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Supplementary Materials for

Recurrent group A *Streptococcus* tonsillitis is an immunosusceptibility disease involving antibody deficiency and aberrant T_{FH} cells

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at www.sciencetranslationalmedicine.org/cgi/content/full/11/478/eaau3776/DC1)

Data file S1 (Microsoft Excel format). Primary data.

Materials and Methods

Tonsils: Fresh palatine tonsils were collected at the time of surgery, at least 6 weeks after the last episode of tonsillitis, with most cases substantially further from the last episode of tonsillitis and antibiotic treatment. Tonsils were collected in RPMI and stored at 4ºC till they were picked up and processed. Tonsils were processed that same day with an average amount processing time of 2 hours. See Study Design for further details.

Adult Lymph Nodes. Fresh lymph nodes were acquired from patients undergoing staging sentinel lymph node biopsy for early-stage breast cancer at University Hospital Southampton, United Kingdom, in whom said staging analysis demonstrated the absence of lymphatic metastasis. All patients had provided informed consent for tissue donation for the purpose of clinical research study (UKCRN ID: 11947) according to protocols approved by The National Research Ethics Service following regional ethics committee review (South Central England).

Cell processing. Tonsillar mononuclear cells were obtained by homogenizing the tissue using a wire mesh, passage through a cell strainer, and isolation via Ficoll density gradient using Histopaque 1077 (Sigma). Whole blood was obtained from La Jolla Institute for Immunology's (LJI) in-house normal blood donor program. Informed consent was obtained from all donors under protocols approved by LJI's IRB VD-057. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque 1077. Plasma was saved after density gradient centrifugation. Cells were washed and suspended in fetal bovine serum (FBS) containing 10% dimethyl sulfoxide, and cryopreserved in liquid nitrogen.

Single cell suspensions of lymph node-derived cells were obtained from freshly excised axillary and mesenteric nodes following enzymatic digest (0.15 Wünsch units/ml Liberase DL (Roche), 800 Kunitz units/ml DNAse I (Sigma)) over 1 hour at 37°C followed by passage through a wire mesh and 70μm cell strainer (BD Falcon). Cells were suspended in complete RPMI 1640 (cRMPMI; Gibco + 25 mM HEPES (Sigma), Penicillin/Streptomycin (Sigma), L-Glutamine (Sigma), sodium pyruvate (Gibco)) and cryopreserved (50% decomplemented human Ab serum (Sigma), 10% Dimethyl Sulfoxide (Sigma)) in liquid nitrogen until use.

Antibodies and flow cytometry. Cells were labeled with fixable viability dye eFluor 780 (Thermo Fisher Scientific). FACS staining buffer consisted of 0.5% Bovine serum albumin (BSA) in phosphate buffered saline (PBS). Primary stains and flow cytometry for leukocyte phenotyping (**Fig. 1A**) were done using fresh cells. Anti-human antibodies for surface staining of fresh tonsils are listed here, by company. Thermo Fisher Scientific: CD19 e780 (clone HIB19), CD14 e780 (clone 61D3), CD16 e780 (clone eBioCB16), CD3 e780 (clone UCHT1), CD25 PE-Cyanine 7 (clone BC96), PD-1 PE (clone eBioJ105), CD38 PE-cyanine 7 (clone HIT2), ICOS PerCP-eFluor710 (clone ISA-3), CD27 PerCP-eFluor710 (clone O323), CD45RO FITC (clone UCHL1); Biolegend: CD20 BV570 (clone 2H7), CD19 AF700 (clone HIB19), CXCR5 BV421 (clone J252D4); BD Biosciences CD3 AF700 (clone UCHT1) and CD4 APC (clone RPA-T4) (**table S2**). Total cell numbers are not available, since part of the tonsil is always retained by the Pathology Department as fixed tissue for diagnostic purposes. For cohort 2, usable flow cytometry was not available from all fresh specimens and thus a few data points are absent. Cells were acquired on a BD Fortessa and analyzed using FlowJo Software, version 9.9.6.

Antibodies used for AIM assays are listed here, by company. Thermo Fisher Scientific: CD19 e780 (clone HIB19), CD14 e780 (clone 61D3), CD16 e780 (clone eBioCB16), OX40 FITC (clone Ber-ACT35), CD25 PE-Cyanine 7 (clone BC96), CD4 PerCP-eFluor710 (clone SK3); Biolegend: CD45RA BV570 (clone HI100), CXCR5 BV421 (clone J252D4), PD-1 BV785 (clone EH12.2H7), PD-L1 PE (clone 29E.2A3), CCR7 APC (clone G043H7) (**table S3**). Cells were acquired on a BD Fortessa and analyzed using FlowJo Software, version 9.9.6.

Antibodies for the proliferation assay using HLA class II cell lines are listed here, by company. Thermo Fisher Scientific: OX40 FITC (clone Ber-ACT35), CD25 PE-Cyanine 7 (clone BC96), CD4 PerCP-eFluor710 (clone SK3); Biolegend: PD-1 BV785 (clone EH12.2H7), PD-L1 PE (clone 29E.2A3) (**table S4**). Annexin V APC Apoptosis Detection kit was utilized (Thermofisher). Cells were acquired on a BD Fortessa and analyzed using FlowJo Software, version 9.9.6.

Antibodies for the granzyme B assays are listed here, by company. Thermo Fisher Scientific: CD19 e780 (clone HIB19), CD14 e780 (clone 61D3), CD16 e780 (clone eBioCB16), OX40 PE (clone Ber-ACT35), CD25 PE-Cyanine 7 (clone BC96), CD4 PerCP-eFluor710 (clone SK3); Biolegend: CD45RA BV570 (clone HI100), CXCR5 BV421 (clone J252D4), PD-1 BV785 (clone EH12.2H7), Granzyme B Alexa Fluor 647 (clone GB11), and Alexa Fluor 647 Mouse

IgG1, κ Isotype Control (clone MOPC-21) (**table S5**). Cells were acquired on a BD Fortessa and analyzed using FlowJo Software, version 9.9.6.

Antibodies for sorting GC-T_{FH} and B cells are listed here, by company. Thermo Fisher Scientific: CD19 e780 (clone HIB19), CD14 e780 (clone 61D3), CD16 e780 (clone eBioCB16), CD8 e780 (clone RPA-T8), CD4 PerCP-eFluor710 (clone SK3), CD38 APC (clone HIT2); Biolegend: CD45RA BV570 (clone HI100), CXCR5 BV421 (clone J252D4), PD-1 BV785 (clone EH12.2H7), CCR7 BV650 (clone G043H7), CD20 BV570 (clone 2H7) (**table S6**). Cells were sorted on the BD FACSAria III or BD FACSAria Fusion. Cells were acquired on a BD Celesta and analyzed using FlowJo 9.9.6.

Antibodies for staining after a 5 day *in vitro* culture are listed here by company. Thermo Fisher Scientific: CD4 PerCP-eFluor710 (clone SK3), OX40 PE (clone Ber-ACT35), CD25 PE-Cyanine 7 (clone BC96), Biolegend: CD45RA BV570 (clone HI100), CXCR5 BV421 (clone J252D4), PD-1 BV785 (clone EH12.2H7), CD20 BV570 (clone 2H7), Granzyme B Alexa Fluor 647 (clone GB11), Perforin FITC (clone B-D48) (**table S7**). Cells were acquired on a BD Celesta and analyzed using FlowJo Software, version 9.9.6.

Antibodies for sorting for the cytotoxicity assay are listed here, by company. Thermo Fisher Scientific: CD14 e780 (clone 61D3), CD16 e780 (clone eBioCB16), CD4 PerCP-eFluor710 (clone SK3), CD8 PeCy7 (RPA-T8), PD-1 PE (clone eBioJ105), CD38 APC (clone HIT2), CD19 AF488 (HIB19); Biolegend: CCR7 BV650 (clone G043H7), CXCR5 APC (clone J252D4); BD Biosciences: CD45RA PE-CF594 (clone H100) (**table S8**). Cells were sorted on the BD FACSAria III or BD FACSAria Fusion. Cells were acquired on a BD Celesta and analyzed using FlowJo Software, version 9.9.6.

Histology. A small section was taken from each tonsil, fixed in 10% zinc formalin fixative for 24 hours at room temperature and transferred to 70% ethanol. For each tonsil, the LJI microscopy core prepared a paraffin embedded section and an H&E stain. Slides were viewed using a Nikon Eclipse 80i. Images of three different locations on the same slide were taken (10X objective) and averaged per tonsil. The number of GCs and GC area were determined using ImageJ (NIH). Immunohistochemistry was performed by HistoTox Labs, Inc. Each tissue was sectioned, mounted on a slide, and stained separately for CD20 (clone H1), Ki67 (clone SP6), CD4 (cloneEPR6855), and PD-1 (clone D4W2J).

Immunofluorescence microscopy. A small section was taken from each tonsil, fixed in in 4% paraformaldehyde at 4°C for 2 hours, washed three times in PBS for 10 minutes each wash and placed in a 30% sucrose gradient for at least 18 hours at 4°C until the tissue sunk. The tissue section was washed in PBS and embedded in OCT compound using methylbutane and liquid nitrogen. Embedded tissues samples were stored at -80°C. Tissue sections were prepared by the LJI Microscopy core. For staining, slides were dried on the grill of the tissue culture hood for 30 minutes, washed in PBS x 2 for 10 minutes, and blocked with 10% FBS containing 0.5% Triton X-100 for 1 hour at room temperature. Antibodies were from Biolegend: CD4 Alexa Fluor 488 (clone RPA-T4) and Granzyme B Alexa Fluor 647 (clone GB11) and Isotype Control Alexa Fluor 647. Slides were stained overnight at 4°C. The next morning, slides were washed in PBS x 2 x 10 minutes, counterstained with Hoechst 3342 for 10 minutes, and washed in PBS x 2 x 10 minutes. Slides were then mounted in Prolonged Gold. Slides were visualized using Olympus FluoView FV10i Confocal.

HLA typing. Genomic DNA was isolated from frozen tonsillar mononuclear cells using standard techniques (REPLI-g, Qiagen). Typing was performed at Murdoch University (Perth, Western Australia) *(59)*. We performed typing on the entire cohort (Cohot 1 + Cohort 2); however, a small numbers of samples did not have sufficient DNA amplification to yield HLA results. HLA typing for normal healthy donors from the San Diego area was obtained from the Sette lab *(60, 61)*.

Superantigen binding assay. Recombinant SpeA produced in *E. coli* (Toxin Technology) was biotinylated using an EZ-Link Sulfo-NHS Biotinylation kit (Thermofisher) following manufacturer's protocol. Biotinylated recombinant SpeA was incubated for 30 minutes at 4°C in FACS buffer using cell lines expressing different HLA class II receptors *(62, 63)*. DQB1*03:02 and DQB1*06:02 were expressed on the RM3 line. Cells were washed twice in FACS buffer. Streptavidin Alexa Fluor 647 (Biolegend) was used as a secondary stain at 1:1000 for 15 minutes on ice. Cells were also labeled with fixable viability dye eFluor 780 (Thermo Fisher Scientific). Cells were fixed in 2% paraformaldehyde and acquired on BD FACS LSRII. Data were analyzed using FlowJo 9.9.4 and histograms generated using FlowJo 10.2.

Superantigen stimulation assay. Antigen Presenting Cells (APCs): HLA class II cell lines, obtained from the Sette lab (LJI) *(62, 63)*, were cultured in R10 media containing RPMI, penicillin/streptomycin, L-Glutamax, 10% FBS, MEM non-essential amino acids, and sodium pyruvate. For HLA DRB1-expressing L cell lines, the selection media included 200μg/mL G418. Prior to use of the HLA DRB1 cell line, 100μg/mL butyric acid was added overnight to induce expression of the HLA DRB1 receptor. For HLA DQB1-expressing RM3 cell lines, the selection media included 12 μg/mL blasticidin + 700 μg/mL G418. The number of APCs was optimized in pilot experiments, using 5,000 cells per well of DRB1-expressing cell lines and 25,000 cells per well of DQB1-expressing cell lines. APCs were irradiated in a 96 well flat bottom tissue culture plate. $CD4^+$ T cells: Cryopreserved PBMCs containing the HLA receptor of interest were thawed and purified using the EasySep Human $CD4^+$ T cell enrichment kit, according to manufacturer's protocol to 95 to 98% purity. $CD4^+$ T cells were Cell Trace Violet (CTV) labeled and cultured at 100,000 cells per well. rSpeA was added to the well at different concentrations. As a control, $CD4^+$ T cells alone were incubated with rSpeA in media consisting of RPMI + 10% Human AB sera (Off the clot, Gemini) + penicillin/streptomycin + L-Glutamax. After 5 days, cells were analyzed for upregulation of activation marker OX40 and CTV.

Antigen-specific $CD4^+$ T cell assays. Tonsillar mononuclear cells were cultured at $1x10^6$ cells/well in AIM-V media in a 96 well round bottom plate for 18 hours. For the GAS-specific $CD4^+$ T cell assay, cells were left unstimulated or stimulated with 10 μg/mL heat-inactivated, antibiotic-killed GAS *(22)*. As a comparison, cells were also stimulated with 10 μg/mL antibiotic-killed GAS or 10 μg/mL antibiotic-killed GAS deficient in SpeA, all from the same strain. The Nizet laboratory (UCSD) provided GAS strain M1T1 5448, originally isolated from a patient with necrotizing fasciitis and toxic shock syndrome *(64)*. A nonpathogenic Streptococcaceae, *Lactococcus lactis* NZ9000 *(65)*, was used as a negative control. For the AIM assay, tonsillar cells were stimulated with 10 μg/mL antibiotic-killed *Lactococcus*. Bacteria were cultured in 100 mL Todd-Hewitt broth (Difco) statically at 37°C to OD600 0.6. Tissue culture grade penicillin/streptomycin (Invitrogen) was added to 1% and incubated for 1 hour. Cells were pelleted by centrifugation for 10 min at 4000xg, washed once and suspended in PBS. Samples were plated on Todd-Hewitt agar to confirm bacterial killing *(66)*. Total protein was quantified

by bicinchonic acid assay (Pierce) for use as antigen. To inactivate superantigen, antibiotic-killed GAS was heat-treated at 65°C for 20 min. For the SpeA AIM assay,1 μg/mL of rSpeA was used as stimulus.

Intracellular cytokine staining for granzyme B expression. Tonsillar mononuclear cells were cultured at $1x10^6$ cells/well in AIM-V media in a 96 well round bottom plate for 24 hours. Cells were either left unstimulated or stimulated with 1 μg/mL SpeA (Toxin Technology). At 20 hours, GolgiPlug was added prior to harvesting the cells at 24 hours for analysis, according to manufacturer's protocol (BD Biosciences). Cells were permeabilized using the BD Cytofix/Cytoperm kit for intracellular cytokines.

Intranuclear staining. Tonsillar mononuclear cells were cultured at $1x10^6$ cells/well in AIM-V media in a 96 well round bottom plate for 24 hours. Cells were permeabilized using the eBioscience Transcription Factor buffer staining set (Thermofisher). FOXP3 PE (clone 236A/E7, Thermofisher) and HELIOS PE/Dazzle (clone 22F6, Biolegend) were used.

RNA sequencing. Tonsillar mononuclear cells were cultured at $1x10^6$ cells/well in AIM-V media in a 96 well round bottom plate for 18 hours. Cells were stained using antibodies listed in Supplementary Table 2. Cells were sorted on the BD FACSAria III or BD FACSAria Fusion for $CD25^+OX40^+$ GC-T_{FH} cells. From 10 donors, cell numbers obtained ranged from 10^4 to 10^5 cells. As described previously, total RNA was purified using a miRNAeasy micro kit (Qiagen) and quantified *(67)*. Standard quality control steps were included to determine total RNA quality using Agilent Bioanalyzer (RNA integrity number (RIN) > 8.5; Agilent RNA 6000 Pico Kit). Purified total RNA (0.25 to 5 ng) was amplified following the Smart-Seq2 protocol. cDNA was purified using AMPure XP beads (1:1 ratio; Beckman Coulter). From this step, 1 ng cDNA was used to prepare a standard Nextera XT sequencing library (Nextera XT DNA sample preparation kit and index kit; Illumina). Samples were sequenced using a HiSeq2500 (Illumina) to obtain 50-bp single-end reads. Both whole-transcriptome amplification and sequencing library preparations were performed in a 96-well format to reduce assay-to-assay variability. Quality control steps were included to determine total RNA quality and quantity, including the optimal number of PCR preamplification cycles (15 cycles), and fragment size selection. Samples that failed quality

control were eliminated from further downstream steps. Barcoded Illumina sequencing libraries (Nextera; Illumina) were generated utilizing the automated platform (Biomek FXp). Libraries were sequenced on the HiSeq2500 Illumina platform to obtain 50-bp single-end reads (TruSeq Rapid Kit; Illumina), generating a median of ~13.6 million mapped 50 bp reads per sample).

RNA-Seq analysis. The single-end reads that passed Illumina filters were filtered for reads aligning to tRNA, rRNA, adapter sequences, and spike-in controls. The reads were then aligned to UCSC hg19 reference genome using TopHat (v 1.4.1) *(68)*. DUST scores were calculated with PRINSEQ Lite (v 0.20.3) and low-complexity reads (DUST > 4) were removed from the BAM files. The alignment results were parsed via the SAMtools to generate SAM files. Read counts to each genomic feature were obtained with the htseq-count program (v 0.6.0) using the "union" option. After removing absent features (zero counts in all samples), the raw counts were converted to RPKM values and filtered by setting a cutoff value of 1. Multiplot Studio in the GenePattern suite (http://www.broadinstitute.org/cancer/software/genepattern/) was employed to generate the volcano plot with RPKM values (**Fig. 6A**). The raw counts were then imported to R/Bioconductor package DESeq2 to identify differentially expressed genes among conditions. DESeq2 normalizes counts by dividing each column of the count table (samples) by the size factor of this column. The size factor is calculated by dividing the samples by geometric means of the genes. This brings the count values to a common scale suitable for comparison. P-values for differential expression are calculated using Wald test that estimates the significance of coefficients in a fitted negative binomial generalized linear model (GLM). These p-values are then adjusted for multiple test correction using Benjamini Hochberg algorithm to control the false discovery rate. Cluster analyses including principal component analysis (PCA) and hierarchical clustering were performed using standard algorithms and metrics. Hierarchical clustering was performed using complete linkage with Euclidean metric. TPM values were used to generate supplemental figures 3C, 6, and 8. The RNA-seq data are available in the Gene Expression Omnibus (GEO) database under the accession number GSE123977.

Sorting GC-T_{FH} and B cells for granzyme B expression. Tonsillar mononuclear cells were sorted (antibodies listed in Supplementary Table 6) for GC-T_{FH} ($CXCR5^{hi}PD\text{-}1^{hi}$ of $CD45RA^{-}CD4^{+}$) and non-B_{GC} cells ($CD20^{+}CD38^{-}$) to serve as APCs. Cells were plated at 75,000 GC-T_{FH}

and 75,000 B cells in 96 well round bottom plates in media containing 10% human sera (RPMI + penicillin/streptomycin + L-glutamax) **+** IL-7 (final concentration 4ng/mL). Cells were either left unstimulated or stimulated with 1 μg/mL SpeA. After a 5 day *in vitro* culture, cells were harvested and stained for granzyme B (**table S7**).

Cytotoxicity assay. Tonsillar mononuclear cells were sorted (**table S8**) for GC-T_{FH} ($CXCR5^{hi}PD\text{-}1^{hi}$ of $CD45RA^{-}CD4^{+}$), mT_{FH} ($CXCR5^{+}PD\text{-}1^{+}$ of $CD45RA^{-}CD4^{+}$), non-T_{FH} ($CXCR5^{-}$ of $CD45RA^{-}CD4^{+}$), naive $CD4^{+}$ ($CCR7^{+}CD45RA^{+}$), and $CD8^{+}$ T cells as effector cells. Tonsillar mononuclear cells were also sorted for autologous $CD19^{+}CD38^{-}$ B cells to serve as target cells. B cells were labeled with CTV and cultured at a 2:1 ratio of 2 effector cells to 1 target cell in media containing 5% human sera (RPMI + penicillin/streptomycin + L-glutamax) *(69)*. Cells were plated at 50,000 target cells to 100,000 effector cells in 96 well round bottom plates. Cells were either left unstimulated or stimulated with 1 μg/mL SpeA. As a control, B cells were also left unstimulated or stimulated with 1 μg/mL SpeA. After 40 hours of incubation, cells were harvested and the number of CTV^{+} cells was quantified by flow cytometry *(70)*. Cells were plated at least in triplicate, depending on how many GC-T_{FH} cells were sorted from each tonsil. Killing capacity for GC-T_{FH} cells was determined by averaging the absolute counts of CTV-labeled B cells co-cultured with unstimulated GC-T_{FH} or naïve $CD4^{+}$ T cells = B. The absolute cell count of CTV-labeled B cells co-cultured with SpeA-stimulated effector cells was then determined for each well = A. Equation: % Killing Capacity = [1 – (A/B)]*100. In some experiments, a blocking antibody to Fas Ab (EMD Millipore, clone ZB4 or isotype control) or FasL Ab (R&D, clone 100419 or isotype control) were co-cultured during the cytotoxicity assay.

ImageStream. Images were acquired on a 2-camera ImageStream MkII imaging flow cytometer (Amnis, Seattle) with 60X objective and Inspire software version 200.1. The cytometer passed all ASSIST performance checks prior to image acquisition. FITC (Ch02, 480-560 nm), PE (Ch03, 560-595 nm) PE-CF594 (Ch04, 595-642 nm), PerCP-eFluor710 (Ch05, 648-745 nm) and PE-Cy7 (Ch06, 745-780 nm) were excited at 488nm (200 mW). BV421 (Ch07, 435-505 nm) and BV510 (Ch08, 505-570 nm) were excited at 405 nm (120 mW). APC (Ch11, 640-745 nm) and APC-eFluor780 (Ch12, 745-780 nm) were excited at 642 nm (150 mW). 10,000 single, in-focus, dump-negative, CD3-positive events were acquired per sample. Data were compensated and

analyzed with IDEAS software version 6.2 using the default masks and feature set.

ELISA. Plasma from RT and non-RT children was tested for IgG, Streptolysin O (SLO) IgG and SpeA IgG. Samples from cohort 2 were used, as that was the cohort for which blood samples were collected. To determine IgG titer, human IgG antibody was coated (1:5000 dilution in PBS) overnight. To determine SLO IgG titer, recombinant Streptolysin O (Abcam) produced in *E. coli* was coated at 1 μg/mL. To determine SpeA IgG titer, recombinant SpeA produced in *E. coli* (Toxin Technologies) was coated at 1 μg/mL. Plates were coated overnight at 4°C. PBS + 0.05% Tween was used for all washes. Plates were blocked with PBS containing 0.2% Tween and 1% BSA at room temperature for 90 minutes. For IgG, human IgG isotype control (Invitrogen) was used as a standard. For SpeA and SLO, pooled plasma from normal healthy human donors was utilized as a standard to establish relative units (RU) of SpeA and SLO IgG in RT and non-RT plasma. A monoclonal mouse anti-human IgG antibody conjugated to HRP (Hybridoma Reagents Laboratory) was used as the secondary antibody.

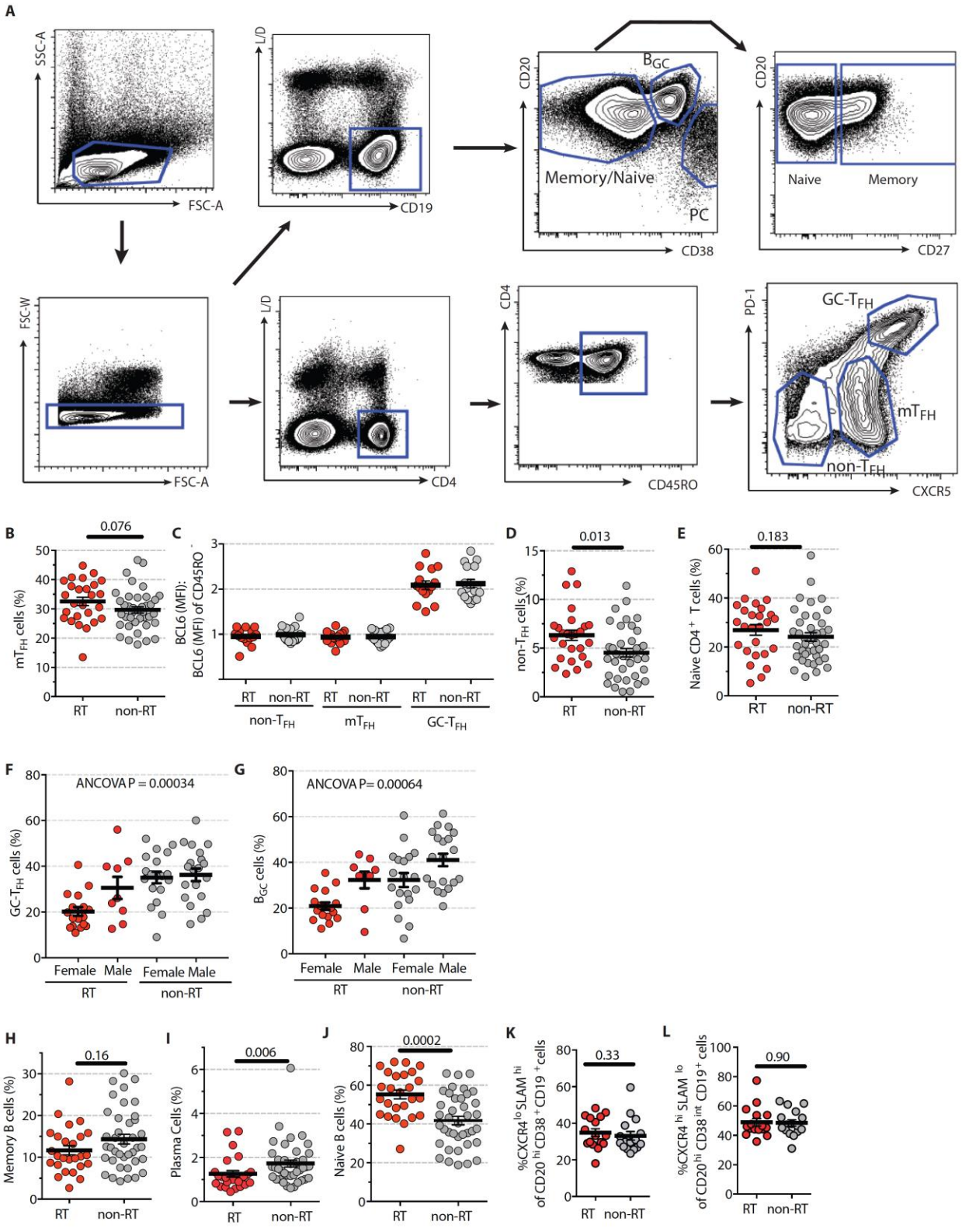


Figure S1. RT and non-RT tonsillar immunophenotyping of cohort 1. Immunophenotyping analysis of Cohort 1 of RT and non-RT patients. **(A)** Gating strategy for tonsillar $CD4^+$ T cells

and B cells. **(B)** mT_{FH} $CD4^+$ T cell ($CXCR5^+PD\text{-}1^+CD45RO^+CD4^+$) frequencies quantified as % of total $CD4^+$ T cells. **(C)** BCL6 expression by GC-T_{FH} cells from RT (n=15) and non-RT tonsils (n=16), P=0.98. The MFI of BCL6 for GC-T_{FH}, mT_{FH}, and non-T_{FH} was normalized to the MFI of BCL6 in $CD45RO^-$ $CD4^+$ T cells. **(D)** Non-T_{FH} $CD4^+$ T cell ($CXCR5^-CD45RO^+CD4^+$) frequencies quantified as % of total $CD4^+$ T cells. **(E)** Naive ($CD45RO^-CD4^+$) frequencies quantified as % of total $CD4^+$ T cells. **(F)** GC-T_{FH} cell frequencies by gender. **(G)** B_{GC} cell frequencies by gender. Statistical significance determined by ANCOVA. **(H)** Memory B cell ($CD27^+CD20^+CD19^+$) frequencies quantified as % of total $CD19^+$ B cells. **(I)** Plasma cell ($CD38^{hi}CD20^{hi}CD19^+$) frequencies in RT tonsils quantified as % of total $CD19^+$ B cells. **(J)** Naive B cell ($CD27^-CD20^+CD19^+$) frequencies quantified as % of total $CD19^+$ B cells. **(K)** Dark zone GC B cell ($CXCR4^{lo}SLAM^{hi}$) frequencies in B_{GC} as % of $CD20^{hi}CD38^+CD19^+$ cells. **(L)** Light zone GC B cell ($CXCR4^{hi}SLAM^{lo}$) frequencies in B_{GC} as % of $CD20^{hi}CD38^+CD19^+$ cells. Statistical significance determined by Mann-Whitney test (b-d, g-i, k-l).

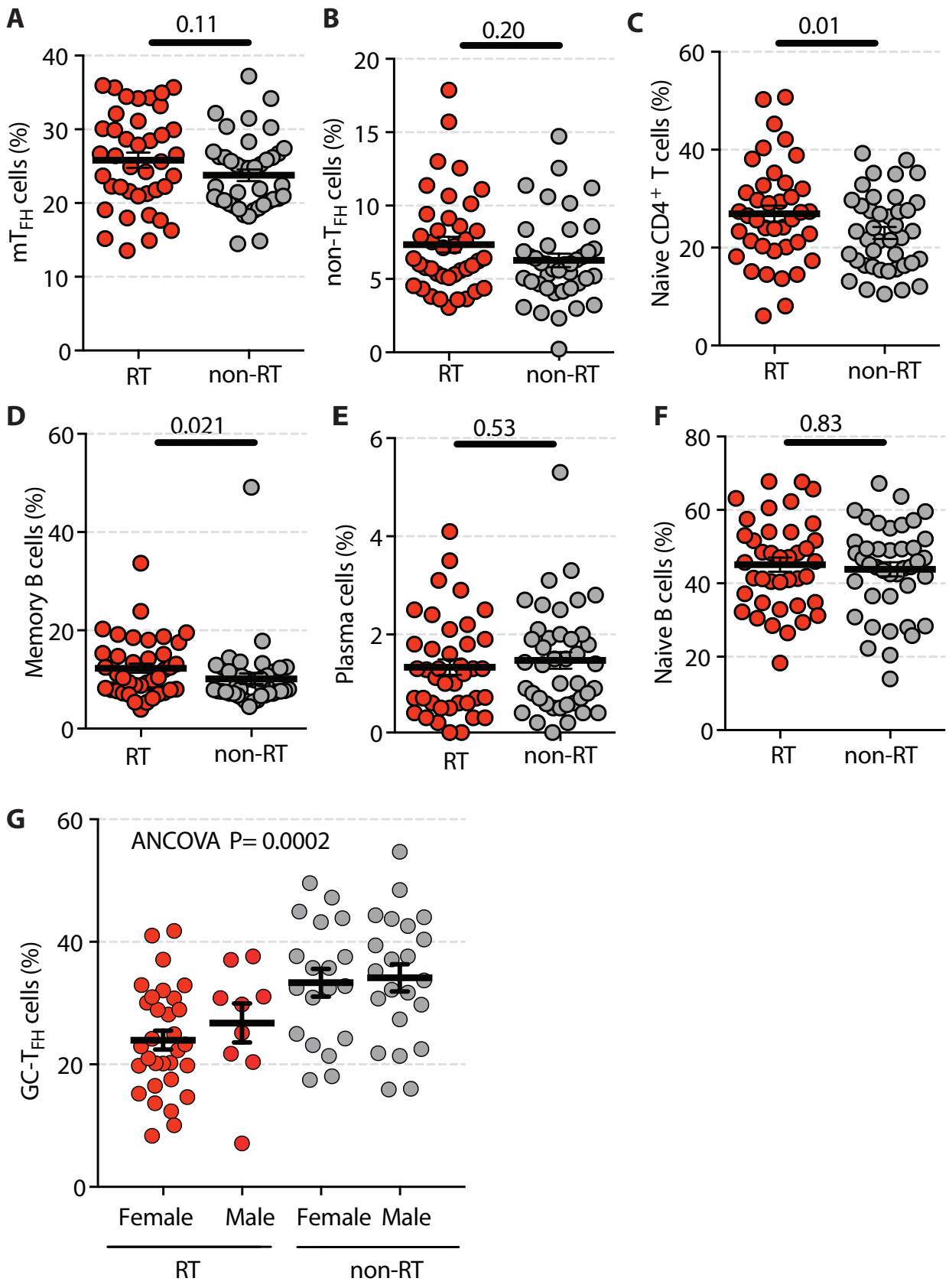

A
0.11
mT$_{FH}$ cells (%)
40
30
20
10
0
RT
non-RT
B
0.20
non-T$_{FH}$ cells (%)
20
15
10
5
0
RT
non-RT
C
0.01
Naive CD4$^+$ T cells (%)
60
40
20
0
RT
non-RT
D
0.021
Memory B cells (%)
60
40
20
0
RT
non-RT
E
0.53
Plasma cells (%)
6
4
2
0
RT
non-RT
F
0.83
Naive B cells (%)
80
60
40
20
0
RT
non-RT
G
ANCOVA P= 0.0002
GC-T$_{FH}$ cells (%)
60
40
20
0
Female
Male
Female
Male
RT
non-RT

Figure S2. RT and non-RT tonsillar immunophenotyping of cohort 2. Immunophenotyping analysis of cohort 2 of RT and non-RT patients. **(A)** mT_{FH} $CD4^+$ T cells ($CXCR5^+PD\text{-}1^+CD45RO^+CD4^+$) frequencies quantified as % of total $CD4^+$ T cells. **(B)** non-T_{FH} $CD4^+$ T cell ($CXCR5^-CD45RO^+CD4^+$) frequencies quantified as % of total $CD4^+$ T cells. **(C)** Naive ($CD45RO^-$) $CD4^+$ T cell frequencies quantified as % of total $CD4^+$ T cells. **(D)** Memory B cell ($CD27^+CD20^+CD19^+$) frequencies quantified as % of total $CD19^+$ B cells. **(E)** Plasma cell ($CD38^{hi}CD20^{hi}CD19^+$) frequencies quantified as % of total $CD19^+$ B cells. **(F)** Naive B cell ($CD27^-CD20^+CD19^+$) frequencies quantified as % of total $CD19^+$ B cells. **(G)** GC-T_{FH} frequencies by gender. Statistical significance determined by ANCOVA.
Statistical significance determined by Mann-Whitney test (a-f).

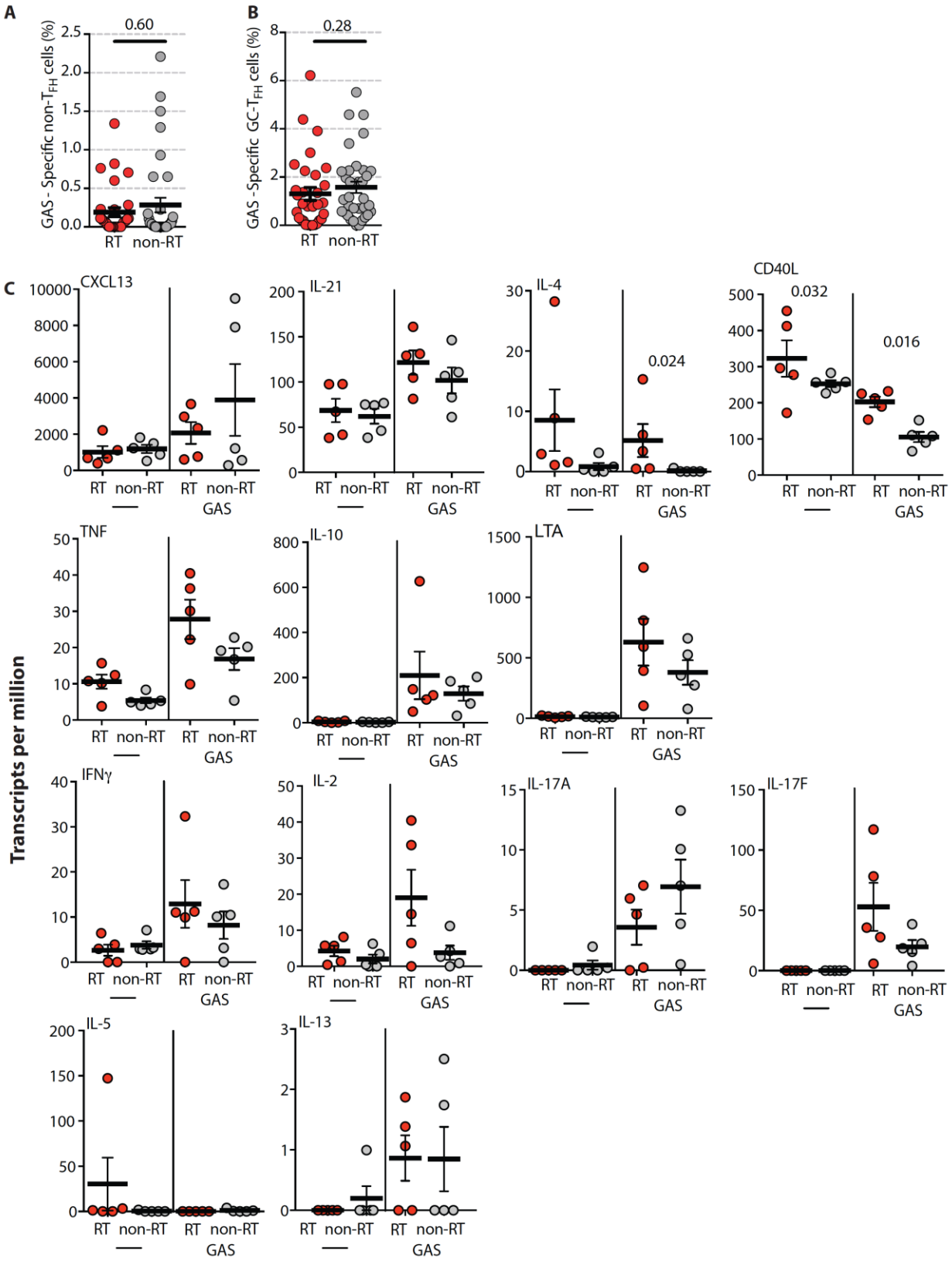
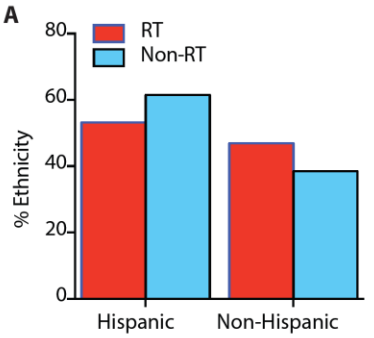

A
GAS - Specific non-T$_{FH}$ cells (%)
0.60
RT non-RT
B
GAS -Specific GC-T$_{FH}$ cells (%)
0.28
RT non-RT
C
Transcripts per million
CXCL13
IL-21
IL-4
0.024
CD40L
0.032
0.016
TNF
IL-10
LTA
IFNγ
IL-2
IL-17A
IL-17F
IL-5
IL-13
RT non-RT RT non-RT
GAS

Figure S3. GAS-specific $CD4^+$ T cells by AIM assay. (A) GAS-specific non-T_{FH} cell frequencies, quantified as % of total $CD4^+$ T cells, between RT (n=31) and non-RT (n=35) tonsils. **(B)** GAS-specific GC-T_{FH} cell frequencies, quantified as % of total $CD4^+$ T cells, between RT (n=31) and non-RT (n=35) tonsils. **(C)** Cytokine and chemokine transcript expression of sorted unstimulated (AIM^-) and GAS-specific (AIM^+) GC-T_{FH} cells determined by RNA-seq from RT (n=5) and non-RT (n=5) tonsils. Statistical significance determined by Mann-Whitney test (a-c).



B

HLA Allele	RT		Non-RT		*P*	GP		*P*	Non-RT+GP		*P*
	+	-	+	-		+	-		+	-	
DQB1*06:02	12	126	28	164	0.12	40	202	**0.042**	68	366	**0.048**
DRB1*01:01	13	125	11	179	0.28	13	233	0.14	24	412	0.11
DRB1*07:01	11	127	13	177	0.83	21	225	1.00	34	402	1.00
DRB1*01:01+GC^{lo}	5	25	11	179	**0.049**	13	233	**0.034**	24	412	**0.031**
DRB1*07:01+GC^{lo}	6	24	13	177	**0.029**	21	225	0.094	34	402	**0.034**

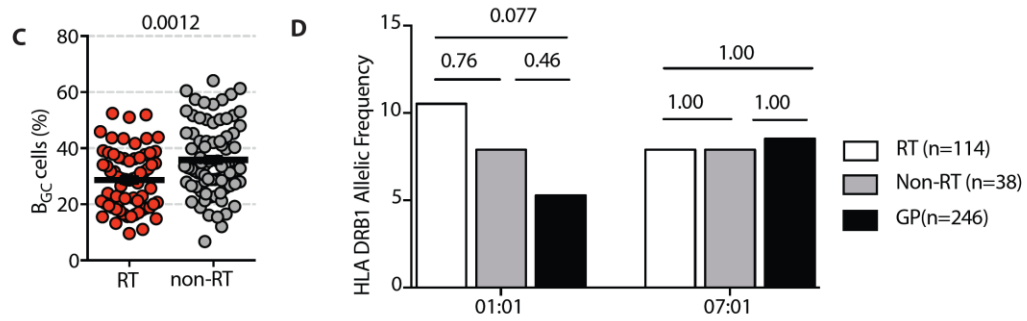


Figure S4. HLA typing of entire tonsillar cohort. (A) Percentage of Hispanic and non-Hispanic RT and non-RT children. Statistical significance determined by Fisher's Exact Test. **(B)** Allelic frequencies in RT, non-RT, GP, and GP+non-RT individuals for HLA class II alleles of interest. P values represent comparison between RT and non-RT, RT and GP, and RT and GP+non-RT. Statistical significance determined by Fisher's Exact Test. **(C)** Tonsillar B_{GC} cell frequencies from the entire cohort (cohorts 1 and 2). Statistical significance determined by Mann-Whitney test. **(D)** HLA DRB1*01:01 and HLA DRB1*07:01 allelic frequencies in non-RT children with tonsils with the lowest quartile of GC activity, defined as lowest combined frequencies of GC-T_{FH} and GC B cells (grey bar, n=19), RT tonsils (white bar, n=114), and GP (black bar, n=246). Statistical significance determined by Fisher's Exact Test.

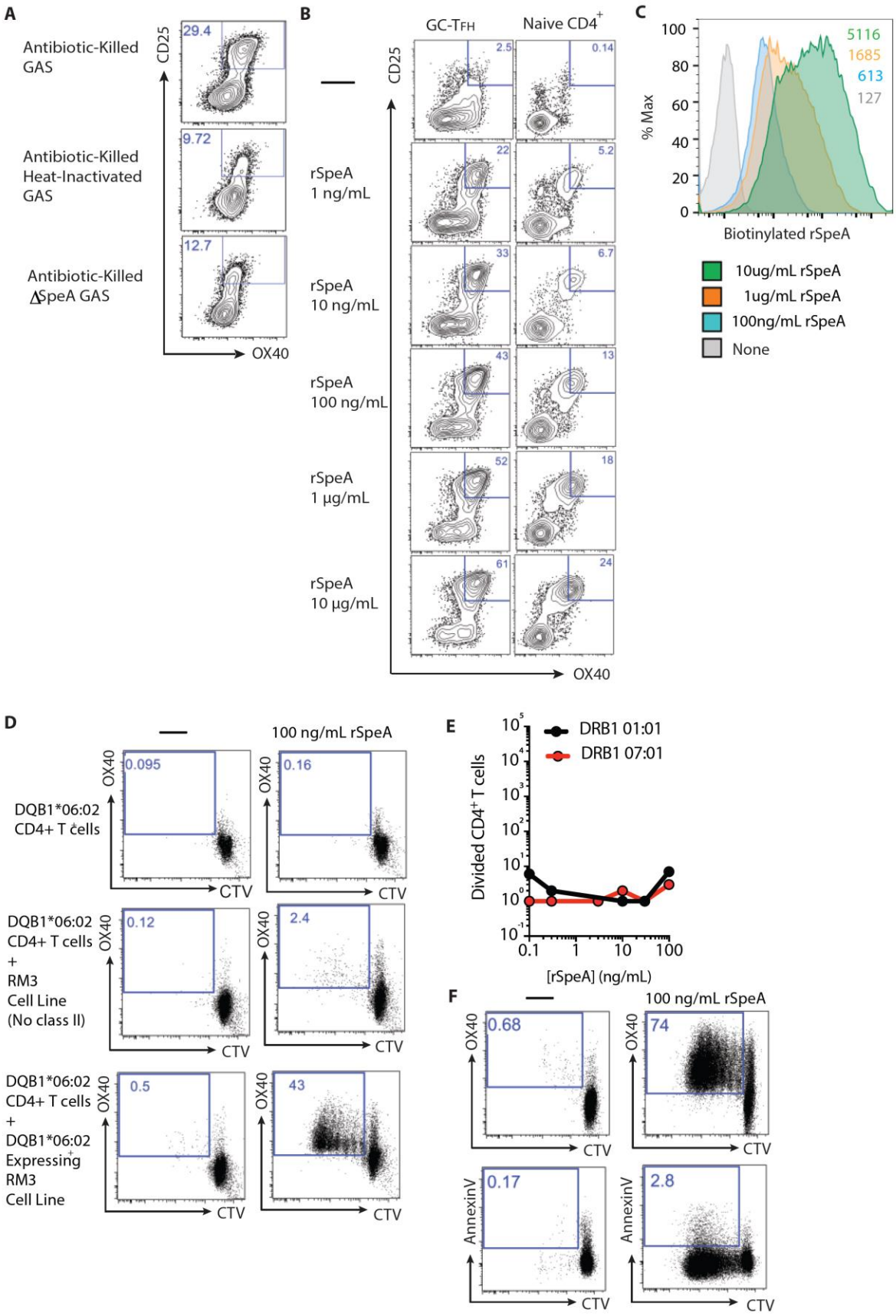

A
Antibiotic-Killed GAS
Antibiotic-Killed Heat-Inactivated GAS
Antibiotic-Killed ΔSpeA GAS
CD25
OX40
29.4
9.72
12.7
B
GC-TFH
Naive CD4+
CD25
OX40
rSpeA 1 ng/mL
rSpeA 10 ng/mL
rSpeA 100 ng/mL
rSpeA 1 µg/mL
rSpeA 10 µg/mL
2.5
0.14
22
5.2
33
6.7
43
13
52
18
61
24
C
% Max
Biotinylated rSpeA
5116
1685
613
127
10ug/mL rSpeA
1ug/mL rSpeA
100ng/mL rSpeA
None
D
100 ng/mL rSpeA
DQB1*06:02 CD4+ T cells
DQB1*06:02 CD4+ T cells + RM3 Cell Line (No class II)
DQB1*06:02 CD4+ T cells + DQB1*06:02 Expressing RM3 Cell Line
OX40
CTV
0.095
0.16
0.12
2.4
0.5
43
E
DRB1 01:01
DRB1 07:01
Divided CD4+ T cells
[rSpeA] (ng/mL)
F
100 ng/mL rSpeA
OX40
CTV
AnnexinV
0.68
74
0.17
2.8

Figure S5. SpeA-responsive GC-T_{FH} cells. (A) Flow cytometry gating of AIM^{+}GC-T_{FH} cells following stimulation with antibiotic-killed GAS, heat-inactivated, antibiotic-killed GAS, and antibiotic-killed GAS lacking SpeA. **(B)** Dose response of GC-T_{FH} cells and naive $CD4^{+}$ T cells to recombinant SpeA (rSpeA). **(C)** Dose-dependent binding of biotinylated rSpeA to an RM3 cell line expressing HLA DQB1*06:02. **(D)** Flow cytometry plots of unstimulated and SpeA-stimulated HLA DQB1*06:02 $CD4^{+}$ T cells alone (top panel), HLA DQB1*06:02 $CD4^{+}$ T cells co-cultured with the RM3 cell line, and HLA DQB1*06:02 $CD4^{+}$ T cells co-cultured with the RM3 cell line expressing DQB1*06:02. **(E)** Magnetically sorted total $CD4^{+}$ T cells PBMCs of HLA DRB1*01:01^{+} donors, co-cultured with SpeA (recombinant SpeA, rSpeA) and a cell line expressing HLA DRB1*01:01 compared to HLA DRB1*07:01^{+} donors, co-cultured with SpeA (recombinant SpeA, rSpeA) and a cell line expressing HLA DRB1*07:01. Statistical significance determined by Mann-Whitney test. **(F)** Flow cytometry plots of unstimulated and SpeA-stimulated DQB1*06:02 $CD4^{+}$ T cells co-cultured with an RM3 cell line expressing HLA DQB1*06:02 and subsequently stained with Annexin V.

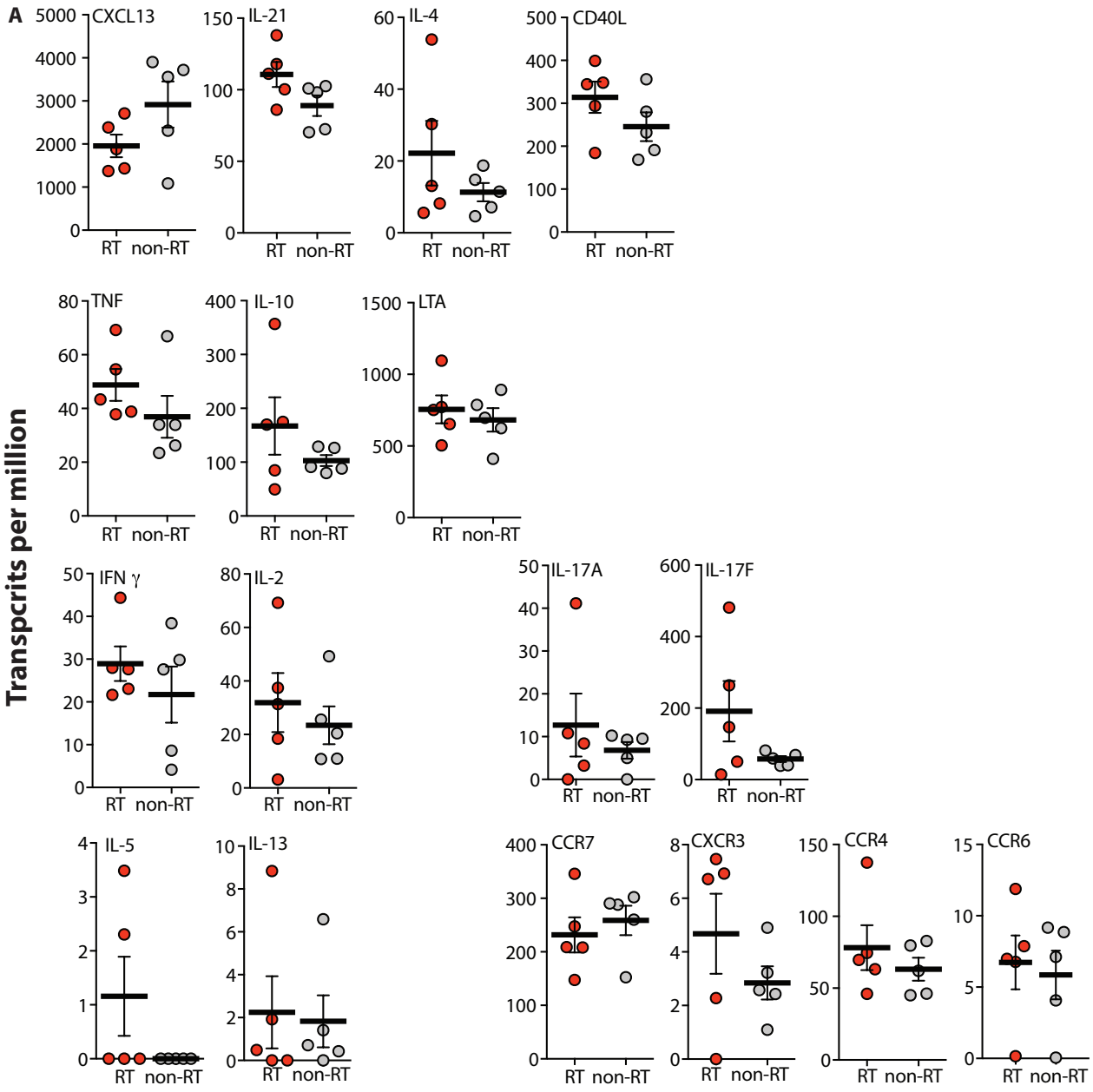


Figure S6. SpeA-responsive GC-T_{FH} cells by AIM assay. Comparison of RNA-seq counts by SpeA-responsive (AIM^+) GC-T_{FH} cells from RT tonsils (n=5) and non-RT tonsils (n=5). Transcripts of T_{FH} (CXCL13, IL-21, IL-4, and CD40L), immunomodulatory (TNF, IL-10, lymphotoxin), Th1 (IFN gamma and IL-2), Th17 (IL-17A and IL-17F), and Th2 (IL-5 and IL-13) cytokines and chemokine receptors (CCR7, CXCR3, CCR6, CCR4) from RT and non-RT tonsils. Statistical significance determined by Mann-Whitney test.

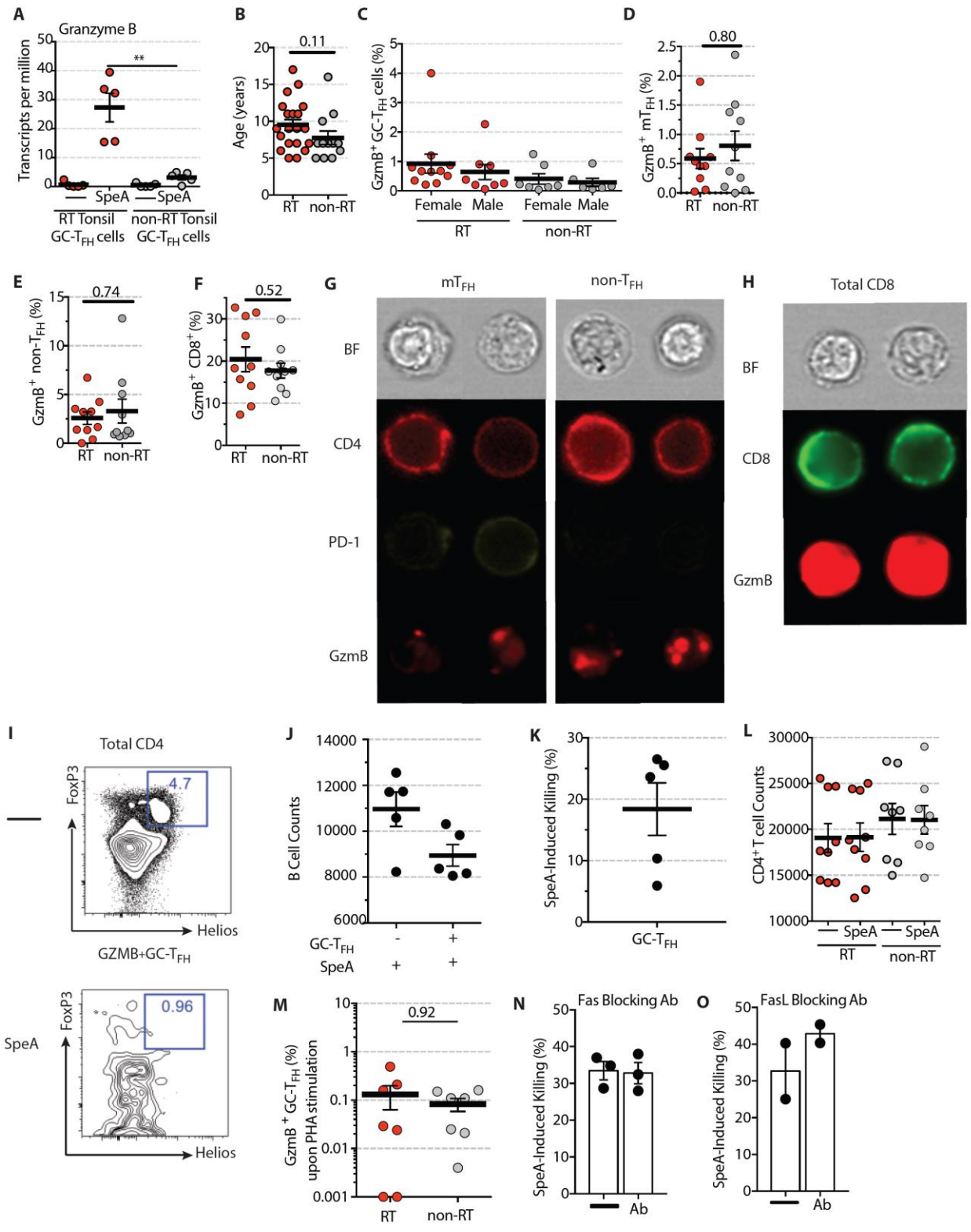


Figure S7. SpeA induced GzmB production. (A) Granzyme B RNA-seq counts in RT tonsils and non-RT tonsils. Comparison of RNA-seq counts by SpeA-responsive (AIM$^+$) GC-T$_{FH}$ cells

from RT tonsils (n=5) and non-RT tonsils (n=5). Statistical significance determined by Mann-Whitney test. **(B)** Ages of children tested for **Fig. 6C.** Statistical significance determined by ANCOVA. **(C)** Gender of children tested for **Fig. 6C.** Statistical significance determined by ANCOVA. Granzyme B expression by **(D)** mT_{FH} cells, **(E)** non-T_{FH} cells, and **(F)** $CD8^+$ T cells from RT tonsils and non-RT tonsils following SpeA stimulation, as measured by flow cytometry. Statistical significance determined by Mann-Whitney test. **(G)** ImageStream examples of granzyme B expression by SpeA-responsive mT_{FH} cells, non-T_{FH} cells, and **(H)** $CD8^+$ T cells from an RT tonsil. **(I)** Percentage of T follicular regulatory (T_{FR}) cells ($FoxP3^+Helios^+$) from unstimulated total $CD4^+$ T cells, SpeA-stimulated total $CD4^+$ T cells, and granzyme B^+ GC-T_{FH} cells. **(J)** B cell counts following 40h co-culture with GC-T_{FH} cells, unstimulated or stimulated with SpeA. A representative donor is shown. **(K)** SpeA-induced cytotoxicity by GC-T_{FH} ($CXCR5^{hi}PD\text{-}1^{hi}CD45RA^-CD4^+$) from the same donor as in **(J)** of autologous non-GC B cells ($CD19^+CD38^-$). **(L)** Cell counts of remaining GC-T_{FH} cells following co-culture with B cells, left unstimulated or stimulated with SpeA. GC-T_{FH} cells from 8 RT and 8 non-RT children are shown. (**M**) Granzyme B^+ GC-T_{FH} cells following stimulation with PHA. **(N)** SpeA-induced GC-T_{FH} cell killing of B cells in the presence of monoclonal antibodies blocking Fas or **(O)** FasL. Statistical significance determined by Mann-Whitney test.

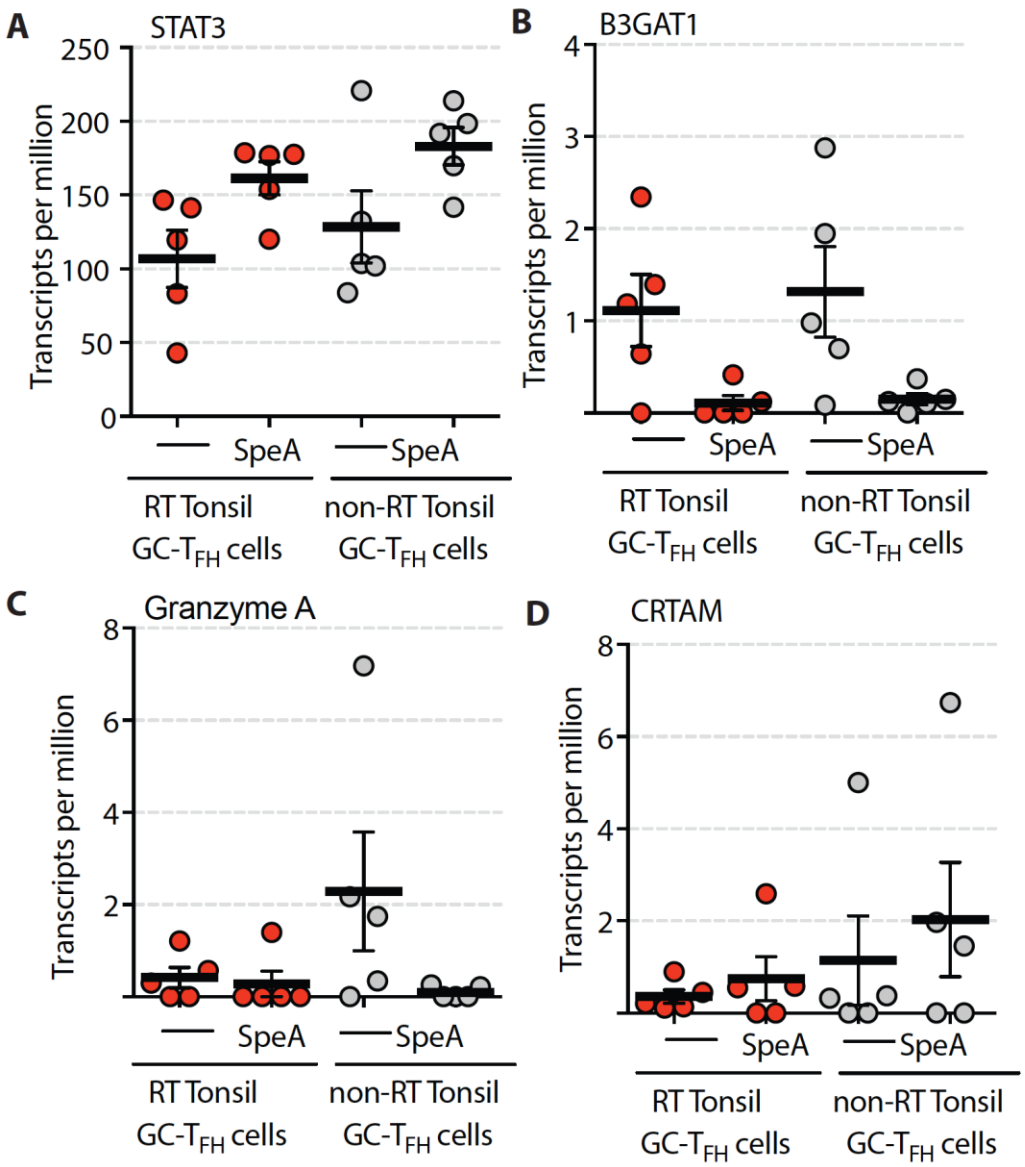


Figure S8. SpeA-responsive GC-T_{FH} cells by AIM assay. Comparison of RNA-seq counts by SpeA-responsive (AIM^+) GC-T_{FH} cells from RT tonsils (n=5) and non-RT tonsils (n=5). **(A)** STAT3 counts from RT and non-RT tonsils. **(B)** B3GAT1 counts from RT and non-RT tonsils. **(C)** Granzyme A counts from RT and non-RT tonsils. **(D)** CRTAM counts from RT and non-RT tonsils. Statistical significance determined by Mann-Whitney test.

Table S1. RNA-seq analysis. RNA-seq analysis of gene expression by SpeA stimulated GC-T_{FH} cells from RT tonsils compared to non-RT tonsils, presented as reads per kilobase of transcript per million mapped reads (RPKM). Gene expression by SpeA-stimulated GC-T_{FH} cells is plotted against *P* values (RT over non-RT tonsils) relative to fold change in (RT over non-RT tonsils). *P* values determined by Benjamini-Hochberg adjusted t-test.

Fold Change > 2

Gene	Fold Change	P-value
GZMB	7.641012092	4.59E-05
LOC284385	2.317810397	0.002559366
PPOX	2.218139609	0.002785651
SNTB1	2.296645977	0.003729742
DCLRE1B	2.150753657	0.005042583
CDT1	2.480776303	0.009692611
LOC100128420	3.079868872	0.010444279
WEE1	3.335473996	0.011568611
NUF2	2.994568266	0.012846426
DECR2	2.316402308	0.013369701
PKIA	2.224832426	0.014374529
LGALS1	2.184116223	0.017846288
SLC35E3	2.040700451	0.020975003
UBOX5	2.068927748	0.021686391
IL22	3.055735984	0.023171022
C4orf34	2.071640813	0.02426651
MZB1	2.476828678	0.025647493
KLF3	2.080842079	0.025762039
SCGB3A1	2.994882237	0.026494406
RPPH1	2.467434663	0.030118042
ATF7IP2	2.054984595	0.03198134
CENPW	4.133223197	0.032880987
PAGE5	2.67613822	0.033396207
UBE2C	2.224833707	0.036300563
IMPA2	2.417129555	0.038225742
MAD2L1	2.828229429	0.038561592
TPX2	2.498344606	0.040002708
GRAP2	2.963261168	0.040914453
AURKA	2.520344445	0.041535268
BUB1	2.020926143	0.042653122
CENPK	2.522201984	0.044391545

DEPDC1B	2.280002346	0.046123652
CDC20	2.203022094	0.046429125
NAPSB	2.047931039	0.05148833
ZNF155	2.564138295	0.057275938
POLR3G	2.024205673	0.063231312
BIRC5	2.060498011	0.065009278
ZNF367	2.33910587	0.068694987
ZADH2	2.188883403	0.069122086
PANK3	2.219992474	0.071876863
HES4	2.025890893	0.083303056
CCL28	2.951966957	0.084301037
SLA2	2.232905509	0.08607985
KIAA0101	2.697930082	0.086727426
TIMP1	2.12029238	0.089767548
MS4A1	2.050547847	0.092444783
PTPN22	2.492185037	0.093908842
PYCARD	2.09885381	0.095960315
ERAP2	2.327480291	0.09698552
PDIA5	2.17676995	0.098763327

Fold Change < 0.5

Gene	Fold Change	P-value
SNORA20	0.219296741	6.86E-04
LZTFL1	0.407304672	0.001213133
SNORA29	0.24175469	0.00164243
SERTAD3	0.317708017	0.004597311
SNORD50A	0.285222272	0.005097441
ZNF468	0.443980171	0.008219553
CCDC64	0.466200146	0.009384882
SNORD56	0.316767722	0.010037785
LOC100507217	0.477196454	0.010294377
SNORA24	0.17610984	0.010422507
PABPC1L	0.421650144	0.010641913
ZNF616	0.450959584	0.010884979
TSPAN12	0.365231965	0.011161333
ZNF79	0.376680402	0.013191762
TAC1	0.296441433	0.01440709
PON3	0.450586281	0.014425667
CAV1	0.386177054	0.014875716
STK39	0.400496892	0.020831134

ZNF582	0.349598263	0.021525763
SNORD59B	0.24883353	0.024130146
C11orf96	0.45170587	0.025603799
HLA-DRB5	0.319043215	0.0305416
ZCCHC11	0.44333271	0.031315628
FGL2	0.488200331	0.032907045
TRIM24	0.479570725	0.035403023
SGCE	0.428370999	0.035905141
ZNF600	0.375312948	0.036229017
BEX5	0.438532397	0.040577234
MIR1322	0.486818912	0.043176712
TP53BP1	0.498082342	0.04757955
ZNF193	0.469979672	0.048261952
MYO6	0.468293495	0.051242255
SPRYD7	0.438181147	0.057517773
HACE1	0.468504172	0.057639005
MIR1204	0.30467294	0.060005741
LINC00467	0.450750221	0.060299993
LOC100506713	0.499940492	0.06359757
UTS2	0.244531668	0.063861328
MIR3128	0.391822063	0.064393945
ECHDC2	0.486166254	0.067159481
MIR4434	0.338995038	0.067435871
SNORA64	0.335854109	0.06784237
LRCH3	0.483478566	0.068098779
ZCCHC4	0.483444356	0.071369221
IFITM3	0.254985075	0.07588393
LOC100506668	0.479312519	0.07763664
FAAH	0.49772865	0.078759892
NNAT	0.363155402	0.085979177
RWDD3	0.434290161	0.087550555
C7orf25	0.41965916	0.099025109

Table S2. Flow cytometry antibodies for fresh tonsil stain.

T_{FH} Stain

Antibody	**Clone**	**Vendor**
Live/Dead e780		ThermoFisher
CD19 e780	HIB19	ThermoFisher
CD14 e780	61D3	ThermoFisher
CD16 e780	eBioCB16	ThermoFisher
CD8a e780	RPA-T8	ThermoFisher
CD3 AF700	UCHT1	BD Biosciences
CD4 APC	RPA-T4	BD Biosciences
CD25 PeCy7	BC96	ThermoFisher
ICOS PerCP-eFluor710	ISA-3	ThermoFisher
PD-1 PE	eBioJ105	ThermoFisher
CD45RO FITC	UCHL1	ThermoFisher
CXCR5 BV421	J252D4	Biolegend

B cell Stain

Live/Dead e780		ThermoFisher
CD14 e780	61D3	ThermoFisher
CD16 e780	eBioCB16	ThermoFisher
CD3 e780	UCHT1	ThermoFisher
CD20 BV570	2H7	Biolegend
CD19 AF700	HIB19	Biolegend
CD38 PE-cyanine 7	HIT2	ThermoFisher
CD27 PerCP-eFluor710	O323	ThermoFisher

Table S3. Flow cytometry antibodies for AIM assay.

Antibody	**Clone**	**Vendor**
Live/Dead e780		ThermoFisher
CD19 e780	HIB19	ThermoFisher
CD14 e780	61D3	ThermoFisher
CD16 e780	eBioCB16	ThermoFisher
CD8a e780	RPA-T8	ThermoFisher
CD45RA BV570	HI100	Biolegend
CCR7 APC	G043H7	Biolegend
CD25 PeCy7	BC96	ThermoFisher
OX40 FITC	BER-ACT35	Biolegend
PD-L1 PE	29E.2A3	Biolegend
CXCR5 BV421	J252D4	Biolegend
PD-1 BV785	EH12.2H7	Biolegend
CD4 PerCP-eFluor710	SK3	ThermoFisher

Table S4. Flow cytometry antibodies for PBMC proliferation assay.

Antibody	**Clone**	**Vendor**
Live/Dead e780		eBioscience
CD25 PeCy7	BC96	eBioscience
OX40 FITC	BER-ACT35	Biolegend
PD-L1 PE	29E.2A3	Biolegend
PD-1 BV785	EH12.2H7	Biolegend
CD4 PerCP-eFluor710	SK3	ThermoFisher

Table S5. Flow cytometry antibodies for GzmB detection.

Antibody	**Clone**	**Vendor**
Live/Dead e780		ThermoFisher
CD19 e780	HIB19	ThermoFisher
CD14 e780	61D3	ThermoFisher
CD16 e780	eBioCB16	ThermoFisher
CD3 AF700	UCHT1	BD Biosciences
CD45RA BV570	HI100	Biolegend
CXCR5 BV421	J252D4	Biolegend
PD-1 BV785	EH12.2H7	Biolegend
CD4 PerCP-eFluor710	SK3	ThermoFisher
CD25 PeCy7	BC96	ThermoFisher
OX40 PE	BER-ACT35	Biolegend
CD8a BV650	RPA-T8	Biolegend
Granzyme B AF647	GB11	Biolegend
Mouse IgG1, κ Isotype Control AF647	MOPC-21	Biolegend

Table S6. Flow cytometry antibodies used for sorting GC-T_{FH} and non-B_{GC} cells for GzmB expression after 5-day in vitro culture.

$CD4^+$ T cell Sorting

Antibody	**Clone**	**Vendor**
Live/Dead e780		ThermoFisher
CD19 e780	HIB19	ThermoFisher
CD14 e780	61D3	ThermoFisher
CD16 e780	eBioCB16	ThermoFisher
CD8a e780	RPA-T8	ThermoFisher
CD4 PerCP-eFluor710	SK3	ThermoFisher
CCR7 BV650	G043H7	Biolegend
CD45RA BV570	HI100	Biolegend
CXCR5 BV421	J252D4	Biolegend
PD-1 BV785	EH12.2H7	Biolegend

Non-B_{GC} cell Sorting

Live/Dead e780		ThermoFisher
CD14 e780	61D3	ThermoFisher
CD16 e780	eBioCB16	ThermoFisher
CD8a e780	RPA-T8	ThermoFisher
CD3 e780	UCHT1	ThermoFisher
CD38 APC	HIT2	ThermoFisher
CD20 B570	2H7	Biolegend

Table S7. Flow cytometry antibodies for GzmB detection from sorted GC-T_{FH} cells.

Antibody	**Clone**	**Vendor**
Live/Dead e780		ThermoFisher
CXCR5 BV421	J252D4	Biolegend
PD-1 BV785	EH12.2H7	Biolegend
CD4 PerCP-eFluor710	SK3	ThermoFisher
CD20 B570	2H7	Biolegend
CD25 PeCy7	BC96	ThermoFisher
OX40 PE	BER-ACT35	Biolegend
Granzyme B AF647	GB11	Biolegend
Perforin FITC	B-D48	Biolegend

Table S8. Flow cytometry antibodies used for sorting for cytotoxicity assay.

$CD4^+$ T cell Sorting

Antibody	**Clone**	**Vendor**
Live/Dead e780		ThermoFisher
CD19 e780	HIB19	ThermoFisher
CD14 e780	61D3	ThermoFisher
CD16 e780	eBioCB16	ThermoFisher
CD45RA PE-CF594	HI100	BD Biosciences
CXCR5 APC	J252D4	Biolegend
PD-1 PE	eBioJ105	ThermoFisher
CD4 PerCP-eFluor710	SK3	ThermoFisher
CCR7 BV650	G043H7	Biolegend
CD8a PeCy7	RPA-T8	ThermoFisher

Non-B_{GC} cell and $CD8^+$ T cell Sorting

Live/Dead e780		ThermoFisher
CD14 e780	61D3	ThermoFisher
CD16 e780	eBioCB16	ThermoFisher
CD8a PeCy7	RPA-T8	ThermoFisher
CD38 APC	HIT2	ThermoFisher
CD19 AF488	H1B19	ThermoFisher
CD4 PerCP-eFluor710	SK3	ThermoFisher