

# Interleukin-10 influences susceptibility to experimental autoimmune thyroiditis independently of the H-2 gene

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**Abstract.** Both BALB/c and C57BL/6 mice are relatively resistant to experimental autoimmune thyroiditis (EAT) due to their histocompatibility (H-2) genetic background; however, susceptibility to EAT is also influenced by other genetic factors. Given the curative effect of interleukin-10 (IL-10) on thyroiditis, in the present study, we investigated whether IL-10 functions as a non-H-2 genetic factor that influences the development of EAT in mice with an EAT-resistant genetic background. In this study, we observed that the development of EAT could be induced in both C57BL/6 IL-10-deficient (*IL-10*<sup>-/-</sup>) and BALB/c *IL-10*<sup>-/-</sup> female mice following immunization with mTg, which indicated that IL-10 may be a non-H-2 factor that affects susceptibility to EAT. However, the role of the H-2 factor remained dominant, as the incidence of EAT was low and its severity was mild. We further investigated the underlying pathogenic mechanisms of EAT in *IL-10*<sup>-/-</sup> female mice. We found that Th1 cells, Th17 cells, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells, and their associated cytokines were all involved in the development of EAT. The absence of IL-10 promoted the polarization of pathogenic cells and the production of associated cytokines, and suppressed the proliferation of protective T cell clones. Together, these factors may contribute to the development of EAT in *IL-10*<sup>-/-</sup> mice. In conclusion, our data demonstrate that IL-10 plays a critical role in the susceptibility to EAT, and a better understanding of the role of IL-10 in autoimmune thyroiditis may facilitate the development of novel strategies for the treatment of autoimmune thyroid diseases.

## Introduction

Experimental autoimmune thyroiditis (EAT), a murine model of Hashimoto's thyroiditis (HT) in humans, can be induced by challenging susceptible mice with thyroglobulin (Tg) emulsified in complete Freund's adjuvant (CFA). EAT is characterized by thyroid lymphocytic infiltration that eventually leads to the destruction of the thyroid follicles (1,2). The mechanisms responsible for thyroid destruction, although not yet completely understood, appear to involve activated Tg-specific T cells and their associated cytokines in the thyroid microenvironment that play a critical role in the pathogenesis of thyroid lesions (3). Previous studies have revealed a positive correlation between susceptibility to EAT and the histocompatibility (H-2) genes, as mice with H-2k or H-2s genetic backgrounds are susceptible, whereas mice with H-2b or H-2d genetic backgrounds are resistant (4,5). However, the H-2 genotype is not the only genetic influence involved in this model, and non-H-2 factors also play important roles in the development of EAT (6).

Interleukin (IL)-10 was first described as a cytokine synthesis inhibitory factor produced by T helper (Th) 2 cells (7). It is now known that IL-10 can also be expressed by many immune cells, including the Th1, Th2 and Th17 cell subsets, as well as regulatory T (Treg) cells and B cells. IL-10 can act as an immunosuppressive cytokine that plays a critical role in various autoimmune diseases (8). IL-10-deficient (*IL-10*<sup>-/-</sup>) mice can spontaneously develop chronic enterocolitis (9). The role of IL-10 has also been described in autoimmune thyroiditis, as Batteux *et al* (10) observed that the systemic administration of IL-10 had curative effects on EAT. In a granulomatous experimental autoimmune thyroiditis (G-EAT) model, IL-10 has been shown to promote the resolution of G-EAT through a mechanism involving an anti-apoptotic effect (11). A previous study indicated that BALB/c (H-2d) and C57BL/6 (H-2b) mice were resistant to EAT, and the mechanisms responsible for the tolerance were mainly related to the H-2 genetic background. However, it remains unclear as to whether other factors also influence the susceptibility to EAT. Given the beneficial effect of IL-10 on thyroiditis, in the present study, we sought to obtain direct evidence of the role of IL-10 in promoting tolerance to EAT. To this end, we used *IL-10*<sup>-/-</sup> mice to examine the significance of endogenously produced IL-10.

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## Materials and methods

**Ethics statement.** All animal care and experimental procedures were performed according to the Guidelines for Animal Experimentation with the approval of the Animal Ethics Committee of China Medical University, Shenyang, China.

**Mice.** Wild-type (wt) BALB/c $IL-10^{+/+}$  and C57BL/6  $IL-10^{+/+}$  mice were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). BALB/c  $IL-10^{-/-}$  and C57BL/6  $IL-10^{-/-}$  mice were all obtained from the Jackson Laboratory, Inc. (Bar Harbor, ME, USA). The  $IL-10^{+/+}$  and  $IL-10^{-/-}$  mice were crossed to generate  $IL-10^{+/-}$  mice, which were then intercrossed to produce  $IL-10^{+/+}$  and  $IL-10^{-/-}$  offspring. The female littermates between 6 and 8 weeks of age were used for the experiments presented herein. CBA/J female mice (susceptible to EAT; Beijing HFK Bioscience Co., Ltd.) between 6 and 8 weeks of age were also used. One hundred mice were used in this study, and all animals were maintained under specific pathogen-free conditions at the Animal Research Center of China Medical University.

**Genotyping of mice.** The  $IL-10^{+/+}$  and  $IL-10^{-/-}$  mice were genotyped by polymerase chain reaction (PCR) using tail genomic DNA. The following 3 primer sets were used: mutant primer, 5'-CCACACGCGTCACCTTAATA-3'; common primer, 5'-CTTGCACTACCAAAGCCACA-3'; and wild-type primer, 5'-GTTATTGTCTTCCCGGCTGT-3'. The PCR conditions were as follows: 35 cycles of 94°C for 30 sec, 64°C for 1 min, and 72°C for 1 min.

**Induction of EAT.** Mouse Tg (mTg) was prepared as previously described (12). We emulsified mTg 1:1 in CFA for immunization on day 0, and in incomplete Freund's adjuvant (IFA) for challenge on day 7. CFA suspensions contained 1 mg/ml of *Mycobacterium tuberculosis*, strain H37Ra (Sigma-Aldrich, St. Louis, MO, USA). There were 2 series of experiments. In the first,  $IL-10^{-/-}$  mice were immunized subcutaneously (s.c.) with varying doses of mTg (100 or 200  $\mu$ g); the susceptible CBA/J mice were used as a positive control and were immunized with 100  $\mu$ g mTg to induce EAT, as previously described (13); the control group was administered PBS and adjuvant. All mice were sacrificed by exsanguination on day 28 and the optimal dose of mTg was determined based on the serum Tg antibody (TgAb) titers. In the second series of experiments,  $IL-10^{-/-}$  mice (BALB/c or C57BL/6) were randomly divided into 2 groups as follows: the mTg group was immunized s.c. with the optimal dose of mTg; and the control group was immunized s.c. with PBS and the adjuvant.  $IL-10^{+/+}$  mice (BALB/c or C57BL/6) were treated in a manner similar to the  $IL-10^{-/-}$  mice. All mice were sacrificed on day 28 after the first immunization.

**Measurement of serum TgAb production.** We assayed mTg-specific serum IgG antibodies in duplicate by a solid-phase enzyme-linked immunosorbent assay (ELISA) as previously described (14). In brief, 96-well cell culture microplates (Corning Inc., Corning, NY, USA) were coated with 10  $\mu$ g/ml mTg overnight at 4°C. After washing with PBS-Tween 20 (PBS-T), the free protein binding sites were blocked by the addition of 1.5% BSA for 30 min at 37°C. Sera from individual mice, diluted in PBS-T at ratios of 1:100, 1:500 and 1:1,000, were

incubated for 2 h at 37°C and were then washed extensively. Peroxidase-labeled goat anti-mouse IgG (1:2,000 dilution, Sigma-Aldrich), was added as a secondary antibody and the colorimetric reaction was developed by the addition of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate. Plates were read using an Infinite F200pro fluorescence microplate reader (Tecan Group Inc., Mannedorf, Switzerland) at 450 nm.

**Histological evaluation of autoimmune thyroiditis.** Thyroid glands were removed 28 days after the mTg challenge. Infiltration indexes were evaluated on 5- $\mu$ m-thick sections stained with hematoxylin and eosin (H&E). Histological grades of thyroiditis were assessed by 3 blinded scorers who analyzed the thyroid specimens. The histological sections were scored as previously described (4): 0, no infiltration; 0.5, extensive infiltration, 0-10% of total area; 1, extensive infiltration, 10-20% of total area; 2, extensive infiltration, 20-40% of total area; 3, extensive infiltration, 40-80% of total area; and 4, extensive infiltration, >80% of total area.

**Antibodies, cell isolation and flow cytometry.** Antibodies against surface or intracellular mouse proteins included the following: CD16/CD32 (Mouse BD Fc Block, 2.4G2), CD19 (1D3), CD5 (53-7.3), CD1d (1B1), CD4 (RM4-5), interferon (IFN)- $\gamma$  (XMG1.2), IL-4 (11B11), IL-17A (TC11-18H10), CD25 (PC61), and Foxp3 (MF2.3) monoclonal antibodies (mAbs; all from BD Biosciences, San Jose, CA, USA). Single-cell suspensions of splenocytes were generated by mashing tissues through 200- $\mu$ m mesh stainless steel screens and then removing erythrocytes with lysis buffer. We incubated  $2 \times 10^6$  cells with anti-CD16/CD32 for 15 min to block Fc-receptor binding. Surface staining was performed using CD4-FITC, CD25-APC, CD19-APC, CD5-FITC and CD1d-PE mAbs according to the manufacturer's instructions. For intracellular cytokine detection, the cells were stimulated with leukocyte activation cocktail in the presence of GolgiPlus (BD Biosciences) for 6 h at 37°C before staining with fluorophore-conjugated anti-IFN- $\gamma$ , anti-IL-4 and anti-IL-17A using a Cytofix/CytoPerm Plus kit (BD Biosciences). Intracellular Foxp3 staining was performed using an anti-mouse/rat Foxp3-PE staining kit (BD Biosciences) following the manufacturer's instructions. Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences) with CellQuest software and data were analyzed using FlowJo software (Treestar Inc., Ashland, OR, USA).

**Reverse transcription-quantitative PCR (RT-qPCR).** Total RNA was prepared from the splenocytes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was reverse transcribed using a PrimeScript<sup>TM</sup> RT Master Mix and subjected to amplification by RT-qPCR using SYBR<sup>®</sup> Premix Ex Taq<sup>TM</sup> II (both from Takara, Otsu, Japan). RT-qPCR was performed using standard protocols on a Lightcycler480 System (Roche, Basel, Switzerland). Primer sequences are listed in Table I. PCR parameters were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec, 60°C for 20 sec, and 65°C for 15 sec. At the end of the PCR cycles, a melting curve analysis was performed. Samples were run in triplicate and data were analyzed using Lightcycler480 Real-Time analysis software.

Table I. Sequences of primers used for RT-qPCR.

Gene	Primer sequences	Product length (bp)
<i>Gapdh</i>	F: ACTCCACTCACGGCAAATTC R: TCTCCATGGTGGTGAAGACA	171
<i>T-bet</i>	F: GTTCAACCAGCACCAGACAGAG R: TGGTCCACCAAGACCACATC	135
<i>IFN-<math>\gamma</math></i>	F: CGGCACAGTCATTGAAAGCCTA R: GTTGCTGATGGCCTGATTGTC	199
<i>GATA3</i>	F: GGATGTAAGTCGAGGCCCAAG R: ATTGCAAAGGTAGTGCCCGGTA	117
<i>IL-4</i>	F: ACGGAGATGGATGTGCCAAAC R: AGCACCTTGGAAGCCCTACAGA	83
<i>ROR-<math>\gamma</math>t</i>	F: TCTGCAAGACTCATCGACAAGG R: CACATGTTGGCTGCACAGG	80
<i>IL-17A</i>	F: CAGCAGCGATCATCCCTCAAAG R: CAGGACCAGGATCTCTTGCTG	302
<i>Foxp3</i>	F: CACCCAGGAAAGACAGCAACC R: CAAGAGCTCTTGTCCATTGA	313
<i>TGF-<math>\beta</math></i>	F: GTGTGGAGCAACATGTGGAAGTCTA R: CGCTGAATCGAAAGCCCTGTA	174

F, forward; R, reverse.

**Statistical analysis.** Data are expressed as the means values  $\pm$  standard deviation (SD). An unpaired two-tailed Student's t-test was used to detect statistically significant differences between the 2 groups. Differences between 3 or more groups were analyzed by analysis of variance (ANOVA). Values were compared using the  $\chi^2$  square test. Data were analyzed using the SPSS 16.0 software package (SPSS Inc., Armonk, NY, USA). A P-value  $<0.05$  was considered to indicate a statistically significant difference.

## Results

**Genotyping of mice.** *IL-10*<sup>+/-</sup> (BALB/c or C57BL/6) mice were generated from intercrosses between *IL-10*<sup>+/+</sup> female and *IL-10*<sup>-/-</sup> male mice, and then mated with each other to produce *IL-10*<sup>+/+</sup> and *IL-10*<sup>-/-</sup> offspring. Genotypes were confirmed by PCR (Fig. 1). A 137-bp band was detected in the *IL-10*<sup>+/+</sup> (BALB/c or C57BL/6) mice, whereas a 312-bp band was observed in the *IL-10*<sup>-/-</sup> (BALB/c or C57BL/6) mice. As expected, both bands were detected in the *IL-10*<sup>+/-</sup> (BALB/c or C57BL/6) mice.

**Determination of the optimal dose for mTg immunization.** Previous studies have demonstrated that thyroiditis-susceptible CBA/J mice respond robustly to immunization with 100  $\mu$ g mTg emulsified in CFA (15,16). In the first series of experiments, CBA/J mice were used as a positive control. As shown in Fig. 2A, in the C57BL/6 *IL-10*<sup>-/-</sup> mice, the group treated with 200  $\mu$ g mTg had higher titers of serum TgAb than the control group (1.44 $\pm$ 0.08 vs. 0.07 $\pm$ 0.01,  $P<0.001$ ) or the group

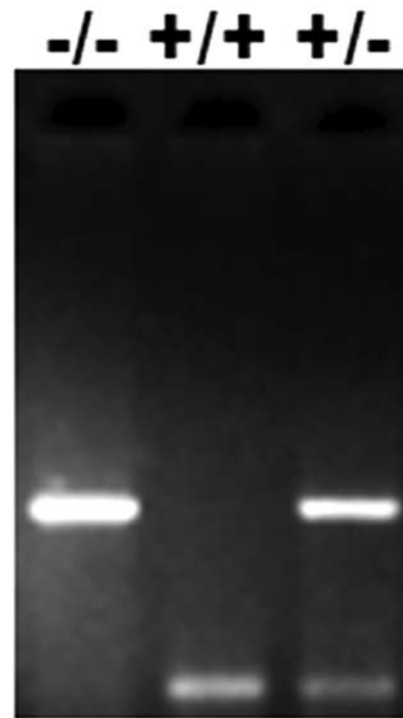


Figure 1. Genotyping of *IL-10*<sup>+/-</sup>, *IL-10*<sup>+/-</sup> and *IL-10*<sup>-/-</sup> mice by PCR using tail genomic DNA. Primer sequences are listed in Table I.

treated with 100  $\mu$ g mTg (1.44 $\pm$ 0.08 vs. 0.56 $\pm$ 0.12,  $P<0.001$ ). Additionally, the levels of antibody (titers) of serum TgAb in the group treated with 200  $\mu$ g group mTg were much more similar to those of the CBA/J positive control group (1.44 $\pm$ 0.08 vs. 1.69 $\pm$ 0.06,  $P=0.021$ ).

Experiments using the BALB/c *IL-10*<sup>-/-</sup> mice yielded results similar to those obtained with the C57BL/6 *IL-10*<sup>-/-</sup> mice. As shown in Fig. 2B, the group treated with 200  $\mu$ g mTg had higher titers of serum TgAb than the control group (1.07 $\pm$ 0.15 vs. 0.05 $\pm$ 0.01,  $P<0.001$ ) or the group treated with 100  $\mu$ g mTg (1.07 $\pm$ 0.15 vs. 0.46 $\pm$ 0.05,  $P<0.001$ ). Additionally, the levels of antibody (titers) of serum TgAb in the group treated with 200  $\mu$ g mTg were more similar to those of the CBA/J positive control group (1.07 $\pm$ 0.15 vs. 1.69 $\pm$ 0.06,  $P<0.01$ ).

**Effects of *IL-10* deficiency on susceptibility to EAT.** In the first series of experiments, we confirmed that 200  $\mu$ g mTg was an optimal dose for immunization. Therefore, in the second series of experiments, *IL-10*<sup>-/-</sup> and *IL-10*<sup>+/-</sup> female mice were immunized with 200  $\mu$ g mTg to induce EAT. As shown in Table II, in the C57BL/6 mice, the *IL-10*<sup>-/-</sup> mTg group had a higher titer of serum TgAb than the *IL-10*<sup>-/-</sup> control group (0.93 $\pm$ 0.2 vs. 0.13 $\pm$ 0.02,  $P<0.001$ ) or the *IL-10*<sup>+/-</sup> mTg group (0.93 $\pm$ 0.2 vs. 0.38 $\pm$ 0.07,  $P<0.001$ ). Histological data indicated that lymphocytic infiltration was present in some *IL-10*<sup>-/-</sup> female mice in the mTg group, but no significant infiltration was observed in the other 3 groups (Fig. 3A-D).

Similar results were obtained using BALB/c mice. As summarized in Table II, the *IL-10*<sup>-/-</sup> mTg group had a higher titer of serum TgAb than the *IL-10*<sup>-/-</sup> control group (0.93 $\pm$ 0.16 vs. 0.15 $\pm$ 0.01,  $P<0.001$ ) or the *IL-10*<sup>+/-</sup> mTg group (0.93 $\pm$ 0.16 vs. 0.46 $\pm$ 0.18,  $P<0.001$ ). Following the analysis of histological

Table II. Increased susceptibility to EAT in the absence of IL-10 in mice with an EAT-resistant genetic background.

Mouse strain	Experimental group (number of mice) <sup>a</sup>	Immunization	Anti-mTg IgG <sup>b</sup> (mean OD <sub>450</sub> ± SD)	Thyroid lymphocytic infiltration score <sup>c</sup>					Incidence of EAT (%)
				0 <sup>+</sup>	1 <sup>+</sup>	2 <sup>+</sup>	3 <sup>+</sup>	4 <sup>+</sup>	
<i>IL-10</i> <sup>+/+</sup> C57BL/6	mTg group (8)	mTg + adjuvant	0.38±0.07 <sup>d</sup>	8	0	0	0	0	0/8 (0) <sup>d</sup>
	control group (8)	PBS + adjuvant	0.14±0.01 <sup>d</sup>	8	0	0	0	0	0/8 (0) <sup>d</sup>
<i>IL-10</i> <sup>-/-</sup> C57BL/6	mTg group (12)	mTg + adjuvant	0.93±0.2	8	3	1	0	0	4/12 (25)
	control group (8)	PBS + adjuvant	0.13±0.02 <sup>d</sup>	8	0	0	0	0	0/8 (0) <sup>d</sup>
<i>IL-10</i> <sup>+/+</sup> BALB/c	mTg group (8)	mTg + adjuvant	0.46±0.18 <sup>e</sup>	8	0	0	0	0	0/8 (0) <sup>e</sup>
	control group (8)	PBS + adjuvant	0.14±0.02 <sup>e</sup>	8	0	0	0	0	0/8 (0) <sup>e</sup>
<i>IL-10</i> <sup>-/-</sup> BALB/c	mTg group (12)	mTg + adjuvant	0.93±0.16	9	3	0	0	0	3/12 (33.3)
	control group (8)	PBS + adjuvant	0.15±0.01 <sup>e</sup>	8	0	0	0	0	0/8 (0) <sup>e</sup>

<sup>a</sup>mTg group: administered mTg [200 µg subcutaneously (s.c.)] in complete Freund's adjuvant (CFA) on day 0, and in incomplete Freund's adjuvant (IFA) on day 7; control group: administered PBS (s.c.) in CFA on day 0, and in IFA on day 7; all mice were sacrificed on day 28. <sup>b</sup>Mean OD<sub>450</sub>±SD of serum; 8-12 individual mice per group. Serum was diluted at 1:1,000. <sup>c</sup>Histological grades of thyroiditis were scored as described in the Materials and methods. <sup>d</sup>P<0.01 vs. the *IL-10*<sup>-/-</sup> mTg group; <sup>e</sup>P<0.01 vs. the BALB/c *IL-10*<sup>-/-</sup> mTg group. EAT, experimental autoimmune thyroiditis.

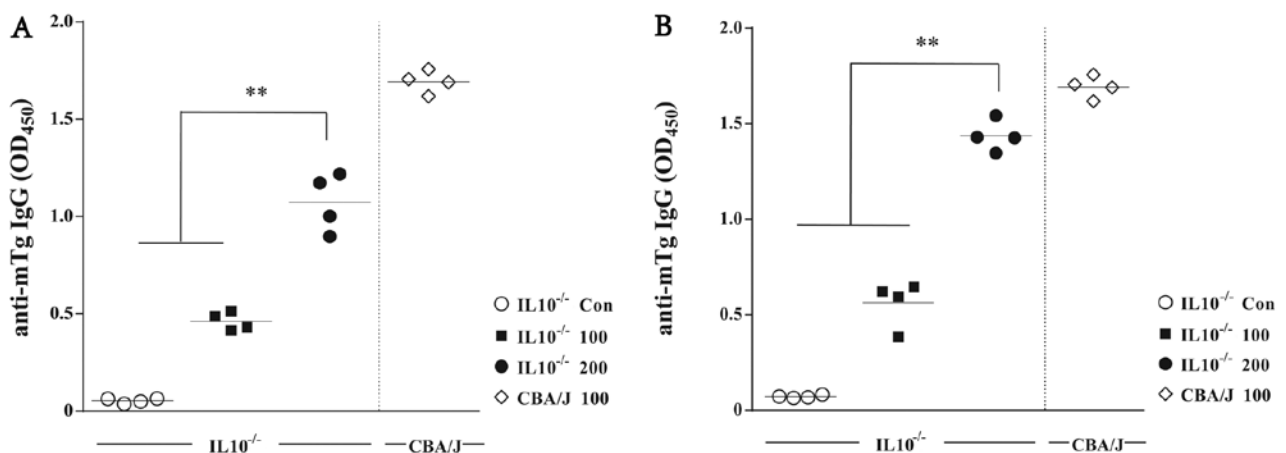


Figure 2. In the first series of experiments, *IL-10*<sup>-/-</sup> mice were immunized subcutaneously (s.c.) with different doses of mTg (100 or 200 µg); the susceptible CBA/J strain was used as a positive control immunized with 100 µg mTg to induce EAT; the control group received PBS and adjuvant. The immunization protocol is described in the Materials and methods. Serum anti-mTg IgG titers (mean OD<sub>450</sub> ± SD) from 4 mice per group were measured by ELISA; serum was diluted 1:500; \*\*P<0.01 vs. the *IL-10*<sup>-/-</sup> 200-µg mTg group. (A) The serum anti-mTg IgG titers of *IL-10*<sup>-/-</sup> C57BL/6 and CBA/J female mice. (B) The serum anti-mTg IgG titers of *IL-10*<sup>-/-</sup> BALB/c and CBA/J female mice.

sections, lymphocytic infiltration was observed in some *IL-10*<sup>-/-</sup> female mice in the mTg group, while no significant infiltration was present in the other 3 groups (Fig. 3E-H).

EAT is indicated by high titers of serum TgAb and thyroid lymphocytic infiltration (4). The data presented in Table II indicate that only 4 of the 12 C57BL/6 *IL-10*<sup>-/-</sup> female mice showed signs of thyroiditis and the severity was mild; overall, the incidence of EAT was 33.3%. Furthermore, 3 of the 12 BALB/c *IL-10*<sup>-/-</sup> female mice showed signs of thyroiditis and the severity was also mild; overall, the prevalence of EAT was 25%. Taken together, these findings indicate that IL-10 deficiency can affect susceptibility to EAT in resistant strains, at least to a certain extent.

*An increased percentage of CD19<sup>+</sup> B cells may contribute to the production of high serum titers of TgAb in IL-10<sup>-/-</sup> mice.* In this

study, we observed significantly increased serum TgAb titers in the *IL-10*<sup>-/-</sup> mTg group. It is known that B cells play a critical role in antibody production (17). Therefore, we first analyzed by flow cytometry the proportion of splenic CD19<sup>+</sup> B cells in the C57BL/6 mouse groups (Fig. 4A). As shown in Fig. 4C, in the C57BL/6 mice, the *IL-10*<sup>-/-</sup> mTg group had a significantly higher percentage of splenic CD19<sup>+</sup> B cells than the *IL-10*<sup>-/-</sup> control group (38.9±3.9% vs. 32.4±5.35%, P=0.023) or the *IL-10*<sup>+/+</sup> mTg group (38.9±3.9% vs. 32.2±6.39%, P=0.014). Correlation analysis was further performed to confirm the association of serum TgAb titers and the proportion of CD19<sup>+</sup> B cells, and there was a significantly positive correlation for the 2 variables (r=0.719, P=0.045; Fig. 4E).

We also analyzed by flow cytometry the proportion of splenic CD19<sup>+</sup> B cells in the BALB/c mouse groups (Fig. 4B). The experiments using BALB/c mice yielded results similar

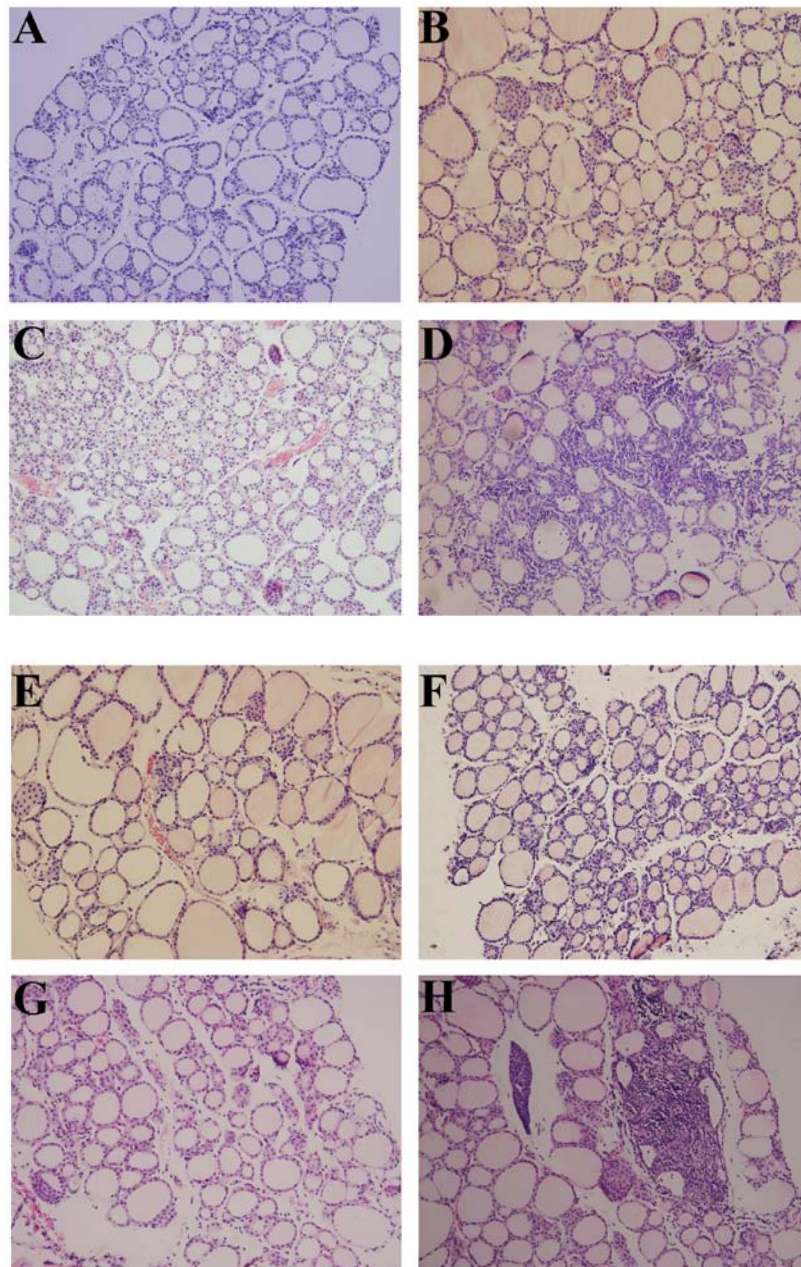


Figure 3. Mice were sacrificed on day 28 to obtain thyroid tissue. Representative photomicrographs of H&E-stained thyroid sections from the different groups are shown (magnification, x200). Thyroid lymphocytic infiltration was only observed in the C57BL/6 *IL-10*<sup>-/-</sup> and BALB/c *IL-10*<sup>-/-</sup> mTg groups. (A) C57BL/6 *IL-10*<sup>+/+</sup> control group, (B) C57BL/6 *IL-10*<sup>+/+</sup> mTg group, (C) C57BL/6 *IL-10*<sup>-/-</sup> control group, (D) C57BL/6 *IL-10*<sup>-/-</sup> mTg group, (E) BALB/c *IL-10*<sup>+/+</sup> control group, (F) BALB/c *IL-10*<sup>+/+</sup> mTg group, (G) BALB/c *IL-10*<sup>-/-</sup> control group and (H) BALB/c *IL-10*<sup>-/-</sup> mTg group.

to those obtained with the C57BL/6 mice. Indeed, the *IL-10*<sup>-/-</sup> mTg group had a markedly increased proportion of splenic CD19<sup>+</sup> B cells than the *IL-10*<sup>-/-</sup> control group ( $38.8 \pm 4.54\%$  vs.  $32.7 \pm 2.57\%$ ,  $P=0.013$ ) and the *IL-10*<sup>+/+</sup> mTg group ( $38.8 \pm 4.54\%$  vs.  $34.1 \pm 3.07\%$ ,  $P=0.028$ ) (Fig. 4D). There was also a significant positive correlation between the serum TgAb titers and the percentage of CD19<sup>+</sup> B cells ( $r=0.758$ ,  $P=0.048$ ; Fig. 4F).

*Increased percentage of Th1 cells and the mRNA expression of T-box expressed in T cells (T-bet) and IFN- $\gamma$  in the absence of IL-10 during the development of EAT.* Previous studies have reported that CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T (Th1) cells play an important role in the pathogenesis of autoimmune thyroiditis (18). Thus, we investigated changes in the percentage of Th1 cells to elucidate

the possible pathogenic mechanisms responsible for the development of EAT in the absence of IL-10. The *IL-10*<sup>-/-</sup> (C57BL/6 and BALB/c) mTg groups were subdivided into the *IL-10*<sup>-/-</sup> non-EAT and *IL-10*<sup>-/-</sup> EAT groups. In the C57BL/6 mice, as shown in Fig. 5A, the percentage of Th1 cells in the *IL-10*<sup>-/-</sup> EAT group was significantly higher than that in the *IL-10*<sup>-/-</sup> non-EAT group ( $8.57 \pm 1.86\%$  vs.  $4.09 \pm 0.79\%$ ,  $P<0.01$ ), the *IL-10*<sup>-/-</sup> control group ( $8.57 \pm 1.86\%$  vs.  $2.47 \pm 1.02\%$ ,  $P<0.01$ ), and the *IL-10*<sup>+/+</sup> mTg group ( $8.57 \pm 1.86\%$  vs.  $2.74 \pm 1.66\%$ ,  $P<0.01$ ). The mRNA expression levels of the Th1 transcription factor, *T-bet*, and the Th1 cell-associated cytokine, *IFN- $\gamma$* , were also measured. As summarized in Fig. 6A, the mRNA expression of *T-bet* was markedly upregulated in the *IL-10*<sup>-/-</sup> EAT group compared with the *IL-10*<sup>-/-</sup> non-EAT group ( $1.4 \pm 0.1$  vs.  $0.71 \pm 0.13$ ,  $P<0.01$ ), the

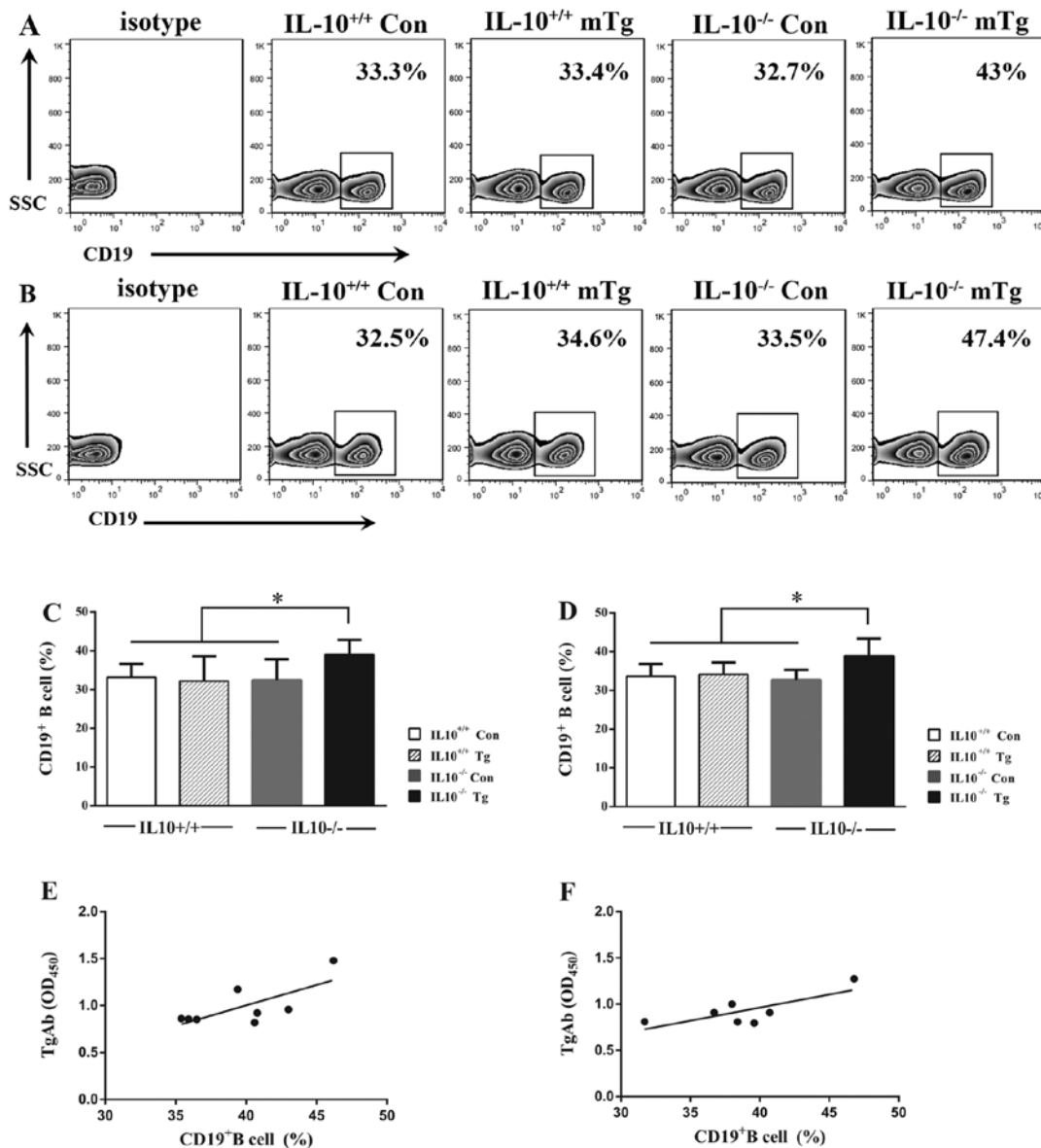


Figure 4. Flow cytometric analysis of spleen CD19<sup>+</sup> B cells in *IL10*<sup>+/+</sup> and *IL10*<sup>-/-</sup> mice with or without mTg immunization. Frequencies of CD19<sup>+</sup> B cells from indicated groups in C57BL/6 mice are shown. (A) Representative histograms indicate the frequencies of CD19<sup>+</sup> B cells for one mouse. (C) Bar graphs show mean frequencies of CD19<sup>+</sup> B cells (n≥6 mice/group). The frequencies of CD19<sup>+</sup> B cells from indicated groups in BALB/c mice are shown. (B) Representative histograms indicate the frequencies of CD19<sup>+</sup> B cells for one mouse. (D) Bar graphs show mean frequencies of CD19<sup>+</sup> B cells (n≥6 mice/group). The frequencies of CD19<sup>+</sup> B cells positively correlated with the level of TgAb in both *IL10*<sup>-/-</sup> (E) C57BL/6 and (F) BALB/c mice. The isotype control acted as a negative control for CD19<sup>+</sup> B cells subsets. MTg group was immunized with 200 μg mTg; control group was immunized with the PBS and adjuvant. All experiments were performed at least 3 times, \*P<0.01.

*IL10*<sup>-/-</sup> control group (1.4±0.1 vs. 0.62±0.1, P<0.01), and the *IL10*<sup>+/+</sup> mTg group (1.4±0.1 vs. 0.65±0.09, P<0.01). The *IL10*<sup>-/-</sup> EAT group also exhibited significantly increased mRNA expression levels of *IFN-γ* compared with the *IL10*<sup>-/-</sup> non-EAT group (0.89±0.13 vs. 0.24±0.02, P<0.01), the *IL10*<sup>-/-</sup> control group (0.89±0.13 vs. 0.18±0.05, P<0.01), and the *IL10*<sup>+/+</sup> mTg group (0.89±0.13 vs. 0.21±0.04, P<0.01).

We obtained similar results using BALB/c mice. As shown in Fig. 5B, the *IL10*<sup>-/-</sup> EAT group had a higher percentage of Th1 cells than the *IL10*<sup>-/-</sup> non-EAT group (6.6±0.75% vs. 3.38±0.41%, P<0.01), the *IL10*<sup>-/-</sup> control group (6.6±0.75% vs. 2.8±0.75%, P<0.01), and the *IL10*<sup>+/+</sup> mTg group (6.6±0.75% vs. 2.92±0.76%, P<0.01). As shown in Fig. 6B, the *IL10*<sup>-/-</sup> EAT group showed significantly increased mRNA expression levels of *T-bet*

compared with the *IL10*<sup>-/-</sup> non-EAT group (1.4±0.15 vs. 0.8±0.1, P<0.01), the *IL10*<sup>-/-</sup> control group (1.4±0.15 vs. 0.6±0.19, P<0.01), and the *IL10*<sup>+/+</sup> mTg group (1.4±0.15 vs. 0.66±0.23, P<0.01). The mRNA expression of *IFN-γ* was also markedly upregulated in the *IL10*<sup>-/-</sup> EAT group compared with the *IL10*<sup>-/-</sup> non-EAT group (0.71±0.05 vs. 0.23±0.01, P<0.01), the *IL10*<sup>-/-</sup> control group (0.71±0.05 vs. 0.18±0.06, P<0.01), and the *IL10*<sup>+/+</sup> mTg group (0.71±0.05 vs. 0.17±0.05, P<0.01).

Apart from the Th1 cell clones, we also investigated changes in the Th2 cell population and measured the mRNA expression levels of its key transcription factor, GATA binding protein 3 (*GATA3*), and the stereotypical cytokine, IL-4. In the C57BL/6 mice, there were no significant differences in the percentage of Th2 cells between the *IL10*<sup>-/-</sup> EAT



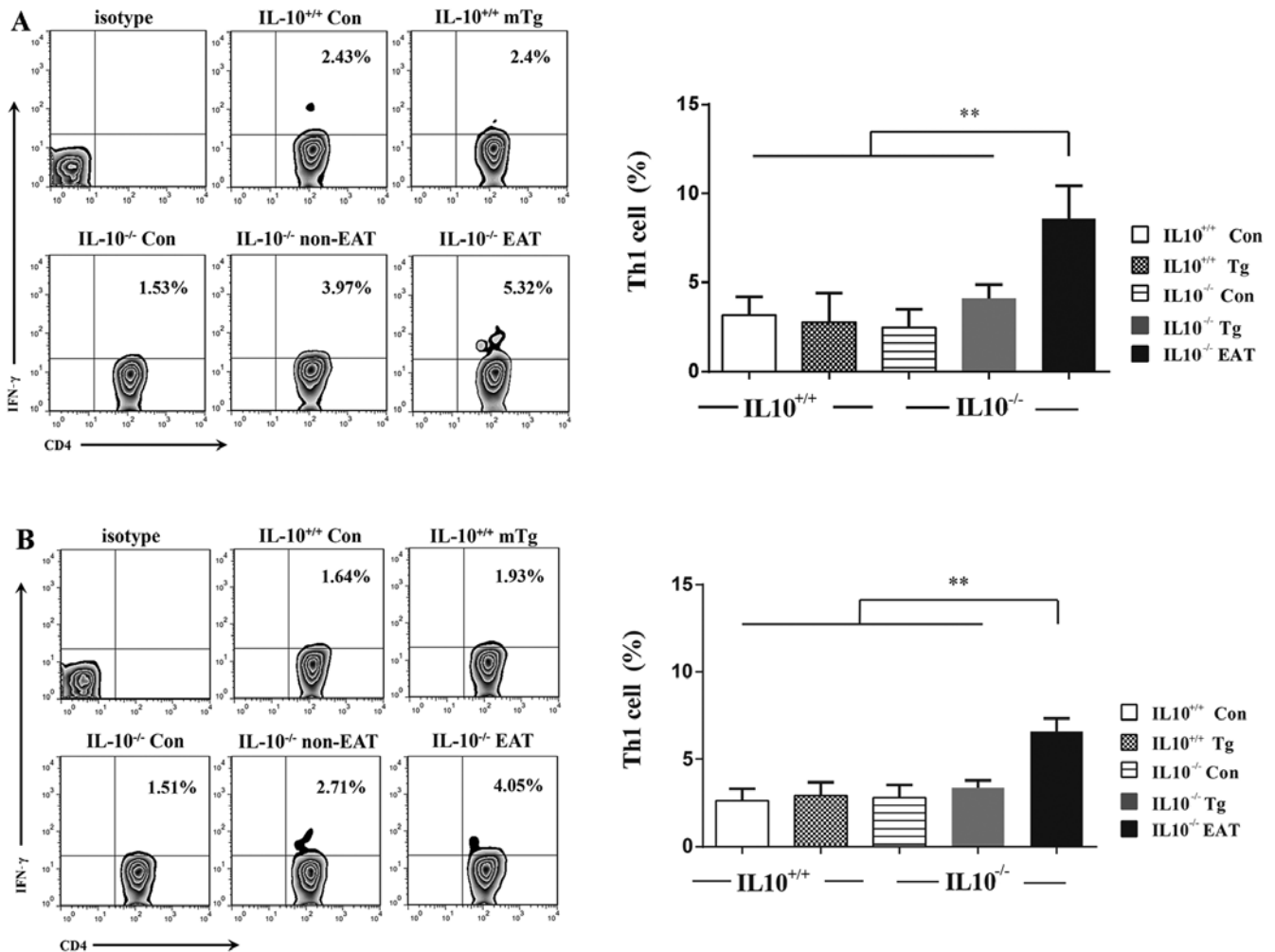


Figure 5. Flow cytometric analysis of splenic CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> T (Th1) cell subsets in *IL-10*<sup>+/+</sup> and *IL-10*<sup>-/-</sup> mice with or without mTg immunization. (A) The frequencies of Th1 subsets from the indicated groups of C57BL/6 mice are shown. Representative histograms (left) indicate the frequencies of Th1 cells in one mouse and the bar graphs (right) show the mean frequencies of Th1 cells ( $n \geq 4$  mice/group). (B) The frequencies of Th1 cells from the indicated groups of BALB/c mice are shown. Representative histograms (left) indicate the frequencies of Th1 cells in one mouse and the bar graphs (right) show the mean frequencies of Th1 cells ( $n \geq 3$  mice/group). The isotype control served as a negative control for Th1 cells. The mTg group was immunized with 200  $\mu$ g mTg; the control group was immunized with PBS and adjuvant. The EAT group was defined as mice in which EAT was present in all mice immunized with 200  $\mu$ g mTg; the non-EAT group was defined as mice in which EAT was absent in all mice immunized with 200  $\mu$ g mTg. All experiments were performed at least 3 times; \*\* $P < 0.01$ .

group, the *IL-10*<sup>-/-</sup> non-EAT group, the *IL-10*<sup>-/-</sup> control group, and the *IL-10*<sup>+/+</sup> mTg group ( $1.38 \pm 0.85\%$  vs.  $1.11 \pm 0.92\%$  vs.  $1.22 \pm 0.24\%$  vs.  $1.85 \pm 0.54\%$ ,  $P > 0.05$ ) (data not shown). Similarly, there were no significant differences in the mRNA expression levels of *GATA3* between the *IL-10*<sup>-/-</sup> EAT group, the *IL-10*<sup>-/-</sup> non-EAT group, the *IL-10*<sup>-/-</sup> control group, and the *IL-10*<sup>+/+</sup> mTg group ( $1.1 \pm 0.2$  vs.  $1.02 \pm 0.15$  vs.  $1.06 \pm 0.14$  vs.  $0.94 \pm 0.08$ ,  $P > 0.05$ ) (Fig. 6A). We found no statistically significant differences in the mRNA expression levels of *IL-4* between the *IL-10*<sup>-/-</sup> EAT group, the *IL-10*<sup>-/-</sup> non-EAT group, the *IL-10*<sup>-/-</sup> control group, and the *IL-10*<sup>+/+</sup> mTg group ( $0.52 \pm 0.08$  vs.  $0.49 \pm 0.12$  vs.  $0.54 \pm 0.14$  vs.  $0.45 \pm 0.12$ ,  $P > 0.05$ ) (Fig. 6A).

Similar results were obtained using BALB/c mice. There were no significant differences in the percentage of Th2 cells between the *IL-10*<sup>-/-</sup> EAT group, the *IL-10*<sup>-/-</sup> non-EAT group, the *IL-10*<sup>-/-</sup> control group, or the *IL-10*<sup>+/+</sup> mTg group ( $1.45 \pm 0.25\%$  vs.  $1.26 \pm 0.39\%$  vs.  $0.9 \pm 0.18\%$  vs.  $1.17 \pm 0.7\%$ ,  $P > 0.05$ ) (data not shown). Similarly, there were no significant differences in the mRNA expression of *GATA3* between the *IL-10*<sup>-/-</sup> EAT group,

the *IL-10*<sup>-/-</sup> non-EAT group, the *IL-10*<sup>-/-</sup> control group, or the *IL-10*<sup>+/+</sup> mTg group ( $1.06 \pm 0.14$  vs.  $1.05 \pm 0.12$  vs.  $0.99 \pm 0.11$  vs.  $0.96 \pm 0.13$ ,  $P > 0.05$ ) (Fig. 6B). We found no statistically significant differences in the mRNA expression levels of *IL-4* between the *IL-10*<sup>-/-</sup> EAT group, the *IL-10*<sup>-/-</sup> non-EAT group, the *IL-10*<sup>-/-</sup> control group, or the *IL-10*<sup>+/+</sup> mTg group ( $0.53 \pm 0.09$  vs.  $0.54 \pm 0.06$  vs.  $0.54 \pm 0.1$  vs.  $0.51 \pm 0.12$ ,  $P > 0.05$ ) (Fig. 6B).

*An increased percentage of Th17 cells and mRNA expression of retinoic acid receptor-related orphan receptor (ROR)- $\gamma$ t and IL-17, but a reduced percentage of Treg cells and mRNA expression of Foxp3 are involved in the development of EAT under IL-10-deficient conditions.* In our previous study, we suggested that CD4<sup>+</sup>IL-17<sup>+</sup> T (Th17) cells are involved in the development of autoimmune thyroiditis (19). Accordingly, we measured changes in the percentage of Th17 cells in the development of EAT in the absence of IL-10. In the C57BL/6 mice, compared with the *IL-10*<sup>-/-</sup> control group, a significantly increased percentage of Th17 cells was observed in the

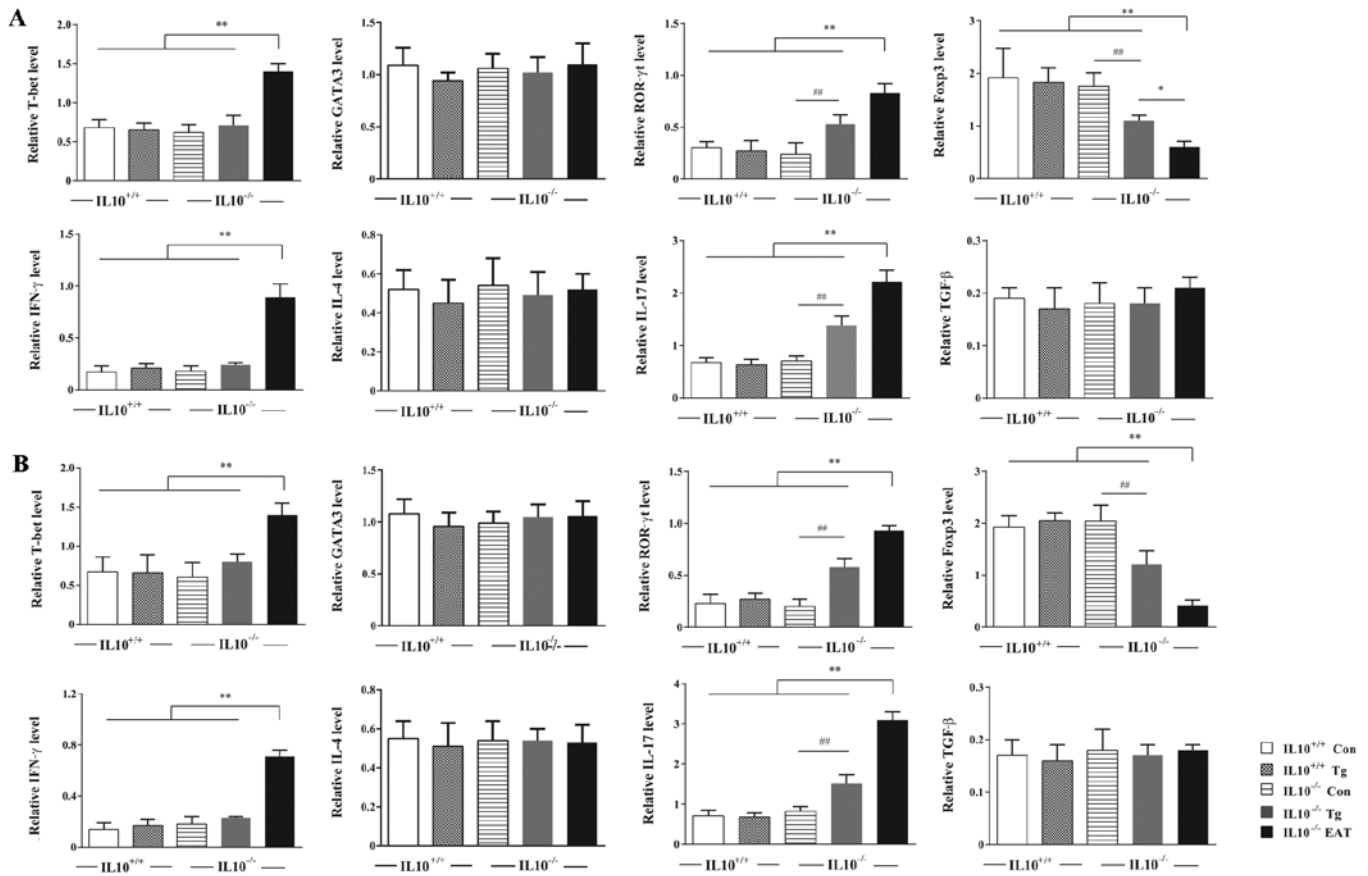


Figure 6. RT-qPCR of the Th1 cell-specific transcription factor, T-box expressed in T cells (*T-bet*), interferon (IFN)- $\gamma$  (a Th1 cell cytokine), the Th2 cell-specific transcription factor GATA binding protein 3 (*GATA3*), interleukin (IL)-4 (a Th2 cell cytokine), the Th17 cell-specific transcription factor retinoic acid receptor-related orphan receptor- $\gamma$ t (*ROR- $\gamma$ t*), IL-17A (a Th17 cell cytokine), the Treg cell-specific transcription factor forkhead box P3 (*Foxp3*), and transforming growth factor- $\beta$  (*TGF- $\beta$* , a Treg and Th17 cell-associated cytokine) mRNA expression in *IL-10*<sup>+/+</sup> and *IL-10*<sup>-/-</sup> mice with or without mTg immunization. Expression of transcription factors and cytokines from the indicated groups of (A) C57BL/6 or (B) BALB/c mice are shown. The mTg group was immunized with 200  $\mu$ g mTg; the control group was immunized with PBS and adjuvant. The EAT group was defined as mice in which EAT was present in all mice immunized with 200  $\mu$ g mTg; the non-EAT group was defined as mice in which EAT was absent in all mice immunized with 200  $\mu$ g mTg. All experiments were performed at least 3 times; \**P*<0.05, \*\**P*<0.01; \*\*\**P*<0.001, #*P*<0.05, ##*P*<0.01, ###*P*<0.001.

*IL-10*<sup>-/-</sup> non-EAT group (3.56 $\pm$ 1.09% vs. 4.94 $\pm$ 0.9%, *P*=0.034; Fig. 7A). Furthermore, the prevalence of Th17 cells in the *IL-10*<sup>-/-</sup> EAT group further increased to 7.39 $\pm$ 1.31%, which was significantly higher than in the *IL-10*<sup>-/-</sup> non-EAT group (*P*<0.01), the *IL-10*<sup>-/-</sup> control group (*P*<0.01), and the *IL-10*<sup>+/+</sup> mTg group (7.39 $\pm$ 1.31% vs. 1.25 $\pm$ 0.63%, *P*<0.01). The mRNA expression of the Th17 transcription factor, *ROR- $\gamma$ t*, and *IL-17* was also measured. As summarized in Fig. 6A, compared with the *IL-10*<sup>-/-</sup> control group, a markedly increased mRNA expression of *ROR- $\gamma$ t* was observed in the *IL-10*<sup>-/-</sup> non-EAT group (0.24 $\pm$ 0.11 vs. 0.53 $\pm$ 0.09, *P*<0.01), and the mRNA expression of *ROR- $\gamma$ t* in the *IL-10*<sup>-/-</sup> EAT group further increased to 0.83 $\pm$ 0.09, which was higher than that in the *IL-10*<sup>-/-</sup> non-EAT group (*P*<0.01), the *IL-10*<sup>-/-</sup> control group (*P*<0.01), and the *IL-10*<sup>+/+</sup> mTg group (0.83 $\pm$ 0.09 vs. 0.27 $\pm$ 0.1, *P*<0.01). We also detected a significant difference in the mRNA expression of *IL-17* between the *IL-10*<sup>-/-</sup> non-EAT group and the *IL-10*<sup>-/-</sup> control group (1.38 $\pm$ 0.18 vs. 0.71 $\pm$ 0.09, *P*<0.01). The mRNA expression of *IL-17* in the *IL-10*<sup>-/-</sup> EAT group further increased to 2.22 $\pm$ 0.22, which was significantly higher than that in the *IL-10*<sup>-/-</sup> non-EAT group (*P*<0.01), the *IL-10*<sup>-/-</sup> control group

(*P*<0.01), and the *IL-10*<sup>+/+</sup> mTg group (2.22 $\pm$ 0.22 vs. 0.63 $\pm$ 0.11, *P*<0.01).

Experiments using BALB/c mice yielded results similar to the C57BL/6 mice. Compared with the *IL-10*<sup>-/-</sup> control group, a significantly increased percentage of Th17 cells was observed in the *IL-10*<sup>-/-</sup> non-EAT group (3.58 $\pm$ 1.71% vs. 5.8 $\pm$ 1.03%, *P*=0.012), and the prevalence of Th17 cells in the *IL-10*<sup>-/-</sup> EAT group further increased to 9.13 $\pm$ 1.09%, which was significantly higher than that in the *IL-10*<sup>-/-</sup> non-EAT group (*P*<0.01), the *IL-10*<sup>-/-</sup> control group (*P*<0.01), and the *IL-10*<sup>+/+</sup> mTg group (9.13 $\pm$ 1.09% vs. 2.03 $\pm$ 1.21%, *P*<0.01; Fig. 7B). As shown in Fig. 6B, compared with the *IL-10*<sup>-/-</sup> control group, a markedly increased mRNA expression of *ROR- $\gamma$ t* was detected in the *IL-10*<sup>-/-</sup> non-EAT group (0.2 $\pm$ 0.07 vs. 0.58 $\pm$ 0.08, *P*<0.01), and the mRNA expression of *ROR- $\gamma$ t* in the *IL-10*<sup>-/-</sup> EAT group further increased to 0.93 $\pm$ 0.05, which was higher than that in the *IL-10*<sup>-/-</sup> non-EAT group (*P*<0.01), the *IL-10*<sup>-/-</sup> control group (*P*<0.01), and the *IL-10*<sup>+/+</sup> mTg group (0.93 $\pm$ 0.05 vs. 0.27 $\pm$ 0.06, *P*<0.01). A significant difference was also observed in the mRNA expression of *IL-17* between the *IL-10*<sup>-/-</sup> non-EAT group and the *IL-10*<sup>-/-</sup> control group (1.5 $\pm$ 0.22 vs. 0.82 $\pm$ 0.12,



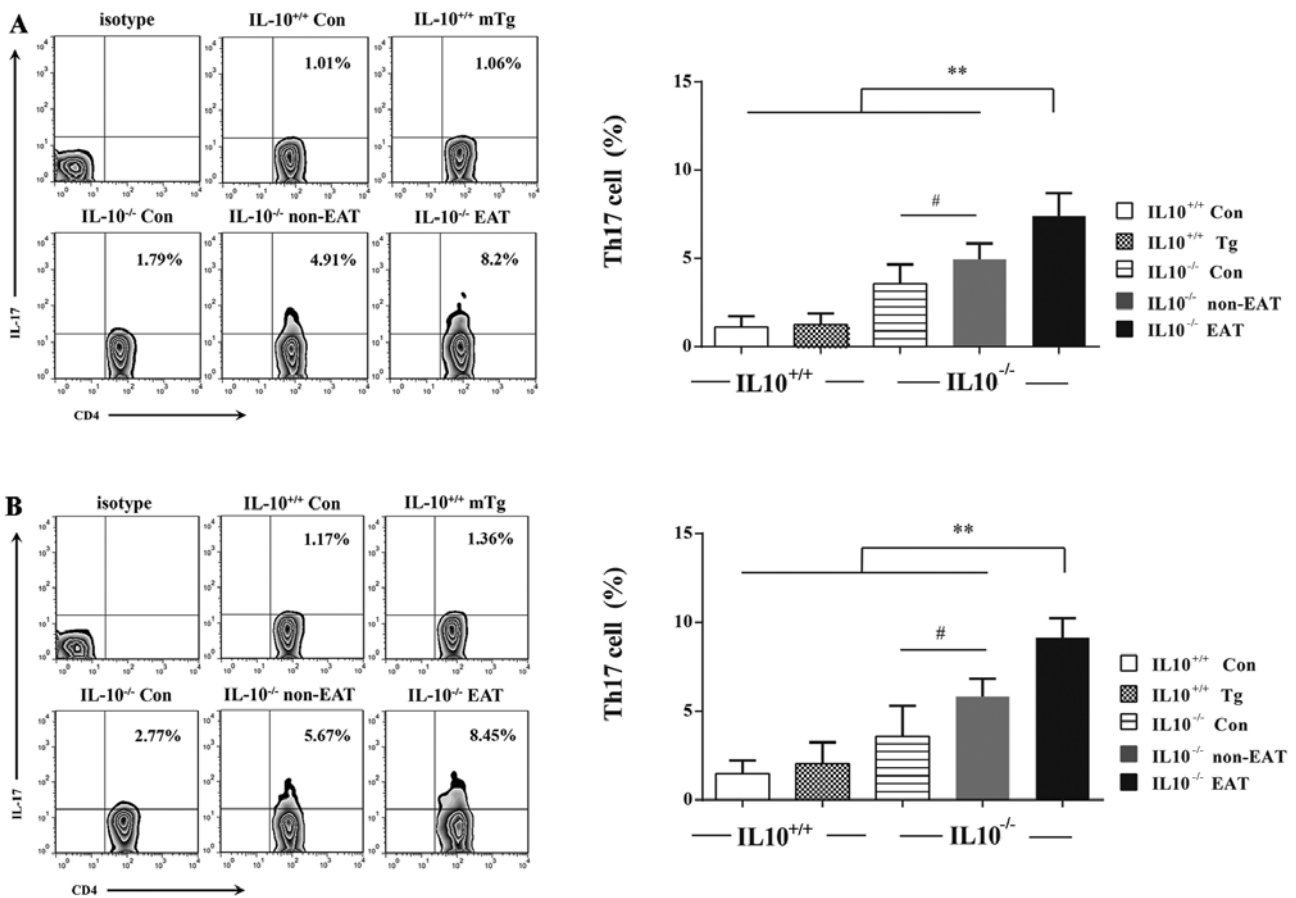


Figure 7. Flow cytometric analysis of splenic CD4<sup>+</sup>IL-17<sup>+</sup> T (Th17) cell subsets in *IL-10*<sup>+/+</sup> and *IL-10*<sup>-/-</sup> mice with or without mTg immunization. (A) The frequencies of Th17 cell subsets from the indicated groups of C57BL/6 mice are shown. Representative histograms (left panel) indicate the frequencies of Th17 cell subsets for one mouse and the bar graphs (right) show the mean frequencies of Th17 cells (n≥4 mice/group). (B) The frequencies of Th17 cell subsets from the indicated groups of BALB/c mice are shown. Representative histograms (left) indicate the frequencies of Th17 cells for one mouse and the bar graphs (right) show the mean frequencies of Th17 cells (n≥3 mice/group). The isotype control served as a negative control for Th17 cells. The mTg group was immunized with 200  $\mu$ g mTg; the control group was immunized with PBS and adjuvant. The EAT group was defined as mice in which EAT was present in all of the mice immunized with 200  $\mu$ g mTg; the non-EAT group was defined as mice in which EAT was absent in all of the mice immunized with 200  $\mu$ g mTg. All experiments were performed at least 3 times; \*P<0.01; \*\*P<0.05.

P<0.01), and the mRNA expression of *IL-17* in the *IL-10*<sup>-/-</sup> EAT group further increased to  $3.1\pm0.21$ , which was significantly higher than in the *IL-10*<sup>-/-</sup> non-EAT group (P<0.01), the *IL-10*<sup>-/-</sup> control group (P<0.01), and the *IL-10*<sup>+/+</sup> mTg group ( $3.1\pm0.21$  vs.  $0.68\pm0.1$ , P<0.01).

The protective role of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells had been demonstrated to affect the development of thyroid autoimmune diseases (20). Therefore, we measured changes in the percentage of Treg cells in the development of EAT. In the C57BL/6 mice, compared with the *IL-10*<sup>-/-</sup> control group, a significantly reduced percentage of Treg cells was observed in the *IL-10*<sup>-/-</sup> non-EAT group ( $3.48\pm0.58\%$  vs.  $2.25\pm0.68\%$ , P=0.03), and the percentage of Treg cells in the *IL-10*<sup>-/-</sup> EAT group further decreased to  $1.05\pm0.12\%$ , which was significantly lower than that in the *IL-10*<sup>-/-</sup> non-EAT group (P=0.043), the *IL-10*<sup>-/-</sup> control group (P<0.01), and the *IL-10*<sup>+/+</sup> mTg group ( $1.05\pm0.12\%$  vs.  $3.41\pm1.11\%$ , P<0.01; Fig. 8A). The mRNA expression levels of the Treg cell transcription factor, forkhead box P3 (Foxp3), and the associated regulatory cytokine, transforming growth factor- $\beta$  (TGF- $\beta$ ), were also measured. As shown in Fig. 6A, compared with the *IL-10*<sup>-/-</sup> control group, a marked decrease in the mRNA expression of Foxp3

was observed in the *IL-10*<sup>-/-</sup> non-EAT group ( $1.76\pm0.25$  vs.  $1.1\pm0.11$ , P<0.01), and the mRNA expression of Foxp3 in the *IL-10*<sup>-/-</sup> EAT group further decreased to  $0.6\pm0.11$ , which was lower than that in the *IL-10*<sup>-/-</sup> non-EAT group (P=0.027), the *IL-10*<sup>-/-</sup> control group (P<0.01), and the *IL-10*<sup>+/+</sup> mTg group ( $0.6\pm0.11$  vs.  $1.83\pm0.28$ , P<0.01). On the contrary, there were no significant differences in the mRNA expression of TGF- $\beta$  between the 5 groups (P>0.05).

Similar results were obtained in the BALB/c mice. Compared with the *IL-10*<sup>-/-</sup> control group, a significantly reduced percentage of Treg cells was observed in the *IL-10*<sup>-/-</sup> non-EAT group ( $6.91\pm1.1\%$  vs.  $4.51\pm0.58\%$ , P=0.042), and the percentage of Treg cells in the *IL-10*<sup>-/-</sup> EAT group further decreased to  $1.95\pm0.36\%$ , which was significantly lower than in the *IL-10*<sup>-/-</sup> non-EAT group (P=0.041), the *IL-10*<sup>-/-</sup> control group (P<0.01), and the *IL-10*<sup>+/+</sup> mTg group ( $1.95\pm0.36\%$  vs.  $5.74\pm2.42\%$ , P<0.01; Fig. 8B). As summarized in Fig. 6B, compared with the *IL-10*<sup>-/-</sup> control group, a markedly decreased mRNA expression of Foxp3 was observed in the *IL-10*<sup>-/-</sup> non-EAT group ( $2.04\pm0.31$  vs.  $1.21\pm0.26$ , P<0.01), and the mRNA expression of Foxp3 in the *IL-10*<sup>-/-</sup> EAT group further decreased to  $0.42\pm0.1$ , which was lower than that in the *IL-10*<sup>-/-</sup>

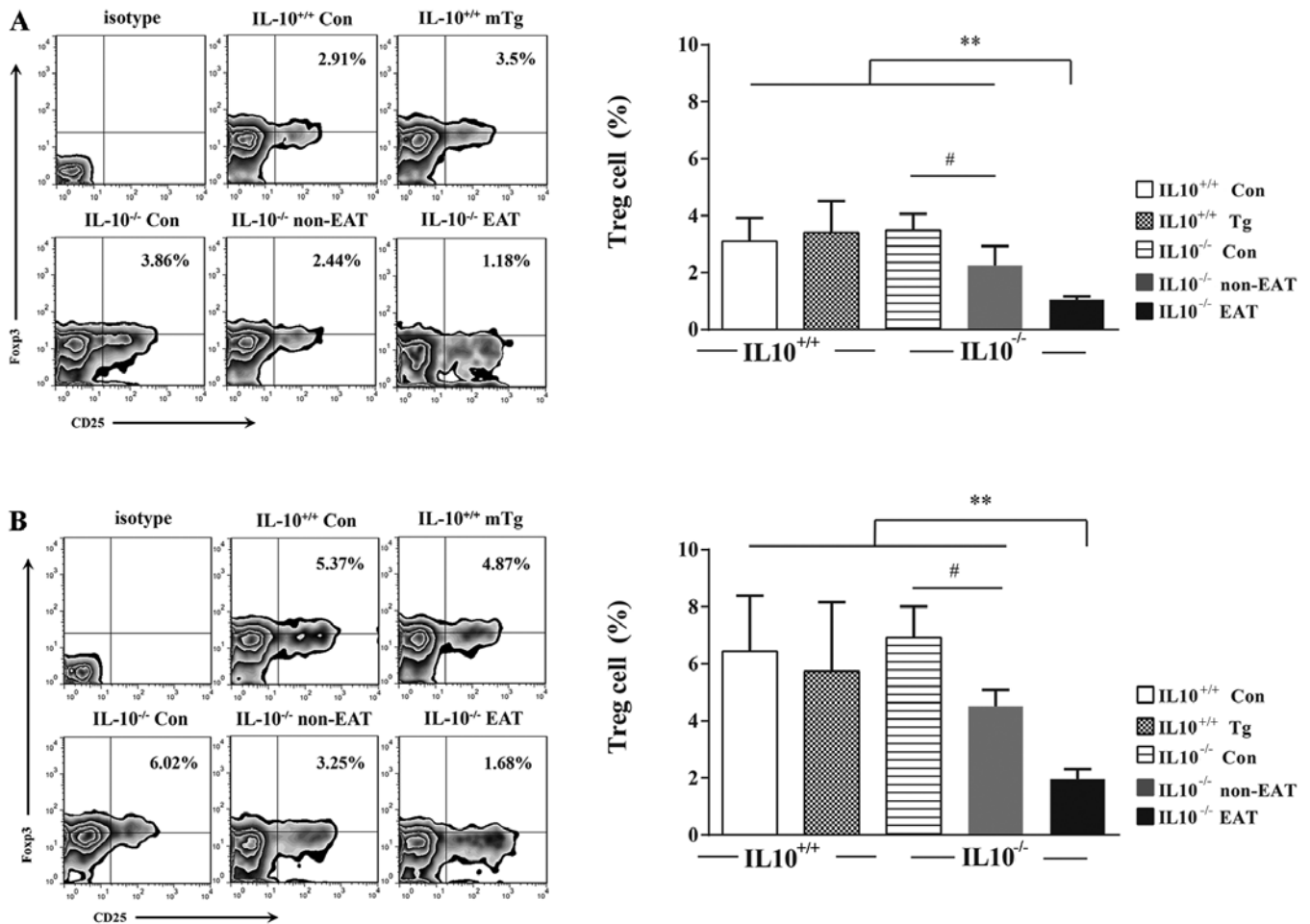


Figure 8. Flow cytometric analysis of splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells (nTreg cells) in *IL-10*<sup>+/+</sup> and *IL-10*<sup>-/-</sup> mice with or without mTg immunization. Splenocytes were gated on CD4<sup>+</sup> T cells, and then CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cell subpopulations were further analyzed within the total CD4<sup>+</sup> T cell population. (A) The frequencies of nTreg cells from the indicated groups of C57BL/6 mice are shown. Representative histograms (left) indicate the frequencies of nTreg cells for one mouse and the bar graphs (right) show the mean frequencies of nTreg cells ( $n \geq 4$  mice/group). (B) The frequencies of nTreg cells from the indicated groups of BALB/c mice are shown. Representative histograms (left panel) indicate the frequencies of nTreg cells for one mouse and the bar graphs (right) show the mean frequencies of nTreg cells ( $n \geq 3$  mice/group). The isotype control served as a negative control for nTreg cells. The mTg group was immunized with 200  $\mu$ g mTg; the control group was immunized with PBS and adjuvant. The EAT group was defined as mice in which EAT was present in all mice immunized with 200  $\mu$ g mTg; the non-EAT group was defined as mice in which EAT was absent in all mice immunized with 200  $\mu$ g mTg. All experiments were performed at least 3 times; \*\* $P < 0.01$ ; # $P < 0.05$ .

non-EAT group ( $P < 0.01$ ), the *IL-10*<sup>-/-</sup> control group ( $P < 0.01$ ), and the *IL-10*<sup>+/+</sup> mTg group ( $0.42 \pm 0.1$  vs.  $2.05 \pm 0.15$ ,  $P < 0.01$ ). By contrast, there were no significant differences in the mRNA expression levels of TGF- $\beta$  between the 5 groups ( $P > 0.05$ ).

## Discussion

In this study, we found that the development of EAT could be induced in *IL-10*<sup>-/-</sup> (BALB/c and C57BL/6) female mice by immunization with mTg. In this system, IL-10 may act as a non-H-2 factor that influences susceptibility to EAT. A previous study established that the genetic control of mTg-specific immune responses showed a significant association with susceptibility to EAT in mice (5). A region located in the H-2 gene, between the K-end and Ir-1, can strongly affect thyroid infiltration (21). Mice with an H-2b or H-2d genetic background, such as BALB/c or C57BL/6 mice, are resistant to EAT. In this study, low titers of TgAb and no thyroid lymphocytic infiltration were observed in the *IL-10*<sup>+/+</sup> (BALB/c and C57BL/6) mTg group, which further indicated resistance to

EAT. By contrast, markedly high titers of TgAb and significant lymphocytic infiltration in the thyroid occurred in some mice in the *IL-10*<sup>-/-</sup> (BALB/c and C57BL/6) mTg group, suggesting that a non-H-2 genetic factor also modified the development of EAT. Beisel *et al* used mTg to immunize mice of the C3H or B10 strains that carry a similar H-2 haplotype; C3H congenic mice had a significantly higher titer of TgAb and more severe thyroid lesions than the B10 strains (6). These observations also suggested that a non-H-2 genetic factor influences the pathogenesis of EAT in addition to H-2 haplotypes. In this study, the development of EAT was induced in mice with an EAT-resistant genetic background in the absence of IL-10, which indicated that IL-10 may be a non-H-2 factor that affects the pathogenesis of EAT. However, the role of the H-2 gene remained dominant, as the overall incidence of EAT was low and disease severity was mild.

A number of studies have demonstrated that IL-10 exerts either protective or suppressive effects in experimental models of autoimmunity (22). A curative effect on EAT was observed by the systemic administration of exogenous IL-10, and endogenous

IL-10 promoted the resolution of granulomatous experimental autoimmune thyroiditis (G-EAT) by an anti-apoptotic mechanism (10,11). These studies suggest that IL-10 plays a protective role in the pathogenesis of EAT. The pathogenic role of Th1 cells and their associated cytokines during the development of EAT has been long established (18,23). In this study, when EAT occurred in *IL-10*<sup>-/-</sup> mice, the percentage of splenic Th1 cells and the mRNA expression of *T-bet* and *IFN-γ* were significantly increased. In a similar experimental autoimmune encephalomyelitis (EAE) model, which is also mediated by Th1 cells, a significant increase in the IL-10 mRNA levels in spinal cord tissue occurred, along with a marked reduction in *IFN-γ* mRNA levels during the remission phase of the disease (24). These findings suggest an antagonistic relationship between IL-10 and Th1 cells. Fiorentino *et al* observed that IL-10 inhibited *IFN-γ* production by Th1 cells by downregulating antigen-presenting cell (APC) function and activating a non-APC-dependent mechanism (25). The absence of IL-10 may provide a favorable context for the proliferation of Th1 cell clones and the production of *IFN-γ* in the presence of mTg. Excessive *IFN-γ* cytokine levels further enhance the expression of MHC II molecules on thyroid epithelial cells and induce epithelial cells to become APCs (26), which is more beneficial for the activation of Th1 clones. Additionally, *IFN-γ* can directly cause the destruction of the thyroid epithelium through Fas-mediated apoptosis (23).

Apart from Th1 cells, Th17 cells also play critical roles in the pathogenesis of various autoimmune and inflammatory diseases (27). Figueroa-Vega *et al* (28) observed an increased percentage of Th17 cells and enhanced synthesis of Th17 cytokines in HT patients. A significant increase in infiltrating Th17 cells in the thyroid was also detected in HT patients (29). Likewise, the pathogenic role of Th17 cells was revealed in a spontaneous autoimmune thyroiditis (SAT) model, as the increased synthesis of IL-17 mRNA was detected in the thyroid glands of *IL-17*<sup>+/-</sup> NOD-H2<sup>h4</sup> mice that had severe thyroiditis. When IL-17 was absent, the incidence of thyroiditis was significantly reduced (30). In our study, when thyroiditis was established in *IL-10*<sup>-/-</sup> mice, the percentage of splenic Th17 cells, along with the mRNA expression of *ROR-γt* and *IL-17*, were significantly increased. Our results also confirmed that Th17 cells and the IL-17 cytokine were linked to the development of EAT. Additionally, the percentage of Th17 cells in the *IL-10*<sup>-/-</sup> non-EAT group was also significantly higher than in the *IL-10*<sup>+/-</sup> mTg group, indicating that IL-10 significantly inhibited the proliferation of Th17 cells. Our results may be explained by the following fact: in a model of inflammatory bowel disease (31), Th17 cells were shown to express IL-10 receptor  $\alpha$  and the specific blockade of IL-10 production by T cells led to a selective expansion of the Th17 population *in vivo*. When mice with established colitis were treated with IL-10, the Th17 cells frequencies were significantly reduced (31).

In the periphery, the critical role of Treg cells in suppressing autoreactive T cells has been previously demonstrated (32). Naturally existing CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells (nTreg cells) play an indispensable role in maintaining peripheral tolerance, and the transcription factor, Foxp3, is required for nTreg cell development and function (33,34). Previously, we observed that NOD-H2<sup>h4</sup> mice with established thyroiditis had fewer nTreg cells and a decreased *Foxp3* mRNA expression in splenocytes compared with healthy controls (14). Therefore, we that changes

occurred in the percentage of nTreg cells and the mRNA expression of *Foxp3* in the current study. Our results indicated that a significant decrease in the number of nTreg cells and a decreased *Foxp3* mRNA expression were present in splenocytes from *IL-10*<sup>-/-</sup> mice with established EAT. Along with previous findings, our study indicated that nTreg cells may exert a protective effect during the development of EAT. A study by Verginis *et al* (20) also supported our findings, as they showed that semi-mature dendritic cells suppress EAT through the selective activation of Tg-specific CD4<sup>+</sup>CD25<sup>+</sup> Treg cells. Gangi *et al* (15) confirmed that the protective effect of Treg cells activated by semi-mature dendritic cells occurred by an IL-10-dependent mechanism. This observation may explain why EAT only occurred in *IL-10*<sup>-/-</sup> mice in our study. Additionally, in another study, *Foxp3* expression was significantly lower in *IL-10*<sup>-/-</sup> *Rag1*<sup>-/-</sup> mice than in *Rag1*<sup>-/-</sup> mice, and IL-10 receptor-deficient Treg cells also failed to maintain *Foxp3* expression (35). This observation suggests that IL-10 may be indispensable for the maintenance of *Foxp3* expression. Notably, IL-10 deficiency impairs both the frequency and function of nTreg cells, which may contribute to the development of EAT.

In conclusion, this study demonstrates that the development of EAT can be induced in mice with an EAT-resistant genetic background in the absence of IL-10 by immunizing mice with mTg emulsified in CFA. Our results indicated that IL-10 acted as a non-H-2 factor that influenced susceptibility to EAT; however, the role of the H-2 gene remained dominant. Th1 cells, Th17 cells and nTreg cells are all involved in the development of EAT. The absence of IL-10 may promote the polarization of pathogenic T cells and suppress the proliferation of protective T-cell clones, which may be one of the mechanisms through which EAT is established in IL-10-deficient mice. Gaining an improved understanding of these potential mechanisms may facilitate the development of novel approaches for the treatment of autoimmune thyroiditis.

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