Interleukin-12 Is the Optimum Cytokine To Expand Human Th17 Cells In Vitro

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Recently, a new lineage of CD4+ T cells in humans and in mice has been reported. This T helper cell secretes interleukin-17 (IL-17) and has been defined as T helper 17 (Th17). Th17 cells express the IL-23 receptor (IL-23R) and play an important pathogenic role in different inflammatory conditions. In this study, our aim was to characterize the optimum conditions for isolation and propagation of human peripheral blood Th17 cells in vitro and the optimum conditions for isolation of Th17 clones. To isolate Th17 cells, two steps were taken. Initially, we negatively isolated CD4+ T cells from peripheral blood mononuclear cells of a normal human blood donor. Then, we isolated the IL-23R+ cells from the CD4+ T cells. Functional studies revealed that CD4+ IL-23R+ cells could be stimulated ex vivo with anti-CD3/CD28 to secrete both IL-17 and gamma interferon (IFN-γ). Furthermore, we expanded the CD4+ IL-23R+ cells for 1 week in the presence of anti-CD3/CD28, irradiated autologous feeder cells, and different cytokines. Our data indicate that cytokine treatments increased the number of propagated cells 14- to 99-fold. Functional evaluation of the expanded number of CD4+ IL-23R+ cells in the presence of different cytokines with anti-CD3/CD28 revealed that all cytokines used (IL-2, IL-7, IL-12, IL-15, and IL-23) increased the amount of IFN-γ secreted by IL-23R+ CD4+ cells at different levels. Our results indicate that IL-7 plus IL-12 was the optimum combination of cytokines for the expansion of IL-23R+ CD4+ cells and the secretion of IFN-γ, while IL-12 preferentially stimulated these cells to secrete predominately IL-17.

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Recent studies have indicated that some of these cells have the ability to produce gamma interferon (IFN-γ) (2). Similarly to Th1 and Th2 cells, Th17 cells require specific cytokines and transcription factors for their differentiation (39). A limited amount of information about the trafficking receptors of Th17 cells is available (1, 2, 31). In the mouse, the differentiation pathway of Th17 cells has been linked to the presence of transforming growth factor β and IL-6, while the maintenance and expansion of these cells seem to be IL-23 dependent (4, 23, 42). Moreover, due to the lack of Th17-specific phenotypic markers, identification and analysis of these cells to date have relied largely on detection of IL-17 mRNA in tissues or measurement of IL-17 protein levels in biological fluids (12, 27). However, recent studies have described the expression of the IL-23 receptor (IL-23R) as a phenotypic marker of Th17 cells (1, 2, 44).

IL-23 consists of a heterodimer of a 40-kDa protein (p40), which is also a component of heterodimeric IL-12, and a protein termed p19. Human p19 and mouse p19 share 70% amino acid sequence identity and are the proteins most closely related to p35, the subunit of IL-12 not shared with IL-23 (28). In humans, IL-12 promotes proliferation of both naive and memory human T cells; however, the proliferative effect of IL-23 is still restricted to memory T cells (11). Although IL-23 is not involved in Th17 differentiation, it plays an important role in maintaining Th17 effector function (38, 42). IL-23 uses the same Jak-Stat signaling molecules as IL-12. However, the compositions of DNA-binding Stat complexes induced by IL-12 and IL-23 exhibit potentially important differences. IL-12 induces a DNA-binding complex containing only Stat4, while IL-23 induces several complexes containing Stat3, Stat1, Stat4, and possibly Stat3/Stat4. These significant differences should...
be expected in the biological responses induced by IL-23 and IL-12 (29). Through the IL-23R, IL-23 activates STAT1, STAT3, STAT4, and STAT5 and can induce IFN-γ, IL-10, and IL-17, depending on the cell type (41).

It is well-known that IL-2 is a T-cell growth factor in vitro (7). Recently, IL-21 has emerged as a key modulator of transforming growth factor β signaling, leading to the reciprocal differentiation of regulatory T cells and Th17 cells (10). IL-21 is a type I cytokine that shares a common cytokine receptor gamma chain (36) with IL-2, IL-4, IL-7, IL-9, and IL-15. These cytokines are critically important for both the maintenance and the function of T and B cells (9). In addition, it was recently reported that IL-15 stimulates the expression of IL-17 by T cells (8).

Most of the information on Th17 cells has been generated using mouse models to understand the differentiation of Th17 cells from naive Th0 cells. However, little information about the propagation of Th17 cells in vitro in humans is known (39). In this study, we optimized the propagation conditions of Th17 cells isolated from human peripheral blood in vitro and determined the optimum conditions for isolation of Th17 clones.

**MATERIALS AND METHODS**

**Subjects.** Discarded whole-blood units from unidentified healthy donors were purchased from Hoxworth Blood Center, University of Cincinnati (Cincinnati, OH). Peripheral blood mononuclear cells (PBMC) were separated using a standard density gradient centrifugation method (Ficoll-Paque; Amersham Pharmacia, Piscataway, NJ). PBMC were either used fresh or cryopreserved at standard density gradient centrifugation method (Ficoll-Paque; Amersham Phar-}

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cells were stimulated for 4 to 6 h in the presence of anti-CD3/CD28 beads or phytohemagglutinin (PHA) (5 mg/ml) and Golgi Stop (BD Biosciences), as described previously (40). Cells were surface stained with anti-CD4–peridinin chlorophyll protein (BD Biosciences catalog no. 550631) for 20 min and then washed. Cells were made permeable with Cytofix/Cytoperm reagents (BD Biosciences) according to the manufacturer's instructions. Cells were stained with FITC-conjugated anti-IL-17 (eBioscience catalog no. 11-7179), anti-IFN-γ–PE (BD Biosciences catalog no. 340452), or isotype controls and analyzed by flow cytometry (FACScan; Becton Dickinson) by gating on CD4+ T cells.

Statistical analysis. Statistical analysis was performed by paired Student's t test using Graph Pad Prism software. Results with a P value of <0.05 were considered significant.

FIG. 1. Flow cytometric analysis of the isolated cells. Cells at a concentration of 1 × 10⁶ cells/ml were stained with biotinylated anti-IL-23R, avidin-FITC, and anti-CD4–PE according to the manufacturers' instructions (A, B, and C). For surface staining of IL-12R, enriched CD4+ IL-23R+ cells were stained with biotinylated anti-IL-23R for 20 min at 4°C and then washed and stained with avidin-FITC according to manufacturer instructions. Isotype control antibodies were used as negative controls. (A) Enriched CD4+ cells. (B) Enriched CD4+ IL-23R+ cells. (C) Remaining CD4+ IL-23R− cells. (D) Graph of the expression of the IL-12R on the isolated CD4+ IL-23R+ cells.

RESULTS

Isolation and phenotypic analysis of CD4+ IL-23R+ T cells from human blood. Negative selection of CD4+ T cells yielded approximately 4 × 10⁶ out of 1 × 10⁷ PBMC with approximately 95% purity, as shown by flow cytometric analysis (Fig. 1A). Positive selection of IL-23R+ cells from CD4+ T cells yielded approximately 1 × 10⁶ out of 1 × 10⁷ CD4+ T cells with approximately 90% purity, as shown by flow cytometric analysis (Fig. 1B). Fewer than 3% of CD4+ IL-23R− cells were positive for the IL-23R (Fig. 1C). CD4+ IL-23R+ cells had a
higher expression of CD4 on the surface than did CD4+/IL-23R- cells (Fig. 1B and C). Additionally, we measured the expression of the IL-12R on the isolated CD4+/IL-23R- cells. As shown in Fig. 1D, more than 90% of the cells expressed the IL-12R.

Functional characterization of CD4+/IL-23R cells. To evaluate the functional activity of the IL-23R cells, levels of IL-17 and IFN-γ secretion by these cells were evaluated after stimulation with anti-CD3/CD28 beads. Our results revealed that IL-17 secretion, as measured by ELISA, was significantly higher (P = 0.0245) in CD4+/IL-23R+ cells than in CD4+/IL-23R- cells (257.3 ± 12 and 98 ± 17 pg/ml, respectively) (Fig. 2A). Additionally, the number of CD4+ IL-23R- cells secreting IFN-γ was significantly lower (P = 0.0188) than the number of CD4+ cells (549.25 ± 69.6 and 2,485 ± 374.8 IFN-γ-secreting cells [ISCs]/10⁶ cells, respectively) (Fig. 2B).

Optimization of the conditions required for propagation of CD4+ IL-23R+ cells. To optimize the conditions for propagation of CD4+ IL-23R+ T cells, we expanded the cells for 2 weeks in the presence of anti-CD3/CD28 beads and different cytokines (IL-2, IL-7, IL-12, and IL-15). The growth curves of the cells under different conditions were analyzed, and the time needed for 50% growth was calculated. IL-12 was the optimum cytokine, with 7.74 days for 50% growth, versus 9.32 days for IL-2, 8.93 days for IL-7, and 8.75 days for IL-15. (In the absence of cytokines, no growth was observed, and 50% cell growth could not be calculated.) To evaluate the best combination of cytokines to propagate CD4+ IL-23R+ cells in the presence of anti-CD3/CD28 beads, we used the optimum concentration of IL-7, IL-15, IL-12, or combinations to determine the optimum conditions for growth. As shown in Fig. 3, addition of a single cytokine (IL-7, IL-12, or IL-15) increased the number of cells 63- to 88-fold. However, IL-7 and IL-15 are antagonistic to each other, and the combination increased the number of cells only 14-fold. This growth was lower than the growth observed with each cytokine alone. In contrast, IL-7 is synergistic with IL-12, and this combination was the optimum for the expansion of CD4+ IL-23R+ cells (99-fold increase).

Functional characterization of the expanded CD4+ IL-23R+ cells. To evaluate the functional activity of the expanded IL-
after stimulation with anti-CD3/CD28 beads (34,130 ± 5,218 ISCs/10^6 cells) compared to nonstimulated cells (4,820 ± 424 ISCs/10^6 cells), as measured by ELISPOT assay (A). Additionally, IL-17 secretion by CD4^+ IL-23R^+ cells stimulated with anti-CD3/CD28 beads (386.5 ± 49.7 ng/ml) was highly significant (P = 0.0029) in comparison to the level for nonstimulated cells (48 ± 12.5 ng/ml), as measured by ELISA (B). Data represent averages and standard deviations. Conc., concentration.

**DISCUSSION**

Th17 cells have been described as a CD4^+ T-cell lineage distinct from Th1, Th2, and regulatory CD4^+ T cells (30). Recent data suggest that Th17 cells play an important role in regulation of the immune responses and induction of autoimmunity (4). To evaluate the role of Th17 cells, a system is needed to optimize the propagation of Th17 cell lines and clones in vitro. In the current study, we isolated differentiated Th17 cells from human PBMC and identified the optimum conditions for the propagation of these cells in vitro. Differentiated Th17 cells were isolated from the PBMC by using immunomagnetic beads to remove all non-CD4 cells. Then, positive selection of IL-23R^+ CD4^+ T cells was accomplished by using a MAb against IL-23R. It has been reported that Th17
cells express specifically IL-23R (1, 2, 44). The frequency of IL-23R⁺ T cells among CD4⁺ T cells is only 1%. The purity of the isolated, enriched CD4⁺ IL-23R⁺ cells was approximately 90%, as measured by flow cytometry. In addition, fewer than 3% of CD4⁺ cells were positive for the IL-23R, which indicates that other cell types, such as NK cells (28), macrophages, and dendritic cells, express the IL-23R (29).

We further evaluated the functional activity of the ex vivo-isolated CD4⁺ IL-23R⁺ cells by measuring the levels of IL-17 and IFN-γ secretion with an IL-17 ELISA and an IFN-γ ELISPOT assay, respectively. Our results indicate that IL-17 secretion is significantly higher in CD4⁺ IL-23R⁺ cells than in CD4⁺ IL-23R⁻ cells. The number of CD4⁺ IL-23R⁺ cells secreting IFN-γ is significantly lower than the number of CD4⁺ cells. These findings suggest that CD4⁺ IL-23R⁺ cells secrete IL-17 and IFN-γ, in accordance with previous studies which suggest that IFN-γ- and IL-17-producing CD4 T cells might represent a common lineage (5, 26). Recently, the existence of remarkable proportions of IL-17- and IFN-γ-producing Th17 cells in the guts of subjects with Crohn's disease has been described (2). It was also demonstrated that IL-12 downregulates IL-17 expression but upregulates IFN-γ expression in Th17 cells. Thus, Th17 cells could secrete IL-17 or IFN-γ in response to the dominant cytokines present in the microenvironment or the receptors involved in the activation. However, other studies have indicated that IFN-γ- and IL-17-producing CD4 T cells are distinct populations of effector cells, each having a unique role in the adaptive immune system; however, the lineage relationships between the two phenotypes were unclear (14).

None of these studies clarified whether the Th17 cells are two subsets, one secreting IFN-γ and another secreting IL-17, or whether they are one type of cell that secretes IFN-γ and/or
IL-17 under different maturation and microenvironmental conditions. To address this question, cloning of Th17 cells may be necessary. However, to clone Th17 cells, we need to understand the optimum conditions necessary for in vitro expansion of these cells. In this study, we characterized the optimum conditions necessary for the proliferation of Th17 cells. Isolated CD4+ IL-23R+ cells were expanded in vitro for 2 weeks in the presence of anti-CD3/CD28 beads and different cytokines (IL-2, IL-7, IL-12, and IL-15). The growth curves of the cells under different conditions were analyzed, and the time needed for 50% growth was calculated. We found that IL-12 was the optimum cytokine, with about 7.7 days for 50% growth. Moreover, our results revealed that the addition of a single cytokine (IL-2, IL-7, IL-12, or IL-15) increased the number of cells 63- to 88-fold.

It is well known that IL-2, IL-7, and IL-15 are members of the IL-21 family. Most cytokines in this family are critically important for both the maintenance and the function of T cells (9). Additionally, it has been reported that IL-21 drives Th17 differentiation (36). In our study, IL-2 had minimal and B cells (9). Additionally, it has been reported that IL-21 was the optimum cytokine, with about 7.7 days for 50% growth. Therefore, IL-7 could compete for the gamma chain of IL-15R and lead to the antagonistic effects.

Regarding IL-17 secretion, individual IL-23, IL-7, and IL-12, as well as the combination of IL-12 plus IL-7, significantly increase IL-17 secretion in comparison to the level for non-stimulated cells. We further characterized the secretion of IL-17 by the expanded CD4+ IL-23R+ T cells by intracellular staining of IL-17 and IFN-γ. IL-17 was produced by 1.2% of the enriched CD4+ IL-23R+ T cells stimulated by anti-CD3/CD28, and in comparison to 0.2% of the unstimulated cells. Our data suggest that unstimulated Th17 does not secrete IL-17 spontaneously but needs stimulation through other T-cell receptors to secrete IL-17. The mechanism of stimulation of Th17 to secrete IL-17 and not IFN-γ is still unknown and needs further investigation, but our data suggest that PHA is more efficient in the stimulation of Th17 to secrete IL-17 than anti-CD3/CD28 (data not shown). Additionally, our intracellular staining data indicate that IFN-γ- and IL-17-secreting cells are distinct populations, because few (<0.01%) CD4+ IL-23R+ cells secrete both IFN-γ and IL-17.

In conclusion, our study provides the optimum conditions for isolation and propagation of human Th17 cells and clones in vitro. In addition, our results indicate that the combination of IL-7 and IL-12 is the optimum for the expansion of cells and that IL-12 preferentially stimulated IL-23R+ CD4+ cells to secrete predominately IL-17.

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