Iran J Public Health, Vol. 49, No.3, Mar 2020, pp.549-556

Original Article

Evaluation of Th1/Th2 Lymphocyte Balance in Peripheral Blood Mononuclear Cells of Patients with Hashimoto's Thyroiditis

Hajar VASEGHI [1], ***Fatemeh ESFAHANIAN*** [2], ****Zohreh JADALI*** [3]

1. *Department of Photo Healing and Regeneration, Medical Laser Research Center, Yara Institute, ACECR, Tehran, Iran*
2. *Department of Endocrinology, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran*
3. *Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*

***Corresponding Author:** Email: zjadali@yahoo.co.uk

(Received 12 Sep 2018; accepted 17 Dec 2018)

Abstract

Background: The role of T cells in the pathogenesis of Hashimoto's thyroiditis is well established, whereas the precise and likely the overlapping contributions of different T-cell subpopulations to thyroid injury are less understood**.** The purpose of this study was to assess the expression pattern of two lineage determining transcription factors, T-bet and GATA-3 that regulate differentiation of T cells into Th1 or Th2 cell fates, respectively. Moreover, the mRNA expression and plasma concentration of *Th1***(***IFN-γ***)** and Th2(IL-4**)** cytokines were analyzed.

Methods: In this case-control study, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine the expression patterns of various transcripts in 20 patients (in Endocrinology Clinic, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran, in 2015) with Hashimoto's thyroiditis (HT) and 22 healthy controls. Plasma IL-4 and IFN-γ concentrations were also measured using enzyme-linked immunosorbent assay.

Results: T-bet gene expression was significantly lower in patients compared to healthy controls (*P*=0.014). The expression of IL-4 mRNAs was significantly increased in the peripheral blood mononuclear cells from patients as compared to normal controls (*P*=0.001). In addition, a marked increase in plasma IL-4 levels were observed in patient group compared to controls (*P*=0.043).

Conclusion: Altered balance between Th1 and Th2 related transcription factors and cytokines may be implicated in the pathogenesis of Hashimoto's thyroiditis.

Keywords: Hashimoto's thyroiditis; T lymphocyte; Autoimmunity; Cytokine

Introduction

Hashimoto's thyroiditis (HT) is **a** common autoimmune disorder resulting from selective destruction of thyroid cells. HT is a multifactorial disease and a combination of genetic, epigenetic, molecular, and cellular elements causes a pathogenic inflammatory response to thyroid gland. It is now accepted that T cells contribute to the pathogenesis of HT (1). The following observations suggest that T-cells are important contributors to thyroid dysfunctions and abnormalities a) T lymphocytes are among the first immune cells to infiltrate and invade the thyroid, promoting the formation of ectopic lymphoid follicles in this organ (2) b) establishment of thyroiditis in normal recipients by adoptive transfer of T cells (3) c) increased percentage of activated peripheral

blood CD4+ T lymphocytes **in** HT patients(4) and d) T-cell-mediated death mechanisms proposed for thyrocyte depletion in HT(5).

Therefore, recent research in HT has largely focused on delineation of T cell subsets responsible for autoimmune responses. Among these subpopulations with broadly different functional and phenotypic properties, great attention has been paid to CD4+ T helper (Th) subsets. The disease process in several autoimmune disorders are driven by the biased development of specific T helper subsets - mainly, T helper type 1 (Th1) and T helper type 2 (Th2) cells - that activate cell-mediated and humoral immune system, respectively. As separate lineages, Th1 and Th2 cells use different transcriptional programs associated with expression and activation of specific transcription factors. The two important transcription factors relevant to Th1 and Th2 lineage commitment are T-box expressed in T cells (T-bet) and GATA binding protein 3(GATA-3). T-bet expression occurs only in differentiating Th1 cells and GATA-3 directs lineage specification of Th2 cells. Each of these subsets has also a unique cytokine profile. For instance, Interferon-gamma (IFN-γ) and Interleukin (IL)-2 are the signature cytokines of Th1 cells, whereas Th2 cells predominantly produce IL-4, IL-5, IL-10, and IL-13. These distinctive patterns of cytokines play a major role in the particular effector functions of these cells (6). Therefore, the expression analysis of Th1/Th2 cytokines and their specific transcription factors provide a better understanding of the pathopshysiological process and effect of treatment in HT patients.

The purpose of the present study was a) to assess the mRNA expression levels of T-bet, GATA-3, IFN-γ, and IL-4 in PBMCs of patients with HT and healthy controls, b) analysis of correlation between T-bet/GATA-3 and IFN-γ/IL-4 to deliniate the potential role of these two transcription factors in IFN-γ, and IL-4 production, respectively c) to determine the profile of Th1 (IFN-γ) and Th2 (IL-4) cytokines in plasma of healthy subjects and patients with HT.

Methods

Subjects

Overall, 20 patients with HT referred to the Endocrinology Clinic, Imam Khomeini Hospital Complex, Tehran University of Medical Sciences, Tehran, Iran, in 2015 were recruited to this case-control study. The patients were examined by endocrinologists and had the biochemical evaluation of their thyroid function. T3, T4, TSH were assayed using enzyme-linked immunosorbent assay (ELISA). All trial participants were women, with a mean age of 31.85 ± 9.17 yr. They were treated with a daily dose of a synthetic thyroid hormone called levothyroxine. The control group consisted of 22 healthy volunteers(5 men, 17 women) with no history of HT or other chronic and autoimmune diseases and their mean ages were 29.18 ± 7.66 yr (men, 25.20 ± 5.63 yr, women, 30.35 ± 7.91 yr).

Informed consent was provided according to the ethical standards given in the Declaration of Research Ethics Board at Tehran University of Medical Sciences and written informed consent was obtained from all participants.

RT-qPCR was performed on all specimens (20 patients with HT and 22 healthy controls) but plasma cytokine levels were measured in 20 HT patients and 20 healthy subjects.

Collection of plasma and Peripheral Blood Mononuclear Cells (PBMCs)

Approximately 5 ml of peripheral blood from each patient and control subject were collected in evacuated glass tubes containing EDTA. All of the blood samples were completely fractionated to plasma and PBMCs components for further analysis. Plasma aliquoted and stored at −20 °C until use. PBMCs were isolated by density separation over a Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) and were further used for RNA extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted from PBMCs according to manufacturer's instruction (GeneALL, Seoul, Korea). The yield and quality of isolated

RNA were checked by gel electrophoresis and optical density measurement using a Nanodrop spectrophotometer (Thermo Scientific, USA). Only high-quality RNA, with the absorbance ratio between 200 and 400 ng/µl, was used in further analysis. cDNA was synthesized with reverse transcription reagent kits (Fermentas, Germany) and was stored at −20 °C.

Determination of mRNA expression by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Synthesized cDNA was subjected to Real-time quantitative PCR (RT-qPCR) analysis to detect mRNA level of T-bet, GATA-3, IL-4, IFN-γ, and β-actin using a SYBR Premix EX Taq II (Takara, Japan) on a RotorGene 6000 (Corbett Life Science, Australia) thermal cycler.

PCR was performed in a total reaction volume of 10 µL including 5 µL of SYBR Premix, 1 µL of cDNA, 0.5 µL of forward primer, 0.5 µL of reverse primer, and 3 µL of double-distilled water. PCR amplification procedure consisted of an initial denaturation step for 2 min at 95 °C, followed by 38-45 cycles of 5 sec at 95 °C (denaturation) and a final step of 25 sec at 62 °C for T-bet, 20 sec at 62 °C for GATA3, 45 sec at 60 °C for IFN-γ, 30 sec at 62 °C for IL-4, and 20 sec at 63 °C for β-actin. Melting curve analysis confirmed the specificity of the amplified product. All primers were obtained from TAG Copenhagen (Denmark). The exact primers sequences utilized in this study was explained before (7). Each reaction was characterized by Ct (cycle threshold) value (number of amplification cycles required for the fluorescent signal to cross the threshold). This value increases with a decreasing amount of initial template. Therefore, the Ct is indirectly proportional to the amount of starting template. The expression level of the target genes was indicated as ΔCt **(**ΔCt = Ct value of the target gene – Ct value of the *β*-actin gene). Statistical analysis were performed on ΔCt values. Lower ΔCt values indicate higher gene expression levels**.**

ELISA

Serum IL-4 and IFN-γ levels were measured by ELISA according to the manufacturer's instruction. Human IL-4 and IFN-γ ELISA reagent Kits were purchased from Bender Med Systems (San Diego, California, USA).

Statistical analysis

The required sample sizes calculated based on information from previous studies using the traditional sample size calculation procedure (8). The minimum number of subjects that need to be enrolled in this study in order to make comparisons between groups was 20 (for 90% power with a 5% level of significance and a two-sided test).

T-tests were used to compare two independent groups (independent-samples t-test). Correlations among pairs of variables were assessed by Pearson test. The level of significance was set at $P<0.05$. Results are expressed as means ± SD. Analysis of data was done using SPSS, ver.11.0 (Inc., Chicago, IL, USA).

Results

The mRNA Levels of T-bet and GATA-3 in PBMCs of patients and controls

As shown in Fig.1, a marked and significant decrease in the mRNA levels of T-bet transcript was observed in patients (11.11±4.29) when compared with normal controls (8.46±1.26) ($P=0.014$).

In contrast, a slightly increased expression of GATA-3 transcripts was observed in patients (11.87±1.88) as compared to normal control subjects (12.34±2.41), although these differences were not statistically significant (Fig.2). Lower ΔCt values indicate higher expression.

The mRNA Levels of IFN-γ and IL-4 in PBMCs of patients and controls

The expression of cytokine mRNAs that are helpful in defining Th1 (IFN-γ) and Th2 (IL-4) lymphokine patterns were evaluated in patients

and normal subjects. In general, patients showed greater expression of Th2 cytokine genes (7.32 ± 1.73) than healthy controls (9.09±1.30) (*P*=0.001).

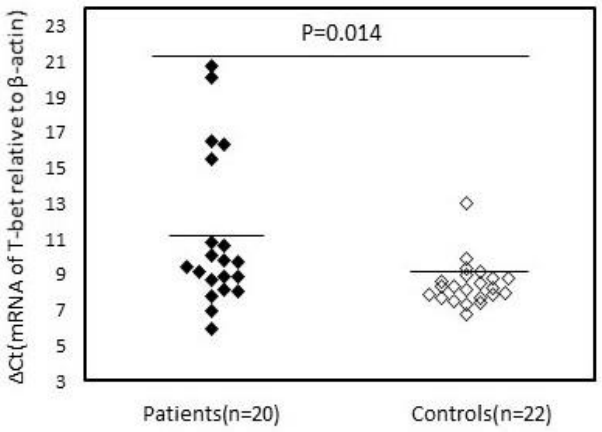

Fig.1: Detection of T-bet mRNA in peripheral blood mononuclear cells from patients with Hashimoto's thyroiditis and healthy persons by reverse transcription-quantitative PCR. T-bet mRNA levels were significantly lower in patients than normal persons. Note that lower ΔCt values indicate higher expression

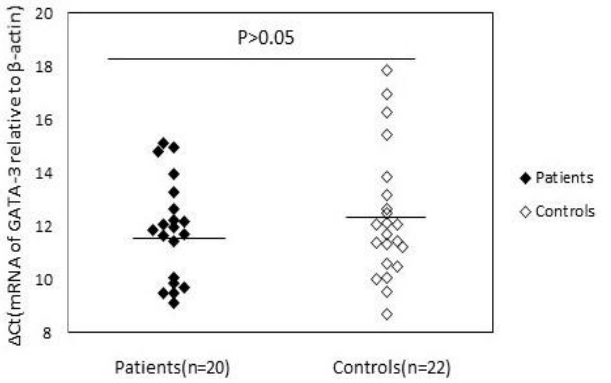

Fig. 2: Detection of GATA-3 mRNA in peripheral blood mononuclear cells from patients with Hashimoto's thyroiditis and healthy persons by reverse transcription-quantitative PCR. GATA-3 mRNA levels were higher in patients than normal persons. However, these differences did not reach statistical significance. Note that lower ΔCt values indicate higher expression

In addition, no significant statistical differences was observed in the mRNA levels of IFN-γ transcript in patients (10.42 ± 1.65) when compared with normal controls (10.17±1.29) (*P*>0.05). The results of cytokine expression levels are presented in Figs. 3 and 4.

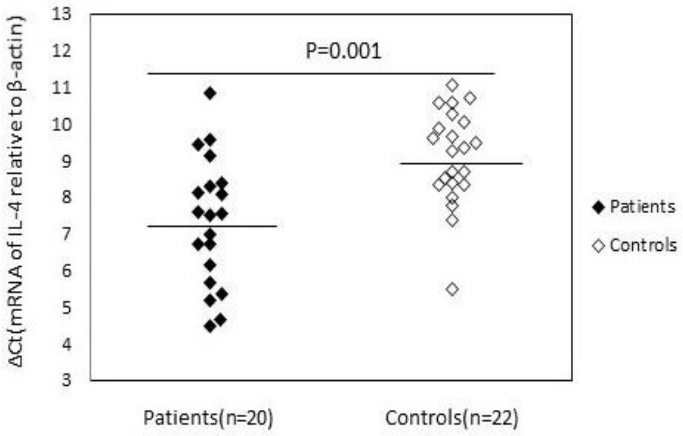

Fig. 3: Detection of IL-4 mRNA in peripheral blood mononuclear cells from patients with Hashimoto's thyroiditis and healthy persons by reverse transcription-quantitative PCR. IL-4 mRNA levels were significantly higher in patients than normal persons. Note that lower ΔCt values indicate higher expression

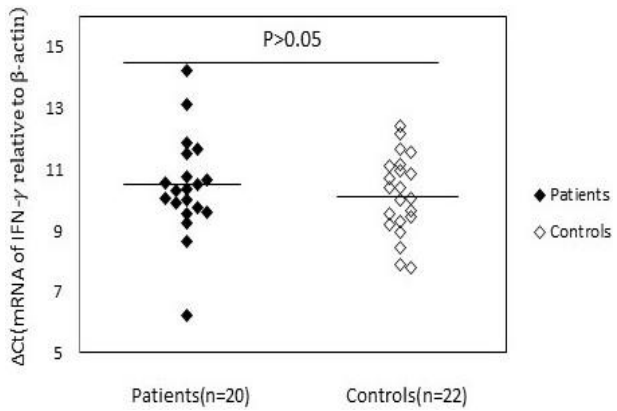

Fig. 4: detection of IFN-γ mRNA in peripheral blood mononuclear cell from patients with Hashimoto's thyroiditis and healthy persons by reverse transcription-quantitative PCR. There was no significant difference in IFN-γ mRNA levels between patients and healthy contros. Not that lower ΔCt values indicate higher expression

ELISA Analysis of IL-4 and IFN-γ Levels

The plasma levels of IL-4 were markedly increased in patients with HT disease (17.03 ±

1.10) versus control subjects (15.79±1.71). Moreover, there was no significant difference in plasma IFN-γ concentration between patients (17.18±1.73) and controls (16.36±1.24). Pearson correlation analysis revealed that IL-4 mRNA levels of HT patients were positively correlated with the plasma IL-4 levels (r =0.504, *P*=0.023). Moreover, in the patient group, there was a significant correlation between plasma IFN-γ concentration and IFN-γ mRNA expression (r =0.818; *P*=0.0001) (Fig.5).

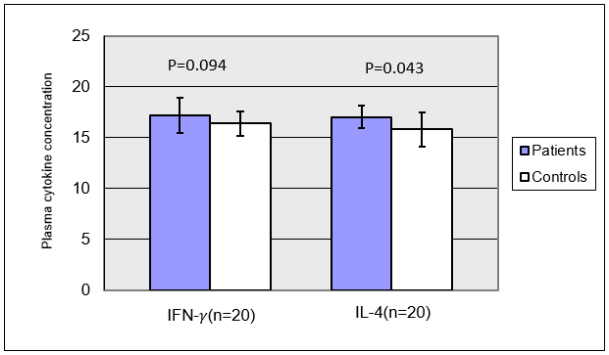

Fig.5: Plasma cytokine levels of patients with Hashimoto's thyroiditis and controls

Discussion

To the best of our knowledge, this is the first report on the simultaneous measurement of the Th1/Th2 cytokine profile in plasma and mRNA expression of Th1/Th2 cytokines and their specific transcription factors in PBMCs of patients with HT.

The results of present study revealed a considerable decrease in T-bet mRNA level in PBMCs of patients compared to normal controls. In contrast, a trend toward higher expression of GATA-3 mRNA was noted in patients compared to controls, though not statistically significant. Moreover, a significant difference was observed between patients with HT and control group regarding the mean mRNA expression of T-bet/GATA-3 ratio (*P*=0.01). These data show a shift toward a Th2 response in patients with HT and suggest a role for altered balance of Th1/Th2 transcription factors in the pathogenesis of disease.

Our results are consistent with previous studies showing reduced expression of T-bet transcripts in PBMCs from patients with HT (9). Nonetheless, there are scientific controversies over the details of this issue (10-12).

In addition to transcription factors, the Th1/Th2 balance can be determined by associated cytokines. Therefore, another part of this research project was devoted to the measurement of plasma level and gene expression of IFN-γ and IL-4. Based on our experimental results, no significant statistical differences were found between the analyzed groups regarding the IFN-γ gene expression and IFN-γ levels in the plasma. In contrast to IFN-γ, IL-4 mRNA levels and plasma concentration of this cytokine were significantly higher in patients than in controls. Furthermore, IFN-γ/IL-4 transcript ratio was significantly decreased in the HT patients when compared with healthy controls (*P*=0.001), confirming the shift toward Th2-mediated immunity. Although these results differ from some published studies (13,14), they are consistent with what other investigators have reported (15,16).

In our opinion, these opposing results with respect to altered cytokine expression and production may be dependent on several factors including host genetic factors (17, 18), different stages of the autoimmune inflammatory response and treatment. A severe and/or chronic Th1 type immune responses in HT patients may shift towards a less polarized profile (Th0) or even to responses specified by the preferential production of Th2 cytokines (19,20). This phenomenon is recognized as immune deviation (19–21), and is accompanied by an increase in the numbers of IL-4-positive cells.

A shift from type 1 to type 2 immunity may act as a compensatory mechanism that can reduce the severity of disease and is consistent with the experimental evidence indicating the role of Th2 cytokines in controlling Th1-mediated autoimmune process (22). The possible functional role of IL-4 in the prevention of thyroid autoimmunity has been supported by multiple lines of evi-

dence (23). Disruption of the IL-4 gene in mice promotes autoimmune disorders such as endocrine autoimmune diseases (24).

Treatment is another important factor considered in patients because it may influence the expression of Th1 and Th2 response. In the present study, all patients were under levothyroxine therapy. Levothyroxine ameliorates the hypothyroidism (normalizes blood levels of TSH, T4, T3) and may have a powerful influence over immune system activity. It seems to modulate autoimmunity reflected by elevated levels of circulating thyroid-specific antibodies and decrease goiter size. The exact mechanism of action for levothyroxine is not fully understood. It may result in a reduction of antigenic substance via a decreased stimulation of thyroid tissue by circulating TSH (25,26). In addition, this drug may exert direct or indirect effect on immune factors in the periphery, such as dendritic cell (DC) subpopulations (27-29).

Although our results highlight the possible importance of Th2 immune responses in HT patients treated with levothyroxine, they also address one of the main limitations of this study. Because the expression of these four genes did not evaluate in thyroid tissues from patients with HT. Small population sample is another limitation of this study that restricts the interpretation of results. Consequently, more studies with larger samples are needed to explore the exact role of Th1/Th2 cells in mediating HT.

We also believe that dichotomization of complex diseases like HT, in terms of Th1 and Th2 patterns, is an oversimplification because the development of a particular type of cell and subsequently the outcome of disease undoubtedly involves the cooperation between different immune cell subsets and factors. Therefore, the role of other cells such as Tregs, Th17, Th10 must certainly be considered (30).

Conclusion

Our results describe a Th2-skewed immune response in patients with HT receiving levothyroxine therapy. This implies the activation of Th2 subpopulation in patients with regard to levothyroxine treatment. Whether this therapeutic approach of HT patients can stimulate the development of protective T-cell subpopulations must be assessed in future researches. More studies are also needed to illuminate alterations in which subgroups of heterogenous populations of T cells are associated with disease duration and how the therapy influences their frequency and function.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

Acknowledgements

This project was conducted with the financial support of Tehran University of Medical Sciences for Research through contract no. 23019.

Conflict of interests

The authors declare that there is no conflict of interests.

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