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Culture Promotes Transfer of Thyroid Epithelial Cell Hyperplasia and Proliferation by Reducing Regulatory T Cell Numbers

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Abstract

IFN- γ ^{-/-} NOD.H-2h4 mice develop a spontaneous autoimmune thyroid disease, thyroid epithelial cell hyperplasia and proliferation (TEC H/P) when given NaI in their water for 7+ mo. TEC H/P can be transferred to IFN- γ ^{-/-} SCID mice by splenocytes from mice with severe (4–5+) disease, and transfer of TEC H/P is improved when splenocytes are cultured prior to transfer. Older (9+ mo) IFN- γ ^{-/-} NOD.H-2h4 mice have elevated numbers of FoxP3⁺ T reg cells, up to 2 fold greater than younger (2 mo) mice. During culture, the number of T reg decreases and this allows the improved transfer of TEC H/P. Co-culture with IL-2 prior to transfer prevents the decrease of T reg and improves their *in vitro* suppressive ability resulting in reduced TEC H/P in recipient mice. Therefore, culturing splenocytes improves transfer of TEC H/P by reducing the number of T reg and IL-2 inhibits transfer by preserving T reg number and function.

Keywords

Thyroid; NOD.H-2h4; TEC H/P; T regulatory Cells; IL-2; Autoimmunity

1. Introduction

Autoimmune thyroid diseases represent a significant category of organ-specific autoimmune diseases that affect ~1.5 % of the population [1], and understanding the immune mechanisms that result in these diseases is important for understanding autoimmune pathology. When given NaI in their drinking water, NOD.H-2h4 mice develop spontaneous (no immunization) autoimmune thyroiditis (SAT) in ~2 mo [2–4]. However, SAT does not develop in IFN- γ ^{-/-} NOD.H-2h4 mice. Rather, they develop a different inflammatory autoimmune disease characterized by extensive hyperplasia and proliferation of thyroid epithelial cells, slow onset (> 7 mo on NaI water), fibrosis, and a more limited lymphocytic infiltrate than in SAT [5]. IFN- γ inhibits TEC H/P both *in vivo* and *in vitro* [6], and inhibits TEC (thyroid epithelial cell) proliferation by upregulation of the cyclin-dependent kinase inhibitors p18 and p21 and downregulation of cyclin D [7].

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Uncontrolled proliferation, hyperplasia and fibrosis of epithelial cells occurs in several autoimmune diseases including systemic lupus erythematosus, systemic sclerosis, rheumatoid arthritis, and autoimmune thyroiditis [8–10]. Thyroid autoimmunity and thyroid hyperplasia are very common [8, 11, 12] and can be associated with an increased risk of thyroid cancer [8, 11, 13]. However, the mechanisms by which inflammatory cells promote epithelial cell hyperplasia and thyroid neoplasia are poorly understood. TEC H/P is an autoimmune disease, as IFN- γ $^{-/-}$ NOD.H-2h4 mice with TEC H/P produce low levels of anti-thyroglobulin autoantibodies, and IFN- γ $^{-/-}$ NOD.H-2h4.SCID mice that lack lymphocytes [5] and IFN- γ $^{-/-}$ TCR $\alpha\beta$ $^{-/-}$ NOD.H-2h4 mice that lack T cells do not develop TEC H/P [14]. TEC H/P in IFN- γ $^{-/-}$ NOD.H-2h4 mice is a well characterized animal model that is useful for increasing our understanding of abnormal cell proliferation and hyperplasia in the context of autoimmunity.

To facilitate studies of the mechanisms by which autoreactive T cells promote thyrocyte proliferation, we developed a transfer model in which splenocytes from donor IFN- γ $^{-/-}$ mice with severe TEC H/P are adoptively transferred to recipient IFN- γ $^{-/-}$ SCID mice [6, 14]. IFN- γ $^{-/-}$ SCID recipients of splenocytes from donors with severe (4–5+) develop severe TEC H/P 1–2 mo later (compared to 7+ mo in donors), whereas recipients of splenocytes from donor mice without TEC H/P do not develop disease [5]. Culture of splenocytes from donors with severe TEC H/P allows a 10 fold reduction in the number of cells required to transfer disease and improves the efficiency of transfer so that the vast majority of recipients develop severe TEC H/P 4 wk after cell transfer [14]. We began this study to determine why splenocyte culture improved disease transfer, and hypothesized that during culture there may be alterations in lymphocyte populations that promote TEC H/P.

Unexpectedly, while characterizing lymphocyte populations before and after culture, we observed that donor mice had elevated numbers of T reg compared to younger mice. T reg decrease during culture presumably because of low production of IL-2, since T reg numbers are preserved when exogenous IL-2 is added to culture and transfer of TEC H/P is inhibited. This indicates that the mechanism by which culture promotes TEC H/P transfer is by loss of T reg in culture.

2. Materials and Methods

2.1 Mice

IFN- γ $^{-/-}$ NOD.H-2h4 and IFN- γ $^{-/-}$ NOD.H-2h4 SCID mice were generated in our animal facility as previously described [5, 15]. To promote development of TEC H/P, mice used as donors were given 0.08 % NaI water for 7–10 months beginning at 7–8 weeks of age unless otherwise noted [5, 15]. Both male and female mice were used, but all mice in an individual experiment were the same sex. IFN- γ $^{-/-}$ NOD.H-2h4 mice expressing eGFP in FoxP3⁺ T reg were generated in our animal facility by crossing previously generated WT FoxP3-GFP NOD.H-2h4 mice [16] with IFN- γ $^{-/-}$ NOD.H-2h4 mice. All animal protocols were approved by the University of Missouri Animal Care and Use Committee.

2.2 *In vitro* cell culture and adoptive transfer of TEC H/P

Adoptive transfer was performed as previously described [14, 17]. Donor mice were given 0.08% NaI in their drinking water for 7 mo. Splenocytes from donor mice were cultured for 72 hrs as previously described [14]. During the 72 hr culture, thyroids from donor mice were scored for TEC H/P severity by histology. Following culture, 3×10^6 splenocytes from mice with severe (4–5+) TEC H/P were transferred i.v. to IFN- γ $^{-/-}$ NOD.H-2h4 SCID mice. Recipient mice were given NaI water, and thyroid histopathology was assessed 28 days later. In some experiments, murine rIL-2 (eBioscience Inc, San Diego, CA) was added to culture at various concentrations as indicated in the figures.

For T reg transfers, T reg from 9–12 mo old IFN- γ $^{-/-}$ NOD.H-2h4 donor mice were labeled with anti-CD25-PE (eBioscience) and enriched using an EasySep PE selection kit (Stemcell Technologies Inc, Vancouver, BC) according to the manufacturer's instructions. Enrichment of T reg was verified by flow cytometry by intracellular stain for FoxP3. Enriched CD25⁺ T cells were added to cultured splenocytes at a ratio of 1:10 (3×10^6 cultured cells + 3×10^5 CD25⁺ T reg) or 1:3 (3×10^6 cultured cells + 1×10^6 CD25⁺ T reg) and transferred i.v. to recipient IFN- γ $^{-/-}$ SCID mice.

2.3 Evaluation of TEC H/P severity scores

Thyroids were removed, and one thyroid lobe was fixed in formalin, sectioned, and stained with H&E as previously described [5, 14, 15]. Thyroid histopathology was scored for the extent of thyroid follicular cell hyperplasia/proliferation using a scale of 0 to 5+ as previously described [5, 15]. Briefly, a score of 0 indicates a normal thyroid, and 0+ indicates mild follicular changes and/or a few inflammatory cells infiltrating the thyroid. A 1+ score is defined as hyperplastic changes or cellular infiltrate of at least 125 cells sufficient to cause replacement of several follicles. A 2+ score represents hyperplastic changes causing replacement or destruction of up to one fourth of the gland, 3+ indicates that one fourth to one half of the gland is destroyed by hyperplastic changes, and 4+ indicates that greater than one half of the gland is destroyed. Thyroids with a score of 5+ have few or no remaining normal follicles. IFN- γ $^{-/-}$ mice with TEC H/P graded 4–5+ have widespread clusters of proliferating thyrocytes with some lymphocyte infiltration, and areas of proliferating thyrocytes surrounded by collagen. All thyroids with mild or severe hyperplasia also have some infiltrating lymphocytes [5, 14].

2.4 Immunohistochemistry

Frozen thyroid sections were stained as previously described [5, 15]. Anti-CD4 (GK-1.5) and anti-FoxP3 (FJK-16s, 1:200, eBioscience) were used as primary Ab. Biotinylated donkey anti-rat IgG (1:500, Jackson ImmunoResearch Laboratories Inc, West Grove, PA) was used as secondary Ab, followed by avidin-HRP binding using a Vectastain Elite PK-6100 kit (Vector Laboratories, Burlingame, CA). Peroxidase activity was visualized using a Vector Nova-Red Substrate Kit (Vector).

2.5 Flow cytometry

For flow cytometric analysis of T reg, cells were stained with anti-CD25-FITC, anti-CD8-PE, anti-CD4-PerCp Cy5.5, and anti-FoxP3-allophycocyanin (eBioscience). Intracellular staining of FoxP3 was performed following surface staining using a FoxP3 intracellular staining kit (eBioscience). Flow cytometry data was collected on a Cyan ADP flow cytometer (Beckman Coulter) and data analyzed using FlowJo for PC Version 7.6 (TreeStar Inc, Ashland, OR).

2.6 Suppression assay

Splenocytes from IFN- γ $^{-/-}$ NOD.H-2h4 mice with TEC H/P were separated into two fractions. One was enriched for CD25⁺ T reg using magnetic beads as above. The other was labeled with CFSE by incubation with 5 μ m CFSE (eBioscience) at room temperature for 10 min. Enriched T reg were added to unsorted CFSE labeled splenocytes at various ratios and cultured at 5×10^5 cells/well in 96 well plates with 1.5 μ g/ml anti-CD3 (eBioscience) for 72 hrs. Following culture, cells were stained with 7-AAD (eBioscience), anti-CD4-PE and anti-CD8-allophycocyanin (Biolegend) and analyzed by flow cytometry.

For suppression assays using FoxP3 GFP mice, T regs from either WT or IFN- γ $^{-/-}$ mice were sorted on the basis of GFP expression using a MoFlo XDP cell sorter (Beckman

Coulter, Brea, CA). The negative fraction was labeled with CFSE as above and FoxP3⁺ T regs were added at varying ratios to cultures stimulated with anti-CD3 as above. After 72 hr cells were stained with anti-CD4 PerCp cy5.5 and anti-CD8 allophycocyanin (Biolegend) and analyzed by flow cytometry.

Some suppression assays were performed by first culturing splenocytes from mice with severe TEC H/P for 72 hrs as above with or without 10 ng/ml recombinant mouse IL-2 (eBioscience). CD25 expressing T reg were then enriched by magnetic beads as above and added at varying ratios to CFSE labeled splenocytes from donor mice with TEC H/P. Mixtures of unlabeled T reg and CFSE labeled splenocytes were stimulated for 72 hrs with 500 ng/ml anti-CD3, and proliferation was measured by flow cytometry as above.

2.7 IL-2 ELISA

Cells from IFN- γ ^{-/-} NOD.H-2h4 mice with TEC H/P were cultured for 72 hr as above with or without addition of 10 ng/ml recombinant mouse IL-2 (eBioscience) as a positive control. IL-2 concentrations in culture supernatants were determined using an IL-2 Ready-Set-Go ELISA kit (eBioscience) according to the manufacturer's instructions.

2.8 Statistical analysis

Mann-Whitney, Kruskal-Wallis, Student's t-test, and ANOVA were performed using analysis software included with GraphPad Prism 6 for Macintosh (GraphPad Software Inc. La Jolla, CA). Groups were considered statistically significant when p values were less than 0.05.

3. Results

3.1 IFN- γ ^{-/-} NOD.H-2h4 mice with TEC H/P have high numbers of CD4⁺ FoxP3⁺ T reg cells

In the transfer model of TEC H/P, 72 hr culture of splenocytes optimizes transfer and allows a ten fold reduction in the number of cells required to transfer severe TEC H/P to recipient SCID mice [14]. We examined lymphocyte populations before and after culture to determine if changes in lymphocyte populations might explain why culture of donor cells promoted TEC H/P. We found that before culture the numbers of T reg cells in donor mice were unusually high, and at the end of 72 hr culture the number of T reg cells was reduced (Fig. 1A). Splenocytes from donor mice with TEC H/P were examined for expression of CD4⁺ and FoxP3⁺ by flow cytometry both before and after 72 hr culture. The percentage of CD4⁺ FoxP3⁺ T reg was significantly ($p < 0.0001$) reduced from 31% to 17% of CD4⁺ cells in 72 hr cultures compared to *ex vivo* splenocytes from the same donors (Fig. 1A).

Since high numbers of T reg in donor mice could affect TEC H/P development, we examined the numbers of T reg cells in young naïve mice and in older mice with TEC H/P. To do this, CD4⁺ FoxP3⁺ T reg in draining cervical LN and spleen were examined by flow cytometry in young (2–3 mo) and old (9–12 mo) IFN- γ ^{-/-} NOD.H-2h4 mice that had been given NaI water or plain water as a control. Old mice (9–12 mo), equivalent in age to donor mice used in all subsequent experiments, had increased percentages of CD4⁺ FoxP3⁺ cells compared to young naïve mice (age 2–3 months) increasing from ~15 to 24 % of CD4⁺ T cells in draining cervical LN (Fig. 1B, E) ($p < 0.001$) and ~19 to 28 % of CD4⁺ cells in spleen (Fig. 1C, E) ($p < 0.01$). 9–12 mo old mice not given NaI water (no TEC H/P) as well as mice with 4–5+ TEC H/P severity scores given NaI water for > 7 mo had similar elevated levels of CD4⁺ FoxP3⁺ T cells (Fig. 1B, C). Total numbers as well as percentages of CD4⁺ FoxP3⁺ T reg were increased ($p < 0.05$). Old mice had ~10×10⁶ CD4⁺ FoxP3⁺ T reg in spleen and young mice had ~4×10⁶ CD4⁺ FoxP3⁺ T reg (Fig. 1D). This indicates that older

IFN- γ ^{-/-} NOD.H-2h4 mice have elevated numbers of T reg that increase independently of NaI or TEC H/P.

3.2 Loss of T reg in culture promotes transfer of TEC H/P

Since the number of T reg are elevated in donor mice and decrease in culture we hypothesized that the decrease in T reg could explain why culture promotes transfer of TEC H/P to recipient SCID mice. We therefore asked if preservation of T reg in culture could inhibit TEC H/P in recipient SCID mice. Effector T cells that transfer TEC H/P do not expand detectably during culture (not shown), and production of IL-2 in culture is low (< 10 pg/ml) as determined by ELISA (Fig. 2A). Since IL-2 is required for T reg function and survival [18] the low concentration of IL-2 might explain why T reg decrease in culture. We therefore tested whether exogenously added IL-2 would preserve T reg numbers during culture. When IL-2 was added to 72 hr cultures of splenocytes from IFN- γ ^{-/-} donor mice, T reg were increased in a concentration dependent manner compared to cultures without IL-2 (Fig. 2B).

Since exogenous IL-2 prevents the loss of T reg in culture, we hypothesized that cells cultured in the presence of IL-2 should transfer less severe TEC H/P. To address this question, splenocytes from mice with severe TEC H/P were cultured with or without 10 ng/ml IL-2 and transferred to SCID recipients. After 28 days, TEC H/P severity in recipients of cells cultured with 10 ng/ml IL-2 was significantly reduced compared to that of cells cultured in the absence of IL-2 (Fig. 2C) ($p < 0.0002$). Since preservation of T reg in culture inhibits the transfer of TEC H/P, these results suggest that the mechanism by which culture of splenocytes improves transfer of TEC H/P is by reduction of T reg.

3.3 *In vitro* and *in vivo* analysis of T reg function

The levels of CD4⁺ FoxP3⁺ T reg in young IFN- γ ^{-/-} NOD.H-2h4 mice are consistent with published observations of young 6 week old NOD and B6 mice [19, 20]. However, CD4⁺ FoxP3⁺ T reg are greatly elevated in older IFN- γ ^{-/-} NOD.H-2h4 mice (Fig 1B, C) at the time they develop severe TEC H/P [5]. This raised the question of whether or not the T reg have suppressive function. To test this, their ability to inhibit T cell proliferative responses *in vitro* was examined (Fig 3A, B). In this assay, CD25⁺ T reg were enriched from spleens of 9–12 mo old mice using magnetic beads. Enriched cells (determined to be 60–70 % FoxP3⁺ by flow cytometry, not shown), were cultured at varying ratios with unsorted, CFSE labeled *ex vivo* splenocytes, and stimulated with anti-CD3. Proliferation of both CD8⁺ and CD4⁺ T cells was inhibited in cultures with high ratios of T reg to responder cells ($p < 0.01$) as measured by dilution of CFSE (Fig 3A). These results indicate that T reg from IFN- γ ^{-/-} NOD.H-2h4 mice have *in vitro* anti-proliferative function when cultured at high ratios with responder cells. CD8⁺ T cells proliferated more than CD4⁺ T cells in response to anti-CD3, which is consistent with CD8⁺ T cells being the primary effector cells in TEC H/P [14]. In order to determine the relative suppressive ability of T reg from IFN- γ ^{-/-} mice T reg from IFN- γ ^{-/-} NOD.H-2h4 mice were compared to T reg isolated from WT NOD.H-2h4 mice. FoxP3⁺ T reg and FoxP3 negative cells were sorted on the basis of GFP expression from spleens of FoxP3 GFP⁺ WT and IFN- γ ^{-/-} mice. FoxP3⁺ T reg were then added to CFSE labeled FoxP3 negative cells at varying ratios and cultures were stimulated with anti-CD3. T reg isolated from either WT or IFN- γ ^{-/-} mice showed similar *in vitro* suppressive function (Fig 3B). T reg from WT and IFN- γ ^{-/-} mice also showed similar ability to suppress proliferation when T reg depleted responder cells were from either WT or IFN- γ ^{-/-} mice and when T reg were derived from young or old mice (not shown).

In order to determine if T reg from mice with TEC H/P have *in vivo* suppressive function, the transfer model was used [14]. Here, following 72 hr culture of splenocytes from donor

IFN- γ $^{-/-}$ NOD.H-2h4 mice, splenic CD25⁺ T reg from old (9–12 mo) IFN- γ $^{-/-}$ given NaI in their drinking water for 7+ mo were enriched *ex vivo* using magnetic beads and mixed at a 1:10 or 1:3 ratio with the cultured cells immediately prior to transfer. Recipient mice were given NaI in their drinking water and thyroids were examined 28 days later. Co-transfer of enriched T reg at a 1:10 ratio with cultured splenocytes did not significantly inhibit development of TEC H/P in recipient mice while development of TEC H/P was significantly ($p < 0.001$) inhibited in recipients of 1:3 T reg:cultured splenocytes (Fig. 3C). Representative H&E stained sections of thyroids showing reduction of TEC H/P severity in IFN- γ $^{-/-}$ SCID recipients when T reg are transferred at high ratios with cultured splenocytes are shown in Fig 3D. These results indicate that the T reg from old (9–12 mo old) IFN- γ $^{-/-}$ NOD.H-2h4 mice have both *in vivo* and *in vitro* suppressive function.

We next determined if T reg were present in thyroids from mice with severe TEC H/P by IHC. Sections of thyroids from donor (Fig. 4A–D) and recipient (Fig. 4E–H) mice with severe TEC H/P were stained with anti-FoxP3 and anti-CD4. FoxP3⁺ T reg (Fig. 4A, B) and CD4⁺ cells (Fig. 4C, D) were present in thyroids of donor mice, and also in thyroids of recipient mice 28 days post transfer (Fig. 4E–H). This indicates that T reg in from IFN- γ $^{-/-}$ NOD.H-2.4 mice with severe TEC H/P can traffic to the thyroid in both donor and recipient IFN- γ $^{-/-}$ SCID mice.

3.4 Culture with IL-2 increases the *in vitro* suppressive function of FoxP3⁺ T reg from mice with TEC H/P

Since IL-2 promotes both T reg homeostasis and function [18, 21], we asked if IL-2 culture increased T reg suppressive function. To test this, we measured *in vitro* suppression by T regs enriched following 72 hr culture with or without IL-2. T reg were enriched, added to freshly isolated splenocytes labeled with CFSE and stimulated with anti-CD3. T reg from IL-2 cultures inhibited proliferation of CD8⁺ cells more effectively than did the same number of T reg enriched from cultures not containing IL-2 (Fig. 5A) ($p < 0.0001$). T reg enriched from IL-2 cultures also had a significantly ($p < 0.01$) increased ability to suppress proliferation of CD4⁺ T cells compared to T reg enriched from cultures without IL-2 (Fig. 5B). These results indicate that in addition to maintaining high T reg percentages, IL-2 increases the *in vitro* suppressive function of T reg.

4. Discussion

T regs are important immune modulators, and alterations in the number or function of T reg can have significant consequences on immune function or autoimmune susceptibility. In both humans [22, 23] and mice [23–25] it has been shown that T regs can increase in number with age. In some reports, T reg function is maintained, and excessive regulation by T reg allows greater susceptibility to infection and cancer, [22, 23, 25–27]. Other reports describe a decline in T reg function [28–31] or number [32–35] and insufficient regulation promotes autoimmunity. These examples illustrate how fine control of T reg number and function is crucial for immune homeostasis. In the TEC H/P model, regulation of T reg homeostasis as the mice age is altered as the number of T reg increase to high levels in LN and spleen and the T reg remain functional. These high numbers of T reg may explain why TEC H/P develops slowly requiring a prolonged exposure to NaI before severe disease occurs.

However, compensatory mechanisms must exist as severe autoimmunity can develop in mice with elevated numbers of functional T regs. One potential avenue for this may be development of resistance of effector cells to suppression by T reg. Changes in T effector cells over time are observed in other autoimmune models and a similar mechanism may also occur in TEC H/P. In a NOD mouse model of diabetes T effector cells can develop

resistance to suppression by T reg that have normal suppressor function and develop autoimmunity [19, 26, 30]. In the TEC H/P model, similar resistance to suppression by effector cells may occur. This may explain why TEC H/P can develop in mice that have substantially elevated numbers of T reg cells.

In multiple autoimmune diseases including EAE, experimental autoimmune uveitis, and collagen induced arthritis, absence or blocking of IFN- γ results in exacerbated disease [36–41]. In the TEC H/P model, IFN- γ deficiency changes the nature of the thyroid autoimmunity that develops. In the absence of IFN- γ , hyperplasia and proliferation of thyrocytes occurs that is not observed in SAT. This has been previously shown to be an effect on the TEC rather than lymphocytes [6]. IFN- γ directly inhibits proliferation of TEC by regulation of cell cycle proteins p18, p21 and cyclin D [7].

IFN- γ may also directly affect T reg function or homeostasis. For example, there is emerging evidence that there are distinct T regulatory cell populations that have similar transcriptional profiles as their target cells [42–44], and IFN- γ has been shown to regulate the expression of T bet and CXCR3 in peripheral T reg [45]. IFN- γ could also directly influence T reg numbers. In two contrasting reports IFN- γ was shown to both promote and inhibit induced T reg (iT reg). In one report, BALB/c or C57BL/6 IFN- γ $^{-/-}$ mice were shown to have increased numbers of iT reg due to enhanced survival of iT reg in the periphery [46]. However, in a contrasting report, IFN- γ deficiency in C57BL/6 mice reduced the number of iT reg [47]. In the TEC H/P model used here, all mice are IFN- γ deficient, so FoxP3 $^{+}$ T reg expand and carry out their function independently of IFN- γ . Older IFN- γ $^{-/-}$ NOD.H-2h4 mice have high numbers of FoxP3 $^{+}$ T reg in LN and spleen, indicating that T reg expand without IFN- γ as the mice age. T reg from IFN- γ $^{-/-}$ mice also suppress TEC H/P *in vivo*, as T reg from IFN- γ $^{-/-}$ mice inhibit TEC H/P following transfer to IFN- γ $^{-/-}$ SCID mice, and FoxP3 $^{+}$ T reg from IFN- γ $^{-/-}$ or WT mice have similar ability to suppress proliferation of CD8 or CD4 T cells *in vitro*.

The primary goal of this study was to understand how 72 hr culture of splenocytes from mice with TEC H/P promotes the transfer of TEC H/P. We found that older IFN- γ $^{-/-}$ mice had elevated numbers of T reg with suppressive function *in vitro* and *in vivo*. During culture, the number of T reg are reduced. However, when exogenous IL-2 is added to these cultures, the population of T reg is maintained, and these cultures have reduced ability to transfer TEC H/P. This indicates that the mechanism by which culture improves the ability to transfer of TEC H/P is via a loss of T reg.

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Abbreviations

TEC	thyroid epithelial cell
TEC H/P	thyroid epithelial cell hyperplasia/proliferation
SAT	spontaneous autoimmune thyroiditis
SCID	severe combined immunodeficient
TCR	T cell receptor
IHC	Immunohistochemistry
IL-2	Interleukin-2

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Highlights

- IFN- γ $^{-/-}$ NOD.H-2h4 mice have elevated numbers of FoxP3⁺ T reg
- The T reg have *in vitro* and *in vivo* function
- Culture reduces T reg and improves transfer of TEC H/P
- Co-culture with IL-2 preserves T reg and reduces TEC H/P transfer

Figure 1. Old mice have elevated CD4⁺FoxP3⁺ T reg in lymph nodes and spleen

A. FoxP3 expression was examined in CD4⁺ splenocytes *ex vivo* or following 72 hr culture. 72 hr cultures had a significant reduction ($p < 0.0001$, ttest) of CD4⁺ FoxP3⁺ T reg compared to *ex vivo* splenocytes. Results are from 6 experiments $n = 34$. B and C. IFN- γ NOD.H-2h4 mice were either given plain water or water supplemented with 0.08 % NaI. FoxP3 expression was examined on CD4⁺ cells recovered from cervical LN (B) and spleen (C) in young (2–3 months) or old (9–12 months) mice. Old mice given either NaI or plain water had significant increases in the percentage of CD4⁺ FoxP3⁺ T reg compared to young mice in both cervical LN ($p < 0.001$, ANOVA) and spleen ($p < 0.01$, ANOVA). D. Total numbers of T reg were increased in spleens of both naïve old mice and old mice given NaI compared to young naïve mice ($p < 0.05$, ANOVA). E. Representative plots of data from A and B. Results shown in A represent 3 experiments, young, $n = 8$; old, $n = 8$; iodine old, $n = 16$, and results shown in B represent 3 experiments, young, $n = 12$; old, $n = 8$; iodine old $n = 22$. Results shown in C represent 2 experiments, young, $n = 6$; old, $n = 5$; iodine old, $n = 6$.

Figure 2. Culture of splenocytes with IL-2 maintains T reg and cells cultured with IL-2 transfer less severe TEC H/P to IFN- γ ^{-/-} NOD.H-2h4 SCID mice

A. Supernatants of splenocytes cultured with or without exogenous IL-2 were examined by ELISA for production of IL-2. IL-2 concentration in culture supernatant was minimal following 72 hr culture. Results are from 2 experiments, $n = 9$ ($p < 0.0001$, t test). B. The loss of CD4⁺ FoxP3⁺ T reg was rescued by addition of exogenous IL-2 in a concentration dependent manner. Reduction of T reg in culture was prevented by addition of 5 or 10 ng/ml IL-2. Results are from 2 experiments, $n = 6$ ($p < 0.05$, ANOVA). C. Recipients of splenocytes cultured with 10 ng/ml IL-2 have a significant reduction in the incidence and severity of TEC H/P compared to recipients of the same cells cultured without IL-2. Results are from 6 experiments, $n = 34$ ($p < 0.0002$, Mann-Whitney test).

Figure 3. High numbers of T reg suppress anti-CD3 mediated proliferation *in vitro* and suppress TEC H/P following transfer to recipient mice

A. CD25 expressing T reg from 9–12 mo old IFN- $\gamma^{-/-}$ NOD.H-2h4 mice were enriched by magnetic beads, added at varying ratios to CFSE labeled splenocytes, and stimulated with anti-CD3 for 72 hrs. CFSE dilution of CD8⁺ and CD4⁺ cells was examined by FACS. Proliferation was significantly inhibited in both CD8⁺ and CD4⁺ populations when the ratio of T reg to splenocytes was greater than 1:64 (CD8, $p < 0.001$ and CD4, $p < 0.01$, ANOVA). B. FoxP3 GFP T reg were sorted by FACS from spleens of WT or IFN- $\gamma^{-/-}$ NOD.H-2h4 mice. T reg were added to 5×10^5 CFSE labeled T reg depleted splenocytes at varying ratio and stimulated with anti-CD3 for 72 hr. Proliferation of CD8 and CD4 T cells was examined by FACS. Suppression of either CD8 or CD4 T cell proliferation was not significantly different between WT or IFN- $\gamma^{-/-}$ T reg. C. Cultured splenocytes (3×10^6) from donor mice with severe TEC H/P were transferred alone or together with 3×10^5 or 1×10^6 CD25 enriched cells to IFN- $\gamma^{-/-}$ SCID recipients. Thyroids were removed 28 days later and scored for TEC H/P severity. TEC H/P was significantly inhibited ($p < 0.001$, Kruskal-Wallis) in recipients of 1:3 CD25 enriched cells but not in recipients of 1:10 CD25 enriched cells compared to mice that received cultured splenocytes alone. D. Selected thyroids from mice shown in C. Scale bar is 0.2 mm. Results shown in A and B are representative of 2 total experiments, $n = 6$, error bars are standard deviation. Results shown in C represent 2 experiments, culture $n = 9$; 1:10 CD25:culture $n = 11$; 1:3 CD25:culture $n = 5$.

Figure 4. FoxP3⁺ T reg are present in Thyroids of mice with severe TEC H/P

Frozen sections of thyroids from donor or recipient mice with severe TEC H/P were examined for FoxP3⁺ (A, B, E, F) and CD4⁺ (C, D, G, H) cells by IHC. FoxP3⁺ T reg are present in both donor (A–D) and recipient (E–H) mice with TEC H/P. Magnification: 100X (A, C, E, G) and 400X (B, D, F, H). Results are representative of 3 experiments, n =9. TEC H/P Severity is 4+ for both donor and recipient thyroids.

Figure 5. Culture with IL-2 increases the *in vitro* function of T reg

A and B. Splenocytes from 9–12 mo old IFN- γ NOD.H-2h4 mice were cultured for 72 hr with or without exogenous IL-2. Following culture, CD25⁺ T reg were enriched from cultures by magnetic beads, added to CFSE labeled *ex vivo* splenocytes, and stimulated with anti-CD3 for an additional 72 hr. Dilution of CFSE was measured by flow cytometry. Proliferation of CD8⁺ cells (A) was reduced when T reg from IL-2 cultures were added at 1:4 (p < 0.0001, t test) and 1:8 (p < 0.01, t test) ratio compared to T reg enriched from cultures without IL-2. Inhibition of CD4⁺ cell proliferation (B) was significantly reduced (p < 0.01, t test) when T reg enriched from IL-2 culture were added at 1:4 ratio compared to T reg enriched from cultures without IL-2. Results in A and B are representative of 3 total experiments, n = 9, error bars are standard deviation.