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Selective addition of $CXCR3^+CCR4^-CD4^+$ Th1 cells enhances generation of cytotoxic T cells by dendritic cells *in vitro*

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Abbreviations: APC, antigen presenting cell; DC, dendritic cell; CTL, cytotoxic T lymphocyte

Abstract

Increasing importance is being given to the stimulation of Th1 response in cancer immunotherapy because its presence can shift the direction of adaptive immune responses toward protective immunity. Based on chemokine receptor expression, $CXCR3^+CCR4^-CD4^+$ T cells as Th1-type cells were investigated its capacity in monocyte-derived dendritic cell (DC) maturation and polarization, and induction of antigen specific cytotoxic T lymphocytes (CTL) *in vitro*. The levels of IL-4, IL-5 and IL-10 were decreased to the basal level compared with high production of IFN-γ, TNF-α, and IL-2 in $CXCR3^+CCR4^-CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 antibodies. Co-incubation of activated $CD4^+$ or $CXCR3^+CCR4^-CD4^+$ T cells with DC ($CD4^+$/DC or $CXCR3^+CD4^+$/DC, respectively) particularly up-regulated IL-12 and CD80 expression compared with DC matured with TNF-α and LPS (mDC). Although there was no significant difference between the effects of the $CXCR3^+CCR4^-CD4^+$ and $CD4^+$ T cells on DC phenotype expression, $CXCR3^+CD4^+$/DC in CTL culture were able to expand number of $CD8^+$ T cells and increased frequencies of IFN-γ secreting cells and overall cytolytic activity against tumor antigen WT-1. These results demonstrated that the selective addition of $CXCR3^+CCR4^-CD4^+$ T cells to CTL cultures could enhance the induction of CTLs by DC *in vitro*, and implicated on a novel strategy for adoptive T cell therapy.

Keywords: adoptive transfer; antigens, differentiation; dendritic cells; immunotherapy, adoptive; maturation; receptors, CCR4; receptors, CXCR3; T lymphocytes, cytotoxic; Th1 cells; Th2 cells

Introduction

Adoptive transfer of *ex vivo* generated cytotoxic T cell lines (CTLs) against tumor associated antigen (TAA) or viral antigen can reconstitute immune system and has shown great promise for eradication of malignancy and control of virus infection (Knutson *et al*., 2005; Mocellin *et al*., 2005; Palucka *et al*., 2005). In contrast to previous studies focused on antitumor responses by $CD8^+$ T cells, recent reports have demonstrated the essential role of $CD4^+$ T cells both in the aspects of APC activation and controlling $CD8^+$ T mediated tumor eradication (Nishimura *et al*., 1999; Surman *et al*., 2000; Janssen *et al*., 2005). In physiology, APCs delivers costimulatory signals to $CD4^+$ T cells for activation, which reciprocally activates APCs via costimulatory molecules, especially CD40-CD40L interaction (Prilliman *et al*., 2002). This interaction eventually primes naïve $CD8^+$ T cells. Mice deficient of functional $CD4^+$ T cells generated lower frequency of antigen specific CTLs and deteriorate antitumor responses (Gao *et al*., 2002). Moreover, it has been reported that activated $CD4^+$ T cells express costimulatory molecules on the surface including CD27, 4-1BB and MHC II which sustains activity of CTLs and long-term survival (Giuntoli *et al*., 2002).

DC has been a choice of APC for its potency in antigen uptake, presentation and delivering costimulatory signals. However, without signals to activate and differentiate to matured form, DCs are more likely to generate tolerance (Lutz *et al*., 2002). Despite the widespread use of maturation cocktail providing danger signals, the limited clinical success of DC vaccines requires improved methodology for sustained production of IL-12 to mediate Th1 polarization. CD40-CD40L interaction has been mainly responsible for IL-12 synthesis and factors including TGF-β and IL-10 inhibit the production (Lyakh *et al*., 2005; Larmonier *et al*., 2007). Resembling the *in vivo* environments, activated $CD4^+$ T cells expressing CD40L used to

mature DC in replacement of maturation reagents and demonstrated augmented secretion of IL-12 indicating type I helper T cells (Th1) polarization capacity of DCs (Sato *et al*., 2004).

Among the subsets of $CD4^+$ T cells, the introduction of Th1 cells enhances interaction between APC and T cells and triggers naïve $CD8^+$ T cells into tumor specific CTLs in the tumor draining lymph nodes. Moreover it has been reported that the preconditioning of PBL towards Th1 significantly augmented vitality and cytotoxic activity by antigen specific $CD8^+$ T cells in cultures (Chattopadhyay *et al*., 2005) and the injection of Th1 cells as adjuvant inhibited accumulation of regulatory T (Tr) cells in tumor draining lymph nodes (Zhang *et al*., 2007), respectively. Th1 and Th2 cells has been distinctively defined based on chemokine receptor expression on the surface, CCR5, CXCR3 and CXCR6, and CCR3, CCR4, CCR7, CCR8, CRTh2 and CXCR5, respectively (Mosmann *et al*., 1989; Mackay *et al*., 1996; Ward *et al*., 1998; Lukacs *et al*., 2001). Despite overlapping expression of chemokine receptors, the classification of Th1/Th2 cells can be based on the representative marker expression of $CXCR3^+$ and $CCR4^-$ and the validity was confirmed with cytokine profile of each subset (Kim *et al*., 2003).

Considering the known capacity of $CXCR3^+$ $CCR4^-CD4^+$ T cells, this study demonstrates that the selective addition of Th1 cells in CTL cultures provides potent maturation signals to DCs and enhances efficiency in generation of CTLs specific for tumor-associated antigen *in vitro*.

Results

Chemokine receptor characterizes $CXCR3^+CD4^+$ T cells as Th1-type cells

To evaluate whether chemokine receptors can be a critical factor defining different helper T cell subsets, $CD4^+$ T cells were isolated from three different healthy individuals for analysis of chemokine receptor expression on the surface and type of cytokine secretion upon stimulation. There were notable differences in frequencies of helper T cells expressing each chemokine receptors. As shown in Figure 1A, a range of 18 to 21% of $CD4^+$ T cells

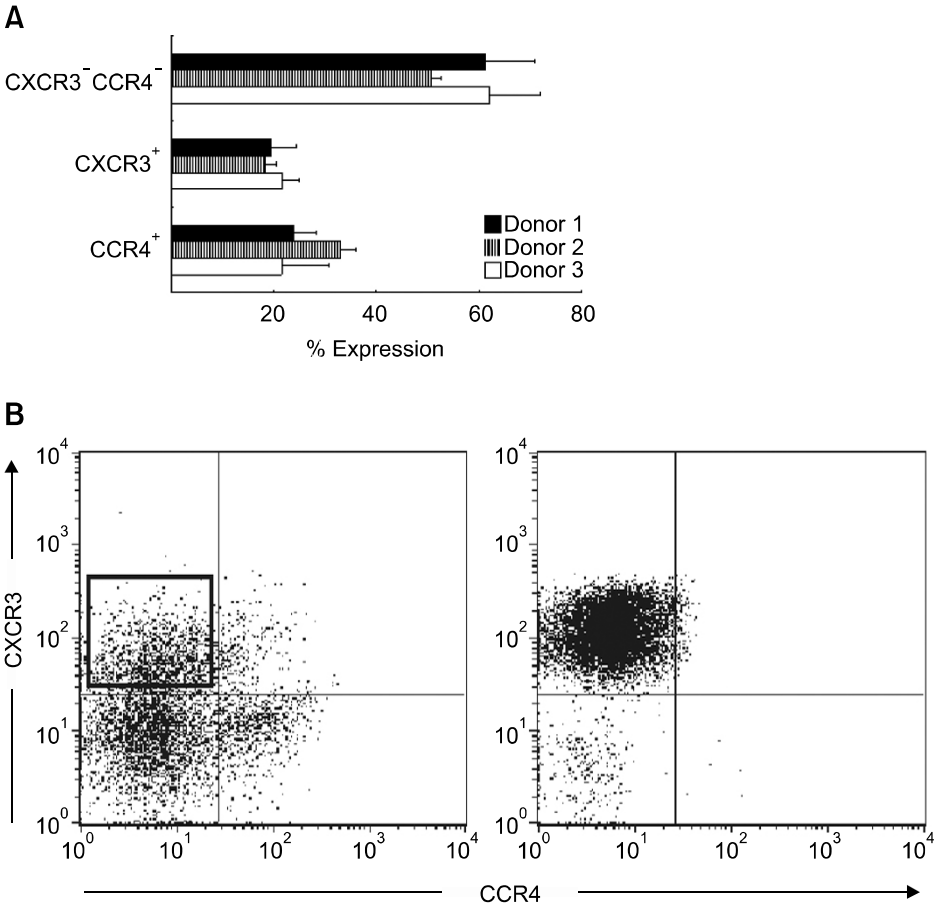

Figure 1. Analysis of chemokine receptor expression of $CD4^+$ T cells. (A) Peripheral $CD4^+$ T cells isolated from three different donors were assessed for surface expression of different chemokine receptors, CXCR3 and CCR4 by flow cytometry. (B) $CXCR3^+CCR4^-CD4^+$ T cells were sorted by FACs VANTAGE and the sorted populations were reexamined for purity ($>$ 95%).

from three individuals expressed CXCR3, a representative molecule of Th1-type subsets, whereas CCR4 positive subpopulations, known to be expressed by Th2-type subsets, ranged from 21 to 33% (Kim *et al*., 2003). A large population within helper T cells still remained undifferentiated without any expression of CXCR3 or CCR4 and a few expressed both (data not shown).

Since there is overlapping of CCR4 expression for both Th1- and Th2-type cells, we preferred CRTH2 as a Th2 marker to measure the expression pattern of cytokines upon activation in subpopulations of CD4$^+$ T cells (Cosmi *et al*., 2000; Kim *et al*., 2001). We isolated subpopulations of CD4$^+$ T cells using CXCR3 and CCR4 for Th1-type cells and CRTH2 for Th2-type cells by VANTAGE sorting (Figure 1B) and tracked comparative cytokine expression after stimulation for 48 h. Although the production of IFN-γ, TNF-α, and IL-2 were not significantly altered between the CD4$^+$ and isolated CXCR3$^+$CCR4$^-$CD4$^+$ T subpopulations, the level of type II cytokines secreted by CXCR3$^+$CCR4$^-$CD4$^+$ T cells including IL-4, IL-5 and IL-10 was significantly decreased down to the basal level (Figure 2). The type of cytokines secreted by CRTH2$^+$CD4$^+$ T cells was mainly IL-4 and IL-5 and IL-10, but IFN-γ and TNF-α were rarely detected after stimulation. Even after Vantage sorting, a small percentage of populations, CXCR3$^-$CCR4$^-$CD4$^+$ T cells, were still present, however the populations were confirmed not to influence the results because of no cytokine expression after activation (data not shown).

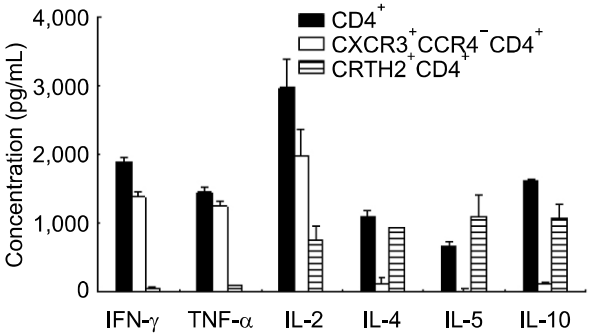

Figure 2. Cytokine profile of CXCR3$^+$CCR4$^-$CD4$^+$ T cells compared to total CD4$^+$ T cells. After VANTAGE sorting based on CXCR3$^+$ expression, cells (5×10^3/well) were stimulated with anti-CD3/CD28 beads (1 bead/1 cell) for 48 h. Comparative cytokine analysis between CD4$^+$ T cells and isolated CXCR3$^+$CCR4$^-$CD4$^+$ T cells was examined by Th1/Th2 CBA. The graphs show the mean of triplicate values ± SD of one representing experiment of three performed from three different donors.

DC activated with CXCR3$^+$CCR4$^-$CD4$^+$ T cells potently induces proliferation of IFN-γ producing and cytolytic CD8$^+$ T cells

To speculate the effect of CXCR3$^+$CCR4$^-$CD4$^+$ T cells, immature DCs were activated with CXCR3$^+$CCR4$^-$CD4$^+$ T cells (CXCR3$^+$CD4$^+$/DC) and used to induce proliferation and cytotoxicity of CD8$^+$ T cells against the tumor antigen, WT-1. Figure 3A shows naive CD8$^+$ T lymphocytes stimulated with CXCR3$^+$CD4$^+$/DC expanded about 2.5 folds against WT-1 antigen compared to DCs treated with either total CD4$^+$ T cells (CD4$^+$/DC) or maturation cocktail (MC) incuding LPS and TNF-alpha (mDC) ($P = 0.031$), indicating superior capacity of CXCR3$^+$CD4$^+$/DC for priming naïve T cells. The expanded populations did not contain non-specific CD8$^+$CD56$^+$ T cells as analyzed by flow cytometry

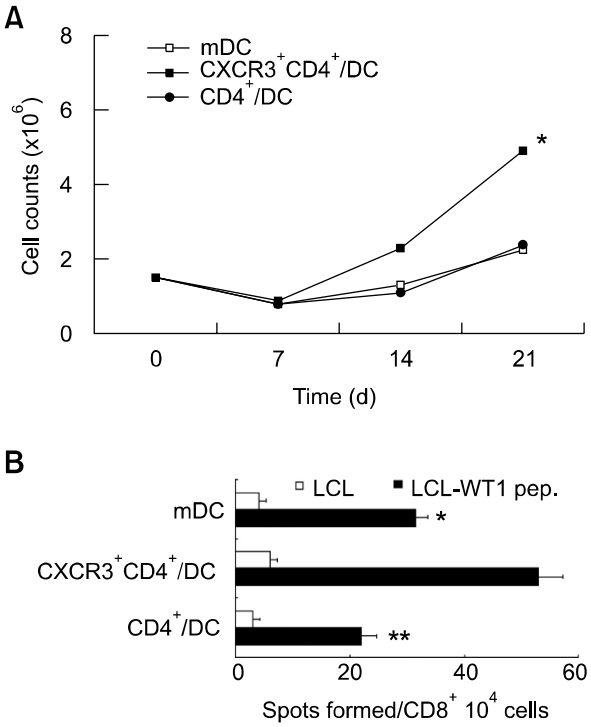

Figure 3. Stimulation of antigen-specific CTLs by CXCR3$^+$CD4$^+$/DCs. Freshly isolated CD8$^+$ T cells were weekly stimulated with autologous DCs pulsed with WT-1 peptide, as described in Methods. (A) The total viable cell count for thrice (21 days for WT-1) stimulation culture was determined by trypan blue dye exclusion. The data is a representative of three independent experiments. An asterisk indicates that the number of spots obtained when CTLs were generated with CXCR3$^+$CD4$^+$/DC was significantly higher than the mDC and CD4$^+$/DC (*$P = 0.031$). (B) The CTLs (as responders) were tested against peptide or empty loaded LCLs as stimulators at a ratio of 10:1. Spots were counted by computer-assisted image analysis. Asterisks indicate that the number of spots obtained when CTLs were generated with CXCR3$^+$CD4$^+$/ DC was significantly higher (*$P = 0.05$ compared to mDC; **$P = 0.0092$ compared to CD4$^+$/DC). A representative IFN-γ ELISPOT experiment of three performed is shown.

(data not shown).

To examine the proliferated T cells are antigen specific, the frequency of IFN-γ secreting $CD8^+$ T cells against WT-1-peptide loaded targets was measured by ELISPOT assay to further assess the relative priming activity of DCs matured with $CXCR3^+CCR4^-CD4^+$ T cells. As shown in Figure 3B, the highest frequency of IFN-γ secreting $CD8^+$ T cells specific for WT-1 was observed from the CTLs generated with $CXCR3^+CD4^+$/DCs (53 ± 4 spots forming cells per 10^4 cells), whereas relatively lower IFN-γ spots were formed by those stimulated with mDC (31.5 ± 2 spots) ($P = 0.05$) and $CD4^+$/DC (22 ± 3 spots) ($P < 0.0092$), respectively.

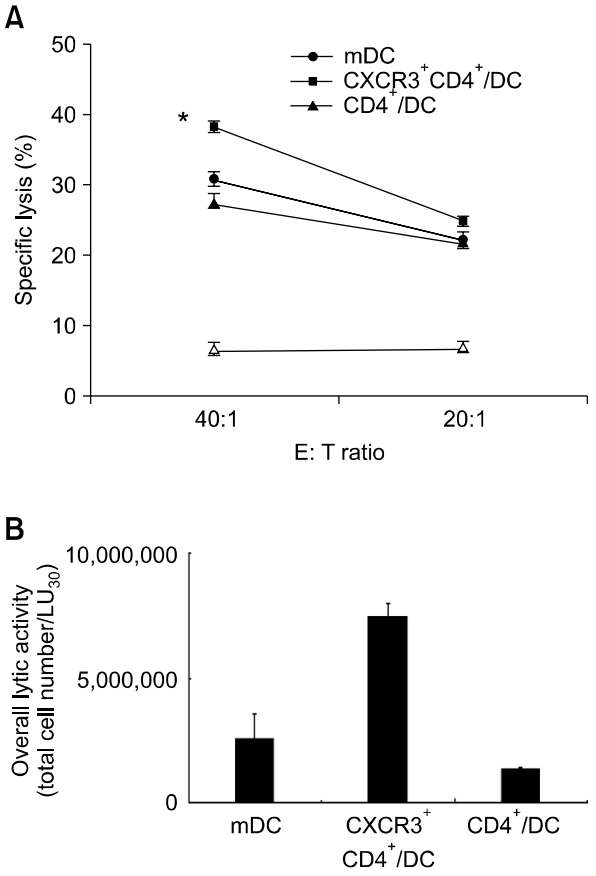

Figure 4. Induction of overall cytolytic activity by $CXCR3^+CD4^+$/DCs. WT-1-specific CTL was generated from normal donors, as described in Methods. (A) Cytotoxicity of T-cells generated with three types of DCs against WT-1 were tested in response to peptide-pulsed LCLs (10 μg/ml) at E:T ratios ranging from 40:1 to 20:1 in a standard 4-h ^{51}Cr release assay. Results are expressed as percentage of specific lysis; the percentage of lysis in the presence of peptide (filled) and the percentage of lysis in the absence of peptide (empty). Asterisk indicates that the cytolytic activity of CTLs generated with $CXCR3^+CD4^+$/DC at E:T ratio of 40:1 was significantly higher than that obtained with $CD4^+$/DC (*P = .04). (B) Lytic units against WT-1 were calculated from cocultures. The data from Figure 3A and 4A were combined to represent the lytic units resulting from the exposure of effectors to targets. One lytic unit is defined as 30% specific lysis. The calculated lytic unit is the yield of effectors (from Figure 3A) divided by the number of cells required to achieve a single lytic unit. One representative experiment of three performed is shown.

The specific lysis of CTLs generated with $CXCR3^+$ $CD4^+$/DCs against WT-1 antigen reached relatively higher than that with mDCs and $CD4^+$/DCs (P = 0.04), respectively, at an effector to target ratio of 40:1 (Figure 4A). When the finding from Figure 3A and 4A were combined to represent the overall lytic activity of the CTLs, the differences in the overall lytic potential induced by $CXCR3^+CD4^+$/DC became even more prominent. The overall lytic units represent total number of $CD8^+$ T cells possessing cytotoxic activity are generated in a single culture. As shown in Figure 4B, the stimulation of $CD8^+$ T cells with $CXCR3^+CD4^+$/DC resulted strongly increased overall effector activity against WT-1 antigen as measured by the total number of lytic units per culture. In contrast, stimulation of effectors with mDC resulted in more than 3 folds lower lytic capacity against WT-1 while $CD8^+$ T cells stimulated with $CD4^+$/DCs elicited about 4 folds lesser capacity than $CXCR3^+CD4^+$/DCs against both WT-1. These results demonstrate $CXCR3^+CD4^+$/DCs efficiently prime naive T cells against certain weakly immunogenic tumor antigens as measured by the total number of lytic units per culture.

Potency of $CXCR3^+CD4^+$/DC in priming $CD8^+$ T cells is due to induction of Th-1 and reduction of immune suppressive cytokine by DC

Given that $CXCR3^+CD4^+$/DC was more efficient in CTL generation (Figure 3 and 4), we attempted to characterize the factors that are responsible for such potency. Focusing on the maturation status of DCs, we used flow cytometry to analyze the expression profile of costimulatory and MHC molecules. After 48 h of incubation, both the number of DC expressing maturation markers and the level of expression intensity were increased compared to iDC (Figure 5A). Among the markers examined, CD80 expression was highly upregulated when cocultured with cellular stimuli, including $CXCR3^+$ $CCR4^-CD4^+$ or $CD4^+$ T cells, than with MC. However, there was no significant difference between the effects of the $CXCR3^+CCR4^-CD4^+$ and $CD4^+$ T cells on phenotypic expression of DC.

Next we examined whether activated $CXCR3^+$ $CCR4^-CD4^+$ T cells influence a pattern of proinflammatory cytokines by DC. As shown in Figure 5B, both of $CXCR3^+CCR4^-CD4^+$ and $CD4^+$ T cells highly induced secretion of Th-1 polarizing cytokine IL-12p70 compared to mDC (P = 0.009 compared to $CXCR3^+CD4^+$/DC and P = 0.03 compared

to CD4$^+$/DC). However, considering the level of IL-10 production by mDC as control, the secretion by CXCR3$^+$CD4$^+$/DC was reduced to half while CD4$^+$/DC remained as mDC, despite CD4$^+$ T cells

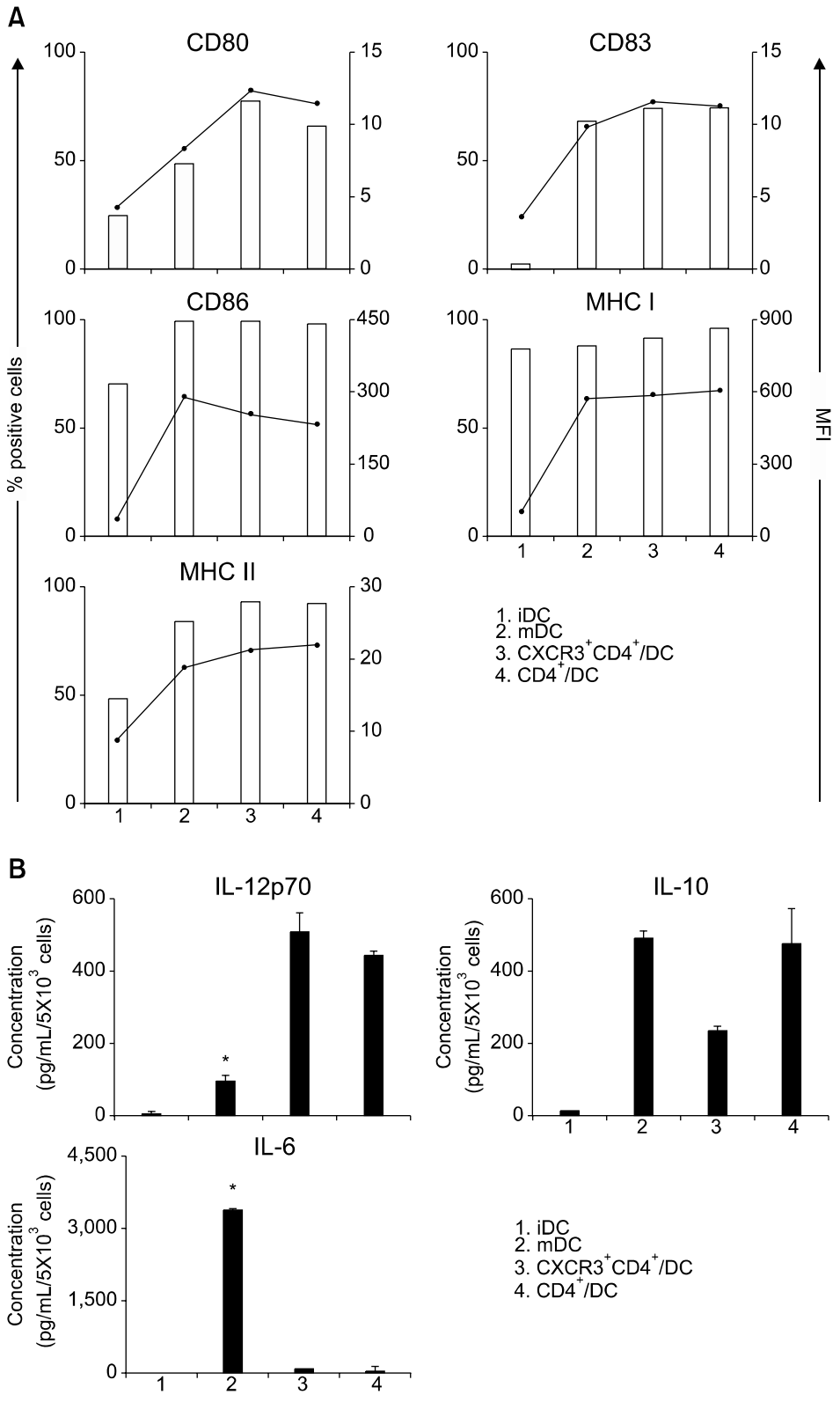

Figure 5. Analysis of maturation markers and type of cytokine secretion by DCs. (A) Immature DCs (1 $\times$ 10^5 cells) were cultured for 48 h with various stimuli. For each marker, expression was plotted for % of marker positive cells (bar graph) and the mean fluorescence intensity (MFI, line) obtained in different conditions. (B) Simultaneous quantification of three cytokines (IL-6, IL-10 and IL-12p70) was performed with CBA assay from 48-h supernatants of DCs (5 $\times$ 10^3 cells). The net concentration is presented here after subtracting the amount secreted by either CXCR3$^+$CCR4$^-$CD4$^+$ or CD4$^+$ T cells themselves. Samples were in triplicate, and the means $\pm$ SD of lymphokine levels are shown. The experiments were conducted at least twice with similar results and a representative set of data is presented. An asterisk indicates statistical significance compared between mDC and CXCR3$^+$CD4$^+$/DC (*P = 0.009 and 0.003 for IL-12 and IL-6, respectively) or CD4$^+$/DC (*P = 0.007 for IL-12 and 0.002 for IL-6, respectively).

significantly secrete IL-10 as shown in Figure 2A. IL-6 was only measured in supernatants of DCs matured with MC and was rarely detectable in those matured with cellular stimuli ($P = 0.007$ compared to $CXCR3^+CD4^+$/DC and $P = 0.002$ compared to $CD4^+$/DC). Because T cells also contribute in lymphokines production, the data is presented after subtracting the cytokine production of T cells alone, so that DC is mainly responsible for cytokine production.

Discussion

In this study, $CXCR3^+CCR4^-CD4^+$ T cells were used to improve CTL generation as well as DC maturation in replacement of typical maturation reagents *in vitro*. Despite the essential role of Th1-conditioning in CTL cultures, there should be more defined protocol in driving Th1-type responses. Here, the parameter used for distinguishing Th1-type subsets from $CD4^+$ T cells was strictly based on the surface expression of $CXCR3^+CCR4^-$chemokine receptor. A small proportion of CXCR3-positive cells (~10%) were found among freshly isolated naive $CD4^+$ T cells, however CXCR3-positive cells were increased to a range of 18 to 21% after seven days without any stimuli given. Although it is not clear of exact mechanism for the change in this receptor expression, *in vitro* culture environments including fetal calf serum and pH might affect T cells. Nevertheless the change did not alter its original property as confirmed by cytokine profiles after activation with magnetic anti-CD3/CD28 beads. Particularly, the levels of type II cytokines including IL-4, IL-5 and IL-10 were significantly decreased to the basal level in $CXCR3^+CCR4^-CD4^+$ T cells (Figure 2). Because there has been no definite surface marker to distinguish Th1 cells from the whole $CD4^+$ T cells yet, $CXCR3^+CCR4^-$ marker was used only to exclude Th2 subpopulations, but not to absolutely purify Th1 cells from the whole $CD4^+$ T cells (Kim *et al*., 2001). $CXCR3^+CCR4^-CD4^+$ T subpopulation may include both Th1 and non-Th2 cells which explains the amount of Th1 cytokines produced by $CXCR3^+CCR4^-CD4^+$ T cells is relatively lower than that of the unsorted $CD4^+$ T cells. However, in spite of the impurity, we assumed that the absence of Th2 cytokines by $CXCR3^+CCR4^-CD4^+$ T cells enhanced the generation of cytotoxic T cells stimulated by DCs *in vitro*.

In addition to efficient contribution to CTL generation (Figure 3 and 4), the use of activated $CXCR3^+CCR4^-CD4^+$ T cells efficiently induced DC differentiation to mature form as determined by upregulation of phenotypic markers and types of cytokine secretion (Figure 5). This approach replaces typically used maturation stimuli including bacterial products, proinflammatory cytokines, and recombinant CD40L (Sato *et al*., 2004; Palucka *et al*., 2005; Voigtlander *et al*., 2006). Because previous studies including our own observation (data not shown) indicated that PGE-2 inhibits IL-12 secretion by DC, we excluded PGE-2 from maturation cocktail and only used LPS and TNF-α. In this way, any external factors that would artificially modulate IL-12 production were avoided, so that a parallel comparison on cytokine pattern of DCs could be done. DC treated with maturation cocktail secreted a significant amount of IL-6, moderate IL-10 and a basal level of IL-12 in contrast to $CXCR3^+CD4^+$/DC that secreted a basal level of IL-6, low IL-10 and high IL-12. IL-6 induces differentiation of bystander DC into a semi-matured state when secreted by semi-matured DC, which inhibit the induction of proinflammatory Th1 responses. Moreover, when T cells are cultured in accumulated IL-6, IL-4 expression and differentiation of Th2-polarized T cell was observed (Frick *et al*., 2006). $CXCR3^+CD4^+$ T cells did not promote IL-10 production compared to the level secreted by mDC and $CD4^+$/DC, while IL-12p70 was continuously secreted in high level. The use of Th1 cells in DC maturation provides an alternative way to overcome the lack of sustained IL-12 synthesis associated with bacterial stimulus.

In spite of the emphasis on the $CXCR3^+CCR4^-CD4^+$ T subpopulations, this purified population did not show dramatic differences in inducing DC maturation compared with total $CD4^+$ T cells (Figure 5). Moreover, unsorted $CD4^+$ T cells induced IL-12 as efficiently as $CXCR3^+CCR4^-CD4^+$ T cells (Figure 5B). Based on the previous study by Hoffmann *et al*, we attribute such observation to the two factors, IFN-γ and CD40L. Despite there is a mixture of population in $CD4^+$ T cells, the activated $CD4^+$ T cells also secrete IFN-γ and express CD40L (data not shown) which induces significant production of IL-12 by DC. A previous study demonstrated that the production of IL-12 was blocked by an anti-CD40L antibody or by separation of the DC and activated T cell fractions by a permeable membrane (Kim *et al*., 2003). However when the idea was tested further for CTL generation, $CXCR3^+CD4^+$/DC used in the CTL culture resulted in a significant increase in the yield of cell numbers, and the overall lytic unit of these CTLs compared to $CD4^+$/DC (Figure 3 and 4). Although the difference in IFN-γ production shown in Figure 3B seems to be statistically significant, the difference in cytotoxity is only minor when CTLs were expan-

ded with CXCR3$^+$CD4$^-$/DCs. We reason the discrepancy that ELISPOT assay is known as more sensitive and quantitative than cytotoxicity assay. Recently, IFN-γ ELISPOT assay has gained increasing popularity for monitoring clinical trials due to its great sensitivity. However IFN-γ ELISPOT alone may not be sufficient because noncytotoxic cells can secrete IFN-γ whereas CTL with lytic activity do not always secrete IFN-γ. This observation is correlated with the previous study that a diverse pool of CTLs with different clonal origins can exert identical cytotoxic effector function against target cells, but exhibit qualitatively different functional behaviors in terms of proliferation and cytokine secretion (Lim *et al*., 2000). In addition to providing Th1 signals to DCs, the costimulatory molecules on the surface of CXCR3$^+$CCR4$^-$CD4$^+$ T cells might have also provided Th1 signals to CD8$^+$ T cells by cross-linking with the known CTL surface molecules such as TCR, CD70 and CD137 (4-1BB) during the CTL culture, resulting in their expansion and increased overall cytolytic function compared to other conditions (Kim *et al*., 2003). It has been reported that stimulation with antibodies specific to MHC class II, CD27 and CD147 enhanced the proliferate response of CTLs to TCR activation (Giuntoli *et al*., 2002). In contrast, two possible factors can be suggested to speculate how the presence of the whole CD4$^+$ T cells exerts these opposing effects on the CTLs. The first is the type of stimulus given to CD4$^+$ T cells. In contrast to previous studies on tetanus toxoid induced T-cell help (Ostankovitch *et al*., 1997; Eiz-Vesper *et al*., 2006), we used anti-CD3/28 magnetic beads to activate freshly isolated CD4$^+$ T cells resulting in nonspecific activation of nonspecific subpopulations. The second is the inhibitory factors produced by the subpopulations including Th2 and regulatory T cells within CD4$^+$ T cells, resulting in reduction of CTL proliferation and the relatively low quality of their effector function (Jin *et al*., 2000; Osada *et al*., 2005).

In this work, we provide evidence that Th1-type cells expressing CXCR3$^+$CCR4$^-$CD4$^+$ molecules make more Th1-type cytokines and influence DC activation, and thus results in enhancement of CD8$^+$ effector T cell activity. To our belief, this is the first study showing that the selective use of Th1-type cells assist DC maturation towards Th1-type response and DC matured by Th1-type cells showed stronger capacity of inducing antigen specific CTLs *in vitro*. However, we realize that the precise mechanism that leads to the enhancement of CD8$^+$ response is not directly addressed here and were only able to present the representative data that IL-12 induces Th1-type response and IL-10 and IL-6 possibly inhibit CD8$^+$ response. However, we can conclude that the effect of Th1 T cells is more likely mediated by both soluble factors secreted by Th1 cells and cell-to-cell interaction, and the cytokine profile of DC was changed towards Th1-type response when activated with CXCR3$^+$CCR4$^-$CD4$^+$ T cells (Figure 5), resulting in enhancement of CD8$^+$ response. In spite of similar changes in the phenotype of the DC, the effect of CXCR3$^+$CD4$^+$/DC on CD8$^+$ response correlates with the previous report that CTL responses can be enhanced *in vitro* when the expression of IL-10 in DC stimulated with LPS and TNF-α was silenced by RNAi instead of blocking anti-IL-10 antibodies even though was no change on MHC class II, CD86, and CD54 expression (Gentao *et al*., 2004). Moreover DC cultured with type I soluble cytokines including IL-12 and IFN-γ induced the differentiation of IFN-γ-producing Th1 cells, which are essential for the generation of CTL against tumor (Steinbrink *et al*., 1997). Nonetheless, we strongly believe that the precise molecular mechanism should be defined further.

The findings here propose that the efficient generation of CTLs result from the ability of CXCR3$^+$CCR4$^-$CD4$^+$ T cells to provide Th1 skewed signals. This work also highlights the importance of a physiological relevant system for CTL generation and APC maturation to augment antitumor responses *in vitro*. Further studies for efficient isolation of Th1 cells, involved immune molecules and *in vivo* animal model should be performed to translate this approach to clinical applications.

Methods

Blood samples and cell separation

PBMC from HLA A2+healthy individuals was isolated by Ficoll density gradient centrifugation (Amersham Pharmacia, Piscataway, NJ). CD14$^+$ monocytes and CD4$^+$, CD8$^+$ T lymphocytes were isolated from PBMCs using marker specific microbeads with AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Each MACS purification was done by positive selection. The purity of subpopulation cells was 95.5% (SD, 0.9%). Consent forms and approval for this study were obtained from the donors and the institutional review board of The Catholic University of Korea, College of Medicine.

Culture conditions and DC generation

Culture experiments were performed in RPMI 1640 with 10% FBS (Sigma-Aldrich, St. Louis, MO). For DC generation, isolated CD14$^+$ cells were cultured for 6 days in RPMI complete medium containing 100 ng/ml GM-CSF (Endogen, Woburn, MA) and 500 U/ml IL-4 (Genzyme, Cambridge, MA). 75 to 80% of the cells showed morpho-

logic and immunophenotypical features of typical DCs, which included the expression of MHC class I and class II molecules and CD86. The medium was replenished with cytokines after 3-4 days. On day 6, final maturation was induced by the addition of either maturation cocktail including 1000 U/ml TNF-α and 1 μg/ml LPS (Sigma-Aldrich, St. Louis, MO), or helper T cells at a ratio of 1:5 in the presence of anti-CD3/CD28 magnetic beads (Dynal Biotech, Lake Success, NY) for an additional 48 h.

Flow cytometry studies

For cell surface staining, cells were processed following standard procedures, and analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). The mAbs to CD4, CD8, CD80, CD83, CD86, HLA-ABC and HLA-DR were purchased from BD Pharmingen (San Diego, CA). After $CD4^+$ T cells isolation using microbeads (Miltenyi, Auburn, CA), cells were stained with fluorescence-conjugated monoclonal antibodies against CXCR3 and CCR4 from BD Pharmingen (San Diego, CA). Populations of cells enriched for $CXCR3^+CCR4^-$ Th1 subsets were isolated by FACs vantage. This produced isolates containing predominantly $CXCR3^{high}/CCR4^{low}$.

Cytokine assay

Immature DCs were stimulated in 96-well culture plates (5×10^3 cells/well in 200 μl vol.) or 24-well culture plates (2×10^4 cells/well in 1 ml vol.) in the presence of maturation cocktail (MC) or helper T cells with anti-CD3/CD28 magnetic beads at a ratio of 1:5 (DC:helper T cells), and cultured for 48 h at 37°C. The 50 μl of cultured supernatants were removed and stored at -20°C, and analyzed for cytokine production with a Cytometric Bead Array (CBA) kit (BD Biosciences, San Diego, CA), Bio-Plex Cytokine Assays (BioRad, CA) or ELISA (Pierce, Rockford, IL) according to the manufacturer's instructions. The lower limit of detection for each cytokine was 20 pg/ml.

CBA analysis

Th1 (IL-2, IFN-γ, TNF-α) and Th2 cytokines (IL-4, IL-5 and IL-10) were quantified simultaneously using a Human Th1/Th2 cytokine Cytometric bead array kit and CBA software (BD Pharmingen, San Jose, CA). This array kit provides a mixture of five microbeads. Inflammatory cytokines (IL-6) was also simultaneously quantified using a CBA Flex Set and FCAP Array software (BD Pharmingen, San Jose, CA). Populations with distinct fluorescent intensities (FL-3) are pre coated with antibodies specific for each cytokine. Fifty microliters of serum and the provided standard cytokines were added to the premixed microbeads in 12 $\times$ 75 mm Falcon tubes (BD Biosciences, San Jose, CA). Mixture of catching beads with samples were incubated for two hours and after the addition of 50 μl of a mixture of PE conjugated antibodies against the cytokines, the mixture was further incubated for 2 h in the dark at the room temperature. This mixture was washed and centrifuged at 500 $\times$ *g* for 5 min and the pellet was resuspended in 300 μl of wash buffer. The FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) was calibrated with setup beads and 1200 events were acquired for each sample. Individual cytokine concentration ratios were indicated by picogram per milliliter.

Generation of WT-1 specific CTLs with autologous DCs

The DCs matured with various stimuli were pulsed with HLA-A02-restricted Wilm's Tumor antigen 1 (WT-1) peptide (RMFPNAPYL) at 10 μg/ml for 2 h, and then irradiated with a dose of irradiation (25 Gy) that entirely prevented outgrowth of matured DCs and added Th cells in the control. The irradiated matured DCs and activated Th cells were seeded into 24-well plates at 1×10^5 and 2×10^5 cells per well. The purified CD8+ T cells were then added to make a 1:10 ratio of DC to T cells per well. After 7 days of coculturing, the stimulated cells were harvested and resuspended at 1×10^6 per well. They were then restimulated with 1×10^5 peptide-pulsed irradiated DCs and 2×10^5 of Th cells. After the second stimulation, every 3 to 4 days, the cells were fed with 10 U/ml interleukin 2 (IL-2; Genzyme, Cambridge, MA) and interleukin 15 (IL-15; R&D Systems, Minneapolis, MN). The cells were harvested either 14 or 21 days later since the initial stimulation and assayed immediately.

IFN-γ ELISPOT assay

The ELISPOT assay was performed as described in the manufacturer's instructions (BD Biosciences, San Diego, CA). Briefly, peptide pulsed autologous Lymphoblastoid cell line (LCL) expressing HLA-A02 as stimulator cells and antigen specific T-cells as responder cells were plated at 1×10^3 and 1×10^4/well, respectively. Responder cell populations were seeded across a range of concentrations to achieve 10 to 100 spots/well so as to facilitate accurate and reproducible counting. All cells were cultured in RPMI-FBS 10% supplemented to a final volume of 100 μl/well. After undisturbed incubation for 20 to 24 h at 37°C, with 5% CO_2, plates were washed 4 times with PBS containing 0.05% Tween 20 (PBS/0.05% Tw). Wells were incubated with biotinylated "detection" antibody against IFN-γ (BD Biosciences, San Diego, CA). The plates were washed 4 times with PBS/0.05% Tw. Avidin-peroxidase-complex (AEC, 100 μl; prepared according to manufacturer's instructions; BD Biosciences, San Diego, CA) was added per well for 1 h at room temperature. The plates were washed 3 times with PBS/0.05% Tw, followed by 3 washes with PBS. AEC substrate (BD Biosciences, San Diego, CA) was prepared according to the manufacturer's instructions and filtered through a 0.45-μM filter prior to use. Per well, 100 μl was added. After 5 to 60 min the reaction was stopped with deionized water and the plates were dried completely prior to membrane removal. The spot number was determined in an independent blinded fashion (AID, Germany). In each experiment, the result was expressed as spots/10000 cells and the results of 3 experiments were used to calculate means and standard deviations.

Cytotoxic assay

The cytotoxic activity of CTLs was measured by a 4 h

^{51}chronium release assay. Briefly, peptide pulsed autologous LCLs (1×10^4 cells) were used as target cells with antigen specific T-cells as effector cells at different effector-target cell ratios (20:1 to 40:1). Experiments were performed in triplicates and the percentage of lysis was calculated as 100% $\times$ (experimental release-spontaneous release)/(maximum release-spontaneous release). Maximum release was obtained by adding 100 μl of 0.2% Triton X-100 to the 100 μl medium containing target cells. Where indicated, data is represented as lytic units 30 (LU_{30}), where one lytic unit is the number of cells derived from the culture necessary to achieve 30% specific lysis of targets. The total number of lytic units was obtained by the formula: [total no. of effectors obtained after the secondary culture]/[no. of effectors required to achieve 30% lysis (LU30)]. SD is derived from the maximum and minimum slopes of lines through the mean $\pm$ SD of the percent specific lysis in the specific region of curve.

Statistical analysis

Control values for each experiment were derived from pooled data on a number of control individuals so that each experiment has a single control value, mean, and standard deviation. Each mean variance was then compared against the control response for each experiment and analyzed for statistical significance using a one-tail unpaired *t* test, assuming unequal variance between the experimental groups. Significant differences are indicated by asterisks.

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