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The Role of Interleukin-23 in Stability of In Vitro T Helper-17 Cells

Marjan Taherian[1], Ali Reza Razavi[1], Maryam Izad[2], Roobina Boghozian[1], Haideh Namdari[2], Mojgan Ghayedi[2], Parisa Rahimzadeh[1], Katayoon Bidad[3], and Eisa Salehi[2]

[1] *Immunology Department, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
[2] *Immunology Department, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*
[3] *Immunology, Asthma and Allergy Research Institute, Tehran University of Medical Sciences, Tehran, Iran*

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ABSTRACT

Interleukin (IL)-17-producing T helper (Th)-17 cells have recently been explained as a distinct population of CD4+ T cells which play an important role in immunity against infectious agents. Establishment of persistent phenotype of Th17 cells and recognition of lineage-deviating factors are of most attractive goals in modern researches in immunology. Although IL-6 and TGF-β are frequently used to differentiate naive T cells to Th17 phenotype in mouse models, the application of IL-23 and its importance in preventing cells from plasticity needs to be more investigated. Our main objective was to evaluate the role of IL-23 in Th17 to Th1 plasticity.

In this research project, we generated in vitro Myelin oligodendrocyte glycoprotein (MOG)-specific Th17 cells in the presence of TGF-β, IL-6, IL-23 and peptide MOG35-55. Th17 development was confirmed by assessment of relevant transcription factors and secreted cytokines by flowcytometry and ELISA, respectively. Th17 to Th1 plasticity was monitored by consecutive samplings in different time points without any extra supplementation of IL-23. Cell culture supernatant was evaluated for Interferon (IFN)-γ secretion and cells were evaluated for intracellular expression of this cytokine.

Our results showed that the employed method was relatively convenient in developing antigen-specific Th17 cells. We also showed that IL-23 deprivation which happens by prolongation of culture period, can convert IL-17 producing cells to IFN-γ secreting Th1 phenotype.

IL-23 can be considered as a Th17 phenotype stabilizing factor for in-vitro developed lineages.

Keywords: CD4-Positive T-Lymphocytes; Interleukin-17; Interleukin-23; Myelin-Oligodendrocyte Glycoprotein; Th 17 Cells

Corresponding Author: Eisa Salehi, PhD; and Ali Reza Razavi, MD; PhD;
Immunology Department, School of Medicine and School of Public Health, Tehran University of Medical Sciences, Tehran, Iran. Tel: (+98 21) 6405 3236, Fax: (+98 21) 664 19536, E-mail: eisalehi@sina.tums.ac.ir and razavial@tums.ac.ir

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INTRODUCTION

CD4+T cells are classified based on their effector functions and transcription factors. T helper (Th)1 and Th2 cells are studied more extensively with T-bet and GATA-3 as their exclusive transcription factors, respectively.[1,2] Th17 cells, as a major effector subset, are recently considered as main players in defense against infectious diseases as well as in development of autoimmune diseases.[3-5] Th17 cells are characterized by the production of IL-17[6,7] and presence of RAR-related orphan receptor gamma (RORγt), as their main transcription factor.[8-10]

It was previously believed that Interferon (IFN)-γ producing Th1 cells were the main pathogenic T cells in several inflammatory and autoimmune diseases such as psoriasis, multiple sclerosis (MS) or its animal model named experimental autoimmune encephalomyelitis (EAE),[11,12] but recent studies showed that Th17 cells can play more important roles in these autoimmune disorders.[13-15]

Cytokine milieu or growth microenvironment is very important in differentiation of T cells to different subtypes. Th17 cells can be derived from naïve T cells in the presence of TGF-β and IL-6. While IL-23 is not necessary for this differentiation,[16] it is required for the maintenance and expansion of Th17 cells.[17,18] The exact role of IL-23 in this lineage stability, however, remains unknown.[3,4] IFN-γ, the main Th1 cytokine and IL-2 have been shown to exert inhibitory effects on Th17 development and differentiation.[6,7,19] On the other hand, TGF-β which is required for Th17 differentiation, inhibits Th1 development.[20] Moreover, IL-12 is essential for Th1 differentiation.[21-23] Recently, many observations have shown remarkable plasticity between cells, including the conversion of Treg cells into Th17 cells and Th17 cells into Th1 cells.[24-28]

Presence of IL-17 and IFN-γ single-producing T cells and IL-17/IFN-γ double-positive T cells are reported by a number of researchers in autoimmune diseases.[29,30] Similar populations have been shown in human in in vitro differentiated Th17 cells.[31,32]

It has been observed that IL-17-producing T cells can begin to secrete IFN-γ when cultured in vitro with IL-12.[25] Thus, regardless of developmental programs that characterize Th17 and Th1 differentiation; there is a plasticity seen between these lineages.

Despite the data in this context, effect of different combinations of cytokines in the lineage differentiation of T cells needs to be more clarified. In the present study, we aimed to investigate the plasticity of in vitro-generated Th17 cells in the presence of TGF-β/IL-6 and IL-23 to generate a model to study autoimmunity.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice were purchased from Animal Production facility of the Pasteur Institute (Tehran, Iran). The mice were housed in conventional facility in cages in a room at 22°C and a 12/12 79 hours light/dark cycle, allowing them free access to food and water. EAE was induced in 8 to 10 week-old mice and then spleen and lymph node cells were isolated for further experiments. All procedures and experiments involving animals were approved by the guidelines of the Animal Ethical Committee of Tehran University of Medical Science.

Culture media and Recombinant Cytokines

Complete culture medium for cell culture experiments consisted of RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum (both from Gibco, UK), 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich). Mouse recombinant cytokines TGF-β, IL-6 and IL-23 were purchased from R&D System, USA.

Induction of EAE Using MOG35–55 as Immunongen

C57BL/6 mice were injected with 200 µL of an emulsion of 300 µg MOG peptide (Alexis, Enzo Life Science, Netherlands) in complete Freund's adjuvant (CFA, Sigma, USA) containing 5 mg/ml of heat-inactivated *Mycobacterium tuberculosis* (H37A; Sigma, USA) subcutaneously on the flank. Additionally, 1 µg Pertussis toxin (Alexis, Enzo Life Science, Netherlands) in 200 µL Phosphate buffered saline was administered intraperitoneally at days 0 (500 ng per mouse) and 2 (500 ng per mouse) post immunization.

Naive T Cell Purification

Immunized mice were sacrificed at the peak of disease about days 21-22. The Lymph nodes and the spleens were isolated and mononuclear cells were separated by Ficoll-Paque (Lymphoflot, Biotest, Germany). $CD4^+CD62L^+$ naive T cells were purified by two sequential MACS

purification steps (Miltenyi Biotec, mouse $CD4^+$ $CD62L^+$ T Cell Isolation Kit II). Purity of isolated T cells was ≥ 95% as assessed by flowcytometry.

In-vitro Differentiation of MOG-specific Th17 Cells

Naive T Cells were stimulated in the presence of 5×10^6 ml^{-1} irradiated splenocytes (4000 rad) as feeder layer, 2 µg/ml anti-mouse CD28 antibody (clone 37.51, eBioscience) and 25 µg/ml of peptide MOG 35–55. IL-6 (30 ng/ml) and TGF-β (3 ng/ml) were added to the cultures as polarizing cytokines. 2 days after exposure, culture medium was supplied with 10 ng/ml IL-23 and MOG peptide to induce Th17 differentiation. All cytokines were purchased from R&D Systems, USA. The differentiation process was monitored daily using a microscope. The cells reached the resting stage after 5 days of culture. As undifferentiated control, T cells were cultured in the presence of irradiated splenocytes and MOG peptide without any polarizing cytokine.

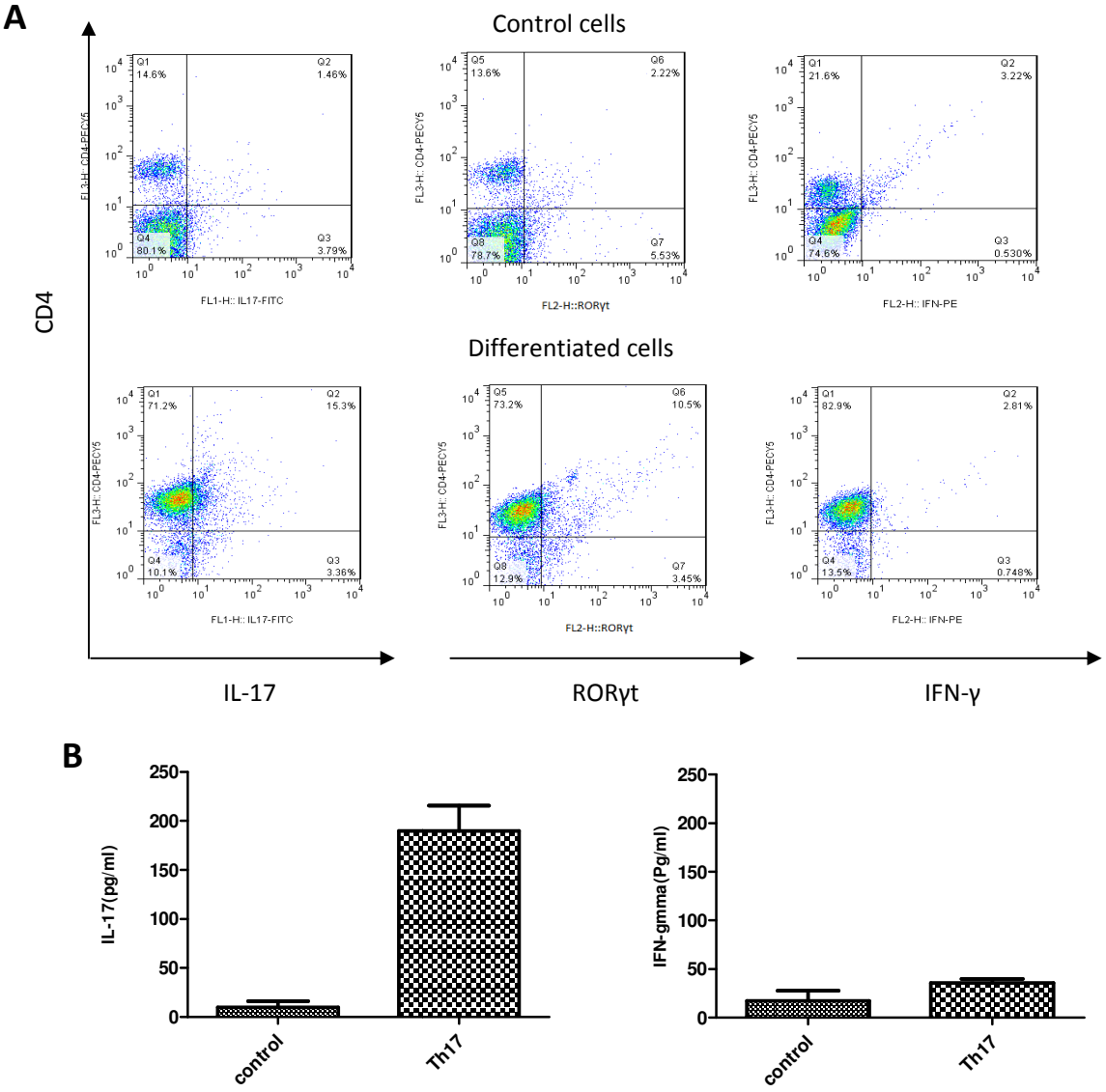

Figure 1. A) Flowctyometric assessment of IL-17, RORγt and IFN-γ in naive $CD4^+CD62L^+$ cells cultured in Th17 polarizing condition containing IL-6, TGF-β and IL-23 as well as in cells cultured in neutral condition. B) Secreted IL-17 and IFN-γ measured in supernatant collected on 5th day of culture by ELISA in IL-17 polarizing or neutral condtions.

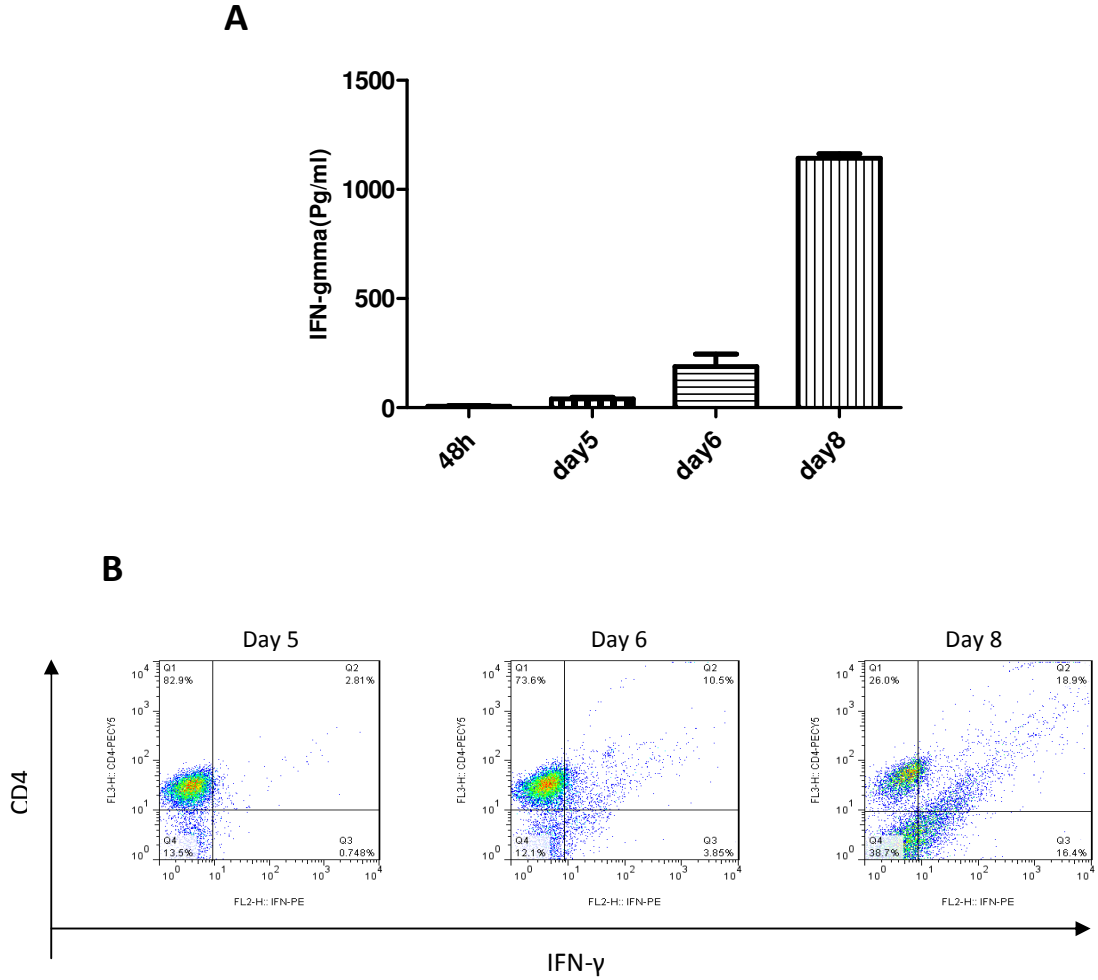

Figure 2. A) CD4$^+$CD62L$^+$ T cells were stimulated with irradiated APCs and peptide MOG and differentiated into Th17 cells with polarizing cytokines including IL-6 and TGF-β. After 48h, culture was supplemented with IL-23 and MOG peptide. Culture supernatant collected on days 2, 5, 6 and 8 and IFN-γ level was measured by ELISA according to the instruction supplied by company.

B) Cells were harvested at days 5, 6 and 8 and IFN-γ producing cells were analyzed after intracellular staining by flowcytometry.

Cytokine Measurement

Level of secreted cytokines (IL-17 and IFN-γ) in cell culture supernatants were measured using Mouse ELISA Ready-SET-GO according to the manufacturer's instructions (eBioscience, USA). To measure intracellular level of IL-17, IFN-γ and RORγt expression, cells were stimulated for 4.5 h with 50 ng/ml PMA (Sigma-Aldrich) and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of Brefeldin-A (GolgiStop; eBioscience) and analyzed by flow cytometry (BD FACSCalibur) .

RESULTS

As shown in figure 1A, Naïve T cells cultured in our polarizing condition preferentially differentiated to Th17 as identified by flowcyotomtric assessment of intracellular IL-17, IFN-γ and RORγt when compared to cells cultured in control neutral condition (15.3% vs. 1.4% for IL-17, 2.8% vs. 3.2% for IFN-γ and 10.5% vs. 2.2% for RORγt, respectively). Secretion of IL-17 but not IFN-γ in cell culture supernatant was significantly higher in polarizing condition than in control neutral condition (Figure 1B).

To investigate the stability or plasticity of cultured cells, Naive cells were cultured in polarizing medium and harvested in different time points. We examined dynamic changes of IFN-γ production both intracellularly and in culture supernatant on days 2, 5, 6 and 8. As depicted in figure 2A, cultured cells tend to secrete more IFN-γ in longer periods of culture. Assessment of intracellular level of IFN-γ by flowcytometry confirmed the deviation of cells from low IFN-γ producer cells to high producers as the culture time was prolonged (figure 2B). IFN-γ producing cells increased from 2.8% in second day to 18.9% of total cells in day 8 of culture.

DISCUSSION

Starting from the naive T cell population, we generated MOG-specific Th17 cell subset in vitro. We tried to establish a convenient protocol for generating MOG-specific Th17 lineage. This kind of differentiated cell population can be used for more investigations including generating animal models for different diseases. In several investigations, naive T cells from a MOG-specific TCR transgenic line (2D2 mice) were utilized for in vitro differentiation of Th17 cells.[33,34] Other investigators tried to generate CNS Ag-specific T cell subsets by isolating T cells from mice and expanding them in vitro with Th17 polarizing cytokines as we did.[35,36]

One of the main differences between this study and previous ones is the starting population used for the differentiation of Th17 cells in vitro. MOG peptide is used as a mere stimulator of specific T cells in our study (instead of Anti CD3 as a pan T cell activator). Thus we expanded only MOG specific T cell clones. After several efforts, we could establish a relatively convenient method for in vitro differentiation of MOG-specific Th17 cell subset.

We found that Th17 cells tended to convert to Th1 phenotype when cell culture continued without replenishment of IL-23. As we substituted the IL-6 and TGF-β continuously in culture, this phenotype change could not be the result of IL-6 and TGF-β deficiency. Studies have shown that presence of IL-6 and TGF-β is sufficient and essential for RORγt and IL-17 expression while IL-23 is clearly not required for initial induction of Th17 cells but it is vital for the maintenance of IL-17 expression.[37] In accordance to our results for in vitro generated Th17 cells, it has been shown that IL-17 expression is dependent on continuous supplementation of TGF- β/IL-6 or IL-23 signaling and can be suppressed by IL-12, IFN-γ and IL-4 signaling[1]. We clearly showed that Th17 cells which mainly produced IL-17 and no considerable IFN-γ in the early days of differentiating culture turned to IFN-γ producing cells when culture period was prolonged. It seems that these IFN-γ producing cells were originated from IL-17 producer cells in in vitro conditions and acquired the ability to produce IFN-γ because of IL-23 degradation as culture time passed. More studies have to be performed to find out if such conversion is due to an inherent plasticity of Th17 cells. Our results showed a critical role for IL-23 in stable expression of IL-17 and was consistent with other *in-vivo* and *in-vitro* studies.[5,37-41]

Although the mechanism by which IL-23 stabilizes Th17 cells is unknown, but IL-23 can induce up-regulation of its receptor,[42] this can increase the responsiveness of the cells to IL-23 and therefore increasing Th17 cell stability. Thus, IL-23 can prevent Th17 cells from differeniating into Th1 cells with the mechanism other than that reported previously in association with inhabition of assembly of a functional IL-12R.[34] It has been shown in *in-vivo* studies that dendritic cells are the main source of IL-23 for persistent expression of IL-17 in some pathologic conditions.[37] As a conclusion, the present study suggests that IL-23 may play an important role in Th17 stability and inhibition of plasticity from Th17 into Th1 cells. This study provides information on a cell line to study autoimmunity in animal models.

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