Recurrent Group A streptococcus tonsillitis is an immunosusceptibility disease involving antibody deficiency and aberrant T follicular helper cells

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One Sentence Summary: Recurrent tonsillitis is a multifactorial disease associated with an aberrant tonsillar germinal center response to Group A Streptococcus.

ABSTRACT

Recurrent Group A Streptococcus (GAS) tonsillitis (RT) is a common indication for tonsillectomy. 'Strep throat' is highly prevalent among children; yet, it is unknown why only some children develop recurrent tonsillitis. To gain insights into this classic childhood disease, we performed phenotypic, genotypic, and functional studies on pediatric GAS RT and non-RT tonsils. GAS RT tonsils had significantly smaller germinal centers, with an underrepresentation of GAS-specific germinal center follicular helper (GC-T_{FH}) $CD4^+$ T cells. RT children exhibited reduced antibody responses to an important GAS virulence factor, streptococcal pyrogenic exotoxin A (SpeA). Risk and protective human leukocyte antigen (HLA) Class II alleles for RT were identified. Finally, SpeA induced granzyme B^+ GC-T_{FH} cells in RT tonsils with the capacity to kill B cells and potential to hobble the germinal center response. These observations suggest that RT is a multifactorial disease and that contributors to RT susceptibility include HLA Class II differences, aberrant SpeA-activated GC-T_{FH} cells, and lower SpeA antibody titers.

INTRODUCTION

'Strep throat' is one of the most prevalent human infections, with an estimated 600 million cases worldwide each year *(1)*. Clinical features of fever, tonsillar swelling or exudates, enlarged cervical lymph nodes, and absence of cough warrant testing for Group A Streptococcus (*S. pyogenes*, GAS) *(2, 3)*. Prompt antibiotic treatment can rapidly clear the infection *(4)*, reducing the risk of GAS-associated syndromes such as acute rheumatic fever and rheumatic heart disease *(3, 5-7)*. Some children, however, develop recurrent tonsillitis (RT) due to GAS *(8, 9)*. Tonsillitis is a substantial healthcare burden and cause of repeated antibiotic usage. There are over 750,000 tonsillectomies performed annually in the United States, with RT being a common indication *(2, 8, 10, 11)*. Tonsils are lymph node-like structures with open crypts evolved for sampling oropharyngeal microbes. As tonsils are a nidus for GAS infection, these lymphoid tissues are anatomically poised to mount a protective immune response to the pathogen *(12, 13)*. It remains a longstanding mystery why some children get GAS RT and others do not.

To attempt to answer this question of why some children are predisposed to RT, we examined immunological characteristics of children (ages 5-18 years old) from the San Diego (SD) area undergoing tonsillectomies for GAS RT or for non-infectious reasons (non-recurrent tonsillitis or non-RT), e.g. sleep apnea. We hypothesized that differences in the GAS-specific tonsillar immune responses may explain a predilection for some children to selectively develop GAS RT.

RESULTS

Germinal center T follicular helper cells and B cells are significantly reduced in RT

RT can be a severe disease, resulting in substantial morbidity and school absences in hundreds of thousands of children per year. By clinical history, RT children in our 1st SD cohort had a mean of 12 tonsillitis episodes compared to 0.4 episodes among non-RT children ($P = 0.0001$, **Fig. 1A**). Multiple epidemiological studies have reported similar asymptomatic GAS carriage rates between RT and non-RT children (18-30%) *(9, 14, 15)*. This suggests that RT may not be due to differences in GAS exposure. We therefore examined the tonsillar immune response in children with RT. We systematically phenotyped tonsillar immune cells from a cohort of children consisting of 26 RT and 39 non-RT children, ages 5-18 (cohort 1, **Table 1A**). Tonsils contain germinal centers, comprised of germinal center T follicular helper cells (GC-T_{FH}), follicular dendritic cells (FDCs), and germinal center B (B_{GC}) cells *(16)*. RT tonsils contained significantly fewer GC-T_{FH} cells

($CD4^+$ $CD45RO^+CXCR5^{hi}PD\text{-}1^{hi}$) compared to non-RT tonsils ($P = 0.0001$, **Fig. 1B**,**C, fig. S1A**). Mantle T_{FH} cell frequencies (mT_{FH}, $CXCR5^+PD\text{-}1^+$, T_{FH} cells outside of germinal centers) were not significantly different ($P = 0.076$, **fig. S1B)**. There was no difference in BCL6 expression by GC-T_{FH} and mT_{FH} cells between RT and non-RT samples (**fig. S1C**). RT tonsils had higher non-T_{FH} cell frequencies ($CXCR5^-$) ($P = 0.013$, **fig. S1D**) and comparable naive $CD4^+$ T cell frequencies ($P = 0.183$, **fig. S1E**). Multivariate analysis demonstrated that the GC-T_{FH} frequencies in RT children were highly significant with or without age ($P = 0.0032$, **Fig. 1D**) or gender ($P = 0.0034$, **fig. S1F**) as a covariate.

T_{FH} cells are a distinct type of $CD4^+$ T cell that provide help to B cells *(17, 18)*. T_{FH} cells are required for germinal centers and thus almost all affinity matured antibody responses to pathogens *(19)*. GC-T_{FH} cells instruct the survival, proliferation, and somatic hypermutation of B_{GC} cells. Paralleling the significant reduction in GC-T_{FH} cells in RT children, RT tonsils exhibited fewer B_{GC} cells compared to non-RT tonsils ($P = 0.0005$, **Fig. 1E**,**F**, **fig. S1A**). This reduction remained statistically significant with or without age ($P = 0.0040$, **Fig. 1G)** or gender ($P = 0.0064$, **fig. S1J**) as a covariate. Memory B cells frequencies were comparable ($P = 0.16$, **fig. S1G**), plasma cell frequencies were lower ($P = 0.006$, **fig. S1H**), and naive B cell frequencies were higher in RT tonsils ($P = 0.0002$, **fig. S1I**).

Histological examination revealed that RT tonsils had smaller germinal centers compared to non-RT tonsils ($P < 0.002$, **Fig. 1H,I**). Germinal center light and dark zones were well defined (**Fig. 1J**). There were no differences in the frequencies of B_{GC} cells in the light ($P = 0.33$, **fig. S1K**) and dark zones ($P = 0.90$, **fig. S1L**). Smaller germinal centers suggested a potential $CD4^+$ T cell defect in RT disease, consistent with the flow cytometry data. However, these differences in GC-T_{FH} cell frequencies and germinal center sizes could not be directly ascribed as RT-associated or non-RT-associated without additional information; thus, we explored additional parameters to establish whether the germinal center differences were associated with RT disease.

RT disease is associated with impaired development of anti-SpeA antibodies

Diminished germinal center activity could potentially result in impaired circulating antibody responses to GAS. Examining antibodies was necessary to test this possibility; however, blood samples are not normally taken during tonsillectomies. Thus, a second cohort of children, from whom blood samples were obtained, was recruited using the same enrollment criteria, i.e. children

ages 5-18 years undergoing tonsillectomies for either RT or non-RT, e.g. sleep apnea (cohort 2, **Table 1B**). Antibody titers were examined against two GAS proteins: streptolysin O (SLO) — the standard GAS serodiagnostic antibody marker — and streptococcal pyrogenic exotoxin A (SpeA), a GAS virulence factor of interest. A simple expectation based on clinical history was that RT children would have higher concentrations of GAS-specific antibodies than non-RT children, since the former group had multiple bouts of tonsillitis (**Fig. 1A**), including experiencing a tonsillitis episode within a few months prior to surgery. However, anti-SLO IgG titers were not elevated in RT children compared to non-RT children ($P = 0.51$, **Fig. 2A**). Detectable and comparable levels of anti-SLO IgG indicated that both RT and non-RT children had all been exposed to GAS. More strikingly, RT children had significantly lower anti-SpeA IgG titers than non-RT patients ($P = 0.024$, **Fig. 2B**). Anti-SpeA IgG titers of RT children were subsequently compared to healthy adult volunteers; average anti-SpeA IgG titers in RT children were less than 10% that of healthy adult volunteers ($P = 0.0008$, **Fig. 2B**). SpeA antibodies have been implicated epidemiologically in immunity against severe systemic GAS infections in adults *(20-23)*. SpeA antibodies have been shown to be protective in a mouse GAS infection model *(24)*. Therefore, impaired production of circulating anti-SpeA IgG in RT children may be associated with RT children's lack of protective immunity against recurrent GAS infections.

The tonsillar cells of cohort 2 were examined by multiparameter flow cytometry (**Fig. 2C-F, fig. S2**). RT children of cohort 2 had significantly lower frequencies of GC-T_{FH} cells than non-RT tonsils ($P < 0.0001$, **Fig. 2C**), which was independent of age ($P = 0.00026$, **Fig. 2D**) and gender ($P=0.0002$, **fig. S2G**). Those results confirmed the observations made in cohort 1. Significant differences in B_{GC} cell frequencies were not observed ($P = 0.24$, **Fig. 2E, F**), suggesting that an RT immunological defect may be directly related to GC-T_{FH} cells.

GAS-specific GC-T_{FH} cells

Phenotypic and histologic analyses of RT tonsils suggested an impairment of $CD4^+$ T cell help to B cells in RT disease. For all subsequent experiments (**Fig. 3-6, fig. S3-8**), samples from both cohort 1 and 2 were used. We next assessed the frequencies of GAS-specific GC-T_{FH} cells in the entire cohort of RT and non-RT children. Antigen-specific GC-T_{FH} cells are difficult to identify by intracellular cytokine staining. The main function of a GC-T_{FH} cell is to provide help to nearby B cells, resulting in stingy cytokine secretion by GC-T_{FH} cells. We therefore developed a cytokine-

independent approach to identify antigen-specific GC-T_{FH} cells by TCR-dependent activation induced markers (AIM), expressed upon recognition of antigen *(25-27)*. We applied the AIM technique to quantify tonsillar GAS-specific $CD4^+$ T cells (**Fig. 3A**). The non-pathogenic Gram-positive bacterium *Lactococcus lactis* was used as a negative control antigen (**Fig. 3A,B**). As children with RT experienced 12 times more tonsillitis episodes than non-RT children (**Fig. 1A**), a simple expectation was that RT tonsils would contain substantially more GAS-specific $CD4^+$ T cells than non-RT tonsils. Instead, GAS-specific antigen-experienced $CD4^+$ T cells ($CD45RA^-$, **Fig. 3C**), GAS-specific non-T_{FH} (**fig. S3A**), and GAS-specific GC-T_{FH} cells (**fig. S3B**) were not significantly elevated in RT tonsils compared to non-RT tonsils. Rather, GAS-specific $CD4^+$ T cells from RT tonsils were skewed away from GAS-specific GC-T_{FH} differentiation, with a lower ratio of GAS-specific GC-T_{FH} to total GAS-specific $CD4^+$ T cells ($P = 0.023$, **Fig. 3D**). Taken together, these data suggested that GAS-specific GC-T_{FH} cell responses were deficient in RT disease.

Using the AIM assay, we performed RNA-sequencing (RNA-seq) on sorted GAS-specific and unstimulated ($CD25^-OX40^-$) GC-T_{FH} cells (**fig. S3C**) to assess if there were functional differences in the GC-T_{FH} cells. There were no notable differences between RT and non-RT GC-T_{FH} cells. More *IL4* mRNA was observed in GAS-specific GC-T_{FH} cells from RT donors (**fig. S3C**), but the overall mRNA levels were low and IL-4 protein was below the limit of detection *(25)*. The data suggested that the RT and non-RT GC-T_{FH} cells were functionally similar by assessment of conventional cytokines of interest.

RT disease is associated with Human Leukocyte Antigen (HLA) Class II alleles

Essentially all children are exposed to GAS during childhood *(28)*. Among children enrolled in this study, RT children were likely to have a significant family history of tonsillectomy ($P = 0.0004$, **Fig. 4A**). This suggested a potential genetic predisposition. Germinal center responses depend on HLA Class II antigen presentation by B cells to GC-T_{FH} cells. Susceptibility to toxic shock syndrome and invasive forms of GAS infection have been inversely associated with HLA DQB1*06:02 *(29)*. DQB1*06:02 has also been associated with protection from the development of rheumatic heart disease *(30, 31)*, the most severe sequela of long-term untreated GAS RT and the leading cause of heart failure in children worldwide *(1, 32)*. We performed HLA typing on the entire tonsillar cohort to test for potential HLA associations with RT that had previously been

associated with other GAS-related diseases. HLA Class II associations frequently require large sample sizes. To increase the sample size, we generated an HLA-typed cohort of ethnically-matched healthy adults from the SD general population (GP) (**fig. S4A**). HLA DQB1*06:02 was significantly less frequent in RT children than in the general population ($P = 0.042$, **Fig. 4B, fig. S4B**). HLA DQB1*06:02 was also significantly less frequent in RT children than the combined groups of non-RT children + general population ($P = 0.048$, **Fig. 4B, fig. S4B**). Of note, there was no difference in the HLA DQB1*06:02 allelic frequency between non-RT children and ethnically matched SD general population ($P = 0.89$) (**Fig. 4B, fig. S4B**). Overall, these data suggest that HLA allele DQB1*06:02 is a protective allele from RT disease, potentially similar to the protective role of DQB1*06:02 against toxic shock syndrome and invasive forms of GAS infection.

HLA alleles DRB1*01:01 *(31, 33)* and DRB1*07:01 have been linked to increased risk for rheumatic heart disease. No significant DRB1*01:01 and DRB1*07:01 allelic associations were observed among all children enrolled in this study (**fig. S4B**). However, given that RT is a multifactorial disease, we considered that a genetic association with disease susceptibility may be more evident in RT children exhibiting the largest germinal center deficits. HLA allelic frequencies were thus examined among children with RT with the lowest quartile of GC-T_{FH} and B_{GC} cells (**Fig. 4C, fig. S4B**, GC^{lo}, **fig. S4C**). These children had significantly higher frequencies of HLA DRB1*01:01 compared to the general population ($P = 0.03$), non-RT children ($P = 0.049$), and the combined control groups ($P = 0.03$, **Fig. 4C, fig. S4B**). Frequencies of HLA DRB1*07:01 were also elevated compare to non-RT children and the combined control groups ($P = 0.03$, $P = 0.03$. **Fig. 4C, fig. S4B**). In contrast, no differences were identified between the non-RT and general population cohort for HLA DRB1*01:01 ($P = 0.85$) or HLA DRB1*07:01 ($P = 0.74$, **Fig. 4C, fig. S4B**). We additionally compared the lowest quartile of GC-T_{FH} and B_{GC} cells from non-RT children to all RT children and the general population and observed no difference in HLA DRB1*01:01 or HLA DRB1*07:01 frequencies, as expected (**fig. S4D**). These data indicate that HLA DRB1*01:01 and DRB1*07:01 are risk alleles for RT. Overall, integration of HLA typing and immunophenotyping data revealed relationships between RT disease, GAS, and germinal center responses.

RT-associated HLA alleles differentially impact $CD4^+$ T cell responses to GAS and the GAS superantigen SpeA

SpeA superantigen is an important GAS virulence factor. Comparison of $CD4^+$ T cell reactivity using an antibiotic-killed wild-type (WT) GAS strain M1T1 5448, with or without heat inactivation, or an antibiotic-killed isogenic SpeA-deficient mutant GAS strain (Δ*speA*), demonstrated that SpeA superantigen-mediated stimulation of $CD4^+$ T cells constituted a major fraction of $CD4^+$ T cell reactivity to in vitro cultured GAS ($P = 0.002$, **Fig. 5A, fig. S5A**). SpeA has provided GAS with an evolutionary advantage *(24, 34, 35)*, associated with the global persistence and dominance of the M1 serotype among throat cultures. GC-T_{FH} cells from RT tonsils bearing a risk HLA Class II allele were less responsive to SpeA stimulation than non-RT tonsils bearing the protective HLA DQB1*06:02 allele ($P = 0.052$, **Fig. 5B, fig. S5B**). While not reaching statistical significance, in light of the small N value we found the results intriguing enough to examine SpeA interactions with human $CD4^+$ T cells in greater detail.

Mechanistic relationships between HLA class II alleles and GAS disease manifestations are unclear *(32)*, but a potential role has been suggested for SpeA *(29, 36, 37)*. We tested binding of SpeA to 19 well-defined single-allele HLA class II expressing cell lines. The highest affinity binding interaction was between SpeA and HLA DQB1*06:02 (**Fig. 5C, fig. S5C**), while moderate binding was observed to cells expressing another DQ allele, DQB1*03:02. Rapid and robust proliferation of HLA DQB1*06:02^+ $CD4^+$ T cells was observed in the presence of the superantigen ($P = 0.0079$, **Fig. 5D, fig. S5D**). In contrast, minimal proliferation was observed for HLA DQB1*06:02^- $CD4^+$ T cells, including HLA DRB1*01:01^+ or DRB1*07:01^+ $CD4^+$ T cells (**Fig. 5D** and **fig. S5E**) with minimal cell death (**fig. S5F**). High affinity interaction of SpeA with the protective allele HLA DQB1*06:02 resulted in robust $CD4^+$ T cell proliferation (**Fig. 5C,D**). Thus, $CD4^+$ T cells from HLA DQB1*06:02^+ individuals exhibit differential interaction with SpeA compared to HLA DQB1*06:02^- individuals.

Granzyme B^+ GC-T_{FH} cells are found in RT disease

While GC-T_{FH} cell frequencies were significantly lower in RT compared to non-RT tonsils (**Fig. 1C,D**., **Fig 2C,D**), BCL6 expression by GC-T_{FH} cells was equivalent on a per cell basis (**fig. S1J**). To identify $CD4^+$ T cell factors potentially involved in SpeA superantigen-associated germinal center abnormalities in RT disease, we performed RNA-seq on SpeA-responsive GC-T_{FH} cells from RT and non-RT tonsils. We observed no difference in T_{FH}, immunomodulatory, Th1, Th2, or Th17 cytokine mRNA expression (**fig. S6**). Strikingly, *GZMB* mRNA, encoding the

cytotoxic effector protein granzyme B (GzmB), was upregulated in RT GC-T_{FH} cells ($P < 0.0079$, **Fig. 6A, fig. S7A, Table S1**). GzmB is secreted by cytotoxic $CD8^+$ T cells and NK cells for killing of target cells. Expression of GzmB by GC-T_{FH} cells is counterintuitive to the B cell help function of GC-T_{FH} cells. We considered that aberrant GzmB expression by GC-T_{FH} cells may result in conversion of a GC-T_{FH} cell from one that helps GC B cells to one that kills GC B cells. This would be a potential mechanism by which GAS could disrupt antibody responses.

To determine if RT GC-T_{FH} cells were capable of GzmB protein expression, four independent approaches were used: (1) flow cytometry of intracellular stained SpeA-stimulated GC-T_{FH} cells, (2) ImageStream imaging cytometry of SpeA-stimulated GC-T_{FH} cells, (3) immunofluorescence microscopy of human tonsillar tissue, and (4) killing of target cells. GC-T_{FH} cell intracellular protein staining confirmed SpeA induced GzmB expression ($P = 0.006$, **Fig. 6B,C**), with no difference based on age (**fig. S7B**) or gender (**fig. S7C**). Perforin expression was also induced by SpeA stimulation (**Fig. 6D**). Consistent with these findings, punctate cytoplasmic GzmB was observed in SpeA stimulated GC-T_{FH} cells from an RT patient by ImageStream (**Fig. 6E,F**). These changes were specific to GC-T_{FH} cells, as there were no differences in the frequencies of $GzmB^+$ mT_{FH} (**fig. S7D,G**), non-T_{FH} (**fig. S7E,G**) or $CD8^+$ T cells (**fig. S7F,H**) between RT and non-RT donors. These $GzmB^+$ GC-T_{FH} cells were not T_{REG} cells (**fig. S7I**). GzmB was also observed histologically in some GC-T_{FH} cells (**Fig. 6G**).

We assessed whether SpeA-stimulated GC-T_{FH} cells were capable of killing B cells. Killing by cytotoxic $CD4^+$ T cells is difficult to demonstrate *in vitro*; nevertheless, killing of B cells by GC-T_{FH} cells was observed in the presence of SpeA (**Fig. 6H**, **fig. S7J,K**). This killing was more profound by RT GC-T_{FH} cells compared to non-RT GC-T_{FH} cells. Bystander cell death was not observed (**fig. S7L**). PHA did not stimulate GzmB expression (**fig. S7M**). B cell killing by RT GC-T_{FH} cells in the presence of GAS SpeA was independent of Fas and FasL (**fig. S7N,O**), and was associated with perforin expression by the $GzmB^+$ GC-T_{FH} cells (**Fig. 6D**).

Lastly, we assessed whether $GzmB^+$ GC-T_{FH} cells were unique to RT. GC-T_{FH} cells from healthy lymph nodes (LN) from patients undergoing a staging LN biopsy were compared to RT and non-RT tonsils. $GzmB^+$ GC-T_{FH} cells were sporadically detected in healthy LNs. Significantly more $GzmB^+$ GC-T_{FH} cells were observed in RT tonsils than healthy LNs, and GzmB expression was specifically induced upon SpeA stimulation ($P = 0.025$, **Fig. 6I**). GC-T_{FH} cells from non-RT tonsils and healthy LNs were indistinguishable (**Fig. 6I**). Collectively these data suggest that SpeA

is capable of deviating GC-T_{FH} cells into $GzmB^+$ $perforin^+$ killer T_{FH} cells and these killer T_{FH} cells are a pathological feature of RT disease.

DISCUSSION

By integrating immune profiling and clinical data with transcriptomic and functional analyses, we revealed immunologic features of GAS recurrent tonsillitis that provide evidence that RT is an immunosusceptibility disease. We observed 1) RT tonsils have significantly lower GC-T_{FH} cell frequencies; 2) RT children have impaired anti-SpeA antibody titers, which have been associated with protective immunity to GAS; 3) specific HLA Class II alleles were associated with RT; and 4) SpeA can induce GzmB expression in GC-T_{FH} cells. While RT is surely a multifactorial disease, these findings suggest that the sporadic nature of RT is linked to HLA-associated genetic susceptibility differences with HLA Class II allelic variants impacting GAS superantigen binding. SpeA perturbation of GC-T_{FH} cells and GC-T_{FH} killing of B_{GC} cells is a parsimonious model to explain key immunological and pathological aspects of RT. Inability to develop protective anti-SpeA immunity may render a child more susceptible to pharyngitis and reinfection due to disruption of GC-T_{FH} cells.

It has been a long-standing mystery why some children get recurrent strep throat. Specific strains of GAS have been proposed as a cause of RT *(2, 38, 39)*. However, previous studies have observed that RT children and non-RT children have similar asymptomatic GAS carriage rates *(9, 14, 15)*. Our pediatric cohorts were recruited from the same geographic area to control for circulating GAS serotypes. Globally, GAS disease burden is high and, in recent decades, the M1 serotype has remained one of the dominant strains *(40, 41)*. It is notable that the M1 serotype possesses a bacteriophage encoding SpeA, and the acquisition of SpeA has been implicated in the dominance of the M1 pandemic strain in the United States *(42, 43)*. Here, we observed that SpeA contributed significantly to GAS superantigen activity on GC-T_{FH} cells.

SpeA superantigen actively skewed GC-T_{FH} cell function, resulting in cytolytic GC-T_{FH} cells. This represents a novel immune evasion mechanism of a pathogen. The ability of SpeA to convert a conventional GC-T_{FH} cell into to a "killer T_{FH}" *in vitro* occurred regardless of whether the GC-T_{FH} cells were from an RT or non-RT tonsil (**Fig. 6H**). However, conversion to $GzmB^+$ $perforin^+$ GC-T_{FH} cells, and cytotoxicity, was more extensive with GC-T_{FH} cells from RT tonsils, and RT children had much lower anti-SpeA antibody responses. Even a small frequency of $GzmB^+$

perforin$^+$ GC-T_{FH} cells may potentially have devastating effects within the confines of a germinal center. We contend that the cytotoxicity scenario is fundamentally different for GzmB$^+$ GC-T_{FH} disruption of germinal centers than it is for cytolytic control of a viral infection. B_{GC} cells are probably the most pro-apoptotic cells in the body. Each B_{GC} cell requires stimulation by a T_{FH} cell every few hours or it will die *(44-46)*. Additionally, unlike most cell types, the B_{GC} cells are all confined to a densely packed space, the germinal center. Equally importantly, GC-T_{FH} cells are constantly making short (~5 min) cognate interactions with B_{GC} cells *(47, 48)*. Thus, in a 24 hour period, ten GC-T_{FH} cells can make cognate interactions with 2,880 B_{GC} cells, and an average germinal center contains only ~1,000 total B_{GC} cells. In contrast, cytolytic killing of virally-infected cells takes much longer cognate interactions, with more resistant cells, over a much greater three-dimensional space *(49)*. Hence, we consider it a reasonable model that it may take very little GzmB to kill a GC B cell and that GzmB$^+$ T_{FH} could serially poison many GC B cells each day.

Our finding of SpeA-induced GzmB$^+$perforin$^+$ GC-T_{FH} cells within tonsils also highlights the plasticity of T_{FH} cells. Granzyme A expressing GC-T_{FH} cells have been described recently in human lymph nodes and tonsils *(50, 51)*. In this study, we observed no significant RNA expression of granzyme A, and no differences in CD57, STAT3, or CRTAM between RT and non-RT tonsils (**fig. S8A-D**). However, we did observe similarities between granzyme B$^+$ GC-T_{FH} and recent reports of CD8$^+$ T cells acquiring T_{FH} phenotypic features *(52, 53)*. CXCR5$^+$ CD8$^+$ T cells have been identified in the context of HIV, SIV, and LCMV chronic infections and have the capacity to migrate into B cell follicles and exhibit cytotoxicity *(54-57)*. Intriguingly, anti-PD1 immunotherapy predominantly rescues exhausted CD8$^+$ T cells via outgrowth of CXCR5$^+$ CD8$^+$ T cells *(57)*. Development of CXCR5$^+$ CD8$^+$ T cells is associated with upregulation of key regulators of T_{FH} differentiation TCF1 and BCL6 *(58)*, and a substantial reduction in the expression of GzmB by the CXCR5$^+$ CD8$^+$ T cells *(57, 59)*. In this study of RT GC-T_{FH} cells, the opposite was observed; downregulation of *TCF1* and its homolog *LEF1* occurred in SpeA-stimulated GC-T_{FH} cells commensurate with GzmB upregulation, suggesting that the TCF1/LEF1 axis may be required for separation of T_{FH} and cytolytic transcriptional programs in both CD4$^+$ and CD8$^+$ T cells. Altogether, the data from this study suggest that conversion of GC-T_{FH} to GzmB$^+$perforin$^+$ T_{FH} cells represents a reciprocal process to the recently described conversion of CXCR5$^-$ GzmB^{hi} CD8$^+$ T cells to CXCR5$^+$ GzmB^{lo} CD8$^+$ T cells.

This study identified risk and protective alleles for GAS recurrent tonsillitis, alleles which have previously been implicated in other clinical presentations of GAS infection. RT disease is associated with a lower frequency of HLA alleles observed to be protective against GAS invasive infection and toxic shock syndrome, and a higher frequency of HLA risk alleles shared with severe autoimmune rheumatic heart disease. Screening for these HLA alleles in children with strep throat may provide a valuable prognostic indicator for susceptibility to recurrent GAS pharyngeal infections.

There are limitations to this study. Tonsillectomy indication was determined by the otolaryngologist at a tertiary referral hospital based on history provided by the referring pediatrician/parent with the tonsils analyzed in an unblinded fashion. There was no culture data for the RT group to determine which GAS serotype was the etiologic agent of strep throat. Additionally, robust statistical associations with HLA Class II alleles frequently require cohorts of 1,000 or more individuals. Those variables will be valuable to assess in future studies.

In a murine HLA class II model of GAS infection, establishment of GAS infection was dependent on SpeA, and immunization with an SpeA toxoid elicited anti-SpeA IgG that was protective against GAS infection *(24, 34)*. Our data indicate that differential binding of SpeA to HLA class II alleles may predict susceptibility of individuals to GAS infection. More broadly, these data support central roles for SpeA and anti-SpeA IgG in tonsillitis pathogenesis and GAS protective immunity, respectively. Strong binding of SpeA to HLA DQB1*06:02 may facilitate the development of SpeA-specific GC-T_{FH} cells to provide help to B_{GC} cells to generate anti-SpeA IgG to neutralize SpeA upon re-exposure. An understanding of this immune evasion strategy may now allow for rational design of countermeasures. An inactivated SpeA toxoid vaccine may be a simple and reasonable candidate for consideration as a strep throat and RT vaccine, as a means to reduce costly RT antibiotics treatments and surgeries per year and reduce childhood strep throat disease burden generally.

In conclusion, we provide evidence that recurrent tonsillitis is a genetic immunosusceptibility disease with a role for SpeA and GC-T_{FH} cells. We have identified correlates of disease both on the side of the pathogen and on the side of the immune system. These findings have several implications, including the plausibility of SpeA as a potential vaccine target for RT and strep throat generally. Finally, the finding of $GzmB^+$ $perforin^+$ GC-T_{FH} cells points to a pathological mechanism of germinal center control.

MATERIALS AND METHODS

Study Design: The goal of the study was to understand why only some children get recurrent GAS-associated tonsillitis. Based on discussions with pediatricians and pediatric otolaryngologists, we restricted recruitment to children ages 5-18 undergoing tonsillectomies, as GAS RT afflicts school aged children. Tonsils were obtained from children at Rady Children's Hospital or the Naval Medical Center, undergoing tonsillectomies for either GAS-associated RT or sleep apnea. Sleep apnea was chosen as the comparator group as: (1) tonsils are never removed from healthy children; (2) partial tonsil biopsies are not feasible due to the small risk of life-threatening oropharyngeal hemorrhage; (3) cadaveric tonsils are not adequate for germinal center research purposes, as there are few live cells to perform functional assays and the quality of the cells is uncertain due to the highly apoptotic nature of germinal centers; (4) pediatric whole body organ donors are extremely rare, and those with tonsils harvested are even rarer; and (5) sleep apnea is another common indication for tonsillectomy but is not associated with a known infectious etiology. Enrollment of children from the same geographic area controlled for circulating GAS strains within the community. Otolaryngologists performing the tonsillectomies determined indication for tonsillectomy (RT or sleep apnea) based on clinical history and laboratory data from referring pediatricians. Inclusion criteria were age and indication for tonsillectomy. Exclusion criteria included active infection, malignancy, autoimmunity, mixed indication (RT/apnea), and age < 5 years old. Substantial effort was made to control for age in enrolling RT and non-RT children. Gender was documented and reported but was not an inclusion criteria.

Cohort 1 (**Table 1A**) consisted of children enrolled at The Naval Medical Center and Rady Children's Hospital. Cohort 2 (**Table 1B**) consisted of children enrolled at Rady Children's Hospital by the same pediatric otolaryngologists as Cohort 1. Cohort 2 consists of children enrolled after amendment of the institutional review board (IRB) to include a blood specimen at the time of tonsillectomy. Characteristics of the total cohort (cohort 1 plus cohort 2) are in Table 1C. Informed consent was obtained from all donors under protocols approved by the IRBs of the University of California, San Diego, Rady Children's Hospital, Naval Medical Center, and La Jolla Institute for Immunology (LJI).

Statistical analysis. All statistical analyses were performed using two-tailed Mann-Whitney test using a nonparametric distribution in GraphPad 7.0, unless otherwise specified. ANCOVA was

performed to evaluate age or gender as a co-variable. Two-tailed Fisher exact test was used to evaluate HLA associations, using GraphPad software or R software version 3.3.1.

Supplementary Materials

Materials and Methods

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Author Contributions: S.C., V.N., and J.M.D. designed the study. M.Bo. and M.Br. enrolled patients and provided clinical specimens. K.Ke., K.Ka., and R.A. assisted with tonsillar cell isolation. K.Ke. measured GC areas and performed immunohistochemistry. J.M.D. designed the AIM assay, performed flow cytometry, confocal microscopy experiments, cell culture assays, and ELISAs and analyzed immunophenotyping, HLA-typing, and RNA-seq data. C.L.A. enrolled

patients and provided HLA-typed PBMCs. A.S. facilitated HLA-typing, provided HLA expressing cell lines, and provided cells for pilot experiments. E.A., C.L., V.N. provided antibiotic-killed GAS, antibiotic-killed *Lactococcus lactis,* and SpeA-deficient GAS. J.M.D. S.R., P.V., and G.S. performed RNA-sequencing. D.L., R.C., and C.O. provided healthy lymph nodes. S.C. and J.M.D. wrote the manuscript with input from all authors.

Competing Interests: The authors declare no competing financial interests. S.C. has an International Patent Application No. PCT/US18/30948, Diagnosis and treatment of infection involving Killer T follicular helper cells, methods of preparation, and uses thereof.

Data and materials availability: The RNA-seq data are available in the Gene Expression Omnibus (GEO) database under the accession number XXX.

Figure 1

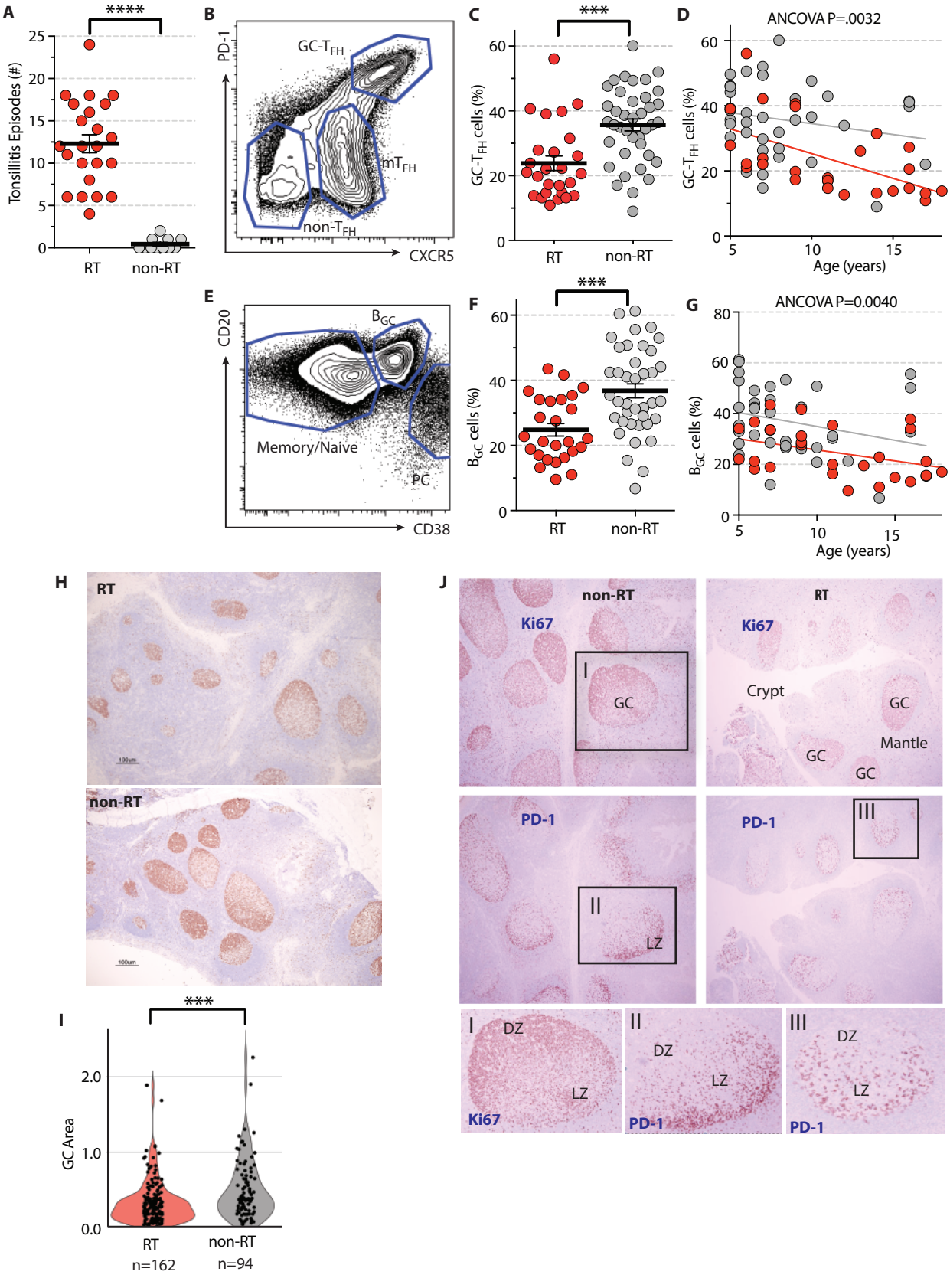
A
****
Tonsillitis Episodes (#)
RT
non-RT
B
PD-1
GC-TFH
mTFH
non-TFH
CXCR5
C
***
GC-TFH cells (%)
RT
non-RT
D
ANCOVA P=.0032
GC-TFH cells (%)
Age (years)
E
CD20
BGC
Memory/Naive
PC
CD38
F
***
BGC cells (%)
RT
non-RT
G
ANCOVA P=0.0040
BGC cells (%)
Age (years)
H
RT
non-RT
J
non-RT
Ki67
I
GC
RT
Ki67
Crypt
GC
Mantle
GC
GC
PD-1
II
LZ
PD-1
III
I
DZ
LZ
Ki67
II
DZ
LZ
PD-1
III
DZ
LZ
PD-1
I
***
GC Area
RT
n=162
non-RT
n=94

Figure 1. RT children have significantly fewer GC-T_{FH} cells. Immunophenotyping analysis of cohort 1 of RT and non-RT patients.

(A) Number of recurrent tonsillitis episodes in RT (n=23) and non-RT children (n=11).

(B) Flow cytometry of GC-T_{FH} ($CXCR5^{hi}PD\text{-}1^{hi}CD45RO^{+}CD4^{+}$), m$T_{FH}$ ($CXCR5^{+}PD\text{-}1^{+}CD45RO^{+}CD4^{+}$), and non-$T_{FH}$ ($CXCR5^{-}CD45RO^{+}CD4^{+}$) cells.

(C) Lower GC-T_{FH} cell frequencies in RT tonsils (n=26) than non-RT tonsils (n=39). GC-T_{FH} cells are quantified as % of total $CD4^{+}$ T cells.

(D) GC-T_{FH} cells by age.

(E) Flow cytometry of B_{GC} cells ($CD38^{+}CD20^{+}CD19^{+}$), plasma cells ($CD38^{hi}CD20^{+}CD19^{+}$), and memory ($CD27^{hi}CD20^{+}CD19^{+}$)/naive ($CD27^{-}CD20^{+}CD19^{+}$) B cells.

(F) Lower GC B cell frequencies in RT tonsils than non-RT tonsils. B_{GC} cells are quantified as % of total B cells.

(G) B_{GC} cells by age.

(H) Representative Ki67 stained sections from RT and non-RT tonsils.

(I) Quantitation of GC areas (μm^2) in RT tonsils (n=21) and non-RT tonsils (n=16). Each data point represents an individual GC.

(J) Staining of B_{GC} cells (Ki67) and GC-T_{FH} cells (PD-1).

**** = $P < 0.0001$, *** = $P < 0.001$, ** = $P < 0.01$. Statistical significance determined by Mann-Whitney tests (a-c, e-f, i) and multivariate ANCOVA (d, g).

Figure 2

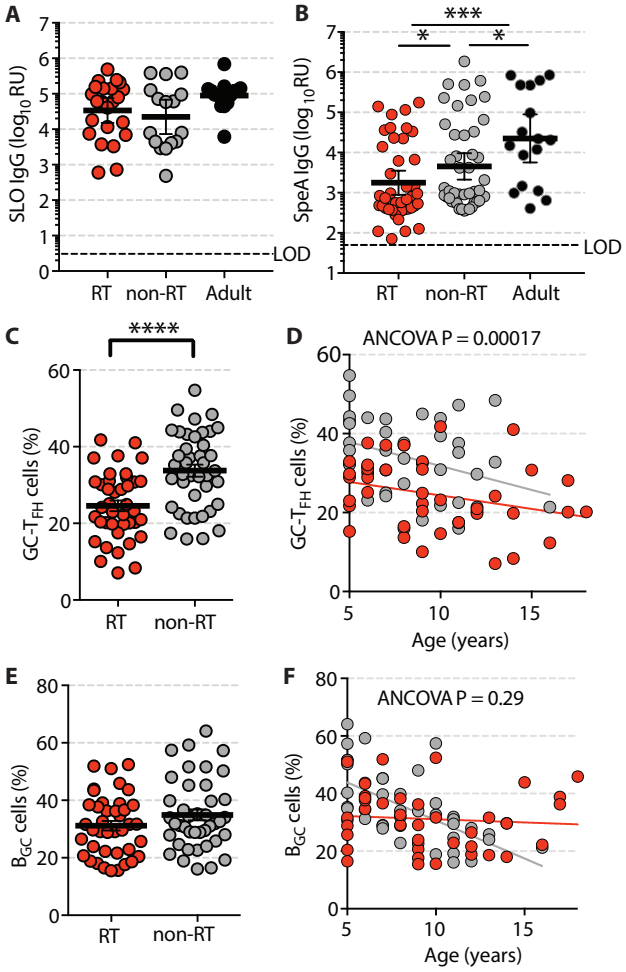
A
SLO IgG (log10RU)
LOD
RT
non-RT
Adult
B
SpeA IgG (log10RU)
***
*
*
LOD
RT
non-RT
Adult
C
****
GC-TFH cells (%)
RT
non-RT
D
ANCOVA P = 0.00017
GC-TFH cells (%)
Age (years)
E
BGC cells (%)
RT
non-RT
F
ANCOVA P = 0.29
BGC cells (%)
Age (years)

Figure 2. RT children have lower circulating anti-SpeA IgG titers. Serological and immunophenotyping analysis of cohort 2 of RT and non-RT patients.

(A) Plasma anti-SLO IgG titers in RT children (n=23), non-RT children (n=16), and normal healthy adults (n=14).

(B) Plasma anti-SpeA IgG titers in RT children (n=42) are significantly lower than non-RT children (n=45) and normal healthy adults (n=17). LOD = Limit of Detection.

(C) Lower GC-T_{FH} cell frequencies in RT tonsils (n=40) than non-RT tonsils (n=41). GC-T_{FH} cells are quantified as % of total $CD4^+$ T cells.

(D) GC-T_{FH} cells by age.

(E) Comparable B_{GC} cell frequencies. B_{GC} cells were quantified as % of total $CD19^+$ B cells.

(F) B_{GC} cells by age.

* $P < 0.05$, *** $P < 0.001$. Statistical significance determined by Mann-Whitney test.

Figure 3

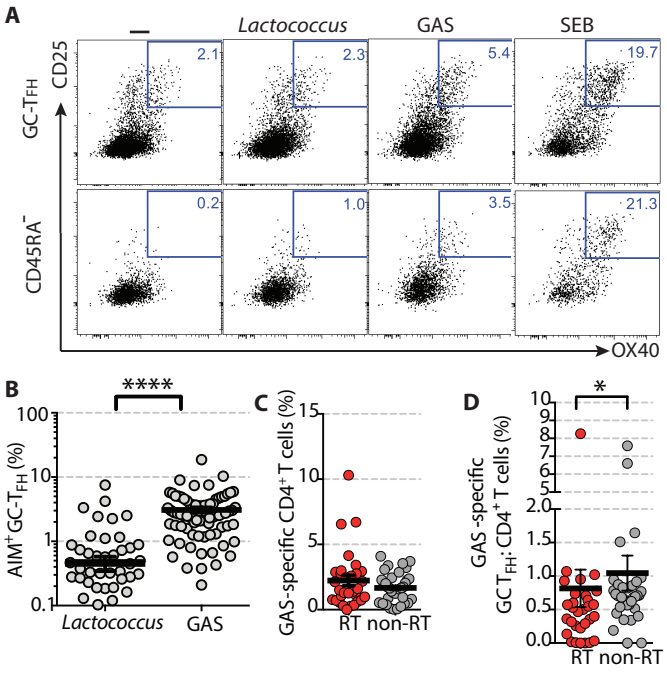
A
—
Lactococcus
GAS
SEB
GC-TFH CD25
CD45RA-
2.1
2.3
5.4
19.7
0.2
1.0
3.5
21.3
OX40
B
****
AIM+GC-TFH (%)
100
10
1
0.1
Lactococcus
GAS
C
GAS-specific CD4+T cells (%)
15
10
5
0
RT non-RT
D
*
GAS -specific GCTFH: CD4+T cells (%)
10
9
8
7
6
5
2.0
1.5
1.0
0.5
0.0
RT non-RT

Figure 3. RT tonsils have reduced GAS-specific GC-T_{FH} cells.

(A) Identification of GAS-specific $CD4^+$ T cells ($CD45RA^-$) and GAS-specific GC-T_{FH} cells ($CD45RA^-CXCR5^{high}PD\text{-}1^{high}$) using $OX40^+CD25^+$ AIM (AIM_{25}). Tonsil cells were left unstimulated or stimulated with 10μg/mL antibiotic-killed *Lactococcus lactis* (a non-pathogenic Gram positive bacteria which served as a negative control), 10μg/mL heat-inactivated, antibiotic-killed GAS, or 1μg/mL staphylococcal enterotoxin B (SEB, positive control) for 18 hours.

(B) Higher frequencies of GAS-specific GC-T_{FH} cells compared to negative control *L. lactis*-specific GC-T_{FH} cells.

(C) Comparable GAS-specific $CD45RA^-CD4^+$ T cell frequencies, quantified as % of total $CD4^+$ T cells, between RT (n=31) and non-RT tonsils (n=35).

(D) RT tonsils exhibit a bias against GAS-specific GC-T_{FH} differentiation. Among total GAS-specific $CD4^+$ T cell (AIM_{25}^+ $CD45RA^-$), the fraction of GAS-specific GC-T_{FH} cells ($CXCR5^{high}PD\text{-}1^{high}$) was smaller in RT tonsils (n=31) compared to non-RT tonsils (n=35).

**** $P < 0.0001$, * $P < 0.05$. Statistical significance determined by paired t-test (b) and Mann-Whitney tests (c,d).

Figure 4

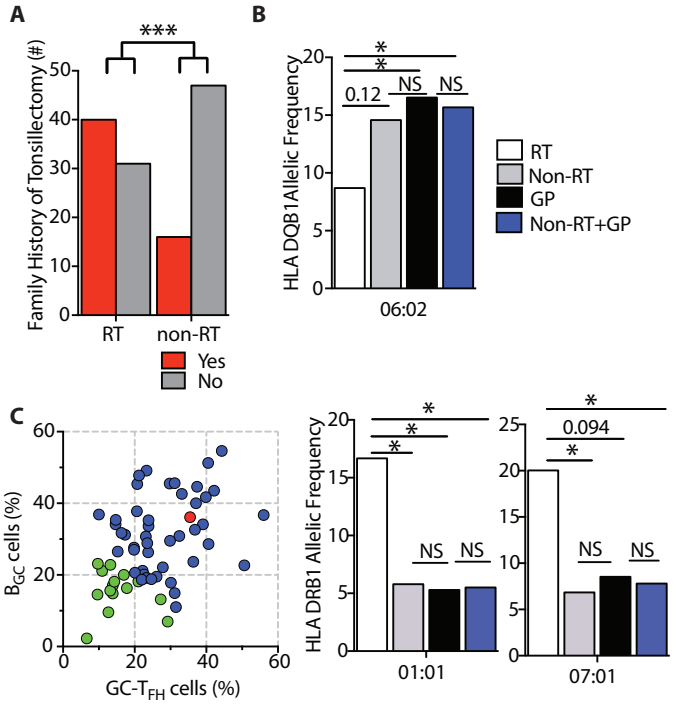
A
***
Family History of Tonsillectomy (#)
50
40
30
20
10
0
RT
non-RT
Yes
No
B
*
*
0.12
NS
NS
HLA DQB1 Allelic Frequency
20
15
10
5
0
06:02
RT
Non-RT
GP
Non-RT+GP
C
60
40
20
0
B_{GC} cells (%)
GC-T_{FH} cells (%)
HLA DRB1 Allelic Frequency
01:01
07:01
0.094
NS

Figure 4. HLA associations identified in RT children.

(A) Family history of tonsillectomy. More RT children have a family history of tonsillectomy than non-RT children. (RT = 71, non-RT = 63).

(B) HLA DQB1*06:02 allelic frequency in non-RT children (grey bar, n=192), RT children (white bar, n=138), ethnically matched adults from the SD general population (GP, black bar, n=242), and non-RT children + GP (blue bar, n=434). HLA DQB1*06:02 frequency is higher in non-RT children compared to GP (black bar) and non-RT children + GP (blue bar).

(C) RT tonsils with the lowest quartile of germinal center activity, defined as lowest combined frequencies of GC-T_{FH} and B_{GC} cells (left panel. Green dots, n=15, GC^{lo} samples, Blue dots, n=46, GC^{lo} samples, Red dot = mean of non-RT GC activity), have a significantly higher frequency of HLA DRB1*01:01 and HLA DRB1*07:01 alleles compared to non-RT tonsils (grey bar, n=190), GP (black bar, n=246), and GP + non-RT tonsils (blue bar, n=436). RT children HLA allele counts (white bar, n=30).

*** $P < 0.001$, * $P < 0.05$. Statistical significance determined by Fisher Exact test (a-c).

Figure 5

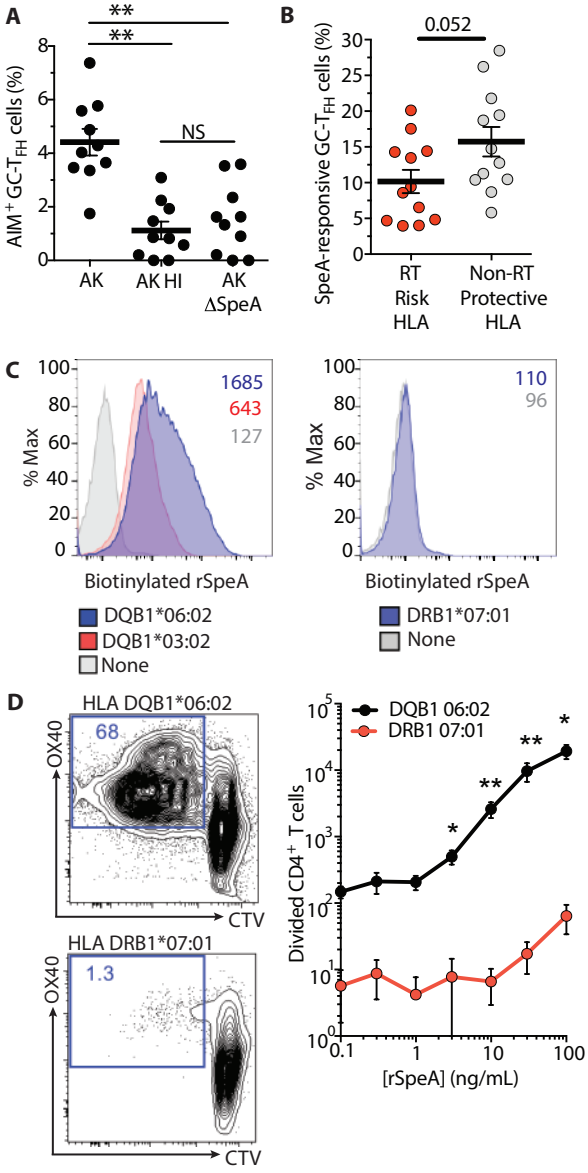
A
**
**
NS
AIM+ GC-TFH cells (%)
AK
AK HI
AK ΔSpeA
B
0.052
SpeA-responsive GC-TFH cells (%)
RT Risk HLA
Non-RT Protective HLA
C
1685
643
127
% Max
Biotinylated rSpeA
DQB1*06:02
DQB1*03:02
None
110
96
DRB1*07:01
None
D
HLA DQB1*06:02
68
OX40
CTV
HLA DRB1*07:01
1.3
DQB1 06:02
DRB1 07:01
Divided CD4+ T cells
[rSpeA] (ng/mL)

Figure 5. HLA Class II associations identified in RT and non-RT children segregate based on preferential GAS superantigen SpeA binding.

(A) Comparison of AIM^+ GC-T_{FH} cells following stimulation with either 10 μg/mL antibiotic-killed GAS (AK), 10 μg/mL antibiotic-killed, heat-inactivated GAS (AK HI), or 10 μg/mL antibiotic-killed SpeA deficient GAS (AK ΔSpeA), n=10. Heat-inactivation of antibiotic-killed GAS inactivates superantigen.

(B) SpeA-responsive GC-T_{FH} cells in tonsils from RT patients with risk HLA alleles (n=12) compared to non-RT patients with protective HLA alleles (n=12). Tonsils were stimulated with 1μg/mL SpeA for 18 hours and background subtracted from unstimulated cells.

(C) Histogram flow cytometric quantitation of SpeA binding. Biotinylated SpeA binds preferentially to HLA DQB1*06:02 > DQB1*03:02 > DRB1*07:01, N=3 experiments.

(D) Total $CD4^+$ T cells from PBMCs of HLA DQB1*06:02^+ donors, co-cultured with SpeA and a cell line expressing HLA DQB1*06:02 proliferated significantly more compared to $CD4^+$ T cells from PBMCs of HLA DRB1*07:01^+ donors, co-cultured with SpeA (recombinant SpeA, rSpeA) and a cell line expressing HLA DRB1*07:01. N=4 experiments.

** $P < 0.01$, * $P < 0.05$ (e). Statistical significance determined by Mann-Whitney test.

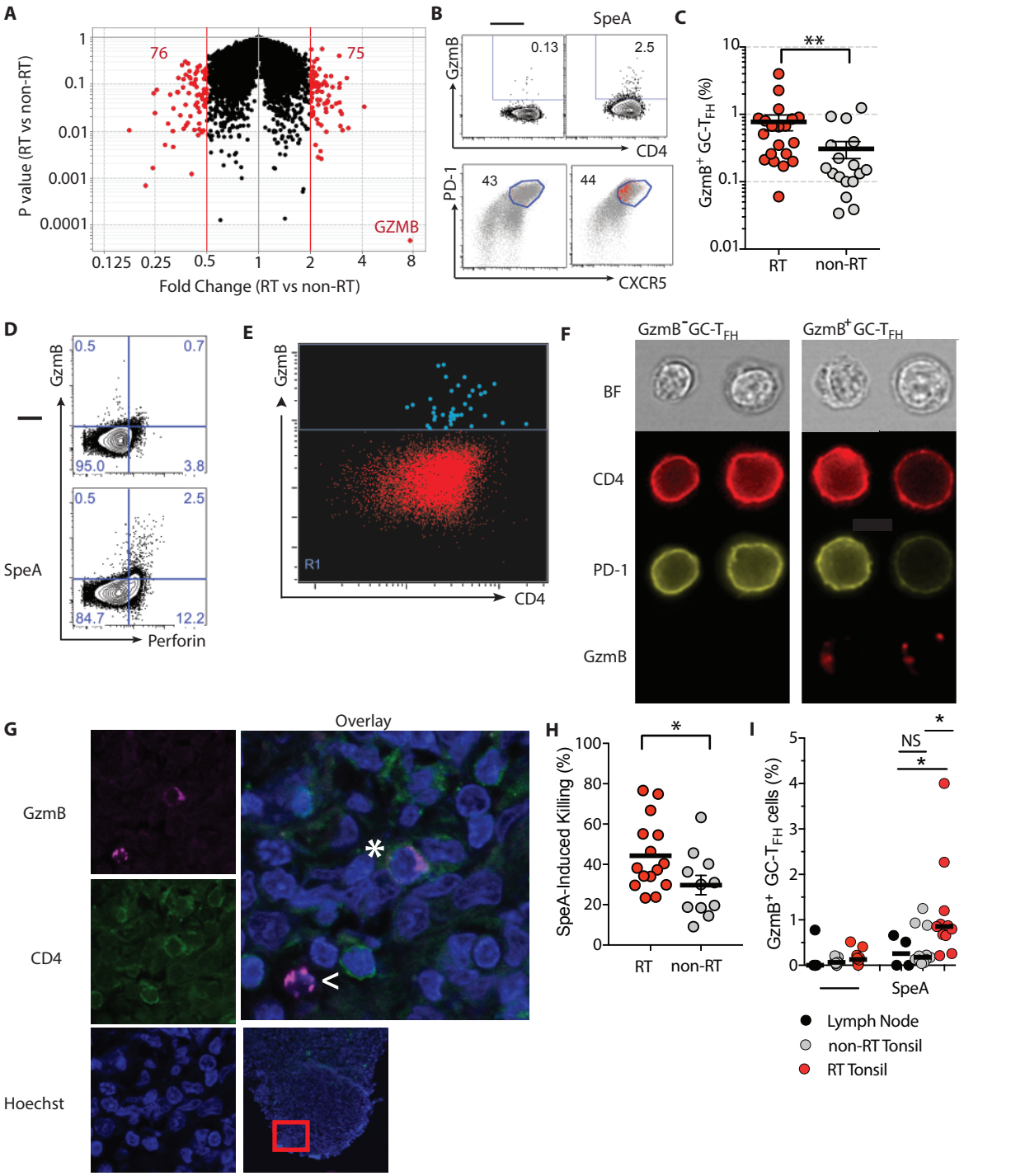
A
P value (RT vs non-RT)
Fold Change (RT vs non-RT)
76
75
GZMB
B
SpeA
GzmB
CD4
PD-1
CXCR5
0.13
2.5
43
44
C
GzmB+ GC-TFH (%)
RT
non-RT
D
GzmB
SpeA
Perforin
E
GzmB
CD4
R1
F
GzmB- GC-TFH
GzmB+ GC-TFH
BF
CD4
PD-1
GzmB
G
Overlay
GzmB
CD4
Hoechst
H
SpeA-Induced Killing (%)
RT
non-RT
I
GzmB+ GC-TFH cells (%)
NS
SpeA
Lymph Node
non-RT Tonsil
RT Tonsil

Figure 6. SpeA stimulation of GC-T_{FH} cells from RT tonsils induces granzyme B.

(A) Volcano plot showing fold change of genes in SpeA-stimulated GC-T_{FH} cells from RT tonsils (n=5) compared to SpeA-stimulated GC-T_{FH} cells from non-RT tonsils (n=5). Red dots denote genes with a < 2 or 2 > fold change. 76 genes exhibited a < 2-fold change and $P < 0.1$, and 75 genes with > 2-fold change and $P < 0.1$.

(B) Intracellular granzyme B expression (%) by GC-T_{FH} cells by flow cytometry. Tonsil cells were stimulated with 1μg/mL SpeA for 24 hours (top). Backgating of the granzyme B^+ GC-T_{FH} cells among total $CD45RA^-$ $CD4^+$ T cells (bottom).

(C) SpeA stimulation revealed significantly more granzyme B^+ GC-T_{FH} cells in RT tonsils (n=20) compared to non-RT tonsils (n=17).

(D) SpeA stimulated GC-T_{FH} cells co-expressed granzyme B and perforin. FACS sorted GC-T_{FH} cells and autologous B cells were cultured +/- SpeA for 5 days and stained for granzyme B and perforin expression. N=3 donors.

(E) ImageStream cytometry plot of granzyme B^+ GC-T_{FH} cells following SpeA stimulation. GC-T_{FH} cells were gated as $CXCR5^{hi}PD\text{-}1^{hi}$ of live $CD45RA^-CD4^+$ T cells.

(F) ImageStream imaging of GC-T_{FH} cells following SpeA stimulation, showing representative granzyme B^- and granzyme B^+ cells.

(G) Confocal microscopy of a granzyme B^+ $CD4^+$ T cell in a germinal center in an RT tonsil (*). A granzyme B^+ $CD8^+$ T cell is also shown for reference (<).

(H) SpeA-stimulated GC-T_{FH} cells are able to kill B cells. GC-T_{FH} cells ($CXCR5^{hi}PD\text{-}1^{hi}CD45RA^-$ $CD4^+$) were co-cultured with autologous CTV-labeled B cells ($CD19^+CD38^-$). Killing was quantified as outlined in the Methods, with controls shown in **Supplementary Figure 8i-k**. N=15 RT and 11 non-RT donors.

(I) Granzyme B expression (%) by GC-T_{FH} cells from healthy lymph nodes and RT and non-RT tonsils. SpeA-stimulated GC-T_{FH} cells from RT tonsils (N=11) expressed more granzyme B than SpeA-stimulated GC-T_{FH} cells from non-RT tonsils (n=11) or healthy lymph nodes (n=4).

** $P < 0.01$, * $P < 0.05$. Statistical significance determined by Mann-Whitney test (c, h, i).

Table 1A: Study participant demographics for cohort 1

	RT (n=26)	Non-RT (n=39)	P-value
Gender (%)			0.21[a]
Female	65.4%	48.7%	
Male	34.6%	51.2%	
Age (mean years)	11	8.31	0.0092[b]

Table 1B: Study participant demographics for cohort 2

	RT (n=40)	Non-RT (n=41)	P-value
Gender (%)			0.0058[a]
Female	77.5%	46.3%	
Male	22.5%	53.7%	
Age (mean years)	9.65	8.39	0.21[b]

Table 1C: Study participant demographics for entire combined cohort

	RT (n=66)	Non-RT (n=80)	P-value
Gender (%)			0.0055[a]
Female	72.7%	47.5%	
Male	27.3%	52.5%	
Age (mean years)	10.18	8.35	0.0024[b]

[a]P-value determined by Fisher Exact using R. [b]P-value determined by Mann-Whitney U test.