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Different Doses of Transforming Growth Factor-β on In vitro Differentiation of Human Naïve CD4+ T Cells to T Helper 17

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ABSTRACT

An appropriate differentiation of distinct human CD4+ T cell subset is critical for manipulating these cells for using in immunity related diseases. Despite various attempts to clarify the role of different factors involved in Th17 differentiation, many crucial contradictions yet remained to be optimized. Although it has been shown that the differentiation of in-vitro Th17 cells culture conditions requires the presence of IL-1beta, IL-23, IL-2, IL-21, IL-6 and TGF-β, the optimum amount of TGF-β regulating in vitro human Th17 cell differentiation is still unclear.

In the current study, a flow cytometric assay was used to evaluate the effect of different concentrations of TGF-β and a combination of IL-1beta, IL-23, IL-2 without using IL-6 on development of Interleukin (IL)-17–producing T helper (Th17) cells.

According to our findings, 0.1 ng/ml of TGF-β significantly increases the expression of IL-17 in comparison to other concentrations of this cytokine.

Results indicated the vital role of TGF-β cytokine in the polarization of human Th17 cells in vitro.

Keywords: CD4+ T cells; Th17 cells; Transforming growth factor-β

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INTRODUCTION

$CD4^+$ effector T helper cells (Th cells) mediated adaptive immune system divide into distinct lineages

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of T helper cells based on the discrete profiles of cytokine production. The initial concept for the existence of distinctive populations of differentiated CD4+ T cells was first reported by Mosmann et al. in 1987,[1] Later on, the mentioned cells were indicated as two main groups of Th1 and Th2 cells.[2] Major cytokines which were produced by Th1 and Th2 cells are IFN-γ and IL-4, IL-5, and IL-13, respectively.[3] In 2003, the third major effector population of CD4+ T cell that could be generated from naïve CD4+ T cells was discovered. These cells were different from Th1 and Th2 cells named as IL-17 producing helper T cell (Th17) due to their distinctive cytokine profiles.[4] Then, it was shown that several cytokines including IL-6, IL-21, IL-23 and IL-1β play an important role in the differentiation of Th17 cells.[5]

The function of TGF-β, as a promotion factor in Th17 cell differentiation, can be complicated by the studies that were carried out in mice lacking this cytokine or its receptor and initiate a systemic autoimmune disease.[9,10] The role of TGF-β, as an inductive or inhibitory factor in the differentiation of human naïve $CD4^+$ T cells to Th17 is difficult to understand. The first study in this context showed that of differentiation, human Th17 cell could be induced in the absence of TGF-β and this cytokine was noted to be as an inhibitor of IL-17 production.[5,11] It was demonstrated that TGF-β is an important factor in the development of human Th17 cells differentiation. However, the role of TGF-β in the differentiation and function of human Th17 cells is still controversial. Different data indicated that TGF-β and a combination of IL-1β and IL-6, IL-21 or IL-23 can be effective cytokines for inducing naïve T cells to become IL-17 producer T cells.[12,13]

In the present study, in order to find the optimum concentration of TGF-β "as an essential cytokine for Th17 cells differentiation", human naïve $CD4^+$Tcells were cultured in the presence of various concentrations of this cytokine; and levels of IL-17 were then evaluated.

MATERIALS AND METHODS

Samples

Individuals, who had no history of autoimmune or other inflammatory diseases in their families, were recruited. All participants were of Iranian Caucasian origin. The study was approved by ethics committee of Tehran University of Medical Science and written informed consent was obtained from each person prior to the study.

Purification of Naïve $CD4^+$ T Cells

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from 50-ml heparinized venous blood using density gradient centrifugation over Lymphoflot (inno-Train, Diagnostic Gmbh, Germany). Subsequently human CD4+ T-cell isolation kit II (MiltenyiBiotec, BergischGladbach, Germany) was put in use for isolation of naïve CD4+ T cells according to the following instructions; CD45RO+ activated/memory T cells and non-CD4+ T cells were magnetically labeled using a cocktail of biotin-conjugated antibodies against CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD36, CD45RO, CD56,CD123, TCRγ/δ, HLA-DR, CD235a (Glycophorine A), and anti-biotin micro-beads. Highly pure naïve CD4+ T cells were then isolated subsequent to the depletion of magnetically labeled cells. Isolation of highly pure naïve CD4+ T cells was confirmed after immune staining with FITC-conjugated anti-CD4 FITC and PE. Cy5-conjugated anti-CD45RA antibodies (BD Biosciences, Franklin Lakes, NJ) and analysis was done using FACSCalibur (Becton, Dickinson) instrument.

Cell Culture and Cytokine Treatment

Naïve CD4+ T cells were stimulated by plate-bound anti-CD3 (2μg/ml) and soluble anti-CD28 Ab (1μg/ml, both obtained from eBioscience). Purified CD4+ T cells were cultured at an initial cell density of 1×10^6 cells/ml in serum-free X-VIVO 20 (Lonza) media on 24-well plates in a 37°C 5% CO2 incubator. Cultures were supplemented by adding the following antibodies and cytokines: 10U/ml IL-2 (BD), 10ng/ml IL-1β (BD), 10ng/ml IL-23 (eBioscience) and different concentrations of TGF-β cytokine (R&D); dose I: 0.1, dose II:0.5, dose III:1 and dose IV:10ng/ml. 1μg/ml anti-IFN-γ and 1μg/ml anti-IL-4 (both from BD) per well were added to inhibit unwanted deviation to Th1 or Th2 subsets. At day 3, supernatants were removed and replaced with fresh media containing all cytokines and antibodies. Cells were then harvested after day 6 for intracellular staining.

Flow Cytometry Analysis of T Cell Population

For intracellular cytokine staining, at the day 6, CD4+ T cells were cultured in X-VIVO20 medium, stimulated with PMA (50 ng/ml, Sigma) and Ionomycin (1 mg/ml, Sigma) for 6 hours in the presence of Brefeldin A (10 mg/ml, BD) to keep enough cytokine for detection inside the cells. Then the cells were harvested, fixed and permeabilized with the corresponding buffers according to the manufacturer's protocol (AbD Serotec) and stained with anti-IL-17A-PE (BD) at room temperature. All analyses were done on BD FACS Calibur flow cytometer. Data analysis was performed with the Flow Jo software.

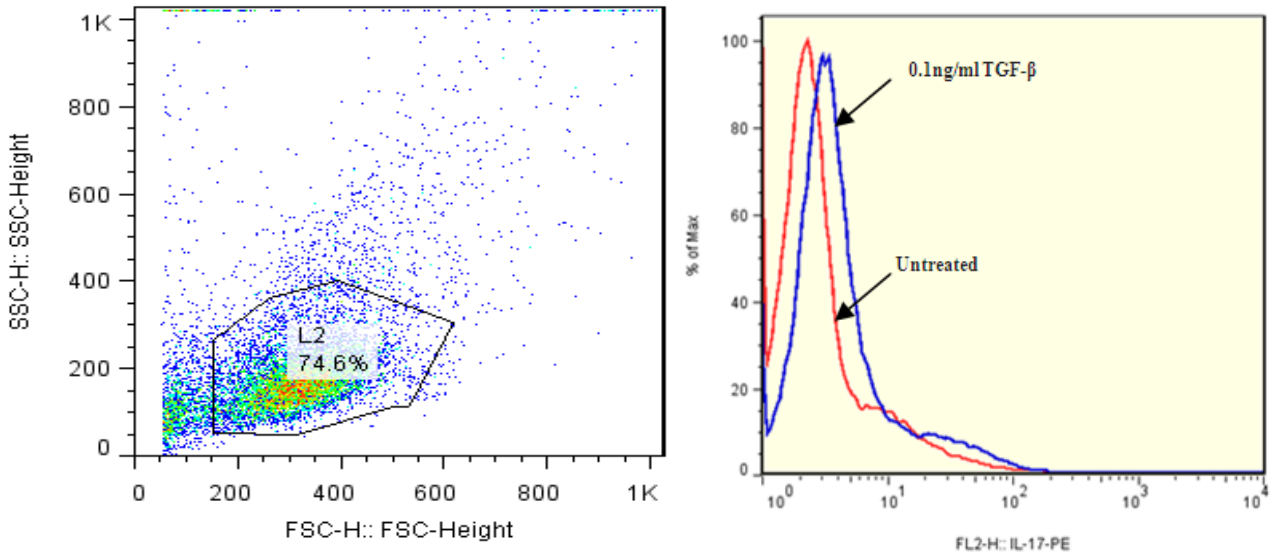
Statistics

To compare the expression of IL-17 in differentiated CD4+ T cells, data were analyzed using Kruskal-wallis. The data values expressed as mean ± SD. *p* values less than 0.05 were considered as a significant difference between groups. SPSS20 software was used for statistical analysis.

RESULTS

Expression of IL-17 in Differentiated Naïve CD4+ T Cells Influenced by Different Doses of TGF-B Cytokine

The effect of different doses of TGF-B was evaluated on IL-17 expression in differentiated CD4+ T cells using flow cytometry. Highly pure naïve CD4+ T cells were isolated from samples and more than 95% purity was confirmed via flow cytometric analysis (data not shown). The effects of four doses of TGF-B cytokine were evaluated on the differentiation of the naïve CD4+ T cells and cultured in serum free X-VIVO20 medium. For this purpose, differentiated naïve CD4+ T cells were gated on a forward vs. side scatter dot Plot (Figure 1a). Then levels of IL-17+ T cells were analyzed in histogram on lymphocyte gated cells (Figure 1b). Subsequently, the relative expression of IL-17 was compared with different concentrations of TGF-β (Figure 2).



(a) **(b)**

Figure 1. Flow cytometric analysis of naïve CD4+IL-17+ T cells population isolated from blood samples. (a) Dot plot of differentiated CD4+ T cells. (b) IL-17 producing CD4+ T cells within the indicated gate were determined using the Flowing software.

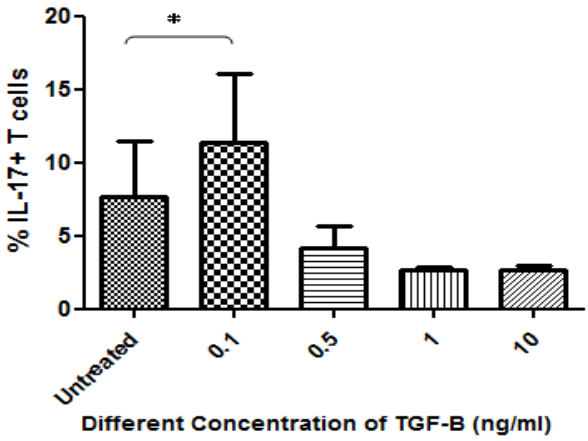


Figure 2. Naïve CD4+ T cells from normal donors (n=6) were activated with plate-bound anti-CD3 and anti-CD28 and cultured for six days with optimal polarizing conditions for human Th17 differentiation (IL-1β, IL-2, IL-23, anti-IFN-γ+anti-IL-4 and different concentration of TGF-β). IL-17 production was assessed by intracellular cytokine staining and flow cytometry analysis. Statistical significance was calculated based on paired student's t-test (n=6)

DISCUSSION

Results indicated that differentiation of human Th17 cells requires the activity of TGF-β. In the studies carried out regarding this issue, the role of TGF-β has been underlined since CD4+ T-cells were cultured in media comprising human or bovine serum, which contains TGF-β.[13,14] In order to verify the function of TGF-β, another research has been carried out in which naïve CD4+ T cells were differentiated into regulatory T-cells in the presence of high concentrations of TGF-β and retinoic acid which express autocrine TGF-β, the major cytokine in maintaining their development. Additionally, lower concentrations of TGF-β in association with IL-6 and IL-1 β promoted the development of Th17 cells expressing autocrine TGF–β and IL-21 resulting in their own development.[15] However, the role of autocrine TGF-β in promoting Th17 development at steady state requires further investigation, particularly regarding to the signals that drive a Th17 cell to produce TGF-β.[16] Moreover, few cases remain unresolved when it comes to the differentiation of human Th17 cells. Although some considerable inconsistency was observed among Th17 cell differentiation conditions and the mechanisms in which these cells were differentiated. In fact, these cells have shown a good potential in a variety of autoimmune disease models in mice and also in human immune disease as well.[17] In the current investigation, low to high levels of TGF-β cytokine treatment in Th17 polarizing condition were employed. The role of TGF-β in the development of such cells is yet remained unresolved. Consequently, providing an optimized condition is crucial for manipulating Th17 cells in autoimmune disease. In this study, the optimum concentration of TGF-β was found to produce a considerable proportion of IL-17+ cells. We also used serum-free medium for differentiation of naïve human CD4+T cells. Cytokine profile consisted of IL-1β, IL-23, and IL-2 with different concentrations of TGF-β in the absence of IL-6. Utilizing a flow cytometric-based approach in this study, we found that the lowest concentration of TGF-β was sufficient to produce IL-17^{+}-T cells compared to the other concentrations of this cytokine. In contrary to our data, Manel et al. showed that high concentration of TGF-β (10ng/ml) was associated with a high proportion of IL-17 in differentiated cells in comparison to other concentrations.

In conclusion, the data in our study indicated the importance of the optimum amount of TGF-β cytokine for providing an optimized condition for Th17 cell differentiation but further research are essential to reveal the polarization conditions for CD4+ Il-17+ T cells precisely for further manipulating of these cells in vitro.

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