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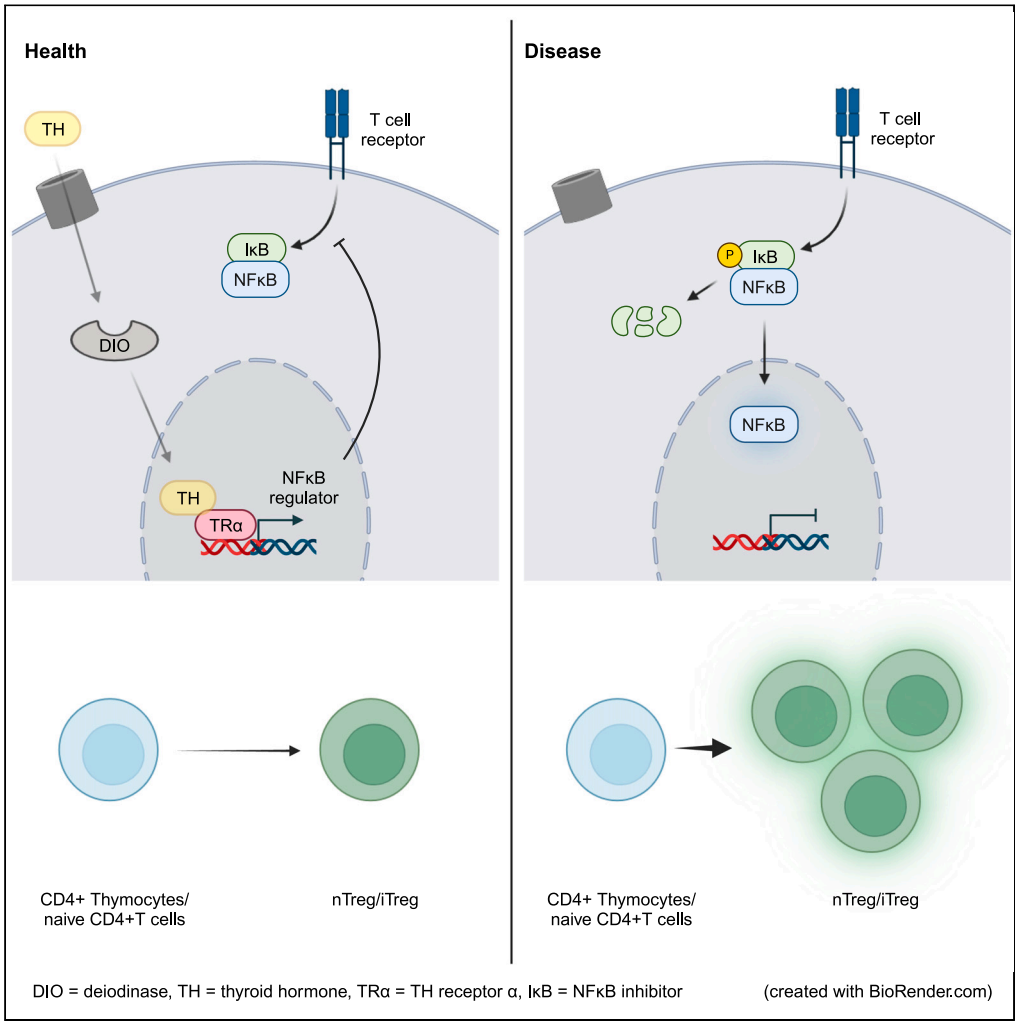

Christina Wenzek, Devon Siemes, G. Sebastian Hönes, ..., Daniel R. Engel, Astrid M. Westendorf, Dagmar Führer

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Highlights

TRα has a significant impact on CD4$^+$ T cells

Lack of canonical TRα action increases regulatory T cell frequencies

Treg lacking canonical TRα action show an activated phenotype

Canonical TRα-mediated TH action limits NF-κB pathway activation

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Lack of canonical thyroid hormone receptor α signaling changes regulatory T cell phenotype in female mice

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SUMMARY

The immune system has emerged as an important target of thyroid hormones (THs); however, the role of TH in T cells has so far remained elusive. In this study, we assessed the effect of TH receptor α (TRα) signaling on activation and function of T cells. Our findings show that lack of canonical TRα action not only increased the frequency of regulatory T cells (Treg) but propelled an activated and migratory Treg phenotype and nuclear factor κB (NF-κB) activation in Treg. Conversely, canonical TRα action reduced activation of the NF-κB pathway previously shown to play a pivotal role in Treg differentiation and function. Taken together, our findings demonstrate that TRα impacts T cell differentiation and phenotype. Given the well-known interaction of inflammation, immune responses, and TH axis in e.g., severe illness, altered TH-TRα signaling may have an important role in regulating T cell responses during disease.

INTRODUCTION

Thyroid hormones (THs) are critical regulators of organ homeostasis and control development, growth, and physiological function of many target tissues in the body.[1] Across tissues and ultimately at the cellular level, local control of TH action is exerted by three steps. First, intracellular availability of TH is regulated by TH transporters that facilitate the uptake and efflux across the cell membrane.[2] Second, TH deiodinases activate or deactivate TH within the cell.[3] Third, TH receptors (TRs) mediate intracellular TH signaling by regulating nuclear TH target gene expression (canonical action) and by cytosolic pathway activation (non-canonical action).[4,5]

Besides well-known target organs, such as the brain, heart, liver, and bone, effects of TH on the immune system have gained increasing interest as THs have been shown to exert pro- and anti-inflammatory actions in innate and adaptive immune cells depending on the cellular and stimulatory context.[6] However, the impact of TH on adaptive immune responses, especially T cell responses, which play a central role in protective immunity, has remained largely elusive. So far, only few studies addressed the impact of THs on T cell immunity with conflicting results. For example, THs were shown to drive T cell proliferation *in vitro* and *in vivo*, improving tumor rejection in murine mammary carcinoma and lymphoma model.[7–9] On the contrary, TH stimulation of the human Jurkat T cell lymphoma cell line and primary human T cells was found to promote T cell apoptosis *in vitro*.[10] Moreover, chronic hyperthyroidism in Graves' disease and in TH-treated mice was associated with reduced frequency of regulatory T cells (Treg), which displayed decreased expression of PD-1.[11]

Previous findings of our group suggested an important role of canonical TRα signaling on T cell immunity,[12] whereby lack of canonical TRα action was associated with an increased frequency of circulating CD4 +T cells.

In this study, we addressed the role of canonical TRα signaling in T cell immunity in more detail. Based on *in vivo* and *in vitro* analyses, we show that lack of canonical TRα action increases frequency of Treg that show an activated and migratory phenotype. In addition, our data indicate an inhibitory effect of TRα-mediated TH action on nuclear factor κB (NF-κB) signaling a central mediator of T cell receptor (TCR) signaling. In sum, our results suggest that TRα-mediated TH signaling limits Treg differentiation and function, with a potential role of reduced NF-κB activation during TCR signaling.

RESULTS

Absence of canonical TRα action increases circulating CD4$^+$ T cell numbers

The intracellular action of THs is mediated by TRα and TRβ, showing overlapping but also isoform specific functions.[4] TR expression was previously demonstrated in the murine BW5147 T cell line,[7] but which TR isoforms are relevant in primary T cells or distinct T cell subsets remained

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elusive. Thus, we initially analyzed the expression of TRs in isolated splenic $CD4^+$ and $CD8^+$ T cells from naive mice by quantitative reverse-transcription PCR (RT-qPCR). We found that both TR isoforms were present in $CD4^+$ and $CD8^+$ T cells, with about 2-fold higher expression of TRα (*Thra*) than TRβ (*Thrb*) (Figure 1A). Additionally, expression of genes encoding for TH transporters *Slc16a2*, *Slc16a10*, *Slc7a8*, *Slco4a1*, deiodinases *Dio1*, *Dio2*, *Dio3*, and integrins *Itgav* and *Itgb3* was analyzed in isolated $CD4^+$ and $CD8^+$ T cells. In both T cells, *Slc16a10*, *Slco4a1*, *Dio2*, which catalyzes intracellular conversion to active T3, *Itgav*, and *Itgb3* were detected (Figures S1A and S1B). Next, to confirm our previous findings on the role of canonical TRα signaling in T cell immunity, we determined the frequency of circulating T cells in blood of transgenic female mice either lacking the TRα (TRαKO) or with exclusive abrogation of canonical TRα action (TRαGS)[13] (Figure 1B). Of note, neither TRαKO nor TRαGS mice display changes in circulating TH concentrations (Figures S3A–S3C).[13] Flow cytometry analyses confirmed an increased frequency of circulating $CD4^+$ T cells in blood of TRαGS mice compared to TRαWT littermates whereas the frequency of circulating $CD8^+$ T cell was decreased (Figures 1C and 1D), resulting in a net shift to a higher CD4/CD8 ration. Interestingly, neither lack of TRα nor lack of canonical TRα action led to differences in splenic T cell pattern both in terms of total T cell numbers (Figure 1E) and in proportion of $CD4^+$ and $CD8^+$ T cell subsets (Figure 1F). Additionally, similar frequencies of double-positive (DP), double-negative (DN), and single-positive (SP) CD4 and CD8 T cells were found in the thymus of TRαWT, TRαKO, and TRαGS mice (Figures 1G and 1H). Hence, our results imply an essential role of canonical TRα action in $CD4^+$ T cell immunity while indicating unaltered conventional T cell development. Therefore, our subsequent analysis focused on TRαGS mice.

Importantly, TRαGS splenic $CD4^+$ T cells did not show differences in gene expression of *Thra* and *Thrb*, *Itgav*, and *Slc16a10* (Figures S1C–S1E). Hence, despite increased expression of *Itgb3* and *Slco4a1*, our results suggest no compensatory effects of TRβ or integrin-mediated TH signaling in TRαGS $CD4^+$ T cells.

$CD4^+$ T cells that lack canonical TRα action do not show enhanced pro-inflammatory properties *in vitro*

Activation of T cells in lymphoid tissue is commonly required for migration of T cells into the circulation. Accordingly, we found elevated levels of lymphoid homing receptor $CD62L^{low}$ and Ki67+$CD4^+$ T cells in spleen of naive TRαGS mice together suggesting $CD4^+$ T cell activation[14] (Figures 2A and 2B). On the contrary, markers of T cell activation were not affected in $CD8^+$ T cells (Figures 2C and 2D).

To define the role of TRα signaling during $CD4^+$ T cell activation in more detail, we stimulated TRαWT and TRαGS total $CD4^+$ T cells with anti-CD3 and anti-CD28 antibodies *in vitro* in the presence of TH in media supplemented with 10% fetal bovine serum (FBS) containing TH. Surprisingly, TRαGS $CD4^+$ T cells showed impaired proliferation compared to TRαWT cells *in vitro* (Figure 2E). To further address the effect of TRα on T cell function, we investigated T helper (Th) cell polarization. No differences were found in frequency of pro-inflammatory Th1 cell subset in spleens of naive TRαGS mice (Figure 2F). Likewise, activation of naive $CD25^-$ $CD4^+$ T cells in a Th1-favoring environment equally induced conversion of TRαWT and TRαGS T cells into Th1 cells *in vitro* (Figure 2G). Moreover, no differences in Th17 cells were found in spleen of naive mice (Figure 2H), however TRαGS $CD4^+$ T cells showed an attenuated differentiation into Th17 cells *in vitro* (Figure 2I). Overall, despite increased frequencies of $CD62L^{low}$ and Ki67+$CD4^+$ T cells in TRαGS mice, $CD4^+$ T cells derived from TRαGS animals showed reduced proliferation and polarization toward Th17 subset *in vitro* compared to WT controls.

Canonical TRα signaling limits the induction of regulatory T cells

Besides pro-inflammatory T cell activation, we addressed the role of TRα in anti-inflammatory Treg responses. Interestingly, naive TRαGS mice showed elevated frequencies of anti-inflammatory Foxp3+Treg in spleen compared to WT littermates (Figure 3A) and CTLA-4+ $CD4^+$ T cells (Figure 3B) also indicating an increased abundance of Treg in naive mice,[15] while anti-inflammatory IL-10 serum concentrations were not altered (Figure 3C). In addition, enhanced conversion of naive TRαGS T cells into induced Treg was found in an *in vitro* polarization assay (Figure 3D). Moreover, naive TRαGS mice displayed an increase of Treg in the thymus (Figure 3E), suggesting enhanced thymic and peripheral Treg differentiation.

No major differences were observed in the innate immune system of naive TRαGS mice (Figure S2A–S2I). Frequencies of monocytes, neutrophils, and dendritic cells (DCs) were neither altered in spleen nor in blood of TRαGS mice. However, a slightly decreased proportion of macrophages was found in spleens of naive mice. Taken together, our results imply an inhibitory effect of local TRα-mediated canonical action in $CD4^+$ T cells and especially on Treg differentiation.

To further characterize the effect of TH-TR signaling in T cell immunity, we used transgenic TRβGS mice with endogenously elevated TH serum concentrations (Figures S3A–S3C),[13,16] which represent a model of increased TH-TRα signaling. Additionally, TRβGS mice show increased levels of thyroid-stimulating hormone (TSH); however, we found no expression of TSH receptor in $CD4^+$ T cells by gene expression analysis of isolated cells. Contrary to TRαGS mice, flow cytometry analyses of naive TRβGS mice revealed a reduced frequency of $CD62L^{low}$ and Ki67+$CD4^+$ T cells (Figures S3D–S3G). Notably, we also found reduced frequencies of Foxp3+ Treg in spleens of TRβGS mice (Figure S3H) further supporting an inhibitory effect of TH-TRα signaling on Treg differentiation.

Canonical TRα signaling shapes the phenotype of regulatory T cells

To further investigate the role of TRα signaling in Treg immunity, gene expression analyses of total RNA from isolated splenic Treg of TRαWT and TRαGS mice were performed. Due to lack of a Foxp3 fluorescent reporter, Treg were isolated based on CD25 surface expression.[17] Flow cytometry analyses confirmed that approximately 93% of $CD25^+$ $CD4^+$ T cells were Foxp3+Treg in both TRαWT and TRαGS mice (Figure 3F). Principal-component analysis (PCA) of RNA-sequencing data showed a clustering of Treg based on the genotype (Figure 3G). Different genes related to Treg activation and effector function (*S100a4*, *GzmB*, and *Rora*) were strongly elevated in TRαGS compared to TRαWT Treg (Figures 3H and 3I). Similarly, overrepresentation analysis (ORA) revealed that differently regulated genes in TRαGS Treg correspond to

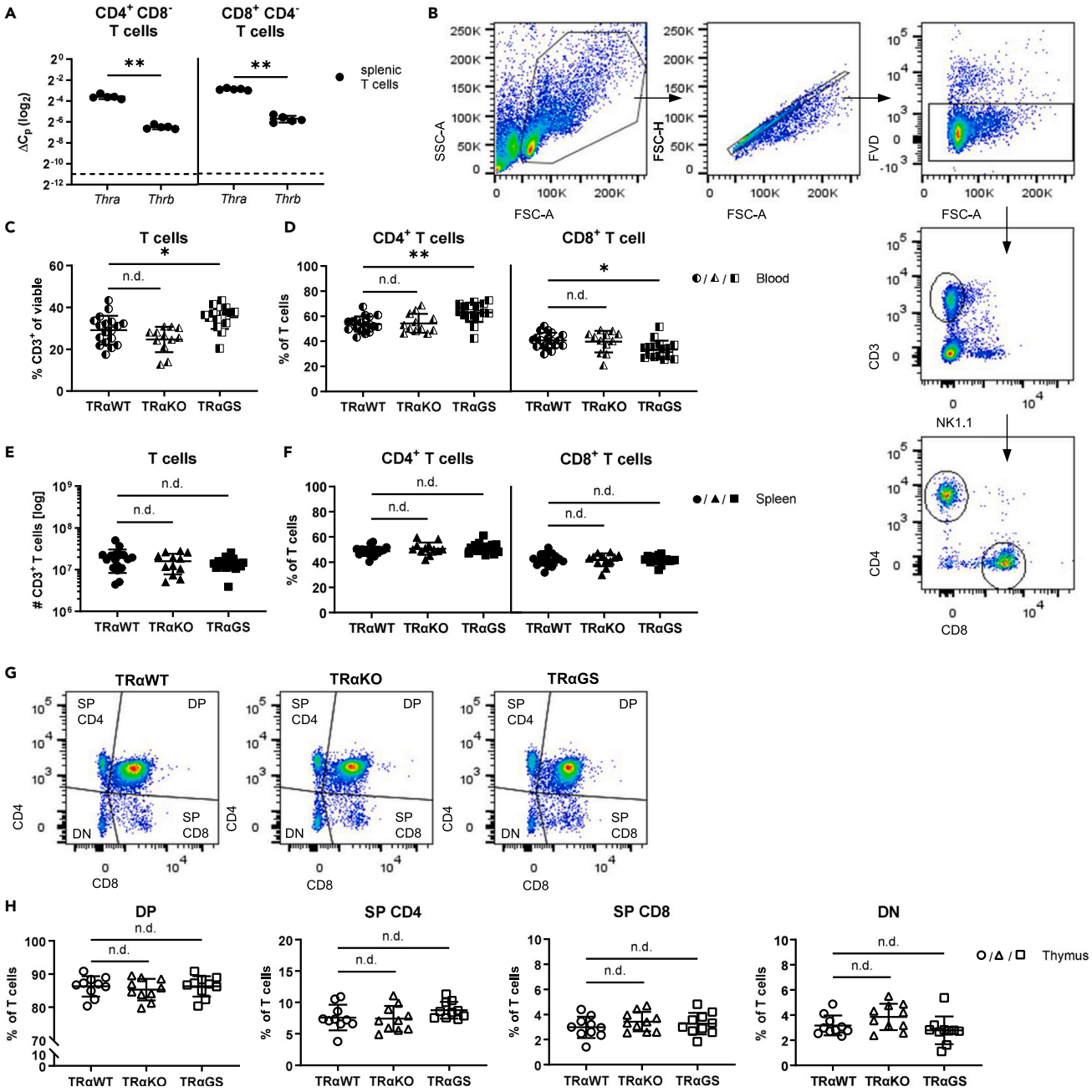


Figure 1. Absence of canonical TRα action increases circulating CD4$^+$ T cell number

(A) TRα (*Thra*) and TRβ (*Thrb*) transcript levels were determined in MACS-enriched CD4$^+$ and CD8$^+$ T cells from spleen of TRαWT mice.

(B) Representative plots show the gating strategy to identify cells of interest starting from exclusion of debris, identifying single cells, viable cells, CD3$^+$ T cells, CD4$^+$ T cells and CD8$^+$ T cells. Naive TRαKO, TRαGS, and TRαWT mice were characterized by flow cytometry to determine (C) the frequency of circulating T cells and (D) proportion of CD4$^+$ and CD8$^+$ T cell subsets in blood.

(E) Moreover, the absolute number of T cells and (F) proportion of CD4$^+$ and CD8$^+$ T cell subsets in spleens was determined by flow cytometry.

(G) Thymic T cells were analyzed by flow cytometry to determine the frequency of (H) double-positive (DP), double-negative (DN), single-positive (SP) CD4 and CD8 T cells in naive mice. Data are shown as mean ± SD pooled from 4 to 7 independent experiments. Each dot represents a biological replicate. For statistical analysis data were tested for normal distribution using D'Agostino and Pearson omnibus normality test. Means were compared by one-way ANOVA or Kruskal-Wallis test. *$p < 0.05$; **$p < 0.01$.

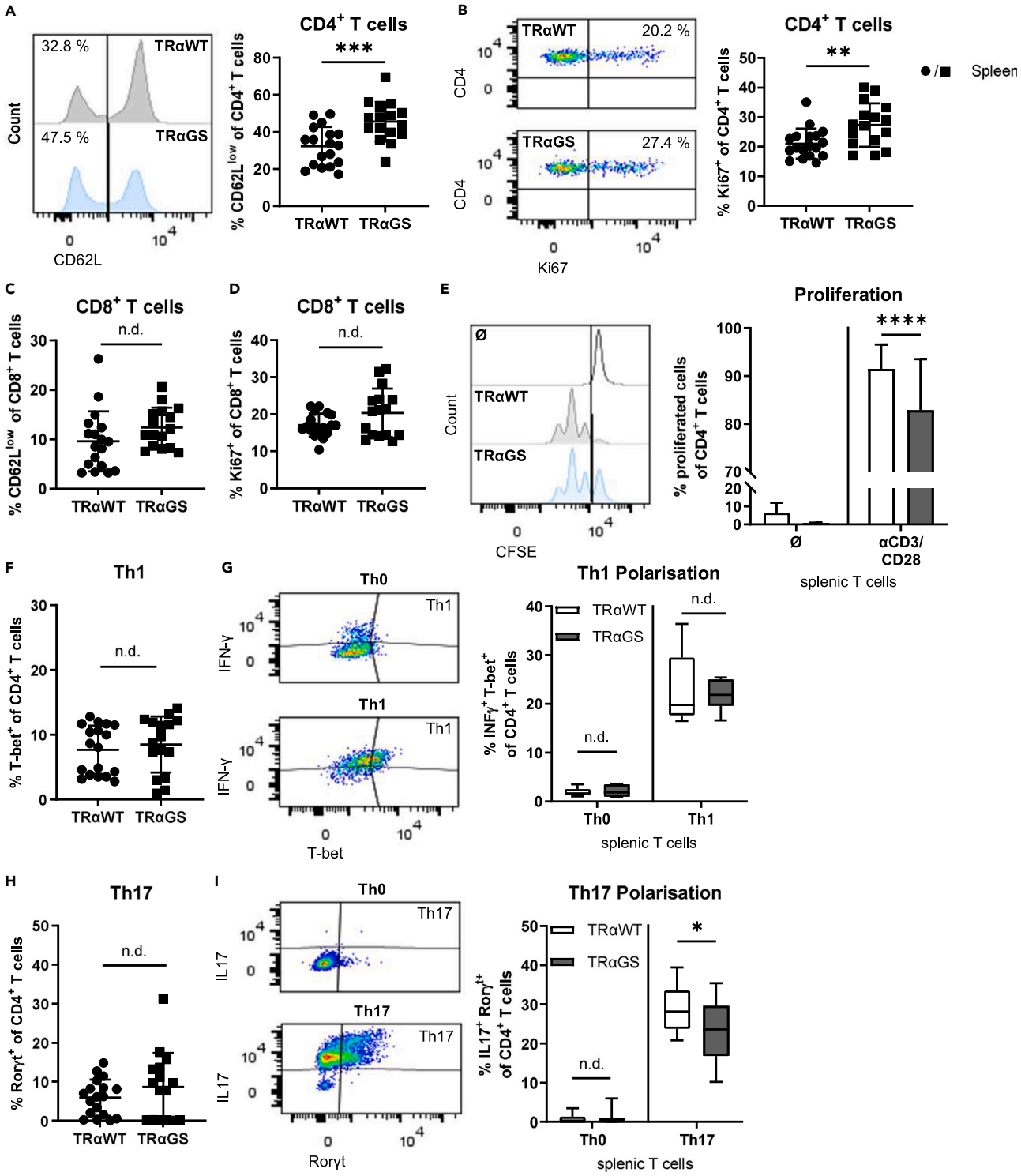


Figure 2. CD4$^+$ T cells that lack canonical TRα action do not show enhanced pro-inflammatory properties *in vitro*

(A–D) (A) Activation of splenic T cells was examined and is shown as representative histogram and frequency of CD62L^{low} CD4$^+$ T cells and (B) Ki67+ CD4$^+$ T cells as well as (C) frequency of CD62L^{low} and (D) Ki67+ CD8$^+$ T cells.

(E) Isolated total splenic CD4$^+$ T cells were stained with CFSE cell tracer and stimulated with anti-CD3, anti-CD28 (1 μg/ml) for 48 h. Representative histogram and frequencies of proliferating T cells are shown.

(F) Percentages of T-bet+ Th1 cells were measured in spleens of naive TRαGS and TRαWT mice by flow cytometry.

Figure 2. *Continued*

(G) *In vitro* polarization of naive CD25$^-$ CD4$^+$ T cells was induced in a Th1 (n = 6) promoting environment and frequency of Th cells was determined at day 5. (H) Likewise, frequency of RORγT+Th17 cell were examined in naive mice and (I) upon *in vitro* polarization of isolated CD25$^-$ CD4$^+$ T cells in Th17 (n = 9–11) promoting environment. Data are shown as mean ± SD pooled from 2 to 7 independent experiments. Each dot represents a biological replicate. For statistical analysis data were tested for normal distribution using D'Agostino and Pearson omnibus normality test. Means of two groups were compared by unpaired t test or Mann-Whitney U test, while comparison of multiple groups was done by two-way ANOVA and post hoc Tukey test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

pathways involved in T cell activation, differentiation, and adhesion (Figure 3J). Hence, RNA-sequencing data of isolated regulatory T cells indicate an important role of TRα-mediated TH signaling in Treg activation and function.

Additionally, we performed RNA-sequencing analysis of isolated naive CD25$^-$ CD4$^+$ T cells from spleen of TRαGS and TRαWT mice. Again, PCA of RNA-sequencing data showed a clustering of CD25$^-$ CD4 T cells based on the genotype (Figure S4A). Genes strongly regulated in TRαGS compared to TRαWT cells included known regulators of T cell immunity *Eomes*, *Bcl2*, and *Tab3* (Figure S4B). ORA showed that differentially regulated genes in TRαGS naive CD4 T cells were mainly involved in lymphocyte homeostasis, apoptosis, and cell adhesion (Figure S4C), but no clear impact on Treg differentiation was observed at naive state. Thus, our results imply a main effect of TH-TRα signaling on homeostasis and migration of naive CD4$^+$ T cells.

To confirm the impact of canonical TRα signaling on Treg activation, we next characterized splenic Foxp3+Treg by flow cytometry. In accordance with the RNA-sequencing data, we observed an augmented frequency of effector (CD62L^{low}, CD44$^+$) Foxp3+Treg in TRαGS compared to TRαWT mice (Figure 3K). Notably, the frequency of effector (CD62L^{low}, CD44$^+$) cells was unaltered in Foxp3- CD4$^+$ T cell population (Figure 3L). Thus, as shown in Figures 2A and 2B, activation of splenic CD4$^+$ T cells found in naive TRαGS mice was rather related to an increase of activated Foxp3+Treg than conventional Foxp3-CD4$^+$ T cells. Moreover, the expression of different functional markers was increased on TRαGS Treg, such as Icos, PD-1, Klrg-1, and St-2 (*Il1rl1*) (Figure 3M). Together, this suggest that canonical TRα-mediated TH action affects the phenotype of CD4$^+$ Treg.

Next, we addressed the role of TRα signaling in Treg function. To this end, CD25$^+$ Treg isolated from TRαWT and TRαGS mice were stimulated with anti-CD3 and anti-CD28 loaded beads *in vitro*. Here, TRαGS Treg showed an impaired proliferation compared to TRαWT cells during *in vitro* expansion (Figure 3N). Nevertheless, TRαGS and TRαWT Treg equally suppressed proliferation of CD4$^+$ T responder cells during co-culture at different ratios (Figure 3O). Interestingly, we observed a reduced frequency of CD25$^+$ cells within Foxp3+Treg population of naive TRαGS mice compared to WT littermates potentially affecting *in vitro* functional analysis of isolated CD25$^+$ Treg (Figure 3P). Similarly, we found an increased frequency of Foxp3+Treg among CD25$^-$ CD4$^+$ T cells in TRαGS mice compared to WT littermates (Figure S4D). Besides direct suppressive function, RNA-sequencing analysis showed an increased expression of genes involved in T cell migration in CD25$^+$ TRαGS Treg compared to TRαWT Treg (Figure 3Q) indicating enhanced Treg recruitment *in vivo*. In sum, although our results imply an enhanced activation of TRαGS Treg, further studies are needed to clarify the impact of TRα on Treg function *in vitro* and *in vivo*.

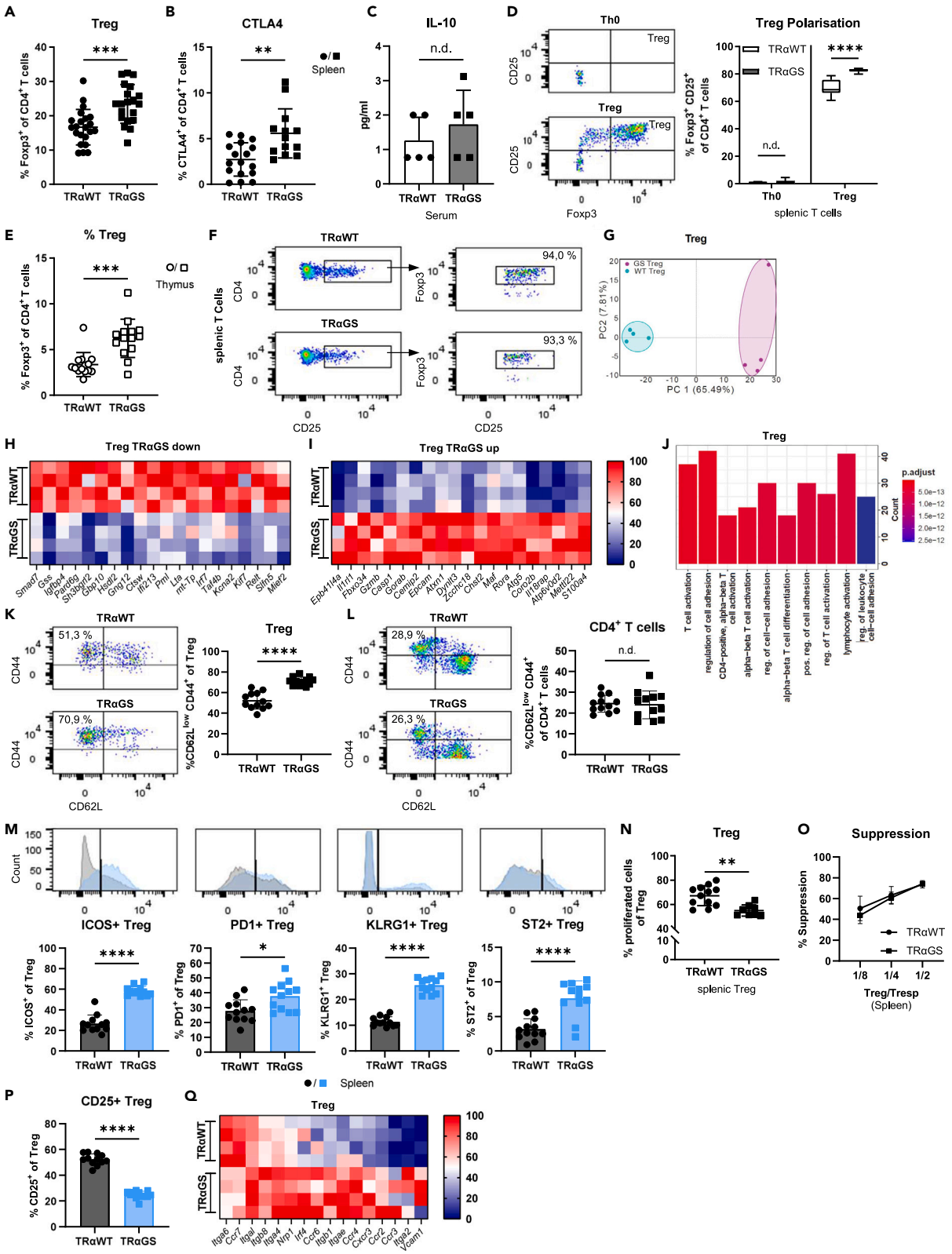
TRα-mediated TH signaling limits NF-κB activation

The differentiation of Treg and their suppressive function highly depend on stimulation of the TCR.[18–20] Signaling of TCR is mediated by different pathways, among which the NF-κB pathway plays a pivotal role in Treg differentiation.[21,22] Interestingly, we found different genes of iκB kinase/NF-κB signaling to be altered in CD25$^+$ TRαGS Treg compared to TRαWT cells during RNA-sequencing analysis (Figure 4A). In order to address the impact of TRα on NF-κB signaling, we measured NF-κB pathway activation in isolated splenic CD4$^+$ T cells of TRαWT and TRαGS mice. At steady state, NF-κB transcription factors are retained in the cytosol through interaction with NF-κB inhibitor (iκB). Activation of TCR and co-stimulatory receptors rapidly induces phosphorylation and degradation of iκB proteins, which are again rapidly restored due to negative feedback.[23,24] Interestingly, iκBα protein levels were decreased in both unstimulated and anti-CD3, anti-CD28 stimulated TRαGS T cells compared to TRαWT T cells, suggesting an increased basal activity of NF-κB signaling in the absence of canonical TRα signaling (Figure 4B). Likewise, naive TRαGS mice displayed increased level of Nur77 in Treg, a marker of TCR signal strength (Figure 4C).

To further elucidate the impact of TRα-mediated TH action on NF-κB signaling, we transiently transfected human Jurkat T lymphoma cells with an NF-κB luciferase reporter. Additionally, cells were transfected with TRαWT, TRαGS, a TRαGR variant or the empty vector. The TRαGR variant is a dominant negative mutant of TRα with abrogated binding of TH to TRα.[13,25] Cells were cultured in medium supplemented with 10% TH-depleted FBS[26] and stimulated with either solvent control, T3, PMA, or T3 and PMA combined. Importantly, in the presence of TH, PMA-induced NF-κB activity was markedly restricted in cells overexpressing TRαWT (~60%). On the contrary, a minor repressive effect was observed in cells overexpressing TRαGS and TRαGR (~20%) (Figure 4D). This residual inhibitory effect on NF-κB was also present in empty vector control and thus, can be attributed to residual action of endogenous TRα, which is expressed in Jurkat cells (data not shown). Taken together, our data demonstrate that TRα-mediated TH action limits NF-κB pathway activation via canonical TRα action as it requires functional DNA and ligand binding. Inversely, enhanced NF-κB activity can be associated with impaired canonical TRα signaling in TRαGS T cells potentially affecting TCR-dependent Treg differentiation.

DISCUSSION

So far, little is known about local TH action in T cells. Previous studies have demonstrated TH deiodination within human lymphocytes,[27] but did not investigate exact mechanisms. Here, we show that TH transporters *Slc16a10* and *Slco4a1* as well as the TH metabolizing enzyme *Dio2*


A
Treg
% Foxp3+ of CD4+ T cells
TRαWT TRαGS
B
CTLA4
% CTLA4+ of CD4+ T cells
Spleen
C
IL-10
n.d.
pg/ml
Serum
D
Th0
Treg
CD25
Foxp3
Treg Polarisation
% Foxp3+ CD25+ of CD4+ T cells
splenic T cells
E
% Treg
Thymus
F
TRαWT
TRαGS
splenic T Cells
CD4
Foxp3
94,0 %
93,3 %
CD25
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GS Treg
WT Treg
PC2 (7.81%)
PC 1 (65.49%)
H
Treg TRαGS down
I
Treg TRαGS up
J
Treg
p.adjust
Count
T cell activation
regulation of cell adhesion
CD4+-positive, alpha-beta T cell activation
alpha-beta T cell activation
reg. of cell-cell adhesion
alpha-beta T cell differentiation
pos. reg. of cell adhesion
reg. of T cell activation
lymphocyte activation
reg. of leukocyte cell-cell adhesion
K
TRαWT
TRαGS
51,3 %
70,9 %
CD44
CD62L
%CD62L low CD44+ of Treg
L
28,9 %
26,3 %
CD4+ T cells
%CD62L low CD44+ of CD4+ T cells
M
Count
ICOS+ Treg
PD1+ Treg
KLRG1+ Treg
ST2+ Treg
% ICOS+ of Treg
% PD1+ of Treg
% KLRG1+ Treg
% ST2+ of Treg
Spleen
N
Treg
% proliferated cells of Treg
splenic Treg
O
Suppression
% Suppression
Treg/Tresp (Spleen)
P
CD25+ Treg
% CD25+ of Treg
Q
Treg

Figure 3. Canonical TRα signaling limits the induction and shapes the phenotype of regulatory T cells

(A) Percentage of Foxp3+ and (B) CTLA4+CD4$^+$ T cells was measured in spleens of naive mice by flow cytometry.

(C) IL-10 was determined in serum of naive mice via Luminex.

(D) Naive CD25$^-$ CD4$^+$ T cells were differentiated into Treg by *in vitro* polarization assay. Exemplary dot plots and frequency of Foxp3+CD25$^+$ induced Treg at day 3 are depicted.

(E) Frequency of Treg was examined in the thymus of naive TRαGS and TRαWT mice.

(F) CD25$^+$ Treg (n = 4) of TRαGS and TRαWT mice were isolated from spleen. Exemplary dot plots show frequency of Foxp3+ cells of CD25$^+$ CD4$^+$ T cells in spleens of naive TRαWT and TRαGS mice.

(G) Transcriptome of isolated Treg was analyzed by RNA-sequencing and principal-component analysis of differently expressed genes was performed assessing changes between genotypes.

(H) Heatmap shows 20 most down and (I) up regulated genes in TRαGS Treg compared to TRαWT cells.

(J) Differently expressed genes were used for overrepresentation analysis and 10 most enriched GO-terms are shown.

(K) Frequency of CD62^{low} CD44$^+$ effector Foxp3+ Treg and (L) CD62^{low} CD44$^+$ effector Foxp3- CD4$^+$ T cells was determined in spleen of naive mice by flow cytometry.

(M) Markers of Treg function were determined on splenic Foxp3+ Treg in naive mice by flow cytometry. (N) Proliferation of splenic CD25$^+$ Treg following *in vitro* expansion for 72 h was examined using CFSE cell tracer.

(O) Suppressive activity of TRαGS and TRαWT Treg was determined in an *in vitro* suppression assay using indicated ratios of T responder cells (Tresp; TRαWT CD4$^+$ T cells) and CD25$^+$ Treg.

(P) Percentage of CD25$^+$ Foxp3+ Treg was analyzed by flow cytometry in spleen of naive mice.

(Q) Differential expression of genes involved in T cell migration from RNA-sequencing analysis of isolated TRαWT and TRαGS Treg. Data are shown as mean ± SD pooled from 1 to 7 independent experiments containing at least 3 biological replicates. For statistical analysis data were tested for normal distribution using D'Agostino and Pearson omnibus normality test. Means were compared by unpaired *t* test or Mann-Whitney U test, comparison of multiple groups was done by two-way ANOVA and post hoc Tukey test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

are expressed in T cells and thus, might function as potential regulators of local TH availability. Similar to the murine BW5147 cell line,[7] we found expression of both TRα and TRβ in primary CD4$^+$ and CD8$^+$ T cells from female mice. Notably, TRα levels were significantly higher in both T cell subtypes compared to TRβ. Complementary roles of abrogated canonical TRα signaling in TRαGS mice and enhanced TH-TRα signaling in TRβGS mice[13,16] implied a dominant role of TRα in CD4$^+$ T cell immunity. Thus, our findings demonstrate an important role of TRα action in CD4$^+$ T cell, especially Treg immunity.

THs have previously been reported to affect T cell proliferation. Elevated T cell numbers were observed at hyperthyroid state following TH treatment in a murine mammary carcinoma model.[8] Inversely, reduced serum TH concentrations during chronic stress were associated with decreased T cell proliferation.[9] This effect was reversed by restoring TH levels. Likewise, our data show increased T cell numbers in hyperthyroid TRβGS mice, whereas proliferation of CD4$^+$ T cells from naive mice lacking canonical TRα action was impaired upon *in vitro* stimulation. Notably, these mice had an elevated proportion of Treg among CD4$^+$ T cells, which could inhibit conventional T cell proliferation *in vitro*. Interestingly, hyperthyroidism related to either Graves' disease in patients or long-term TH treatment of mice was shown to reduce Treg frequencies.[11] Similarly, Treg frequencies increase during chronic stress,[28–30] but the role of TH has not been investigated in this context. In addition to potential direct effects, TH might therefore indirectly promote T cell proliferation limiting Treg responses. Interestingly, we observed an increase of both thymic and induced Treg in the absence of canonical TRα signaling, while hyperthyroid TRβGS mice show reduced Treg frequencies. Conversely, CD4$^+$ T cells lacking canonical TRα action showed diminished Th17 differentiation *in vitro*. The polarization of Th17 cells and Treg are opposing pathways.[31–34] Thus, TRα-mediated TH action might favor pro-inflammatory Th17 responses thereby limiting Treg differentiation.

Besides Treg differentiation, our findings imply an inhibitory effect of TRα on Treg function. RNA-sequencing and flow cytometry analyses revealed an increase of effector Treg in the absence of canonical TRα action, showing elevated expression of functional markers, e.g., Icos, Klrg1, and St-2. Since we did not observe an increase of effector Foxp3-CD4$^+$ T cells, augmented CD62L^{low} and Ki67+CD4$^+$ T cell frequencies found in naive mice lacking canonical TRα signaling were related to an increase in effector Treg rather than conventional T cell activation. Nevertheless, no differences in the suppressive capacity of Treg were observed in the presence and absence of canonical TRα action. Of note, we found reduced expression of IL-2 receptor CD25 on Treg lacking canonical TRα action, which is an essential regulator of Treg homeostasis. Accordingly, decreased proliferation was observed during *in vitro* expansion of Treg lacking canonical TRα action. Moreover, CD25 contributes to the suppressive function of Treg depriving effector T cells of IL-2.[35,36] Thus, reduced CD25 levels might balance the expansion and suppressive capacity of Treg in the absence of canonical TRα signaling *in vitro*. Interestingly, Foxp3 was found to directly induce expression of CD25 in cooperation with NF-κB pathway indicating a potential role of NF-κB in TRαGS Treg phenotype.[37–39] *In vivo*, migratory phenotype of Treg might additionally affect the suppressive function in the absence of canonical TRα signaling, supporting Treg recruitment. Therefore, further studies are needed to clarify the precise effect of TH-TRα signaling on Treg function.

The differentiation of Treg in thymus and periphery are two distinct pathways. While differentiation of natural occurring Treg in the thymus requires stimulation of the TCR by self-antigens,[18] in the periphery Treg are induced following activation of naive CD4$^+$ T cells in the presence of TGF-β and IL-2.[40–42] Interestingly, signaling strength of TCR affects the differentiation of both natural occurring and induced Treg.[19,43] Additionally, TCR signaling is essential for the inhibitory capacity of Treg.[20] Various pathways are involved in signaling cascades downstream of TCR among which NF-κB pathway has a central role in natural Treg differentiation and function.[21,22,44–48] TH have previously been shown to affect NF-κB signaling. Yet, while some studies report an inhibitory effect,[49–51] others suggest a stimulatory action of TH on NF-κB activation.[7,52,53] Of note, different cell types and stimulating conditions have been analyzed in these studies, indicating that the impact of TH on

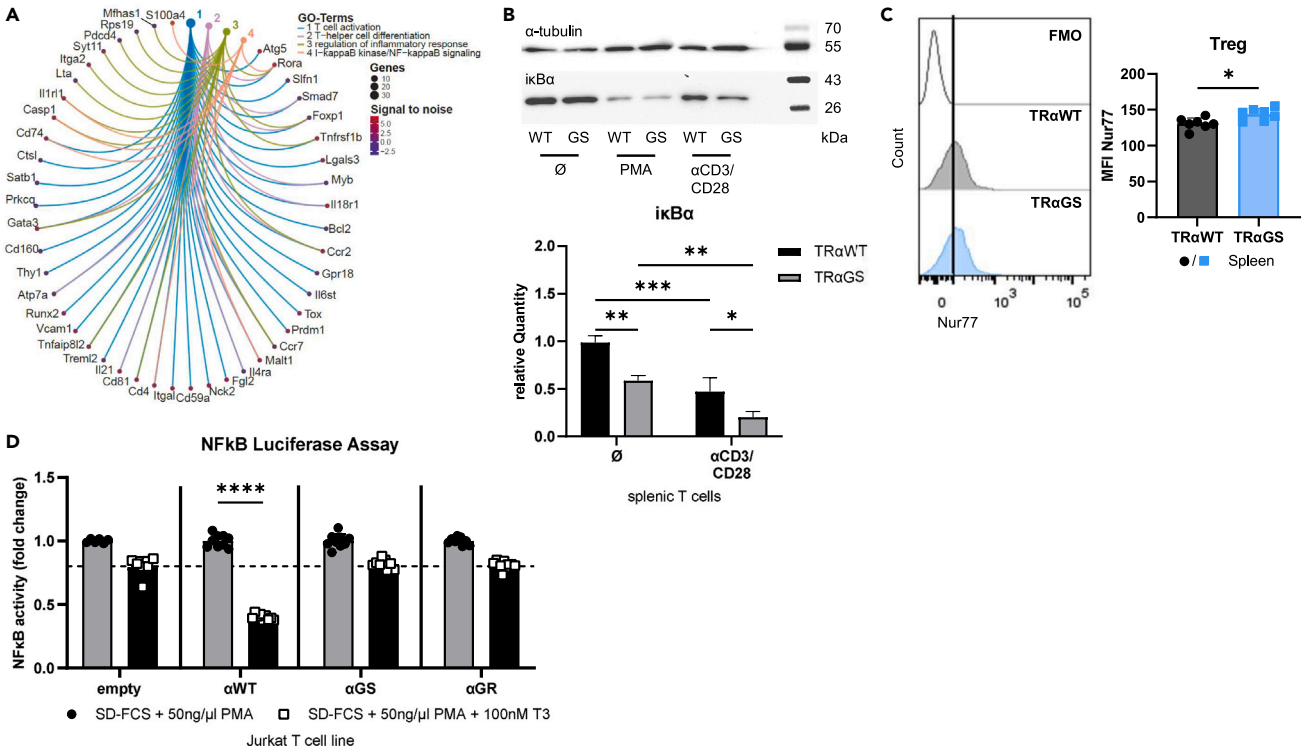

Figure 4. TRα-mediated TH signaling limits NFκB activation

(A) T cell related pathways significantly enriched in TRαGS CD25$^+$ Treg and correlating differently expressed genes of RNA-sequencing analysis are shown.

(B) MACS-enriched CD4$^+$ T cells from spleen were stimulated with anti-CD3, anti-CD28 stimulation (5 µg/ml) for 4 h. For positive control T cells were stimulated with 50 ng/ml PMA and 1 µg/ml Ionomycin for 1 h and for negative control cells were left untreated (Ø). Representative western blot analysis and quantification (n = 3) of iκBα protein levels upon stimulation are shown. α-tubulin was used as loading control.

(C) Protein level of Nur77 was measured in splenic Foxp3+Treg of naive mice by flow cytometry.

(D) NF-κB pathway activation was determined in Jurkat cells which were transfected with NF-κB luciferase reporter and plasmids encoding TRα variants TRαWT, TRαGS, TRαGR, or empty vector control (EV) using dual luciferase system. Cells were treated with 100 nM T3 and 50 ng/mL PMA or solvent control for 24 h and 18 h, respectively. Fold change of NF-κB activity induced by T3 is shown for each condition. Data are shown as mean ± SD pooled from 1 to 3 independent experiments containing at least 3 biological replicates. For statistical analysis data were tested for normal distribution using D'Agostino and Pearson omnibus normality test. Means were compared by unpaired t test or Mann-Whitney U test, comparison of multiple groups was done by two-way ANOVA and post hoc Tukey test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

NF-κB pathway may depend on the cellular and stimulatory context and the microenvironment. Our findings show an inhibitory effect of TRα-mediated TH signaling on NF-κB activation downstream of PMA or TCR stimulation. Due to the limited number of Treg present in spleen, NF-κB activation was assessed in isolated CD4$^+$ T cells but not Treg. However, elevated TCR activation was observed in Treg lacking canonical TRα signaling, indicating enhanced NF-κB activation also in Treg. Previously, Zhong et al. claimed an essential role of TH-induced PD-1 expression in the control of Treg differentiation and function. Interestingly, we found increased frequency of PD-1+CD4$^+$ and CD8$^+$ T cells in spleen of naive GS mice compared to WT littermates. Yet, PD-1 expression is induced upon TCR stimulation[54] and thus, PD-1 might correlate with increased TCR signaling but does not necessarily cause the Treg phenotype. Overexpression of TRα variants in Jurkat T lymphoma cells further demonstrated, that the inhibitory action of TH on NF-κB pathway depended on canonical action of TRα. TRα is a ligand-modulated transcription factor.[4] Binding of THs to TRα induces a switch in cofactors controlling target gene expression. Accordingly, in the presence of TH TRαWT inhibited PMA-induced NF-κB activation. This inhibitory effect was diminished in cells lacking canonical TRα action, as well as cells expressing TRαGR mutant that is incapable of TH binding. Therefore, our results imply that TRα might control expression of an NFκB-regulator in a TH dependent manner.

In conclusion, our findings suggest an inhibitory effect of TRα-mediated TH signaling on NF-κB activation restricting the induction and activation of Treg. Still, further studies are needed to elucidate the impact of TH-TRα signaling on T cell immunity during disease and to finally infer potential clinical implications.

Limitations of the study

Even though our study significantly contributes to a better understanding of the role of TH in T cell immunity in detail using naive mice and isolated T cells, further insights into T cell phenotype will require additional models and a pathophysiological context, for example by

challenging TH-TRα action in the context of infection or acute and chronic disease. Moreover, use of cell-specific mouse models, e.g., CD4-cre or Foxp3-cre, may help to distinguish T cell intrinsic and indirect effects mediated by other cell populations or developmental differences.[13] TH were reported to promote maturation and activation of bone-marrow-derived DCs,[55,56] yet TH action in DCs is mainly mediated by TRβ[57] and no major changes in DC population were found in our studies. While bone marrow chimera may be considered to address TRα action in immune cells, irradiation affects circulating TH levels[58,59] and thus may distort results. *In vitro* analyses addressing Treg function relied on CD25 for isolation of Treg from TRαWT and TRαGS mice in the present study. However, reduced CD25 levels on TRαGS Treg, might obstruct efficient isolation of effector Treg cells by FACS. Thus, to define the impact of TH on Treg function further analysis using, e.g., Foxp3 reporter mice are needed. Finally, further in-depth analysis on the mechanism mediating the interplay of TH and NF-κB will be a topic of future analysis, as will be the analysis of potential sex-specific effects since our study exclusively focused on female mice.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110547.

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AUTHOR CONTRIBUTIONS

D.F. conceived the project. Experiments were designed by D.F. and C.W. C.W. performed the experiments and analyzed data, with D.S. performing analysis of RNA-sequencing data. G.S.H. and L.C.M. initially generated GS mice strains and contributed plasmids used. G.S.H., E.P., N.H., F.K., L.C.M, D.R.E., and A.M.W. provided advice and technical expertise. C.W. and D.F. wrote the manuscript and all authors contributed to the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD3e	BD Biosciences	Cat# 553058; RRID: AB_394591
CD28	BD Biosciences	Cat# 553295; RRID: AB_394764
IL-4 monoclonal antibody; functional grade	Thermo Fisher Scientific	Cat# 16-7041-85; RRID: AB_469209
Mouse IL-2 antibody	R&D Systems	Cat# MAB702-SP
IFN gamma monoclonal antibody; functional grade	Thermo Fisher Scientific	Cat# 16-7311-81; RRID: AB_469242
FITC anti-mouse CD3	BioLegend	Cat# 100204; RRID: AB_312661
Rat anti-CD4 monoclonal antibody; APC	BD Biosciences	Cat# 553051; RRID: AB_398528
Rat anti-CD4 monoclonal antibody; Pacific Blue	BD Biosciences	Cat# 558107; RRID: AB_397030
Rat anti-CD4 monoclonal antibody; PerCP	BD Biosciences	Cat# 561090; RRID: AB_10562560
Rat anti-CD8a monoclonal antibody; PE	BD Biosciences	Cat# 553032; RRID: AB_394570
BV510 anti-mouse CD8a	BioLegend	Cat# 100752; RRID: AB_2563057
PE/Cy7 anti-mouse/human CD11b	BioLegend	Cat# 101215; RRID: AB_312798
BV510 anti-mouse CD11c	BioLegend	Cat# 117353; RRID: AB_2686978
Rat anti-CD19 monoclonal antibody; PE-Cy7	BD Biosciences	Cat# 552854; RRID: AB_394495
Rat anti-CD25 monoclonal antibody; PE-Cy7	BD Biosciences	Cat# 561780; RRID: AB_10893596
Rat anti-CD25 monoclonal antibody; BV510	BD Biosciences	Cat# 563037; RRID: AB_2737969
Rat anti-CD44 monoclonal antibody; FITC	BD Biosciences	Cat# 561859; RRID: AB_10894581
CD62L monoclonal antibody; PE-Cy5	Thermo Fisher Scientific	Cat# 15-0621-82; RRID: AB_468767
Armenian hamster anti-CD152 monoclonal antibody; PE	BD Biosciences	Cat# 553720; RRID: AB_395005
F4/80 monoclonal antibody; PE	Thermo Fisher Scientific	Cat# 12-4801-82; RRID: AB_465923
Armenian hamster anti-ICOS monoclonal antibody; BB515	BD Biosciences	Cat# 565881
Rat anti-IFN-g monoclonal antibody; PE	BD Biosciences	Cat# 554412; RRID: AB_395376
BV421 anti-mouse IL-17A	BioLegend	Cat# 506925; RRID: AB_10900442
FOXP3 monoclonal antibody; APC	Thermo Fisher Scientific	Cat# 17-5773-82; RRID: AB_469457
FOXP3 monoclonal antibody; eFluor™450	Thermo Fisher Scientific	Cat# 48-5773-82; RRID: AB_1518812
Ki-67 monoclonal antibody; eFluor™660	Thermo Fisher Scientific	Cat# 50-5698-82; RRID: AB_2574235
Hamster anti-KLRG1 monoclonal antibody; BV510	BD Biosciences	Cat# 740156; RRID: AB_2739909
Rat anti-Ly6C monoclonal antibody; FITC	BD Biosciences	Cat# 553104; RRID: AB_394628
APC anti-mouse Ly-6G	BioLegend	Cat# 127614; RRID: AB_2227348
NK1.1 monoclonal antibody; eFluor™450	Thermo Fisher Scientific	Cat# 48-5941-82; RRID: AB_2043877
Mouse anti-Nur77 monoclonal antibody; Alexa 647	BD Biosciences	Cat# 566735; RRID: AB_2869837
PE/Cy7 anti-mouse CD279 (PD-1)	BioLegend	Cat# 109110; RRID: AB_572017
ROR gamma (t) monoclonal antibody; APC	Thermo Fisher Scientific	Cat# 17-6988-80; RRID: AB_1633425
Mouse anti-RORgt monoclonal antibody; PE	BD Biosciences	Cat# 562607; RRID: AB_11153137
IL-33R (ST2) monoclonal antibody; PE-Cy7	Thermo Fisher Scientific	Cat# 25-9335-82; RRID: AB_2637464
PE/Cy7 anti-mouse T-bet	BioLegend	Cat# 644824; RRID: AB_2561761
Mouse anti-T-bet monoclonal antibody; BV421	BD Biosciences	Cat# 563318; RRID: AB_2687543
IκBα rabbit mAb	Cell Signaling Technology	Cat# 4812; RRID: AB_10694416
Mouse anti-tubulin; α monoclonal antibody	Millipore	Cat# 05–829; RRID: AB_310035
Anti-mouse IgG; HRP-linked antibody	Cell Signaling Technology	Cat# 7076; RRID: AB_330924

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-rabbit IgG; HRP-linked antibody	Cell Signaling Technology	Cat# 7074; RRID: AB_2099233
Chemicals; peptides; and recombinant proteins		
Human IL-2 recombinant protein	Thermo Fisher Scientific	Cat# PHC0027
Recombinant human TGF-β 1 protein	R&D Systems	Cat# 240-B-002/CF
Recombinant mouse IL-6 protein	R&D Systems	Cat# 406-ML-005/CF
Mouse IL-1 β recombinant protein	Thermo Fisher Scientific	Cat# PMC0814
Recombinant mouse IL-21 protein	R&D Systems	Cat# 594-ML-010/CF
Recombinant mouse IL-23 protein	R&D Systems	Cat# 1887-ML-010/CF
Recombinant mouse IL-12 protein	R&D Systems	Cat# 419-ML-010/CF
Fixable viability dye eFluor780	Thermo Fisher Scientific	Cat# 65-0865-18
Ionomycin	Sigma-Aldrich	Cat# 10634
phorbol 12-myristate 13-acetate (PMA)	Sigma-Aldrich	Cat# P1585
Brefeldin A	Sigma-Aldrich	Cat# B7561
Critical commercial assays		
$CD4^+$ T cell isolation kit; mouse	Miltenyi Biotec	Cat# 130-104-454
Vybrant™ CFDA SE Cell Tracer Kit	Thermo Fisher Scientific	Cat# V12883
Treg Expansion Kit	Miltenyi Biotec	Cat# 130-095-925
Foxp3 Transcription Factor Staining Buffer Kit	Thermo Fisher Scientific	Cat# 00-5523-00
SuperScript III	Thermo Fisher Scientific	Cat# 18080-051
PerfeCTa SYBRGreen Super Mix	QuantaBio	Cat# 95055-02K
Dual-Glo Luciferase Assay System	Promega	Cat# E2980
Deposited data		
RNA-sequencing data	This paper (GEO)	GEO: GSE246289
Experimental models: Cell lines		
Jurkat cell line	Prof. Westendorf	RRID: CVCL_0065
Experimental models: Organisms/strains		
B6; 129-Thratm2Jas/Orl (TRα0/0)	Hönes et al.; 2017[13]	EMMA ID: EM:01358
TRαGS/GS	Hönes et al.; 2017[13]	N/A
TRβGS/GS	Hönes et al.; 2017[13]	N/A
Oligonucleotides		
Primer THRA Fw- GAA AAG CAG CAT GTC AGG GTA Rev- GGA TTG TGC GGC CAA AGA AG	Eurofins	NM_001313983.1
Primer THRB Fw-GGA CAA GCA CCC ATC GTG AA Rev-ACA TGG CAG CTC ACA AAA CAT	Eurofins	NM_001113417.2
Primer PPIA Fw-CTT GGG CCG CGT CTC CTT CG Rev-GCG TGT AAA GTC ACC ACC CTG GC	Eurofins	NM_008907.2
Primer GAPDH Fw-CCT CGT CCC GTA GAC AAA ATG Rev-TGA AGG GGT CGT TGA TGG C	Eurofins	NM_008084.4
Primer HPRT1 Fw-TGG GCT TAC CTC ACT GCT TT Rev-TCA TCG CTA ATC ACG ACG CT	Eurofins	NM_013556.2

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Further Primers for qRT-PCR see Table S1	Eurofins	
Recombinant DNA		
pSI-Check2-hRluc-NFkB-firefly plasmid	Addgene	Cat# 106979; RRID: Addgene_106979
pcDNA™3.1	Thermo Fisher Scientific	Cat# V79020
TRα WT	Hönes et al.; 2017[13]	N/A
TRα71GS	Hönes et al.; 2017[13]	N/A
TRαG291R	Hönes et al.; 2017[13]	N/A
Software and algorithms		
GraphPad Prism v. 9.5.1	GraphPad Prism	RRID: SCR_002798
Microsoft Excel 365	Microsoft	N/A
FlowJo™ v. 10.8	BD Biosciences	RRID: SCR_008520
BD FACSDiva™ v. 9.0	BD Biosciences	N/A
Quantity One 1-D Analysis Software	Bio-Rad	RRID: SCR_014280
Image Lab Software	Image Lab Software	RRID: SCR_014210
Inkscape	Inkscape	RRID: SCR_014479
Other		
BD LSR II Flow Cytometer	BD Biosciences	N/A
BD LSRFortessa™	BD Biosciences	N/A
BD FACSAria™ III	BD Biosciences	N/A
LightCycler® LC480	Roche	N/A
VersaDoc™ MP4000 system	Bio-Rad	Cat# 170-8640
Neon™ Electroporation System	Thermo Fisher Scientific	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christina Wenzek (christina.wenzek@uk-essen.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The RNA sequencing datasets presented in this study have been deposited at online repository Gene Expression Omnibus (GEO) and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. (GSE246289).
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT

Mice

All mice strains used were bred and housed under specific pathogen free (SPF) conditions in the local animal facility of the University Hospital Essen. Mice were housed at 21 ± 1°C on an alternating 12 h light/dark cycle. Standard chow and tap water were provided *ad libitum*. All mouse strains were on a C57BL/6J background. Female, homozygous TRα0/0 (TRαKO) mice, TRαGS/GS (TRαGS) mice, and TRβGS/GS (TRβGS) mice[13] as well as wildtype littermates (TRαWT or TRβWT) were studied at an age of 8–16 weeks. While TRαKO mice lack TRα completely, TRαGS and TRβGS mice have a mutation in the DNA binding domain that impairs the canonical transcription factor function of TRα and TRβ, respectively.

Notably, TRβ plays a central role in negative feedback on TH synthesis. Hence, we have previously shown, that TRβGS mice are hyperthyroid showing elevated levels of TSH, T4 and T3, while TRαGS mice are euthyroid (Figures S3A–S3C).[13] Tissues were collected from naive mice and thus did not require ethics approval by local authorities according to §4 (3) of the German animal welfare act.

Cell lines

Human Jurkat T lymphoblast cell line and was obtained from laboratory of Prof. Astrid Westendorf (University Hospital Essen, Germany). Cells were maintained in RPMI 1640 medium with GlutaMAX (Gibco) supplemented with 10% FBS and 1% Penicillin-Streptomycin (Gibco).

METHOD DETAILS

Isolation of immune cells from murine spleen, blood or thymus

In order to isolate murine, splenic immune cells, spleens were dissected and rinsed with erythrocyte lysis buffer. Cell suspensions obtained were filtered through a 70 μm cell strainer. For isolation of murine peripheral blood leukocytes, blood samples were drawn by cardiac puncture and erythrocyte lysis was performed. For isolation of immune cells from the murine thymus, the tissue was dissected and meshed through a 70 μm strainer in PBS containing 10% FBS and 2 mM EDTA. Subsequently, erythrocytes were lysed. For further use cells were resuspended in PBS containing 2% FBS and 2mM EDTA. Due to the enrichment of lymphocytes in lymphoid tissues, spleen and thymus tissue was collected without prior perfusion.

In vitro proliferation of conventional T cells and Treg

$CD4^+$ T cells were enriched from murine spleens using the $CD4^+$ T cell Isolation Kit (Miltenyi Biotec). Next, cells were labeled with Vybrant CFDA SE Cell Tracer Kit (Invitrogen). Briefly, cells were washed with IMDM without additives. Next cells were stained in IMDM containing 2.5μM CFDA SE dye and incubated for 8 min at 37°C. Staining was stopped adding equal amount of FBS to medium and incubating 5 min at 37°C. For *in vitro* activation 2 x 10^5 labeled cells were seeded on 96-well plate (flat) and activated with 1 μg/ml immobilized anti-CD3 antibody (BD Biosciences) and 1 μg/ml soluble anti-CD28 antibody (BD Biosciences). Proliferation of the cells was examined after 48h by flow cytometry based on the dilution of CFDA SE staining during cell division.

$CD4^+$ $CD25^+$ Treg were isolated using FACSAria III cell sorter (BD Biosciences) and labeled with CFDA SE Cell Tracer (Invitrogen). Expansion of Treg was induced using Treg Expansion Kit (Miltenyi Biotec). Proliferation was determined after 72h by flow cytometry.

During *in vitro* stimulation TH were present in medium, which was supplemented with 10% FBS containing TH.

In vitro T cell polarization assay

Naive $CD4^+$ $CD25^-$ T cells were isolated from MACS-purified splenic $CD4^+$ T cells using FACSAria III cell sorter (BD Biosciences). For polarization, 5 x 10^5 cells were seeded on 48-well plate and stimulated with 1 μg/ml anti-CD3 and 1 μg/mL anti-CD28. Activation in the presence of 10 ng/mL IL-2 (Gibco) and 5 ng/mL TGF-β (R&D Systems) for 72 h was performed to induce Treg. For Th17 polarization, cells were stimulated for 5 days in the presence of 2 ng/mL TGF-β, 50 ng/mL IL-6 (R&D Systems), 20 ng/mL IL-1β (Gibco), 100 ng/mL IL-21 (R&D Systems), 20 ng/mL IL-23 (R&D Systems), 200 ng/mL anti-IL-4 (eBioscience), 200 ng/mL anti-IL-2 (R&D Systems), and 200 ng/mL anti-IFN-γ (eBioscience). Th1 polarization was induced adding 20 ng/mL IL-12 (R&D Systems) and 200 ng/mL anti-IL-4 for 5days. As control (Th0), naive T cells were stimulated with anti-CD3 and anti-CD28 solely.

In vitro Treg suppression assay

To study the suppressive capacity of Treg *in vitro*, $CD4^+$ T cells were enriched by MACS from murine spleens and $CD4^+$ $CD25^+$ Treg were isolated using FACSAria III cell sorter (BD Biosciences). $CD4^+$ T responder cells (Tresp) were purified by MACS from TRαWT littermates and labeled with Vybrant CFDA SE Cell Tracer Kit (Invitrogen). For *in vitro* suppression, 5 x 10^4 responder cells were co-cultured with either 2.5 x 10^4 Treg (1/2), 1.25 x 10^4 Treg (1/4) or 0.625 x 10^4 Treg (1/8). Activation was induced by 1.5 x 10^5 irradiated splenocytes from TRαWT littermates and 1 μg/ml soluble anti-CD3 antibody (BD Biosciences). After 4 days proliferation of responder cells was examined by flow cytometry based on CFDA SE staining. Inhibition of responder cells by Treg was calculated using following formula:

$$\%\ Inhibition = \frac{(\%\ Proliferation\ Tresp - \%\ Proliferation\ Tresp/Treg)}{\%\ Proliferation\ Tresp} \times 100$$

FLOW CYTOMETRY

Isolated leukocytes were stained using marker-specific fluorochrome-labeled antibodies and fixable viability dye eFluor780 (eBioscience). For intracellular staining of proteins Foxp3 Transcription Factor Staining Buffer Kit (eBioscience) was used. In order to analyze intracellular cytokines, cells were stimulated for 4h with 1 μg/mL ionomycin (Sigma-Aldrich) and 10 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) in the presence of 5 μg/mL Brefeldin A (Sigma-Aldrich).

Samples were acquired using LSR II or LSRFortessa (BD Biosciences) and analyzed using FlowJo version 10.8 Software (BD Biosciences).

GENE-EXPRESSION ANALYSIS

Total RNA was isolated from T cells which were enriched by MACS or FACS (RNeasy Kit; Qiagen) and stored at −80°C. An adequate amount of total RNA was reverse transcribed into cDNA with SuperScript III (Invitrogen) and random hexamer primers. qRT-PCR was performed using PerfeCTa SYBRGreen Super Mix (QuantaBio) on a LightCycler LC480 (Roche). Primer sequences used are listed in key resources table and Table S1. According to the MIQE guidelines for qRT-PCR (41), we used a set of three reference genes (GAPDH, PPIA and HPRT1) for accurate

normalization and calculation. Ct values <35 were used for analysis and calculation of the relative change in gene expression by the efficiency-corrected method.[60]

For total transcriptome analysis, concentration and quality of RNA were measured with Qubit (Invitrogen, Waltham, MA,USA) and Agilent Bioanalyzer pico or nano chips (Agilent, SantaClara, CA, USA), depending on the RNA concentration. Library preparation was performed using Lexogens QuantSeq 3′ mRNA-Seq Library Prep Kit FWD (Lexogen, Inc., Greenland, NH, USA). Samples were quantified with Agilent Bioanalyzer DNA HS and Library Quant qPCR and sequenced on a NextSeq500 (Illumina, San Diego, CA,USA). The obtained data were tested for statistical significance by Welch-t-test. The log_2 fold change (log_2FC) was calculated using following formula where x_1 and x_2 are each groups mean. Signal-to-noise-ratio (SNR) was computed as follows with std1 and std2 as the standard deviations of each group.

$$log_2\ FC\ =\ log_2\left(\frac{\overline{x_1}}{\overline{x_2}}\right)$$

$$SNR\ =\ \frac{\overline{x_1}\ -\ \overline{x_2}}{std_1+std_2}$$

For further analysis data were filtered, excluding genes with less than 3 determined values across the groups. Principal component analysis (PCA) was generated using the python packages matplotlib and seaborn. Data were filtered according to p-value <0.05. Heatmaps were generated with GraphPad Prism (v.9.5.1) showing top 40 genes according to SNR and log_2FC. Overrepresentation analysis for analyzing functional enrichments was conducted using terms from the enrichGO function of clusterprofiler (v4.2.0), considering the top 389 genes (~2.5% of all genes) according to absolute SNR. All genes were included in the background distribution and resulting p-values were corrected for FDR with Benjamini-Hochberg procedure to determine the p.adjust-value. Barplot and Cnetplot were created using the enrichplot package (v1.14.1).

IMMUNOBLOTTING

Whole-protein lysates were generated from MACS purified T cells in RIPA buffer (150mM NaCl, 50mM HCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50mM NaF) with cOmplete protease inhibitor (Roche) and PhosSTOP (Roche). Lysis was performed at 4°C under gentle rotation for 30 min. Cell debris were removed by centrifugation (13,000g, 4°C, 30 min) and supernatant was stored at −80°C. For analysis 15-20 μg of samples were separated by SDS/PAGE and transferred to a PVDF membrane (Roth). After blocking with 5% milk in TBS-T (Tris-buffered saline with 0.5% Tween 20) for 1h at RT, membranes were incubated for 16 h at 4°C under gentle agitation with desired primary antibodies against iκBα (Cell Signaling) and α-tubulin (Millipore). Horseradish peroxidase-labeled secondary antibody against mouse IgG (Cell Signaling) and rabbit IgG (Cell signaling) were used for detection with a VersaDoc MP 4000 system (Bio-Rad).

NF-κB DUAL LUCIFERASE ASSAY

1 x 10^5 Jurkat cells were transiently transfected using 500 ng of pSI-Check2-hRluc-NFkB-firefly plasmid (Addgene) and 500 ng plasmid encoding for TRα variants TRα, TRα71GS and TRαG291R or empty vector pcDNA3.[13] Transfection was performed using Neon Electroporation System (Invitrogen) according to the manufacturer's protocol (cell density = 2 x 10^7 cells/ml; 1000 V; 20 ms; 3 pulses). After transfection cells were seeded in RPMI 1640 medium with GlutaMAX supplemented with 10% TH-depleted FBS.[26] Cells were stimulated with 100 nM T3 or solvent control (DMSO) at 24 h and with 50 ng/mL Phorbol-12-myristate-13-acetate (PMA) or solvent control (DMSO) at 30 h post transfection. At 48 h cells were harvested and luciferase activities were determined using Dual-Glo Luciferase Assay System (Promega) and Sirius luminometer (Berthold Detection Systems GmbH). Fold change of PMA-induced NF-κB activity by T3 was calculated for each construct.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

Results were tested for normal distribution using D'Agostino and Pearson omnibus normality test. Means were compared by unpaired t-test, Mann-Whitney U test, one-way ANOVA or Kruskal-Wallis test. Statistics for two groups with multiple variants were done by two-way ANOVA and post hoc Tukey test for multiple comparisons. Statistical analyses were performed using GraphPad Prism v. 9.5.1 for Windows (Graph-Pad Software). For all statistical analyses: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.