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Cutting Edge

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Cutting Edge: Phenotypic Characterization and Differentiation of Human CD8$^+$ T Cells Producing IL-17[1]

Takaaki Kondo,[2] Hiroshi Takata,[2] Fumichika Matsuki, and Masafumi Takiguchi[3]

Although IL-17-producing CD8$^+$ T cells (Tc17 cells) have recently been identified, the detailed information about these cells still remains unclear. In this article we describe a study investigating the phenotype and differentiation of human Tc17 cells. Human Tc17 cells were detected in a minor population of CD8$^+$ T cells and were predominantly found in CD27$^{-/+}$ CD28$^+$CD45RA$^-$ memory subsets. They also expressed CCR6 and a high level of CCR5. Though most Tc17 cells produced IFN-γ, a small part of the Tc17 cell population was IFN-γ negative in some individuals. Analysis of the in vitro induction of Tc17 cells showed that these cells were induced from the CD27$^+$ CD28$^+$CD45RA$^+$ naive subset and that they expressed the CD27$^-$CD28$^+$CD45RA$^-$ and CCR6$^+$ phenotype. These results together indicate that Tc17 cells have a unique phenotype and suggest that they differentiate from the same precursors that differentiate into IFN-γ-producing CD8$^+$ T cells. *The Journal of Immunology,* **2009, 182: 1794–1798.**

Interleukin-17 is a group of inflammatory cytokines (1). IL-17A is the prototype member of this family and is produced by both CD4$^+$ and CD8$^+$ T cells (2). IL-17- producing CD4$^+$ T cells (Th17 cells) were recently identified as a new subset of Th cells (3). Th17 cells are primary effector cells and play important roles in inflammatory and autoimmune diseases (4–6). Naive CD4$^+$ T cells differentiate into Th17 cells by a pathway that is distinct from that for Th1 and Th2 cell differentiation, suggesting that Th17 cells have specific differentiation pathway (6, 7). In contrast, less is known about Tc17 cells, CD8$^+$ T cells that produce IL-17. A study on mice showed that Tc17 cells are involved in Ag-induced contact hypersensitivity (8). Tc17 cells have also been detected in humans, but no further details were reported (9).

Phenotypic classification of functional CD8$^+$ T cells, such as naive, memory, and effector T cells, is very useful in various studies on mouse or human T cells. 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 functions of CD8$^+$ T cells (10, 11). A recent study demonstrated that CD8$^+$ T cells expressing the phenotypes of CD27$^+$CD28$^-$CD45RA$^{+/-}$ and CD27$^-$CD28$^-$CD45RA$^{+/-}$ could be classified into effector memory and effector cells, respectively, whereas naive and memory CD8$^+$ T cells were phenotypically CD27$^+$ CD28$^+$CD45RA$^+$ and CD27$^+$CD28$^+$CD45RA$^-$, respectively (12). A linear differentiation model for human CD8$^+$ T cells has proposed that "naïve" CD27$^+$CD28$^+$CD45RA$^+$ cells progress to "early" CD27$^+$CD28$^+$CD45RA$^-$ cells and that these cells then differentiate into "intermediate" CD27$^-$ CD28$^+$CD45RA$^-$ cells, which then become "late" CD27$^-$ CD28$^-$CD45RA$^{-/+}$ T cells (13). Such a linear differentiation is supported by ex vivo phenotypic analysis of Ag-specific CD8$^+$ T cells in human viral infections, telomere length measurement, perforin/granzyme expression of CD8$^+$ T cells, and in vitro data on naive T cell priming (14, 15). However, there has been no study on the differentiation pathway leading to Tc17 cells.

In the present study, we focused on the analysis of the phenotypes and the differentiation of human Tc17 cells. We report that human Tc17 cells expressed CCR6 and a high level of CCR5 and that they were predominantly found in CD27$^+$ CD28$^+$CD45RA$^-$ and CD27$^-$CD28$^+$CD45RA$^-$ memory subsets. Tc17 cells induced from naive CD8$^+$ T cells in vitro had a phenotype similar to that found in vivo, suggesting that Tc17 cells have a unique phenotype and a unique differentiation pathway among human CD8$^+$ T cells.

Materials and Methods

Blood samples

Blood samples were taken from 12 healthy adult individuals (nine male and three female; mean age ± SD, 27.4 ± 3.4 years). Kumamoto University Ethics Committee (Kumamoto, Japan) approval was received for this study, and the informed consent of all participating subjects was obtained.

Antibodies

mAbs used in this study were obtained from BD Biosciences, DakoCytomation, eBioscience, Beckman Coulter, or Molecular Probes (Cascade Blue).

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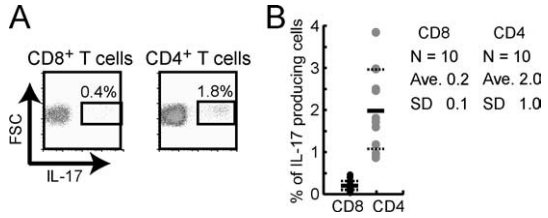

FIGURE 1. IL-17-producing cells among human $CD8^+$ and $CD4^+$ T cells. *A*, IL-17-producing cells in human $CD8^+$ and $CD4^+$ T cell populations. PBMCs isolated from a healthy donor, U-16, were sorted into $CD8^+$ and $CD4^+$ T cells. The sorted cells were cultured for 6 h in F-bottomed 96-well plates containing R10 medium supplemented with PMA (10 ng/ml) and ionomycin (1 μg/ml). IL-17-producing cells were analyzed by flow cytometry. The percentage of IL-17-producing cells is shown in each plot. *B*, Frequency of IL-17-producing cells in $CD8^+$ and $CD4^+$ T cell populations from 10 healthy individuals. The mean percentage and SD of IL-17-producing cells among the $CD8^+$ and $CD4^+$ T cells are presented. Av., Average.

Cell culture

Sorted $CD27^+CD28^+CD45RA^-$ naive $CD8^+$ T cells were stimulated with anti-CD3 mAbs (2 μg/ml) and CD28 mAbs (1 μg/ml) and cocultured in 10% FCS-containing RPMI 1640 (R10) medium at a 1:1 ratio with or without autologous monocytes in 96-well plates. After 5 days in culture, the cells were cultured for a further 4 days in R10 supplemented with human rIL-2 (200 U/ml).

Flow cytometric analysis

Flow cytometric analysis of cell surface markers of and the method used to detect intracellular cytokine production by cells stimulated with PMA/ionomycin were previously described (16). For analysis of Tc17 induction in vitro, cultured cells were stimulated with anti-CD3 mAb (20 μg/ml) and anti-CD28 mAb (20 μg/ml), and then IL-17 production was measured by using a FACSAria flow cytometer.

Cytokine production and ELISA

Purified $CD8^+$ T cell subsets were cultured at 1.4×10^5/well and stimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml) in 96-well U-bottom plates for 48 h. The concentrations of IL-17 and IFN-γ in the supernatants were measured by using human IL-17 or IFN-γ DuoSet (R&D Systems).

Results and Discussion

Frequency of Tc17 cells among human $CD8^+$ T cells

To clarify the secretion of IL-17 from human $CD8^+$ T cells, we first investigated the production of IL-17 from $CD8^+$ and $CD4^+$ T cells isolated from 10 healthy individuals. $CD8^+$ and $CD4^+$ T cells were purified from PBMCs by using magnetic beads, and then the isolated cells were stimulated with PMA and ionomycin. IL-17 production by these cells was investigated by flow cytometry. A representative result of the flow cytometric analysis is shown in Fig. 1*A*. Only 0.4% of the total $CD8^+$ T cells produced IL-17, whereas 1.8% of the total $CD4^+$ T cells did so. The production of IL-17 varied among the 10 individuals, with the percentage of $IL\text{-}17^+CD8^+$ T cells among the total $CD8^+$ T cell population ranging from 0.04 to 0.4% (mean $\pm$ SD, 0.2% $\pm$ 0.1%; Fig. 1*B*). The frequency of $CD8^+$ T cells producing IL-17 was much smaller than that of $CD4^+$ T cells expressing this cytokine (Fig. 1*B*), indicating that the population of Tc17 cells was much smaller than that of Th17 cells.

Human Tc17 cells were predominantly found in $CD27^{+/-}CD28^+$ $CD45RA^-$ subsets

Previous studies showed that human $CD8^+$ T cells can be classified into naive, memory, effector memory, and effector cells by the expression pattern of three cell surface molecules i.e., CD27, CD28, and CD45RA (10, 11). To identify the population producing IL-17, we sorted human $CD8^+$ T cells into six CD27CD28CD45RA subsets and investigated IL-17 secretion from each subset. A representative result from individual U-26 is shown in Fig. 2*A*. Tc17 cells were predominantly found in the $CD27^+CD28^+CD45RA^-$ memory and $CD27^-CD28^+CD45RA^-$ subsets but not in the $CD27^+CD28^+CD45RA^+$ naive and $CD27^-CD28^-$ $CD45RA^{-/+}$ effector subsets. This result was confirmed by the analysis of a total of five individuals in terms of the frequency (Fig. 2*B*) and absolute number (Fig. 2*C*) of Tc17 cells. These results suggest that Tc17 cells are in $CD27^{+/-}$ $CD28^+CD45RA^-$ subsets. Tc17 cells predominantly accumulated in the $CD27^-CD28^+CD45RA^-$ subset (Fig. 2*B*), although the absolute number of Tc17 cells was higher in the $CD27^+CD28^+CD45RA^-$ subset than in the $CD27^-CD28^+$ $CD45RA^-$ one (Fig. 2*C*). The $CD27^-CD28^+CD45RA^-$ subset has not been studied in detail, although this subset is detected in ~2% of human $CD8^+$ T cells (10–12). This subset includes heterogeneous populations expressing perforin and/or granzymes A/B (15). EBV-, human CMV-, hepatitis C virus-, and HIV-specific $CD8^+$ T cells were rarely detected in the $CD27^-CD28^+CD45RA^-$ subset (10–13). These findings imply that this subset may include more Tc17 cells than CTL precursors.

Because most Th17 cells do not produce IFN-γ (9, 17), they are thought to be a subset completely different from Th1 and Th2. Thus, we investigated whether Tc17 cells could produce IFN-γ. Three sorted subsets, i.e., $CD27^+CD28^+CD45RA^-$, $CD27^-CD28^+CD45RA^-$, and $CD27^-CD28^-CD45RA^-$, were stimulated with PMA and ionomycin and then the amounts of IL-17 and IFN-γ produced were measured by flow cytometry. The majority of Tc17 cells in $CD27^+CD28^+$ $CD45RA^-$ and $CD27^-CD28^+CD45RA^-$ subsets from five individuals produced IFN-γ, whereas ~30% of Tc17 cells produced only IL-17 in two of these individuals (Fig. 2*D*). ELISA analysis confirmed that IL-17 was produced in both $CD27^+$ $CD28^+CD45RA^-$ and $CD27^-CD28^+CD45RA^-$ subsets but not in the $CD27^-CD28^-CD45RA^-$ one (Fig. 2*E*). These results suggest that Tc17 cells differentiate from the same precursors that differentiate into IFN-γ-producing $CD8^+$ T cells and that some Tc17 cells differentiate further into specialized Tc17 cells producing only IL-17.

Human Tc17 cells express $CCR5^{high}$ and CCR6

CCR7 is predominantly expressed on naive and central memory $CD8^+$ T cells, and CCR6 is detected on early effector memory ones (16). We recently demonstrated that CCR4 is expressed on central and early effector memory $CD8^+$ T cells and that ~15% of $CCR4^+CD8^+$ T cells are present in the $CD27^-$ $CD28^+CD45RA^-$ subset (our unpublished observation). CCR5 was earlier shown to be expressed on effector memory $CD8^+$ T cells (10). $CCR5^{+/-}CD8^+$ T cells could be divided into three subsets based on their expression level of CCR5, i.e., high expression ($CCR5^{high}$), low expression ($CCR5^{low}$), and no CCR5 ($CCR5^-$). $CCR5^{high}$ cells were found only within $CD27^+CD28^+CD45RA^-$ memory and $CD27^-$ $CD28^+CD45RA^-$ subsets, whereas $CCR5^{low}$ cells were predominantly found in $CD27^+CD28^+CD45RA^-$ memory and $CD27^-CD28^-CD45RA^-$ effector subsets (15). We

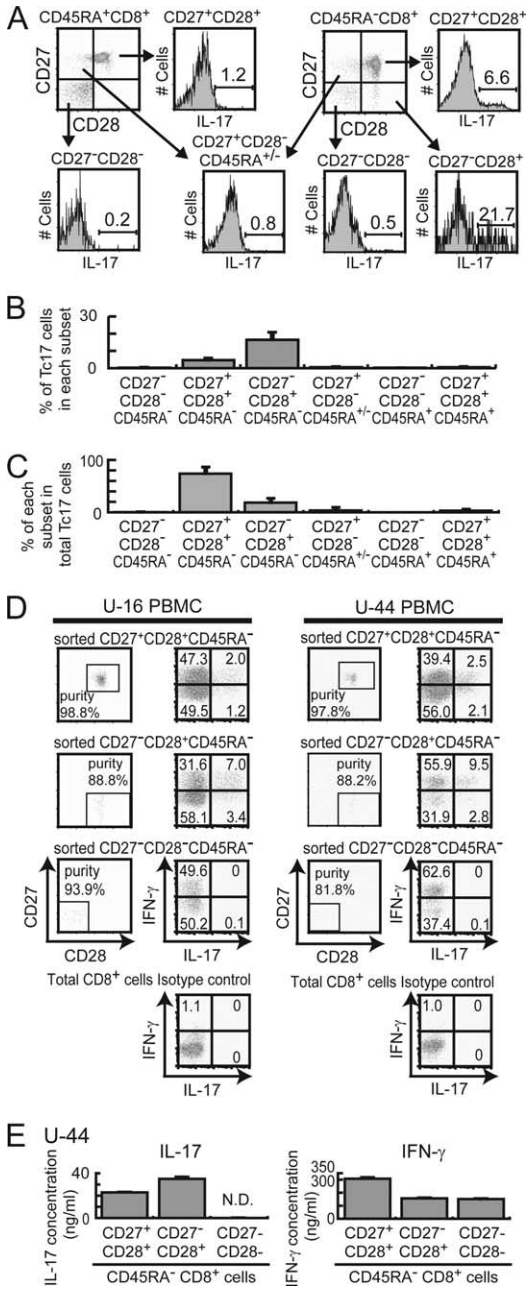

FIGURE 2. Tc17 cells were found only within CD27$^{+/-}$CD28$^+$ CD45RA$^-$ subsets. *A*, Secretion of IL-17 in CD27CD28CD45RA subsets of CD8$^+$ T cells. PBMCs were isolated from healthy donors and then stained with anti-CD8, anti-CD27, anti-CD28, and anti-CD45RA mAbs. Each subset was isolated by using a cell sorter, and six sorted subset cells were stimulated with PMA and ionomycin for 6 h. The percentage of Tc17 cells in each subset from individual U-26 is shown in each plot. *B*, Frequency of Tc17 cells in the CD27CD28CD45RA subsets. The mean percentage and SD of IL-17-secreting cells in each subset from five healthy individuals are shown. *C*, Frequency of cells expressing each CD27CD28CD45RA phenotype in Th17 cells. The mean percentage and SD of IL-17-secreting cells in each subset from five healthy individuals are shown. *D*, Analysis of IL-17 and IFN-γ production in the CD27$^{+/-}$CD28$^+$CD45RA$^-$ and the CD27$^-$CD28$^-$CD45RA$^-$ subsets by flow cytometry. The three subsets were sorted by using a cell sorter, and the sorted cells were stimulated with PMA and ionomycin for 6 h. The percentage of IL-17- and IFN-γ-producing cells in each subset from individuals U-16 and U-44 is shown in each plot. *E*, Analysis of IL-17 and IFN-γ production in the CD27$^{+/-}$CD28$^+$CD45RA$^-$ and the CD27$^-$CD28$^-$CD45RA$^-$ subsets by ELISA. The sorted cells from an individual U-44 were analyzed. The data were shown as mean ± SD from triplicate wells. N.D. indicates a concentration that is lower than the detection limit.

thus investigated the expression of these chemokine receptors on Tc17 cells, because CD27$^{+/-}$CD28$^+$CD45RA$^-$ subsets included cells expressing these chemokine receptors. Tc17 cells expressed the CD45RA$^-$ phenotype (Fig. 2*B*). Most CCR4$^+$CD8$^+$, CCR6$^+$CD8$^+$, and CCR5^{high}CD8$^+$ T cells were included among the cells expressing this phenotype (Fig. 3*A*). Therefore, we focused on CD45RA$^-$CD8$^+$ T cells. We sorted CD45RA$^-$CD8$^+$ T cells expressing or not expressing these chemokine receptors. IL-17 production of sorted cells stimulated with PMA and ionomycin was measured by flow cytometry. A representative result is shown in Fig. 3*B*. We detected IL-17-secreting cells in sorted CCR5^{high} and CCR6$^+$CD45RA$^-$ subsets (mean ± SD for three donors, 16.7 ± 3.0 and 14.7 ± 4.9%, respectively), but not in CCR5^{low}, CCR5$^-$, and CCR6$^-$CD45RA$^-$ subsets from three donors (Fig. 3*C*). In contrast, IL-17-producing cells were not detected in either CCR4$^+$ or CCR7$^+$ subsets (mean ± SD for three donors, 0.6 ± 0.2 and 0.5 ± 0.5%, respectively). More than 90% of the CCR6$^+$CD8$^+$ T cell subset express a high level of CCR5 (16), suggesting that Tc17 cells were predominantly detected in the CCR5^{high} CCR6$^+$CD45RA$^-$ subset.

The results described above suggest that human Tc17 cells are in the CCR5^{high}CCR6$^+$CD27$^{-/+}$CD28$^+$CD45RA$^-$ subsets of CD8$^+$ T cells. Therefore, we next investigated IL-17 production from CD8$^+$ T cells expressing this phenotype. We sorted CD8$^+$ T cells into four subsets, i.e., CCR5^{high}CCR6$^+$ CD27$^-$CD28$^+$CD45RA$^-$, CCR5$^{low/-}$CCR6$^-$CD27$^-$CD28$^+$ CD45RA$^-$, CCR5^{high}CCR6$^+$CD27$^+$CD28$^+$CD45RA$^-$, and CCR5$^{low/-}$CCR6$^-$CD27$^+$CD28$^+$CD45RA$^-$. These sorted cells were stimulated with PMA and ionomycin. A representative result is shown in Fig. 3*D*. As seen in this figure, 44.6 and 27.9% of CCR5^{high}CCR6$^+$CD27$^-$CD28$^+$CD45RA$^-$ and CCR5^{high}CCR6$^+$CD27$^+$CD28$^+$CD45RA$^-$ subsets, respectively, produced IL-17, whereas either of the CCR5$^{low/-}$CCR6$^-$ subsets did not. The same results were found in a different experiment using the same subsets from another individual (data not shown). These results taken together indicate that Tc17 cells were a part of the population of CCR5^{high}CCR6$^+$CD27$^{-/+}$CD28$^+$ CD45RA$^-$CD8$^+$ T cells. Tc17 cells were not detected among CD27$^-$CD28$^-$CD45RA$^{-/+}$ effector CD8$^+$ T cells, suggesting that Tc17 cells, which are effector cells, are different from CD8$^+$ T cells that have the ability to kill target cells. Thus, our findings strongly suggest that Tc17 cells develop via a pathway different from that giving rise to CTL.

Previous studies demonstrated that human Th17 cells are predominantly present in a CCR6$^+$CCR4$^+$ subset or a CCR2$^+$ CCR5$^-$ subset (17, 18). In contrast, Tc17 cells expressed a high level of CCR5 but not CCR4. These results suggest that Tc17 cells have chemotaxic activity different from that of Th17 cells and also that they migrate into the inflammatory sites in response to different chemokines.

In vitro induction of Tc17 cells

Previous studies demonstrated that human Th17 cells are induced from precursors present among naive CD4$^+$ T cells by cell-cell contact with activated monocytes in the context of TCR ligation (19) or by stimulation with anti-CD3 and anti-CD28 mAbs and then culturing them in the medium with IL-1β, IL-6, IL-23, and TGF-β (20). To clarify whether Tc17 cells could differentiate from the naive subset in vitro, we stimulated

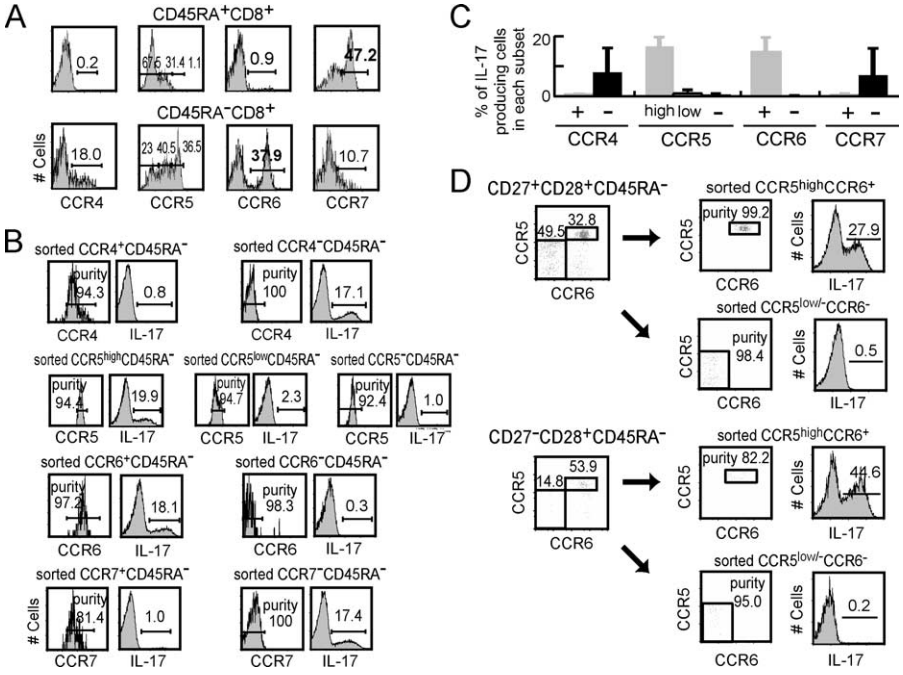

FIGURE 3. Tc17 cells were predominantly found in $CCR5^{high}$ and $CCR6^{+}$ subsets. *A*, PBMCs were stained with anti-CD8, anti-CD45RA, and anti-chemokine receptor mAbs. The frequency of cells expressing each chemokine receptor among $CD45RA^{+}CD8^{+}$ and $CD45RA^{-}CD8^{+}$ cells is shown in each plot. *B*, $CCR4^{+/-}CD45RA^{-}$, $CCR5^{high/low/-}CD45RA^{-}$, $CCR6^{+/-}CD45RA^{-}$, and $CCR7^{+/-}CD45RA^{-}$ subsets were sorted by the cell sorter. Sorted cells were stimulated with PMA and ionomycin for 6 h, and then IL-17-secreting cells were analyzed by flow cytometry. Sorting purities of each subset are shown. The percentage of Tc17 cells in each subset is shown in its respective plot. *C*, Frequency of Tc17 cells in $CCR4^{+/-}CD45RA^{-}$, $CCR5^{high/low/-}CD45RA^{-}$, $CCR6^{+/-}CD45RA^{-}$, and $CCR7^{+/-}CD45RA^{-}$ subsets of $CD8^{+}$ T cells. The percentage and SD of Tc17 cells in each subset from three individuals are shown. *D*, Production of IL-17 in $CCR5^{high}CCR6^{+}$ and $CCR5^{low/-}CCR6^{-}$ subsets of $CD27^{+/-}CD28^{+}CD45RA^{-}CD8^{+}$ T cells. $CCR5^{high}CCR6^{+}$ and $CCR5^{low/-}CCR6^{-}$ subsets of $CD27^{+/-}CD28^{+}CD45RA^{-}CD8^{+}$ T cells from individual U-26 were purified by using the cell sorter. The frequency of expression of CCR5 and CCR6 in the T cells is shown in each plot on the *left*. The percentage of Tc17 cells in each subset is shown in its respective plot on the *right*. Sorting purities of $CCR5^{high}CCR6^{+}$ and $CCR5^{low/-}CCR6^{-}$ subsets of the T cells are shown in each plot in *B* and *D*.

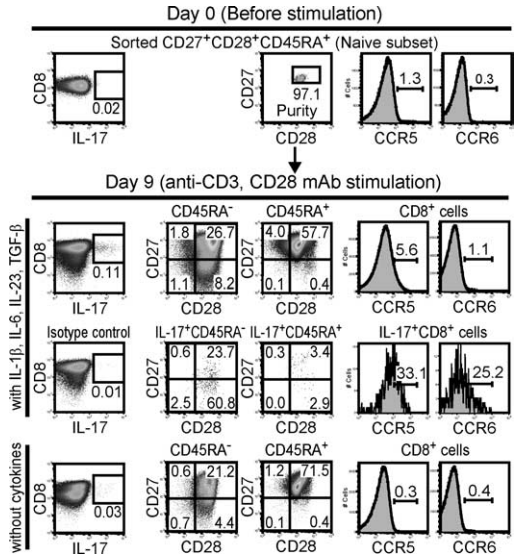

FIGURE 4. In vitro differentiation of Tc17 cells. Naive $CD27^{+}CD28^{+}CD45RA^{+}CD8^{+}$ T cells from individual U-21 were sorted and stimulated with anti-CD3 and anti-CD28 mAbs in the presence or absence of Th17 cell-inducible cytokines. After 9 days in culture, the cells were stimulated with anti-CD3 and anti-CD28 mAbs for 6 h and then stained with anti-IL-17, anti-CCR5, anti-CCR6, anti-CD27, anti-CD28, anti-CD45RA, and anti-CD8 mAbs. The expression of these surface molecules on $CD8^{+}$ T cells and the frequency of Tc17 cells in each subset were analyzed by flow cytometry. The frequency of each subset in the cultured $CD8^{+}$ and Tc17 cell population is shown in its respective plot.

purified $CD27^{+}CD28^{+}CD45RA^{+}$ naive $CD8^{+}$ T cells for 5 days with plates coated with anti-CD3 and anti-CD28 mAbs in the presence or absence of autologous monocytes or in the presence of IL-1β, IL-6, IL-23, and TGF-β and then cultured the cells for a further 4 days in medium supplemented with human rIL-2. The result showed that only 0.01–0.02% of the $CD8^{+}$ T cells cultured with monocytes exhibited IL-17 production (data not shown). In contrast, Tc17 cells were induced from the naive $CD8^{+}$ T cells by culturing the naive cells stimulated with anti-CD3 and anti-CD28 mAbs in the medium with IL-1β, IL-6, IL-23, and TGF-β (Fig. 4). Induced Tc17 cells expressed the $CD27^{+}CD28^{+/-}CD45RA^{-}$ phenotype, and ~30% of these cells expressed CCR5 or/and CCR6 (Fig. 4). The same results were found in an experiment using the naive cells from a different individual.

In conclusion, in this study we showed that human Tc17 cells express a unique phenotype and chemokine receptors and that they differentiate from the same precursors that differentiate into IFN-γ-producing $CD8^{+}$ T cells or CTLs. These findings suggest that Tc17 cells are a unique cell lineage in terms of both function and differentiation.

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Disclosures

The authors have no financial conflict of interest.

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