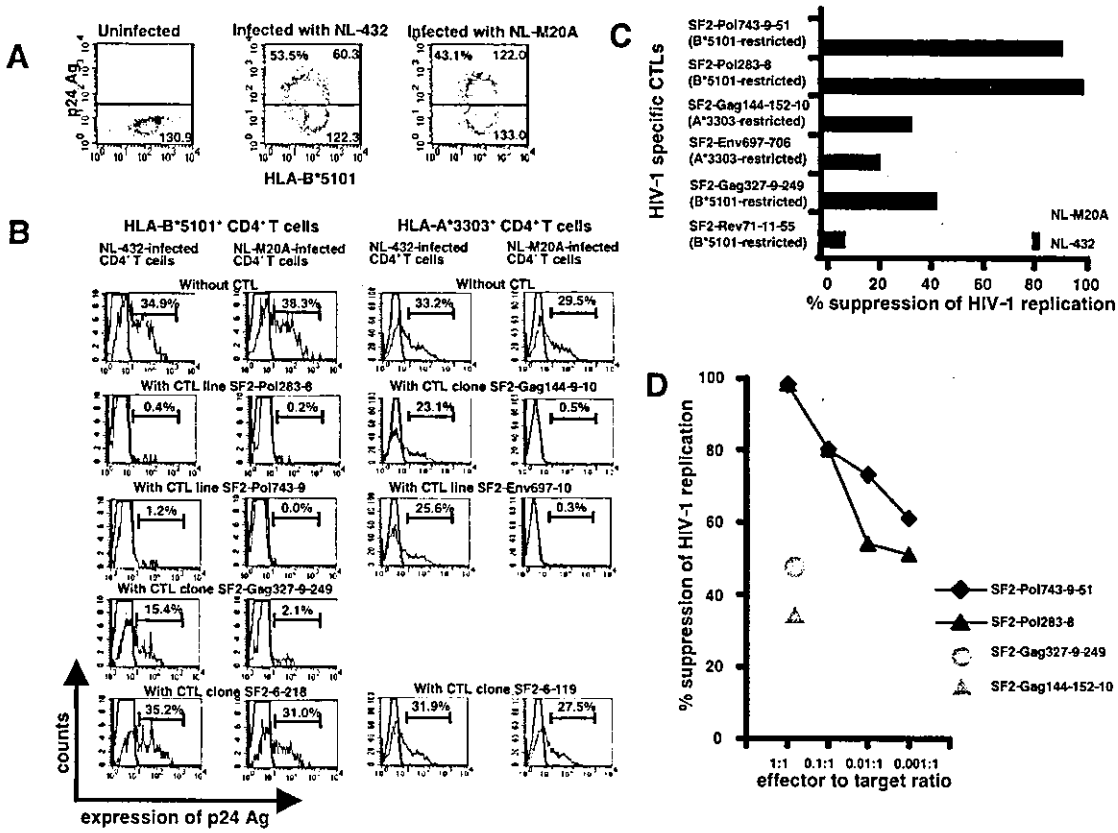

FIGURE 1. Suppression of HIV-1 replication in HIV-1-infected $CD4^+$ T cells by HIV-1-specific CTLs. *A*, Expression of HLA class I molecules on $CD4^+$ T cells infected with HIV-1 NL-432 or NL-M20A. $CD4^+$ T cells from donor U-13 with HLA-B*5101 were cultured and then infected with HIV-1 NL-432 or NL-M20A. On day 3 postinfection, the cells were stained with HLA-B5/B35-specific mAb 4D12 and anti-HIV-1 p24 Ag-specific mAb. The expression of HLA-B*5101 on p24-positive or p24-negative cells is shown as the mean fluorescence intensity (MFI) in each figure. *B*, Number of HIV-1-infected $CD4^+$ T cells in the cultures of HIV-1-infected $CD4^+$ T cells with HIV-1-specific CTLs. $CD4^+$ T cells isolated from donor U-13 were infected with NL-432 or NL-M20A and cocultured with HIV-1-specific CTLs at an E:T ratio of 1:1. The number of HIV-1 p24 Ag^+CD8^- cells in the culture was measured by flow cytometry at the peak of HIV-1 infection. Uninfected and HIV-1-infected $CD4^+$ T cells stained with anti-p24 mAb are shown as bold line and filled histogram, respectively. Percentages of p24 Ag^+ cells are shown in each figure. *C*, Amount of HIV-1 p24 Ags in the cultures of HIV-1-infected $CD4^+$ T cells with HIV-1-specific CTLs. Cultured $CD4^+$ T cells from donors U-13 and U-27(A^*3303^+) were infected with NL-432 or NL-M20A and then cocultured with HIV-1-specific CTLs at an E:T ratio of 1:1. HIV-1 p24 Ags in the supernatant were measured on days 2–7 postinfection by enzyme immunoassay. The percentage of suppression of HIV-1 replication was calculated. *D*, Comparison of the ability of HIV-1-specific CTLs to strongly suppress Nef^+ HIV-1 replication. $CD4^+$ T cells isolated from donor U-13 were infected with NL-432 and then cocultured with the HIV-1-specific CTLs at various E:T ratios. HIV-1 p24 Ag in the supernatant at the peak of infection was measured by enzyme immunoassay, and the percent suppression of NL-432 replication was calculated.

indicate that Nef-mediated HLA class I down-regulation does not affect the ability of these CTLs to kill HIV-1-infected $CD4^+$ T cells. In contrast, the three HIV-1-specific CTLs (SF2-Gag144-152-10 and SF2-Gag327-9-249 CTL clones, and SF2-Env697-706 CTL line) killed NL-M20A-infected $CD4^+$ T cells but failed to kill NL-432-infected $CD4^+$ T cells (Fig. 2*A*), suggesting that Nef-mediated HLA class I down-regulation affected the ability of these CTLs to kill HIV-1-infected $CD4^+$ T cells. These results are consistent with those of a previous study showing that 2 HLA-B*3501-restricted CTL clones killed NL-M20A-infected $CD4^+$ T cells but failed to kill NL-432-infected $CD4^+$ T cells (7).

Next, we investigated the ability of these CTLs to produce IFN-γ and TNF-α after having been stimulated with HIV-1-infected $CD4^+$ T cells (Fig. 2*C*). The total percentages of IFN-γ- and TNF-α-producing cells were ~2–5% and 4–9% in the HIV-1 Pol-specific CTLs stimulated with NL-432-infected and NL-M20A-infected ones, respectively. In contrast, the total percentages of IFN-γ- and TNF-α-producing cells were ~4 and 4–6% in the HIV-1 Gag- and Env-specific CTLs stimulated with $CD4^+$ T cells infected with NL-432 and NL-M20A, respectively. Thus, there was no difference in the number of cytokine-producing cells between these two groups of HIV-1-specific CTLs. These results suggest that the difference in the ability to suppress HIV-1 replication between the two groups results from that in cytolytic activity between them, and that cytokines secreted from the CTLs are partially involved in the suppression of HIV-1 replication.

The SF2-Rev71-11-55 clone failed to produce cytokines after stimulation with either $CD4^+$ T cells infected with NL-432 or those infected with NL-M20A (Fig. 2*C*). This result together with that of suppression of HIV-1 replication indicate that the SF2-Rev71-11 epitope is very weakly presented by HLA-B*5101 in HIV-1-infected $CD4^+$ T cells.

*Ability of four HLA-B*5101-restricted CTLs to recognize HIV-1 epitopes*

It is thought that the ability of CTLs to kill HIV-1-infected cells and to suppress HIV-1 replication is determined by the ability

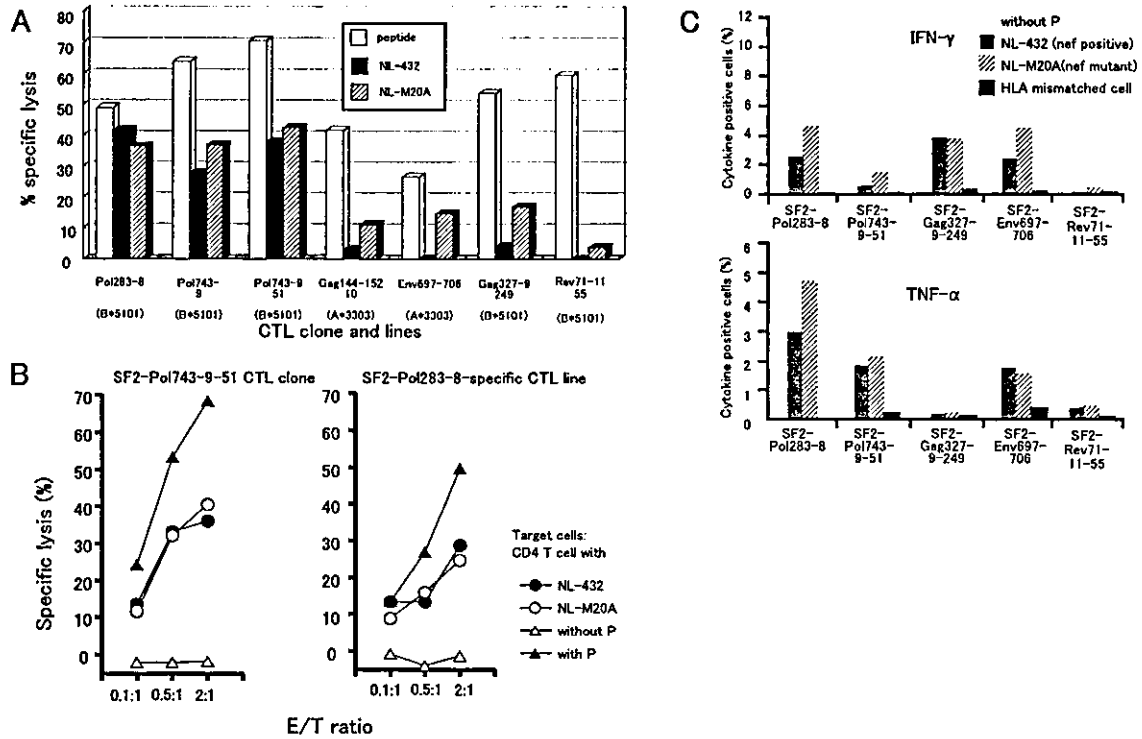

FIGURE 2. Activity of HIV-1-specific CTLs to kill HIV-1-infected CD4$^+$ T cells and to produce cytokines after stimulation with HIV-1-infected CD4$^+$ T cells. *A* and *B*, CD4$^+$ T cells from donors U-13 and U-27 were infected with NL-432 or NL-M20A. On day 3 postinfection, the cells were harvested and used as target cells in the standard ^{51}Cr release assay. Cytotoxic activity of two CTL lines and five CTL clones was examined for CD4$^+$ T cells prepulsed with each epitope peptide (□) and those infected with NL-432 (■) or with NL-M20A (▨) at an E:T ratio of 2:1 (*A*). Cytotoxic activity of SF2-Pol283-8 and SF2-Pol743-9-51 for NL-432-infected CD4$^+$ T cells or NL-M20A-infected CD4$^+$ T cells was examined at E:T ratios of 2:1, 0.5:1, and 0.1:1 (*B*). The specific lysis of the CTLs for CD4$^+$ T cells pulsed with epitope peptide (1 μM) or without peptide was examined by the same assay. Percentages of p24-positive cells in HLA-B*5101-positive CD4$^+$T cells infected with NL-432 and with NL-M20A were 42.0 and 37.0%, respectively. *C*, CD4$^+$ T cells purified from the same donors were infected with NL-432 or NL-M20A. On day 3 postinfection, uninfected CD4$^+$ T cells and NL-432- or NL-M20A-infected CD4$^+$ T cells were mixed with HIV-1-specific CTLs, at an effector-to-stimulator ratio of 1:2 and then incubated for 6 h. Intracellular staining of IFN-γ or TNF-α was performed by using PE-labeled anti-IFN-γ and anti-TNF-α mAbs. The percentages of p24 Ag-positive cells among U-13 CD4$^+$ T cells infected with NL-432 or NL-M20A were 36.6 and 32.1%, respectively, and those among U-27 CD4$^+$ T cells infected with NL-432 or NL-M20A were 33.6 or 31.0%, respectively. The percentages of IFN-γ- and TNF-α-producing cells in the CTLs stimulated with peptide-pulsed CD4$^+$ T cells were as follows: Pol283-8, IFN-γ, 46.3%, TNF-α, 22.0%; Pol745-9-51, IFN-γ, 49.7%, TNF-α, 18.5%; Gag327-9-249, IFN-γ, 17.4%, TNF-α, 0.4%; Env697-706, IFN-γ 33.0%, TNF-α, 31.5%; and Rev71-11-55, IFN-γ, 40.0%, TNF-α, 21.8%.

of TCR to recognize the epitope and by the amount of the epitope presented on the surface of HIV-1-infected cells. We investigated the ability of TCR to recognize the epitope among four HLA-B*5101-retricted CTLs. We measured the ability of the peptides to bind to HLA-B*5101 molecules (BL_{50}) by an HLA-B*5101 stabilization assay, and also measured the ability of CTLs to kill epitope peptide-pulsed cells (LL_{50}, peptide concentration providing a half of maximum percent specific lysis; Table I). A high BL_{50}/LL_{50} ratio indicates a high ability of TCR to recognize the epitope. BL_{50} values of Pol743-9 and Pol283-8 peptides were 10- and 100-fold lower than those of Rev71-11 and Gag327-9, respectively, indicating that the former peptides had higher ability to bind to HLA-B*5101 than the latter ones. In contrast, LL_{50} values of Pol743-9-51 and SF2-Rev71-11-55 CTLs were 6- and 20-fold lower than those of SF2-Pol283-8 and SF2-Gag327-9-249 CTLs, respectively. Thus, the Pol743-9-51and Pol283-8-specific CTLs showed lower BL_{50}/LL_{50} ratio than SF2-Gag327-9-249 and SF2-Rev71-11-55 CTLs (Table I). These results indicate that the ability of TCR of the former CTLs to recognize the epitope was much lower than that of the latter ones. Both Pol743-9-51 and Pol283-8-specific CTLs effectively killed NL-432-infected CD4$^+$ T cells, whereas SF2-Gag327-9-249 and SF2-Rev71-11-55 CTLs failed to kill them. These findings together suggest that the

Table I. *Ability of HLA-B*5101-restricted CTLs to recognize the epitopes*

CTLs	Epitope Peptide	Sequence	(A) Binding Ability of Peptide (BL_{50})	(B) Cytolytic Activity for Peptide-Pulsed Cells (LL_{50})[a]	(A)/(B) Ability of TCR to Recognize the Epitope	Cytolytic Activity for NL-432-Infected Cells (% specific lysis)
Pol743-9-51	Pol743-9	LPPVVAKEI	6.1×10^{-6} M	5.0×10^{-9} M	1,220	36.0
Pol283-8	Pol283-8	TAFTIPSI	6.8×10^{-6} M	3.0×10^{-8} M	227	28.7
Gag327-9-249	Gag327-9	NANPDCKTI	4.0×10^{-4} M	1.0×10^{-7} M	4,000	8.0
Rev71-11-55	Rev71-11	VPLQLPPLERL	5.0×10^{-5} M	5.0×10^{-9} M	10,000	0

[a] LL_{50}, Peptide concentration providing a half of maximum percent specific lysis.

difference in the ability between these CTLs to kill NL432-infected $CD4^+$ T cells is due to that in the number of epitopes presented by HLA-B*5101 on the surface of NL-432-infected $CD4^+$ T cells rather than that in the ability of TCR to recognize the epitope. A recent study also showed that the abilities of HIV-1-specific CTLs to kill cell lines infected with Nef-defective HIV-1 IIIB clone and to suppress replication of this clone were associated with specificity of the CTLs but not with functional avidity of the CTLs (14). Thus, the number of HLA-epitope complex presented on HIV-1-infected $CD4^+$ T cells may be critical for recognition of HIV-1-specific CTLs.

HLA-B57 and -B27 alleles are well-known factors associated with slow progression to AIDS (10). A recent study revealed that HIV-1-specific $CD8^+$ T cells have a high proliferation capacity that is coupled to perforin expression in HLA-B*5701^+ LTNPs but not in HLA-B*5701^+ or HLA-B*5701^- progressors (15), suggesting that HIV-1-specific $CD8^+$ T cells, which have a high proliferation capacity and effector function, control HIV-1 replication in HLA-B*5701^+ LTNPs. However, the mechanism of the association of these HLA alleles with slow progression to AIDS still remains unclear. The present study revealed that the CTLs specific for the two Pol epitopes presented by one of the HLA class I molecules associated with slow progression to AIDS, HLA-B*5101, completely suppressed HIV-1 replication and killed HIV-1-infected $CD4^+$ T cells, implying that these cells effectively control HIV-1 replication in vivo. Because we investigated a limited number of CTLs restricted by HLA alleles that are not associated with slow progression of AIDS in the present and previous studies (8), it still remains unclear that the existence of these CTLs is associated with slow progression of AIDS. Further analysis of HIV-1-specific CTLs restricted by various HLA alleles will clarify the mechanism of the association of these HLA alleles with slow progression to AIDS.

In the present study, we showed that the effect of Nef-mediated HLA class I down-regulation on recognition by HIV-1-specific $CD8^+$ T cells of HIV-1-infected $CD4^+$ T cells vary in epitopes, and particularly demonstrated the existence of HIV-1-specific CTLs that could completely suppress Nef^+ HIV-1 replication and effectively kill primary $CD4^+$ T cells infected with Nef^+ HIV-1. These CTLs are expected to suppress HIV-1 replication in vivo.

Acknowledgments

We thank Dr. Adachi for the generous gift of the NL-M20A clone; Sachi Doki for technical assistance; and Sachiko Sakai for secretarial assistance.

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別紙５

研究成果の刊行に関する一覧表

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Matsushita S, Yoshimura K, Kimura T, Kamihira A, Takano M, Eto K, **Shirasaka T, Mitsuya H**, and **Oka S**.	Spontaneous recovery of hemoglobin and neutrophil levels in Japanese patients on a long-term Combivir® containing regimen.	***J Clin Virol*** (in the press)			
Sakaguchi N, Kimura T, **Matsushita S**, Fujimura S, Shibata J, Araki M, Sakamoto T, Minoda S, and Kuwahara K.	Generation of high-affinity antibody against T cell-dependentantigen in *ganp* gene-transgenic mouse.	***J Immunol*** (in the press)			

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Journal of Clinical Virology xxx (2004) xxx–xxx

JOURNAL OF CLINICAL VIROLOGY

www.elsevier.com/locate/jcv

Spontaneous recovery of hemoglobin and neutrophil levels in Japanese patients on a long-term Combivir® containing regimen

Shuzo Matsushita[a,d,*], Kazuhisa Yoshimura[a,d], Tetsuya Kimura[a], Asako Kamihira[b], Misao Takano[c], Kenichiro Eto[d], Takuma Shirasaka[b], Hiroaki Mitsuya[d], Shinichi Oka[c]

[a] *Division of Clinical Retrovirology and Infectious Diseases, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Kumamoto 860-0811, Japan*
[b] *Department of Immunological and Infectious Diseases, Osaka National Hospital, Osaka 540-0006, Japan*
[c] *AIDS Clinical Center, International Medical Center of Japan, Tokyo 162-8655, Japan*
[d] *Department of Infectious Diseases, Kumamoto University School of Medicine, Kumamoto 860-8556, Japan*

Received 11 August 2004; accepted 3 November 2004

Abstract

Objective: In order to evaluate long-term toxicity of Combivir, we retrospectively reviewed clinical records of HIV-1 infected cases under treatment with Combivir-containing regimen and we analyzed the clinical data compared to other NRTIs-containing regimens.
Study design: A total of 55 patients who were on Combivir and 39 on a control regimen were examined.
Results: After starting treatment with Combivir-containing regimens viral load and $CD4^+$ T-cell count improved as well as the control group. Rates of adverse events in Combivir group and ZDV (400 mg/day) + 3TC group were 50.9% (28/55) and 60% (12/20), respectively. Some of these Japanese patients who started Combivir regimen as a first-line HAART (primary Combivir group) showed some decrease in hemoglobin levels or neutrophil counts within 6 months. However, a significant recovery of these indices of hematological toxicities occurred in patients who continued the regimen for 18–24 months.
Conclusion: Our findings suggest that the safety of 600 mg of ZDV is similar to 400 mg/day of ZDV and the existence of mechanisms that compensate for anemia and for the neutropenia associated with long-term use of Combivir.

Keywords: Combivir; Zidovudine; Lamivudine; Hemoglobin; Neutrophil; Long-term treatment

1. Introduction

Prognosis of HIV infections dramatically improved after introduction of highly active anti-retro viral therapy (HAART). However, the occurrence of adverse events and drug resistance during long-term use of anti-retrovirals are now big issues (Yeni et al., 2002; Dieleman et al., 2002). Present HAART also has a problem to maintain a high adherence because of the pill burden and patients' quality of life is affected. Combivir® is a fixed dose combination tablet containing zidovudine (ZDV) and lamivudine (3TC) (Eron et al., 2000). Each tablet contains 300 mg of ZDV and 150 mg of 3TC and has been widely used as a nucleoside reverse transcriptase inhibitor (NRTI) component of HAART against HIV-1 infection.

HIV infection and AIDS are known to be associated with a significant hematological toxicity, including anemia, neutropenia, and thrombocytopenia (Moses et al., 1998). In addition, studies with zidovudine have shown that this drug may compound the hematological toxicity of HIV and lead to an independent development of anemia and neutropenia (Wilde and Langtry, 1993). Consistent with these observations, the incidence of anemia or neutropenia in mildly or asymptomatic adults treated with zidovudine was between 1.1% and 9.7%, whereas in adults with AIDS or the AIDS related complex it ranged from 15% to as high as 61% (Wilde

* Corresponding author. Tel.: +81 96 373 6536; fax: +81 96 373 6537.
E-mail address: shuzo@kaiju.medic.kumamoto-u.ac.jp (S. Matsushita).

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doi:10.1016/j.jcv.2004.11.002

and Langtry, 1993). In Japan, many physicians prescribe low dose ZDV such as 400 mg/day to avoid drug-induced anemia and neutropenia even though the standard dose of ZDV is 500–600 mg/day in United States (Kimura et al., 1992, 1998). Given the dose-dependent nature of these adverse effects, they are concerned about increased risk of hematological toxicity using Combivir that contains 600 mg of ZDV as the daily dose for Japanese patients who have lower body weights compared to patients in United States. Moreover, long-term consequence of the hematological toxicity resulting from continuous use of Combivir has not been well defined. We retrospectively reviewed clinical records of HIV-1 infected cases under treatment of Combivir-containing regimen used in three hospitals in Japan and we analyzed clinical data cross-sectionally to evaluate long-term toxicity of Combivir.

2. Patients and methods

HIV-1 positive Japanese patients were recruited from Kumamoto University Hospital, Osaka National Hospital and International Medical Center of Japan from June 1999 (after the Combivir launch) until June 2003. The clinical record was investigated in a retrospective manner. All collected cases were separated into four groups, as follows;

Primary Combivir Group (PCV): started Combivir as a first-line HAART.
Secondary Combivir Group (SCV): changed to Combivir from other NRTIs.
Primary Control Group (PCO): started NRTIs (except for Combivir) as a first-line HAART.
Secondary Control Group (SCO): changed to NRTIs except Combivir from other NRTIs.

We checked hemoglobin levels and neutrophil counts to examine the influence on hematological toxicity of ZDV every 6 months. We analyzed the data that could be followed over 18 months for removing various biases such as drop out cases with abnormal laboratory test values. Moreover, we also checked the HIV-RNA, $CD4^+$ T-cell counts and other laboratory test data every 6 months. We also checked any adverse events. This study was done under the approval of the Institutional Review Board of the Kumamoto University Hospital, Japan. All participants provided written informed consent.

3. Results

3.1. Patients' characteristics

Of the 94 data on subjects were 55 who were on Combivir (PCV: 27, SCV: 28) and 39 were on control regimens (PCO: 29, SCO: 10). The NRTIs used in the control group included of 20 cases of ZDV (400 mg/day) + 3TC, 18 cases of stavudine (d4T) + 3TC and one case of d4T + didanosine (ddI). Patients' characteristics are shown in Table 1. A couple of factors are statistically different such as the sex ($p < 0.01$: Fisher's exact test), weight ($p < 0.05$: Student's t-test) and Karnofsky score ($p = 0.0062$: Student's t-test) between Combivir group and control group. Combivir was likely to be used for patients with a higher baseline weight and the males. The mean viral load at baseline in Combivir group was $10^{3.9}$ copies/mL and for the control group was $10^{4.1}$ copies/mL. There was no statistical difference between the groups. The baseline $CD4^+$ T-cell counts in Combivir group were higher than in the control group significantly ($393/mm^3$ versus $263/mm^3$; $p = 0.0101$: Student's t-test). Most patients were prescribed efavirenz (EFV) or nelfinavir (NFV) as a concomitant drug. Fifty-two percent of all patients were on EFV and 16% were taking NFV. The Combivir group had more combination cases with EFV than did the control group, because these two drugs approved for use in Japan at the same period have similar characteristics such as small pill counts and frequency of ingestion.

3.2. Effects on hemoglobin levels

To avoid biases in the data resulting from inclusion of patients with a shorter time follow up, including drop out cases, we focused on the patients that could be followed for over 18 months. Mean hemoglobin levels at baseline of Combivir group (PCV group: 13.9 g/dL, SCV group: 14.2 g/dL) were higher than for the control group (PCO group: 13.1 g/dL, SCO group: 13.7 g/dL) (Fig. 1A). It seems Combivir was likely to give to those with a lesser risk of anemia. We divided patients in PCV group into two sub-groups such as hemoglobin level decreased (sub-group A; $n = 10$) and not changed or increased (sub-group B: $n = 8$) at 6 months after starting Combivir. Fig. 1B shows a trend of hemoglobin levels in sub-group A. Each hemoglobin level at 6, 12, 18 and 24 months after starting treatment decreased significantly compared to baseline ($p < 0.005$, $p < 0.005$, $p < 0.005$ and $p < 0.05$, respectively; Wilcoxon matched pairs signed rank test). However, the decreased hemoglobin levels at 6 months gradually recovered to the baseline level despite continuation of the same regimen. The hemoglobin level at 18, 24 months increased significantly compared to 6-month values ($p < 0.05$ and $p < 0.005$, respectively). On the other hand, the hemoglobin level of sub-group B did not decrease for 18–30 months of follow up period (data not shown). The difference of background between sub-groups A and B was baseline level of hemoglobin and hematocrit. These levels in sub-group A were higher than for sub-group B statistically (14.9 ± 1.2 versus 12.6 ± 0.7; $p < 0.001$, 44.4 ± 3.2 versus 37.4 ± 2.0; $p < 0.001$, Student's t-test).

3.3. Effects on neutrophil counts

The trend of mean neutrophil counts was similar to counts for hemoglobin levels. Mean neutrophil counts of all groups

Table 1
Baseline characteristics

	Combivir group (PCV + SCV) ($n = 55$)	Control group (PCO + SCO) ($n = 39$)	p-value
Sex (male/female)	54/1	32/7	0.00815[a]
Age	35.9 ± 9.5 (22–68)	38.6 ± 10.7 (23–78)	0.2117[b]
Weight (kg)	64.6 ± 10.8 (47.0–91.6)	59.6 ± 11.2 (36.4–81.0)	0.0303[b]
Hemophilia			
Non	48	32	0.562[a]
A	5	7	
B	2	0	
Baseline VL (log)			
<2.6	19	11	0.4432[b]
2.6–3	1	1	
3–4	6	4	
4–5	11	13	
>5	15	10	
Unknown	3	0	
Mean ± S.D.	3.9 ± 1.2	4.1 ± 1.2	
Range	2.6–5.9	2.6–5.9	
Baseline CD4 count			
<200	14	14	0.0101[b]
200–500	25	19	
>500	13	5	
Unknown	3	1	
Mean ± S.D.	393 ± 265	263 ± 179	
Range	1–1132	5–607	
CDC class			
A1	5	3	0.8064[c]
A2	22	17	
A3	6	13	
B1	2	0	
B2	3	0	
B3	2	5	
C1	3	0	
C3	12	11	
Karnofsky score			
20%	0	1	0.0062[b]
40%	0	2	
50%	0	1	
60%	1	0	
70%	0	1	
80%	4	6	
90%	11	8	
100%	39	20	
Mean ± S.D.	95.8 ± 7.9	87.7 ± 19.4	

[a] Fisher' exact test.
[b] Student's t-test.
[c] Wilcoxon 2-sample test.

were over 2000/mm^3 and did not have statistically change from the baseline during the follow up period (Fig. 1C). We separated subjects in the PCV group into two sub-groups as well as for hemoglobin levels to examine the toxicity of Combivir to neutrophils. In the sub-group C ($n = 10$) those with mean neutrophil counts decreased and the sub-group D ($n = 7$) included subjects with no changes or increased neutrophil counts at 6 months after being on Combivir. Fig. 1D shows the trend of the neutrophil counts in sub-group C. Neutrophil counts at 6, 12, 18 and 24 months after starting the treatment decreased significantly compared to baseline ($p < 0.005$, $p < 0.05$, $p < 0.05$ and $p < 0.05$, respectively; Wilcoxon matched pairs signed rank test). However, the decreased neutrophil counts gradually recovered as did hemoglobin levels. The mean neutrophil counts at 18 months increased significantly compared to data at 6 months ($p < 0.05$; Wilcoxon matched pairs signed rank test).

3.4. Effects on other laboratory test value

MCV values at baseline for the secondary treatment group such as SCV group and SCO group were higher than for pri-

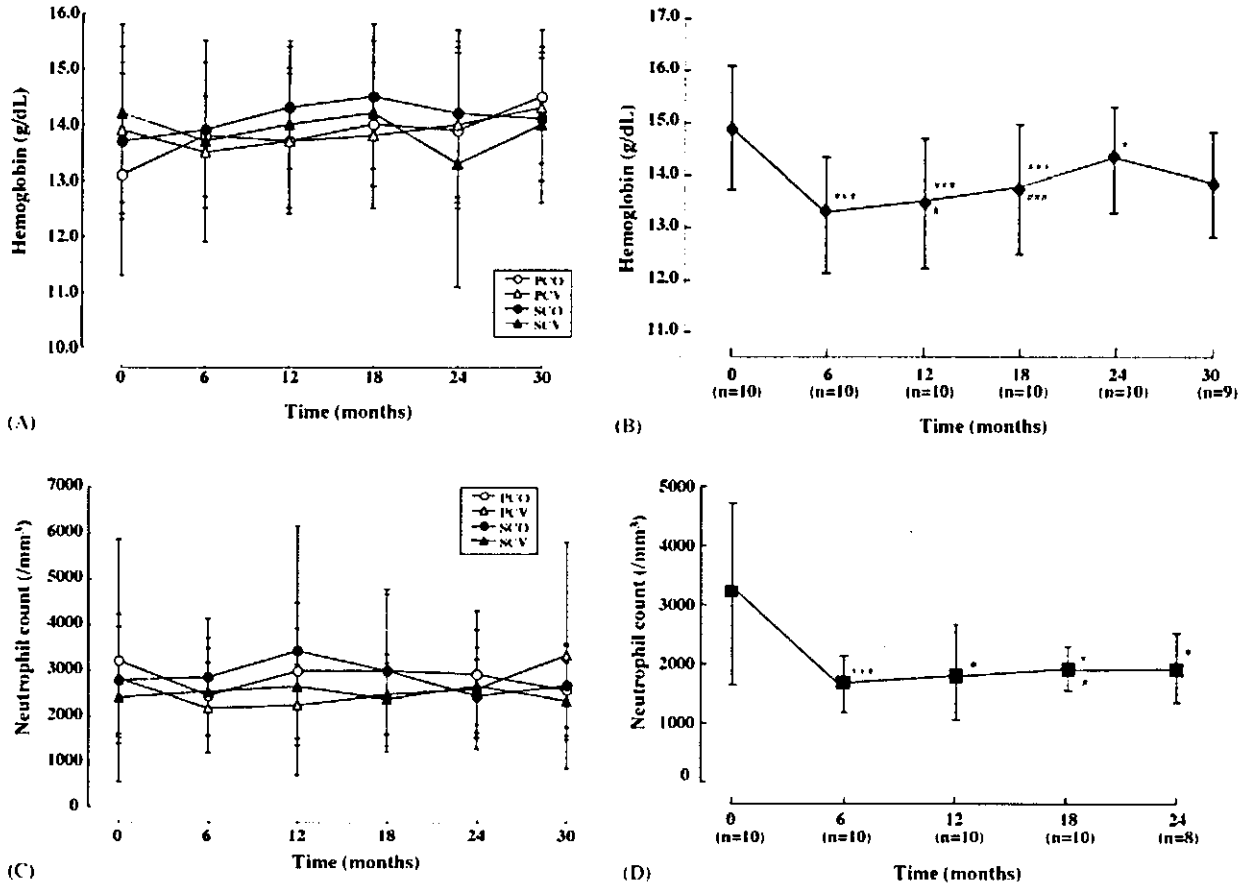

Fig. 1. Recovery after transient suppression of hemoglobin and neutrophil levels in patients with long-term use of Combivir. (A) Mean hemoglobin levels did not change significantly in all groups during each treatment. The baseline hemoglobin level in the Combivir group (PCV + SCV) was higher than in controls (PCO + SCO). (B) Mean hemoglobin levels at 6, 12, 18 and 24 months after start of treatment decreased significantly compared to baseline in the subgroup A of PCV group ($n = 10$). However the decreased hemoglobin level gradually reverted to the baseline levels despite continuation of the same regimen. Hemoglobin levels at 12 and 18 months were significantly high compared to findings at 6 months. (Wilcoxon matched pairs signed rank test; $^{*,\#}p < 0.05$, $^{***,\#\#\#}p < 0.005$). (C) Mean neutrophil counts did not change significantly in all groups during each treatment. (D) Mean neutrophil counts at 6, 12, 18 and 24 months after beginning treatment decreased significantly compared to baseline in sub-group C of PCV group ($n = 10$). However, the neutrophil counts gradually reverted to baseline levels despite continuation of the same regimen. The neutrophil counts at 18 months was significantly high compared to that of 6 months (Wilcoxon matched pairs signed rank test; $^{*,\#}p < 0.05$, $^{***}p < 0.005$).

mary treatment groups such as PCV group and PCO group. It seems ZDV or d4T in the secondary treatment group affected red blood cell counts. However, after starting each treatment, MCV values increased and became high at around 110/mm^3 in all groups (Fig. 2A). Other laboratory test values did showed no notable changes (data not shown).

3.5. Adverse events

The most common adverse events in each group were nausea/vomiting, dizziness and malaise. Anemia was observed in two in the Combivir group and one in the control group. Discontinuing each treatment led to elimination of these adverse effects. The anemia in two in the Combivir group was observed 2 months after their starting treatment, and that in one in the control group was evident as early as the eighth day. The occurrence of anemia in the control group was on ZDV 400 mg/day + 3TC. The frequency of anemia in the Combivir group was 3.6% (2/55) and similar to that in the control group {2.6% (1/39)}. The 20 in the control group on ZDV + 3TC regimen were on a ZDV 400 mg/day. We compared the safety profile of ZDV 600 mg/day to ZDV 400 mg/day. Adverse events rate of Combivir was 50.9% (28/55) and 60.0% (12/20) of AZT + 3TC group. Moreover, the number who discontinued Combivir group was 7 (12.7%) and that in ZDV + 3TC group was 5 (25.0%). In the SCV group, nineteen were changed to Combivir from ZDV 400 mg/day + 3TC. There were six with some adverse events and these were similar to other groups' events. These observations suggest that increasing the ZDV dose to 600 mg/day does not affect the incidence of adverse events. In addition there were no concomitantly used drugs that could affect pharmacokinetic parameters of ZDV and enhance its toxicity.

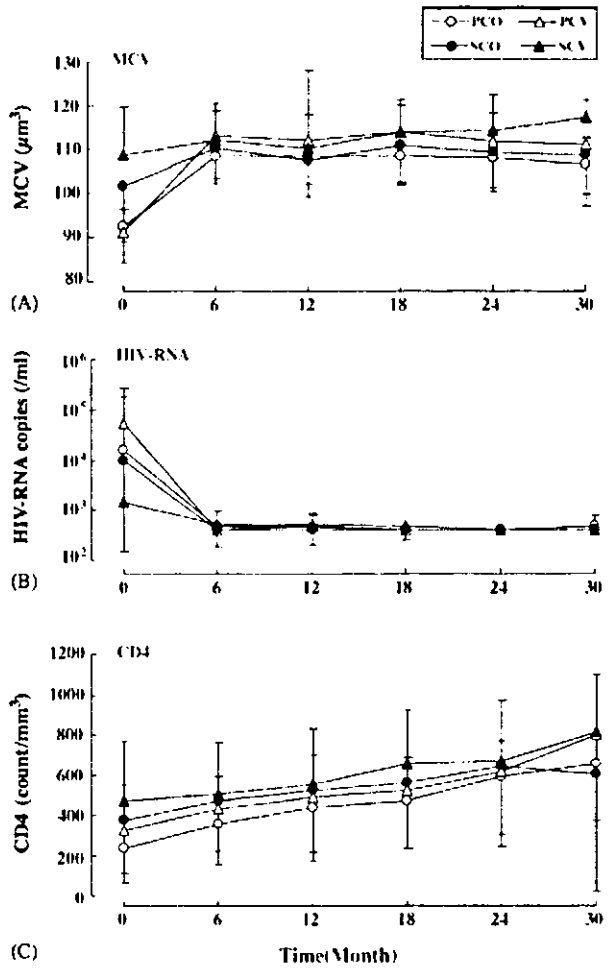

Fig. 2. Changes in MCV values, HIV RNA level and CD4$^+$ T cell counts in each group of patients. (A) MCV values for the secondary treatment group such as SCV and SCO group were higher than for primary treatment groups such as PCV and PCO group at baseline. However, after starting each treatment, MCV values increased and became high at around 110/mm^3 in all groups. (B) Mean HIV RNA level in all groups of treatment decreased compared to baseline significantly ($p < 0.05$–$p < 0.001$; Wilcoxon matched pairs signed rank test). (C) Mean CD4$^+$ T cell counts in all groups of treatment increased significantly compared to the baseline ($p < 0.05$–$p < 0.001$; Wilcoxon matched pairs signed rank test).

3.6. Effects on viral load and CD4$^+$ T-cell counts

Baseline viral load in the primary treatment group (PCV + PCO) was higher than in the secondary treatment group (SCV + SCO). Mean baseline viral loads of each group were $10^{4.6}$ copies/mL (PCV), $10^{4.0}$ copies/mL (PCO), $10^{3.0}$ copies/mL (SCV) and $10^{3.7}$ copies/mL (SCO), respectively. However, after starting each treatment, HIV RNA was not detectable in serum samples from in each group (VL < 50 or <400 copies/mL) (Fig. 2B). Baseline CD4$^+$ T-cell count in the SCV group was 518/mm^3 and higher than other groups (PCV: 304/mm^3, SCO: 345/mm^3, PCO: 277/mm^3) significantly ($p < 0.001$; Student's t-test) (Fig. 2C). This result suggests effective treatment with the previous combination for the SCV group. CD4$^+$ T-cell counts during each treatment increased significantly ($p < 0.05$–$p < 0.001$; Wilcoxon matched pairs signed rank test) and reached over 600/mm^3 at 30 months in all groups (Fig. 2C).

4. Discussion

The nucleoside reverse transcriptase inhibitor (NRTI) was first developed as an anti-HIV drug. However, the appropriate dosage was unclear because this type of drug is only active after being phosphorylated inside cells. A daily dose of 400 mg of ZDV has been widely used in Japan because anemia and neutropenia occurred frequently in cases of ingesting a higher dose (800 mg/day) than did 400 mg/day of ZDV in a clinical trial conducted in Japan (Kimura et al., 1992). Bone marrow toxicity associated with AZT such as macrocytic anemia and neutropenia has been frequently reported for the patients treated with a higher dose of ZDV mono therapy (Richman et al., 1987). Given the dose-dependent nature of these adverse effects, Japanese health care providers have some hesitance to prescribe Combivir that contains 600 mg of ZDV, as the daily dose. Data on four patients with severe anemia associated with Combivir have also been reported (Sibery et al., 2003). To evaluate the long-term toxicity of Combivir, we reviewed clinical records of HIV-1 infected Japanese patients on treatment with Combivir-containing regimen.

The results in this retrospective study showed that anemia and adverse events occurred at comparable frequency in each group of patients. Consistent with previous reports (Hester and Peacock, 1998; Tseng et al., 1998) these adverse events occurred in less than a few months after starting each treatment. The frequency of anemia in the Combivir group was only 3.7% (2/54), and it was similar to that for ZDV 400 mg/day + 3TC group (5.0%) group. In other words there was no difference in these groups with respect to the frequency of anemia by the difference in the dose of ZDV. It is also of note that the efficacy of Combivir was comparable to that of 400 mg of ZDV of four times a day with a twice a day dosing of 3TC. However, we have to take into account the fact that Combivir was prescribed for heavy weight patients. And such may mask the occurrence of adverse events as well as the difference in efficacy.

We observed a certain degree of decrease in hemoglobin levels and neutrophil counts in the subgroups of patients in PCV (subgroups A and C, respectively). Interestingly, a gradual recovery of these hematological toxicities occurred despite the continuation of Combivir containing regimens. The mechanism whereby the risk of hematological toxicity associated with increasing ZDV dosages may be related to the intracellular accumulation of the toxic metabolite zidovudine monophosphate (AZTMP) (Tornevik et al., 1995). AZTMP interferes with both cellular DNA synthesis and exonuclease-catalyzed removal of ZDV from host cell DNA (Sommadossi et al., 1989; Harrington et al., 1993). In addition, at clinically