Single-Cell Analysis of Tfh ICOS and IL-21 Variation Links Local Immune Activation to Anti-Thyroid Antibodies in Graves' Disease

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Abstract

Graves' disease (GD) is an autoimmune cause of hyperthyroidism in which T cell and B cell interactions drive thyroid autoantibody production. To test whether intrathyroid T follicular helper (Tfh) signatures relate to downstream immune activation, we analyzed single-cell RNA sequencing (scRNA-seq) data from GD and healthy thyroid tissue. Data were processed in Scanpy with filtering, normalization to 10,000 reads per cell and log-transformation, PCA (principal component analysis, summarizing gene expression into components that capture variation across cells), and Leiden clustering. Batch effects were addressed using BBKNN (Batch Balanced K-Nearest Neighbors), which builds a batch-balanced neighbor graph by selecting neighbors from each sample or batch. We examined Tfh-cell ICOS and IL21 because ICOS co-stimulation supports Tfh survival and IL-21 secretion, and IL-21 promotes B-cell activation and differentiation. ICOS expression was quantified as normalized ICOS transcript abundance in Tfh cells and summarized per donor. We integrated these signals with a per-sample B-cell activation index and serum anti-thyroglobulin (anti-TG) and anti-thyroid peroxidase (anti-TPO) titers.

At the donor level, mean Tfh ICOS expression was higher in GD than in controls but variable and did not separate groups (Welch's t-test $p = 0.302$; healthy $n = 4$, GD $n = 5$). IL21 expression was low overall and similarly did not separate groups ($p = 0.427$). We therefore stratified GD donors into Tfh $ICOS^+$ and Tfh $ICOS^-$ subgroups. B-cell activation differed across Healthy, Tfh $ICOS^-$, and Tfh $ICOS^+$ groups (ANOVA $p = 0.0013$), with higher activation in Tfh $ICOS^+$ donors. Serum anti-TG (ANOVA $p = 0.0011$) and anti-TPO (ANOVA $p = 0.0289$) were highest in the Tfh $ICOS^+$ subgroup.

Together, these results suggest that heterogeneity in Tfh ICOS expression may mark a subset of GD donors with stronger B-cell activation and higher anti-thyroid antibodies, while highlighting power limits from small donor counts for donor-level comparisons.

Introduction

Graves' disease is an autoimmune disorder and the leading cause of hyperthyroidism.[14,9] It affects roughly 40 million people worldwide and, if untreated, can lead to serious complications such as heart disease, osteoporosis, and vision problems.[2,14] At its core, Graves' disease reflects a loss of immune tolerance to thyroid antigens, leading to the formation of stimulatory autoantibodies that chronically activate the thyroid. The condition is driven by autoantibodies that stimulate the thyroid-stimulating hormone receptor (TSHR), leading to excessive hormone production.[7] Beyond its systemic effects, Graves' disease is marked by altered immune cell infiltration of the thyroid, which sustains chronic inflammation and local autoantibody production.[1,3]

In Graves' disease, CD4+ helper T cells interact with B cells to drive the production of thyroid-targeting antibodies.[3,8] A specialized subset of T cells known as T follicular helper (Tfh) cells in particular provides signals that drive B cells to proliferate and produce high-affinity antibodies.[6] Two key proteins that mediate this process are IL-21, a cytokine produced by Tfh cells that promotes B cell activation and differentiation[18], and ICOS, a co-stimulatory receptor on T cells that enhances their survival and supports IL-21 secretion.[5] Together, these molecules form a signaling pathway that amplifies immune activation within the thyroid, driving the breakdown of self-tolerance and contributing to the autoimmune pathology characteristic of Graves' disease.[3,6]

Although the presence of autoantibodies in Graves' disease is well recognized, the precise mechanisms within the thyroid that drive their production and sustain local immune activation remain poorly understood.[1,3] Specifically, it remains unclear how the expression levels of IL-21 and ICOS differ between thyroid tissue affected by Graves' disease and healthy thyroid tissue. Prior investigations primarily employed bulk RNA sequencing, which aggregates signals from mixed cell populations and thus conceals key transcriptional differences among specific immune cell subsets within the thyroid.[11]

This study addresses this gap by examining IL-21 and ICOS expression in thyroid-infiltrating immune cells using single-cell RNA sequencing (scRNA-seq) data from the published Graves' disease thyroid study by Álvarez-Sierra et al. (Journal of Autoimmunity, 2023), with the dataset provided by the authors upon request.[1] By comparing thyroid tissue from individuals with Graves' disease and healthy controls, this study tests the hypothesis that helper T cells within the thyroid of Graves' disease patients exhibit elevated expression of inflammatory genes such as IL-21 and ICOS, which may enhance local B-cell activation and intensify autoimmune responses. The analysis reveals distinct transcriptional profiles of helper T cells in Graves' disease, highlighting increased IL-21 and ICOS expression in specific subpopulations and suggesting a localized amplification loop that promotes antibody production within the thyroid. Unlike prior scRNA-seq studies that primarily describe immune composition and infiltration, this analysis links Tfh ICOS heterogeneity to two downstream readouts, intrathyroid B-cell activation and serum anti-thyroid antibody titers, using donor-level statistics. Understanding these mechanisms may offer new insights into therapeutic strategies targeting immune pathways in Graves' disease.[1,3]

Methods

Acquisition of raw data

Single-cell RNA sequencing (scRNA-seq) data were obtained directly from the published dataset by Álvarez-Sierra et al.[1] The data was provided as gene-by-cell UMI count matrices generated after preprocessing with Cell Ranger (i.e., read alignment and count matrix generation were performed in the original study), along with thyroid tissue samples from patients diagnosed with Graves' disease and healthy controls, capturing transcriptomic profiles of infiltrating immune populations and thyroid-resident cells with associated metadata describing sample conditions and sequencing quality.[1]

Data processing and analysis

All analyses were conducted in Python using the Scanpy library (v1.11.3).[19] Preprocessing steps followed established scRNA-seq pipelines. Cells expressing fewer than 200 genes and genes expressed in fewer than three cells were excluded, where “genes expressed” denotes the number of unique genes with nonzero counts detected per cell (a standard per-cell QC metric).Cells with greater than 10% mitochondrial gene content were filtered out. Counts were normalized to 10,000 reads per cell and log-transformed. Highly variable genes were identified and retained for downstream analysis, and dimensionality reduction was performed using principal component analysis (PCA).

To correct for inter-sample variation and batch effects, the BBKNN (Batch Balanced KNN) algorithm was applied during neighborhood graph construction.[15] Unsupervised clustering was subsequently performed using the Leiden algorithm. Clusters were annotated on the basis of canonical marker genes (e.g., CXCR5, PDCD1, BCL6, ICOS, and IL21), and naive $CD4^+$ T cells and B cells were computationally isolated for further study. B-cell activation was quantified using a gene-set score computed per B cell from log-normalized expression of MS4A1, CD79A, and CD74. Per-sample activation indices were calculated as the mean score across B cells for each donor and used for all statistical tests.

Differential gene expression analysis was conducted to quantify the expression levels of IL-21 and ICOS, as well as markers of B cell activation, across identified T and B cell subsets. Comparative analysis between Graves’ disease samples and healthy thyroid controls was performed by summarizing expression at the donor level and applying statistical tests to per-sample mean values. Welch’s two-sample t-tests were used for Healthy vs GD comparisons of per-sample mean Tfh-cell ICOS and IL21 expression, and one-way ANOVA with Tukey HSD post-hoc testing was used for comparisons across Healthy, Tfh $ICOS^-$, and Tfh $ICOS^+$ groups. Data visualizations were generated using Scanpy plotting functions. Additionally, data on anti-TPO and anti-TG antibody levels were obtained from Álvarez-Sierra et al. for integration with gene expression findings.[1]

Results

Differential expression of ICOS and IL-21

Quantitative analysis of single-cell expression data revealed variability in ICOS and IL-21 transcript abundance between Graves’ disease (GD) and healthy thyroids (Fig. 1). The mean normalized ICOS expression was higher in GD samples (0.024 ± 0.020 SEM) compared to controls (0.000 ± 0.000 SEM), with a corresponding two-sided Welch’s t-test p-value of 0.302 (per-sample means). In contrast, IL-21 expression values were lower overall and exhibited less dispersion (GD = 0.002 ± 0.001 SEM; Healthy = 0.011 ± 0.010 SEM), with a two-sided Welch’s t-test p-value of 0.427 (per-sample means). Here, SEM refers to the standard error of the mean, representing variability between samples within each group. Together, these results suggest that although ICOS expression tends to increase in GD thyroids, substantial variance between donors limits consistent differentiation from healthy controls.

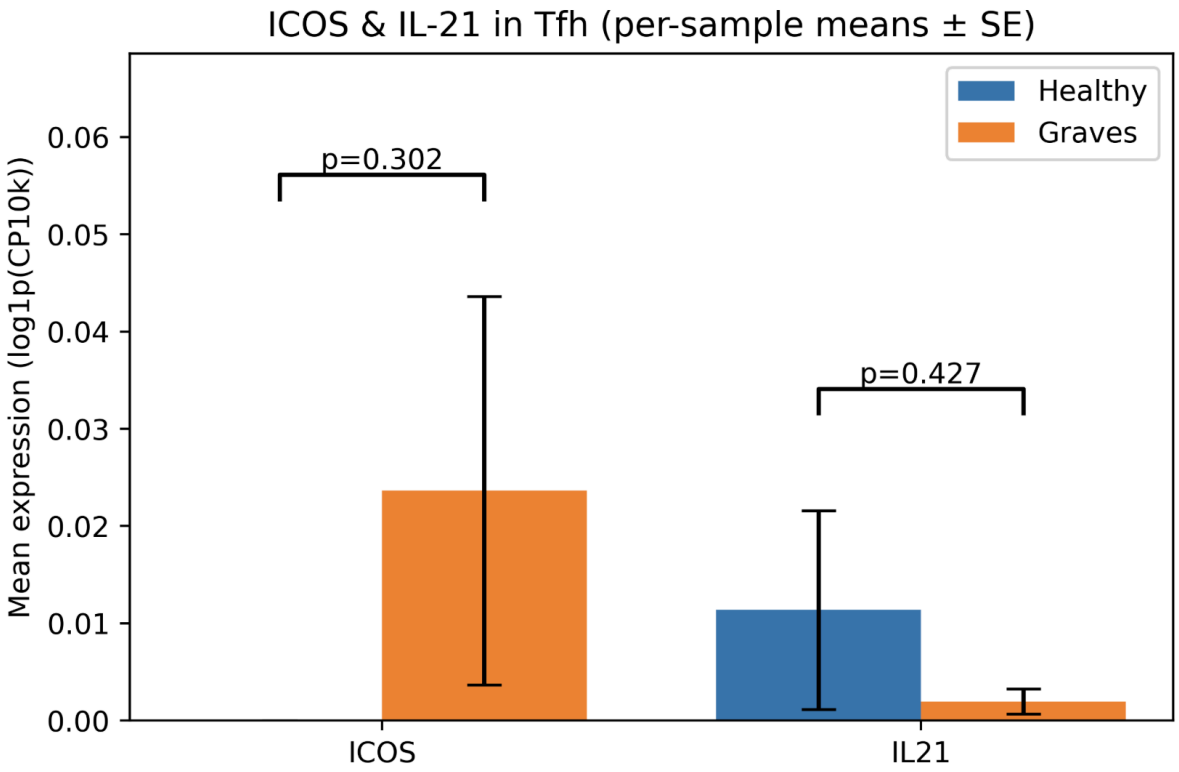


Fig. 1. Expression of ICOS and IL-21 in CD4⁺ Tfh cells from Graves' disease (GD) and healthy thyroids. Bar plots show normalized mean expression per sample (mean ± SEM). Brackets indicate the Healthy vs GD comparison for each gene; p-values are from a Welch's two-sample t-test performed on per-sample mean expression values (Healthy n=4, GD n=5).

B-cell activation in Graves' disease vs healthy controls

Because earlier analyses suggested heterogeneity in ICOS expression across Graves' disease (GD) donors, the GD cohort was stratified into two groups based on whether T-follicular-helper (Tfh) cells showed elevated ICOS expression (Tfh ICOS⁺) or not (Tfh ICOS⁻) (Fig. 2). This grouping was used to test whether variation in Tfh activation across GD individuals tracked with broader immune activity in the thyroid microenvironment, measured here as a per-sample B-cell activation index. Across the three groups (Healthy, Tfh ICOS⁻, Tfh ICOS⁺), ANOVA yielded $p = 0.0013$. Tukey HSD comparisons showed similar B-cell activation between Healthy and Tfh ICOS⁻ donors ($p = 0.935$), while Tfh ICOS⁺ donors exhibited higher B-cell activation than both Healthy ($p = 0.0018$) and Tfh ICOS⁻ donors ($p = 0.0019$). Overall, B-cell activation in GD thyroid tissue clustered most strongly in individuals with elevated Tfh-cell ICOS expression.

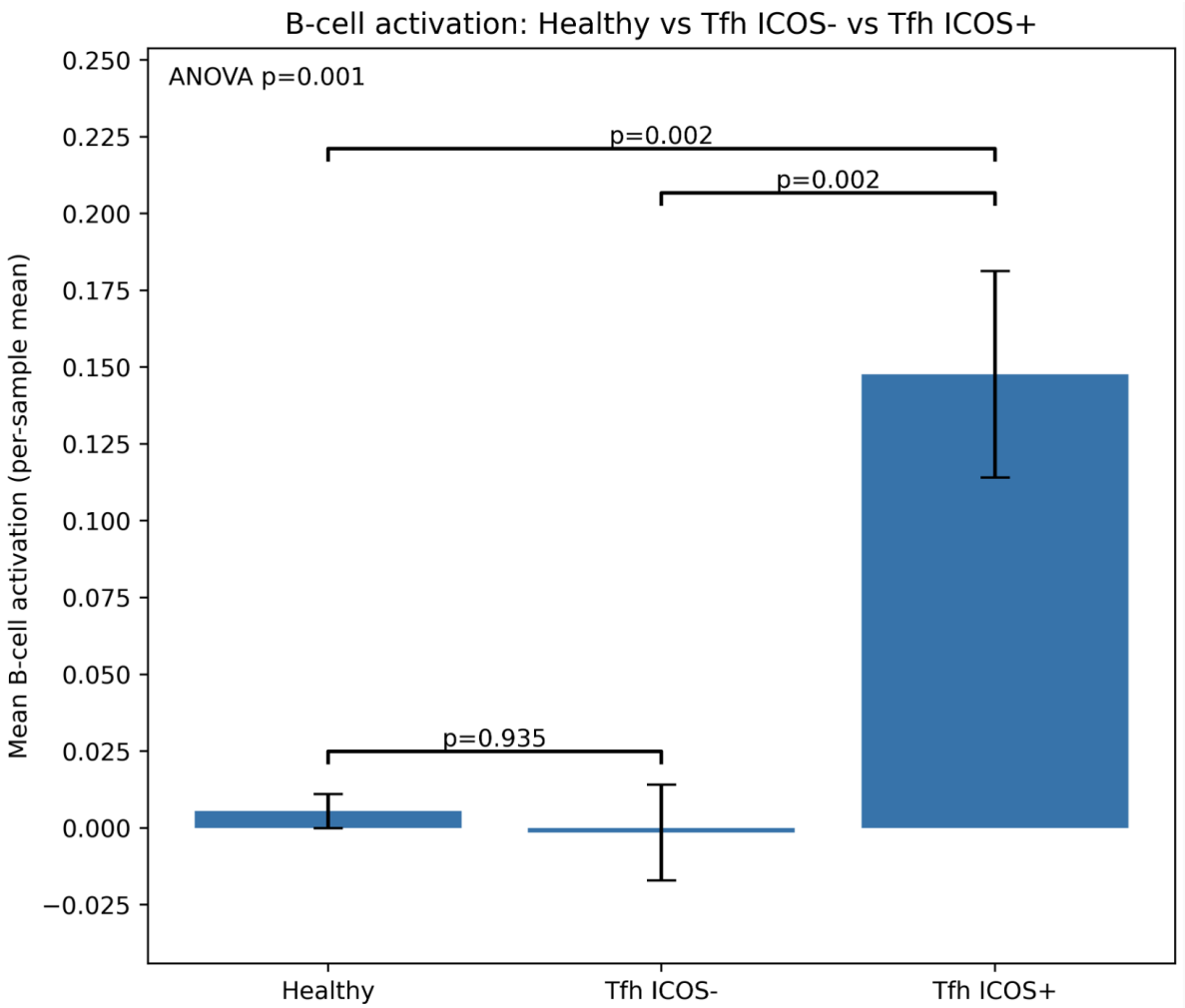


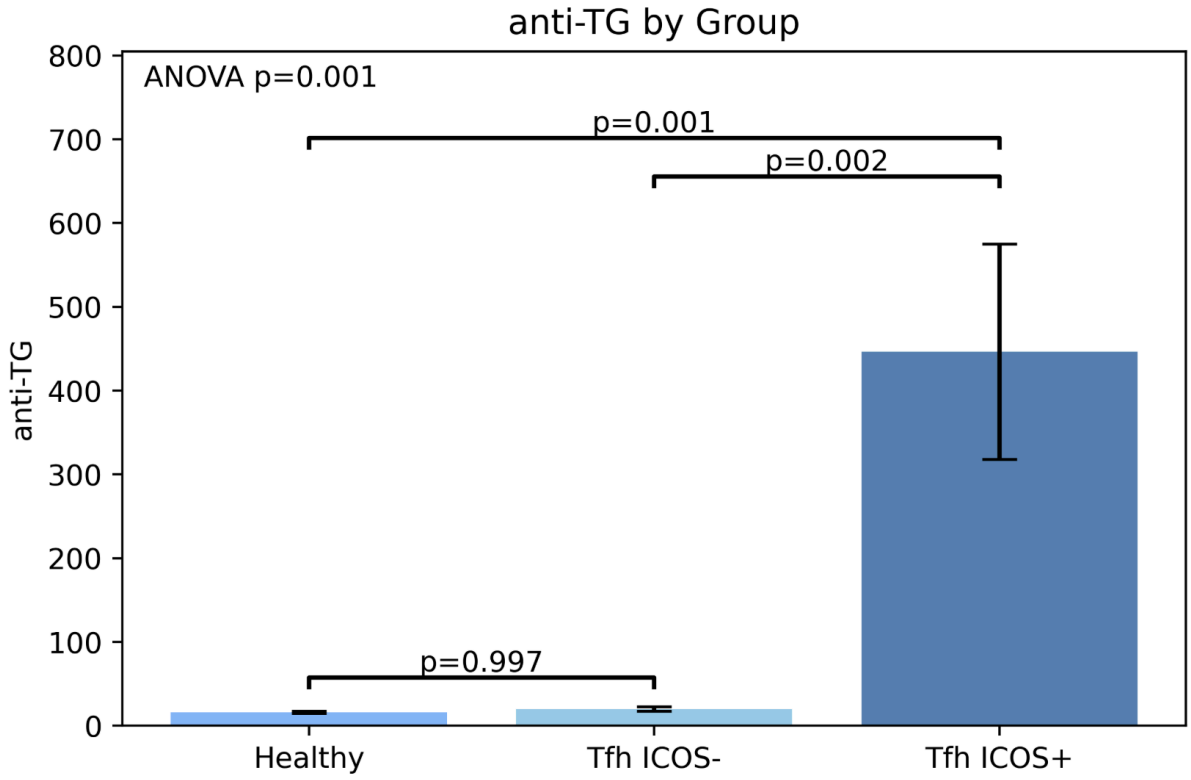
Fig. 2. B-cell activation across Healthy (n = 4), Tfh ICOS⁻ (n = 3), and Tfh ICOS⁺ (n = 2) donor groups. Bars show per-sample mean B-cell activation index (mean ± SEM). Overall group differences were assessed by one-way ANOVA (p = 0.0013), followed by Tukey HSD pairwise comparisons (Healthy vs Tfh ICOS⁻ p = 0.935; Healthy vs Tfh ICOS⁺ p = 0.0018; Tfh ICOS⁻ vs Tfh ICOS⁺ p = 0.0019).

Association between ICOS⁺ status and circulating anti-TPO and anti-TG antibodies

To evaluate systemic correlations of ICOS-driven activity, anti-TPO and anti-TG antibody titers, which serve as markers of thyroid-directed autoimmunity, were compared among healthy controls, Tfh ICOS⁻ GD donors, and Tfh ICOS⁺ GD donors (Fig. 3). A one-way ANOVA across these three groups yielded p = 0.0289 for anti-TPO. Post-hoc testing showed that Tfh ICOS⁺ donors exhibited higher anti-TPO levels than healthy controls (p = 0.0249), whereas Tfh ICOS⁻ donors were similar to the control group (p = 0.618). The comparison between Tfh ICOS⁺ and Tfh ICOS⁻ donors yielded p = 0.084.

For anti-TG, a separate one-way ANOVA across the same three groups yielded p = 0.0011. Pairwise comparisons indicated that Tfh ICOS⁺ donors had elevated anti-TG titers relative to

both healthy controls (p = 0.0013) and Tfh ICOS$^-$ donors (p = 0.0018), while Tfh ICOS$^-$ and healthy donors did not differ (p = 0.997). Together, these findings suggest that increased ICOS expression within the Tfh compartment of GD donors corresponds with higher systemic anti-thyroid antibody levels, linking local T-cell co-stimulatory signaling to enhanced autoimmune activity.



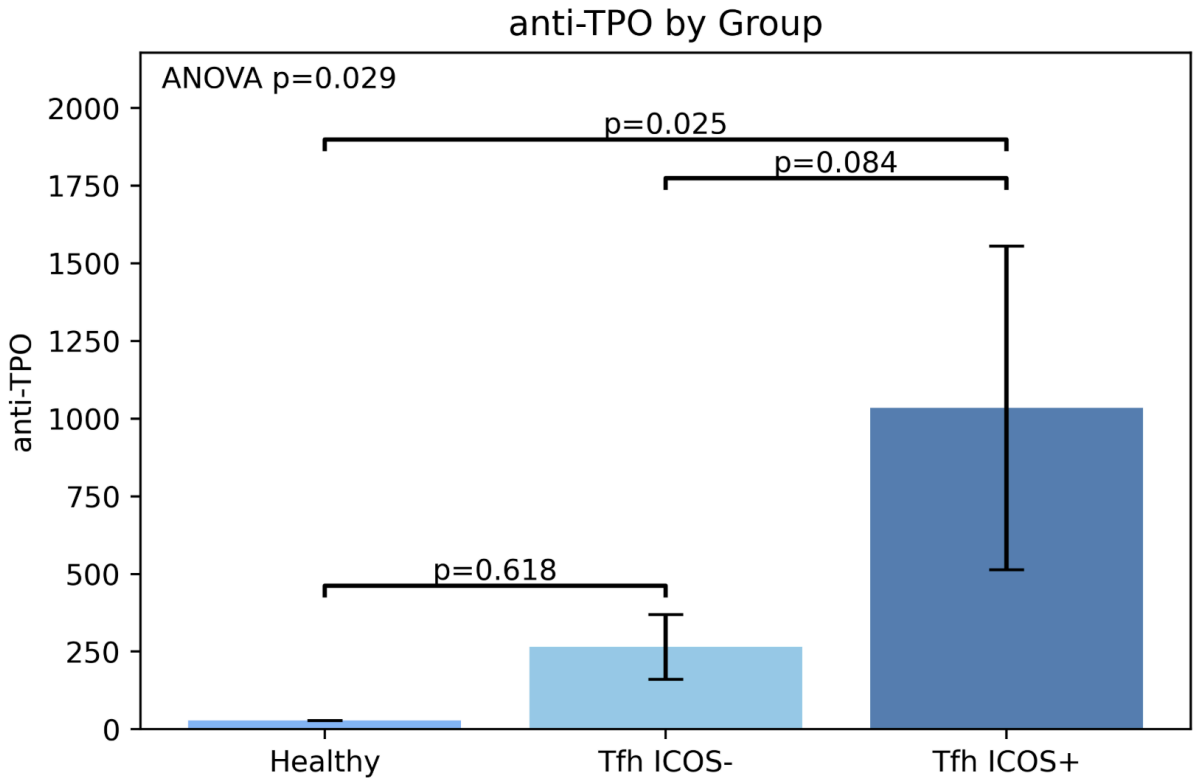


Fig. 3. Serum anti-TPO and anti-TG titers in healthy controls (n = 4), Tfh ICOS⁻ (n = 3), and Tfh ICOS⁺ (n = 2) groups. Bars show group means ± SEM for anti-TG (top) and anti-TPO (bottom). One-way ANOVA was followed by Tukey HSD post-hoc testing; p-values are shown above brackets.

Discussion

The present analysis investigated thyroidal immune interactions in Graves' disease (GD) by integrating single-cell expression of ICOS and IL-21 with a thyroid B-cell activation index and circulating anti-thyroid antibody titers. Across donors, mean Tfh-cell ICOS and IL21 expression did not clearly separate GD from healthy controls. However, stratifying GD donors by elevated Tfh-cell ICOS expression identified a subset with higher intrathyroid B-cell activation and higher circulating anti-TG and anti-TPO titers than both Tfh ICOS⁻ donors and healthy controls. A key strength of this approach is its cell-type-specific resolution, which allows immune signaling to be evaluated within defined compartments rather than averaged across bulk tissue.[11] In addition, stratifying GD donors based on elevated Tfh-cell ICOS expression provides a practical way to capture meaningful heterogeneity within the disease group and test whether differences in T-cell activation align with downstream B-cell responses.[6]

Despite variation across donors, several consistent patterns emerged. GD samples with high Tfh-cell ICOS expression tended to show higher mean B-cell activation and elevated anti-TG and anti-TPO titers relative to both Tfh ICOS⁻ GD donors and healthy controls. This pattern aligns with prior evidence that ICOS-mediated co-stimulation supports T follicular helper activity and downstream B-cell responses.[5,6] In earlier studies, this link was shown through experimental

disruption of the ICOS pathway in mouse models and through human ICOS-deficiency immunophenotyping, which revealed a marked reduction in $CXCR5^{+}CD4^{+}$ germinal-center helper T cells; in contrast, the present analysis evaluates co-variation between Tfh ICOS expression, intrathyroid B-cell activation, and serum autoantibody titers in GD thyroid tissue.[4,20] Together, the parallel increase in thyroidal activation markers and systemic autoantibody levels supports a model in which intensified local T–B cell interactions in the thyroid contribute to the broader autoimmune phenotype characteristic of GD.[3]

This study adds to existing literature by linking local immune activation states to systemic antibody readouts at single-cell resolution. Prior work, including Álvarez-Sierra et al. (2023), established broad immune infiltration and cellular composition in GD thyroid tissue.[1] Building on that foundation, the present analysis emphasizes quantitative relationships between $ICOS^{+}$ Tfh activity, B-cell activation, and circulating anti-thyroid antibodies, helping clarify how variation in co-stimulatory signaling within the thyroid may map onto systemic autoimmune activity.[1,3]

At the same time, several limitations shape how strongly these findings can be generalized. The number of available donor datasets was small, and subdividing GD donors into Tfh $ICOS^{+}$ and Tfh $ICOS^{-}$ groups further reduced group sizes, which increases sensitivity to donor-specific effects and inflates uncertainty around group means. High within-group dispersion also reduced the detectability of differences that appeared visually prominent, such as elevated ICOS expression in GD thyroid tissue. More broadly, this reflects a recurring challenge in autoimmune single-cell studies: substantial biological heterogeneity between individuals can obscure reproducible signals when sample size is constrained.[11]

Future work should therefore prioritize both replication and validation using independent experimental approaches. A larger and more diverse cohort of healthy and GD thyroid samples would improve power, better characterize variability across disease presentations, and test whether the ICOS-stratified patterns observed here persist across donors. In parallel, validating these relationships at the protein and tissue-organization level would strengthen the biological interpretation. For example, single-cell multi-omic approaches that jointly measure surface proteins and transcripts (such as CITE-seq) could directly test whether elevated ICOS mRNA corresponds to elevated ICOS protein within Tfh cells.[17] In addition, spatial transcriptomics could be used to map where $ICOS^{+}$ Tfh cells and activated B cells localize within thyroid tissue and whether these signals co-occur in organized immune aggregates resembling tertiary lymphoid structures or germinal-center–like regions.[13,21] Together, these follow-ups would help determine whether the observed ICOS-associated signature reflects a robust disease axis and would move the analysis closer to a mechanistic understanding of how local co-stimulatory pathways relate to systemic autoantibody production in Graves' disease.

The scRNA-seq dataset analyzed in this study was generated by Álvarez-Sierra et al. and was accessed upon request from the authors. A detailed description of processing steps, parameters, and software versions is provided in the Methods, and code can be shared upon request.

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