PEDIATRICS

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

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"Strep throat" is highly prevalent among children, yet it is unknown why only some children develop recurrent tonsillitis (RT), a common indication for tonsillectomy. To gain insights into this classic childhood disease, we performed phenotypic, genotypic, and functional studies on pediatric group A *Streptococcus* (GAS) RT and non-RT tonsils from two independent cohorts. GAS RT tonsils had smaller germinal centers, with an underrepresentation of GAS-specific CD4$^+$ germinal center T follicular helper (GC-T$_{FH}$) 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. Lastly, SpeA induced granzyme B production in GC-T$_{FH}$ cells from RT tonsils with the capacity to kill B cells and the 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 (LNs), and absence of cough warrant testing for group A *Streptococcus* (GAS, also known as *Streptococcus pyogenes*) (*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) because of GAS (*8*, *9*). Tonsillitis is a substantial health care burden and cause of repeated antibiotic usage. RT can be a severe disease, resulting in substantial morbidity and school absences in hundreds of thousands of children per year. There are more than 750,000 tonsillectomies performed annually in the United States, with RT being a common indication (*2*, *8*, *10*). Tonsils are LN-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 (*11*, *12*). It remains a long-standing 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 to 18 years) from the San Diego (SD) area undergoing tonsillectomies for GAS RT or for noninfectious reasons, e.g., sleep apnea (non-RT). 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 reduced in RT disease

By clinical history, RT children in our first SD cohort had a mean of 12 tonsillitis episodes in total 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 to 30%) (*9*, *13*, *14*). 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 to 18 years (cohort 1; Table 1). Tonsils contain germinal centers (GCs), composed of germinal center T follicular helper (GC-T$_{FH}$) cells, follicular dendritic cells, and germinal center B (B$_{GC}$) cells (*15*). T$_{FH}$ cells are a distinct type of CD4$^+$ T cells that provide help to B cells (*16*, *17*). T$_{FH}$ cells are required for GCs and thus almost all affinity-matured antibody responses to pathogens (*18*). GC-T$_{FH}$ cells instruct the survival, proliferation, and somatic hypermutation of B$_{GC}$ cells. RT tonsils contained a significantly reduced frequency of GC-T$_{FH}$ cells (CD4$^+$CD45RO$^+$CXCR5^{hi}PD-1^{hi}) compared to non-RT tonsils (*P* = 0.0001; Fig. 1, B and C, and fig. S1A). Mantle T$_{FH}$ cell frequencies (mT$_{FH}$; CXCR5$^+$PD-1$^+$, T$_{FH}$ cells outside of GCs) were not significantly different (*P* = 0.076; fig. S1B). There was no difference in

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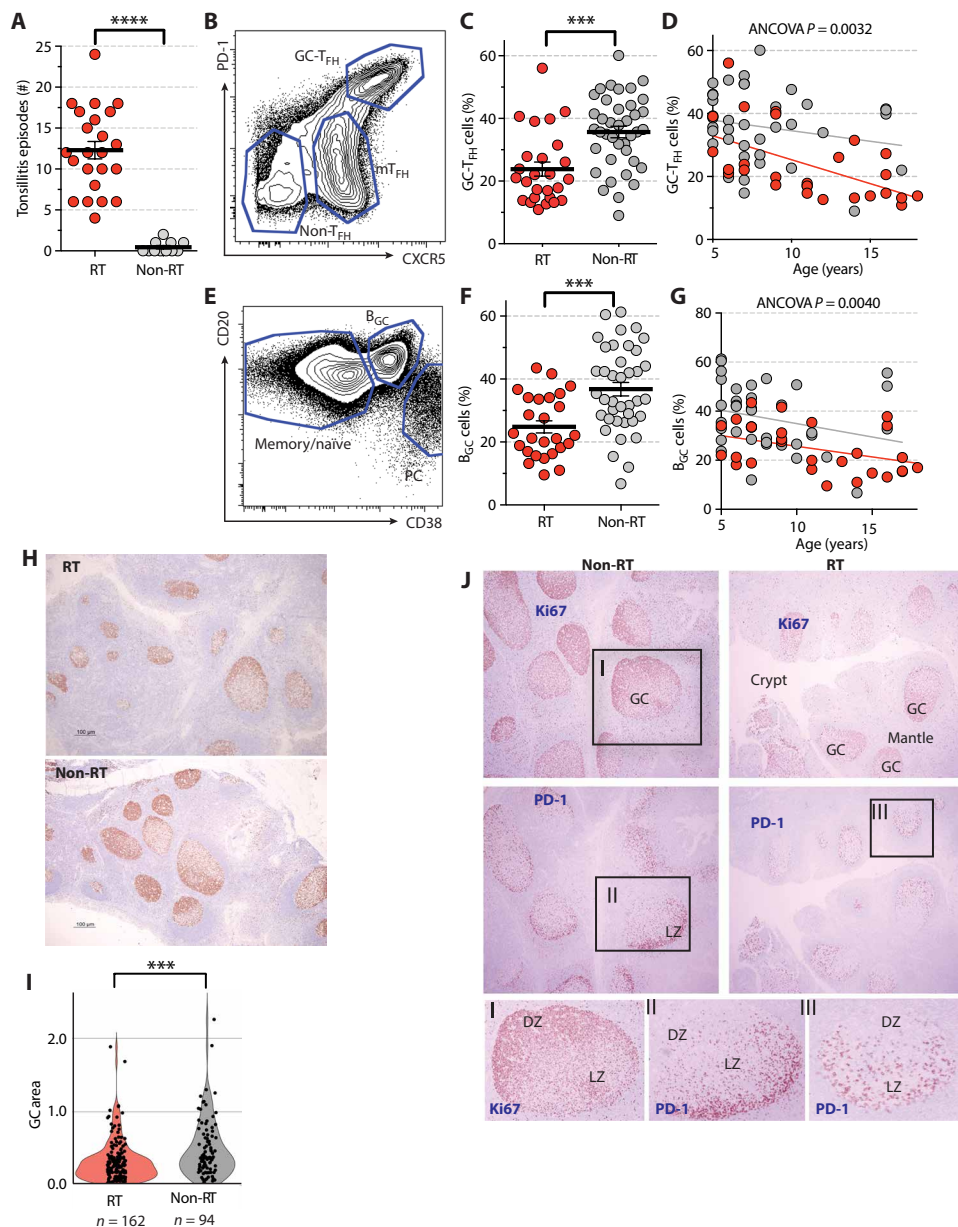Dan *et al.*, *Sci. Transl. Med.* 11, eaau3776 (2019)   6 February 2019

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Fig. 1. RT children have fewer GC-T$_{FH}$ cells in their tonsils. Immunophenotyping analysis of cohort 1 of patients with and without RT. (**A**) Number of RT episodes in RT children ($n = 23$) and non-RT children ($n = 11$). (**B**) Flow cytometry of GC-T$_{FH}$ (CXCR5^{hi}PD-1^{hi}CD45RO$^+$CD4$^+$), mT$_{FH}$ (CXCR5$^+$PD-1$^+$CD45RO$^+$CD4$^+$), and non-T$_{FH}$ (CXCR5$^-$CD45RO$^+$CD4$^+$) cells. (**C**) GC-T$_{FH}$ cell frequencies in RT tonsils ($n = 26$) and non-RT tonsils ($n = 39$), quantified as percentage of total CD4$^+$ T cells. (**D**) GC-T$_{FH}$ cells by age. (**E**) Flow cytometry of B$_{GC}$ cells (CD38$^+$CD20$^+$CD19$^+$), plasma cells (PC; CD38^{hi}CD20$^+$CD19$^+$), and memory (CD27^{hi}CD20$^+$CD19$^+$)/naïve (CD27$^-$CD20$^+$CD19$^+$) B cells. (**F**) B$_{GC}$ cell frequencies in RT and non-RT tonsils, quantified as percentage of total B cells. (**G**) B$_{GC}$ cells by age. (**H**) Representative Ki67-stained sections from RT and non-RT tonsils. m.(**I**) Quantitation of GC areas (in 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 [programmed cell death protein 1 (PD-1)]. Insets: Enlarged versions of representative GCs stained for Ki67 or PD-1. II and III show PD-1$^+$ GC-T$_{FH}$ cells in representative GCs from a non-RT tonsil and an RT tonsil, respectively. ****$P < 0.0001$, ***$P < 0.001$. Statistical significance was determined by Mann-Whitney tests (A to C, E, F, and I) and multivariate analysis of covariance (ANCOVA; D and G). DZ, dark zone; LZ, light zone.

parable naïve 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.

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. 1, E and F, and fig. S1A). This reduction remained statistically significant with or without age ($P = 0.0040$; Fig. 1G) or gender ($P = 0.0064$; fig. S1G) as a covariate. Memory B cell frequencies were comparable ($P = 0.16$; fig. S1H), plasma cell frequencies were lower ($P = 0.006$; fig. S1I), and naïve B cell frequencies were higher in RT tonsils ($P = 0.0002$; fig. S1J).

Histological examination revealed that RT tonsils had smaller GCs compared to non-RT tonsils ($P < 0.002$; Fig. 1, H and I). GC 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 GCs suggested a potential CD4$^+$ T cell defect in RT disease, consistent with the flow cytometry data. However, differences in GC-T$_{FH}$ cell frequencies and GC sizes could not be directly ascribed as RT associated without additional information; thus, we explored additional parameters to establish whether the GC differences were associated with RT disease.

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

Diminished GC 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 to 18 years undergoing tonsillectomies for either RT or non-RT, e.g., sleep apnea (cohort 2; Table 2). 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). A simple expectation based on clinical history was that RT children would have higher concentrations of GAS-specific

B cell lymphoma 6 (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 com-

Table 1. Study participant demographics for cohort 1.

	RT ($n = 26$)	Non-RT ($n = 39$)	P value
Gender (%)			0.21*
Female	65.4%	48.7%	
Male	34.6%	51.2%	
Age (mean years)	11	8.31	0.0092†

*P value determined by Fisher's exact test using R. †P value determined by Mann-Whitney U test.

antibodies than non-RT children because the former group had multiple bouts of tonsillitis, including experiencing a tonsillitis episode within a few months before surgery. However, anti-SLO immunoglobulin G (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. RT children had significantly lower anti-SpeA IgG titers than non-RT children ($P = 0.024$; Fig. 2B). The 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). Anti-SpeA antibodies have been implicated epidemiologically in immunity against severe systemic GAS infections in adults (*19*, *20*). SpeA antibodies have been shown to be protective in a mouse GAS infection model (*21*). 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. 2, C to F, and fig. S2). RT tonsils of cohort 2 had significantly lower frequencies of GC-T_{FH} cells than non-RT tonsils ($P < 0.0001$; Fig. 2C), which were 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. 2, E and F), suggesting that an RT immunological defect may be directly related to GC-T_{FH} cells.

RT CD4+ T cells are skewed away from GAS-specific GC-T_{FH} cells differentiation

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 (Figs. 3 to 6 and figs. S3 to S8), samples from both cohorts 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 used our previously developed cytokine-independent approach to identify antigen-specific GC-T_{FH} cells by T cell receptor–dependent activation-induced markers (AIMs), expressed upon recognition of antigen (*22*–*24*). We applied the AIM technique to quantify tonsillar GAS-specific CD4+ T cells (Fig. 3A). The nonpathogenic Gram-positive bacterium *Lactococcus lactis* was used as a negative control antigen (Fig. 3, A and B). Because children with RT experienced 12 times more tonsillitis episodes than non-RT
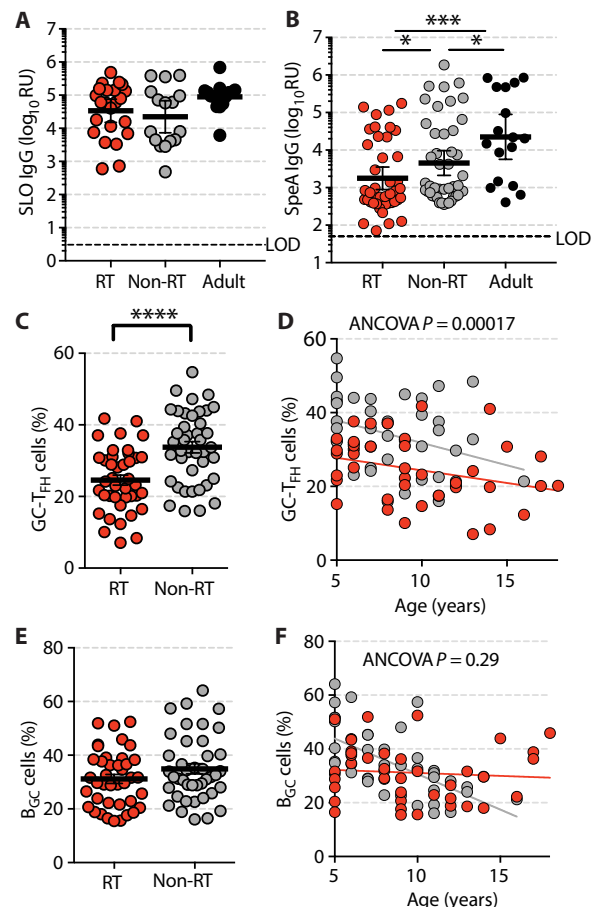

Fig. 2. RT children have lower circulating anti-SpeA IgG titers. Serological and immunophenotyping analysis of cohort 2 of patients with and without RT. (**A**) Plasma anti-SLO IgG titers in RT children ($n = 23$), non-RT children ($n = 16$), and normal healthy adults ($n = 14$). LOD, limit of detection; RU, relative units. (**B**) Plasma anti-SpeA IgG titers in RT children ($n = 42$), non-RT children ($n = 45$), and normal healthy adults ($n = 17$). (**C**) GC-T_{FH} cell frequencies in RT tonsils ($n = 40$) and non-RT tonsils ($n = 41$), quantified as percentage of total CD4+ T cells. (**D**) GC-T_{FH} cells by age. (**E**) B_{GC} cell frequencies in RT and non-RT tonsils, quantified as percentage of total B cells. (**F**) B_{GC} cells by age. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$. Statistical significance was determined by Mann-Whitney test.

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} cells (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} cell differentiation, with a lower ratio of GAS-specific GC-T_{FH} cells to total GAS-specific CD4+ T cells ($P = 0.023$; Fig. 3D). 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 whether 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
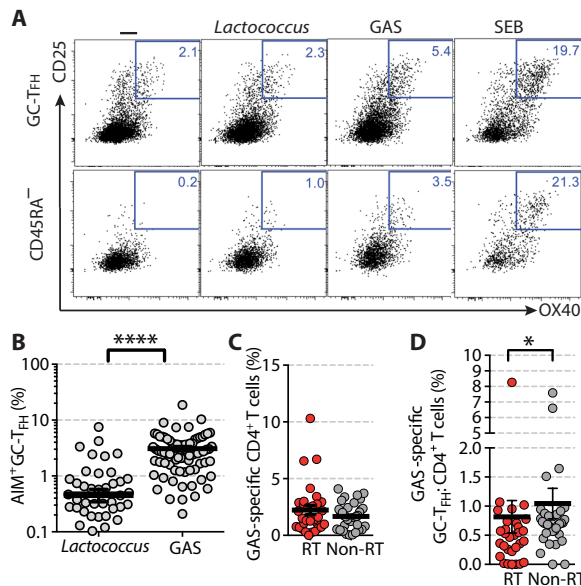
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Fig. 3. RT tonsils have reduced GAS-specific GC-T$_{FH}$ cells. (**A**) Identification of GAS-specific CD4$^+$ T cells (CD45RA$^-$) and GAS-specific GC-T$_{FH}$ cells (CD45RA$^-$CXCR5^{hi}PD-1^{hi}) using OX40$^+$CD25$^+$ AIM (AIM$_{25}$). Tonsil cells were left unstimulated or stimulated with antibiotic-killed *L. lactis* (a nonpathogenic Gram-positive bacteria that served as a negative control; 10 μg/ml), heat-inactivated, antibiotic-killed GAS (10 μg/ml), or staphylococcal enterotoxin B [SEB (positive control); 1 μg/ml] for 18 hours. (**B**) GAS-specific GC-T$_{FH}$ cell frequencies. (**C**) GAS-specific CD45RA$^-$CD4$^+$ T cell frequencies, quantified as percentage of total CD4$^+$ T cells, in RT tonsils (n = 31) and non-RT tonsils (n = 35). (**D**) Fraction of GAS-specific GC-T$_{FH}$ cells (CXCR5^{hi}PD-1^{hi}) among total GAS-specific CD4$^+$ T cell (AIM$_{25}$$^+$ CD45RA$^-$) in RT tonsils (n = 31) and non-RT tonsils (n = 35). ****P < 0.0001, *P < 0.05. Statistical significance was determined by paired t test (B) and Mann-Whitney tests (C and D).

Fig. 4. HLA class II associations identified in RT children. (**A**) Family history of tonsillectomy (RT = 71 and non-RT = 63). (**B**) HLA DQB1*06:02 allelic frequency in non-RT children (gray bar, n = 192), RT children (white bar, n = 138), ethnically matched adults from the SD GP (black bar, n = 242), and non-RT children and GP (blue bar, n = 434). NS, not significant. (**C**) Left: HLA DRB1*01:01 and HLA DRB1*07:01 allelic frequencies in RT tonsils with the lowest quartile of GC activity, defined as the lowest combined frequencies of GC-T$_{FH}$ and B$_{GC}$ cells [green dots, n = 15 (GC^{lo} samples); blue dots, n = 46 (GC^{lo} samples); red dot, mean of non-RT GC activity]. Right: HLA DRB1*01:01 and HLA DRB1*07:01 allelic frequencies in non-RT tonsils (gray bar, n = 190), GP (black bar, n = 246), and GP and non-RT tonsils (blue bar, n = 436). RT children HLA allele counts (white bar, n = 30). ***P < 0.001, *P < 0.05. Statistical significance was determined by Fisher's exact test (A to C).

mRNA abundance was low, and interleukin-4 (IL-4) protein was below the limit of detection (*22*). 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 class II alleles

Essentially, all children are exposed to GAS during childhood (*25*). Among children enrolled in this study, RT children were likely to have a significant family history of tonsillectomy (P = 0.0004; Fig. 4A), suggesting a potential genetic predisposition. GC responses depend on human leukocyte antigen (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 (*26*). The DQB1*06:02 has also been associated with protection from the development of rheumatic heart disease (*27, 28*), the most severe sequela of long-term untreated GAS RT and the leading cause of heart failure in children worldwide (*1, 29*). We performed HLA typing on the entire tonsillar cohort to test whether HLA associations previously associated with other GAS-related diseases may have relevant associations with RT disease. 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 GP (P = 0.042; Fig. 4B and fig.
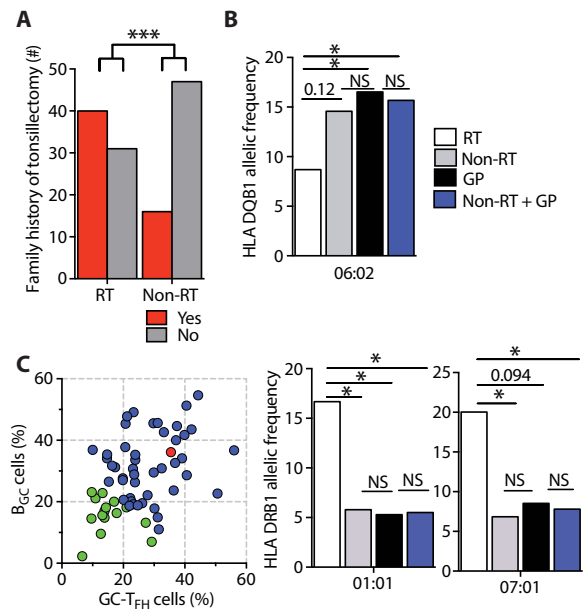
S4B). HLA DQB1*06:02 was also significantly less frequent in RT children than the combined groups of non-RT children and GP (P = 0.048; Fig. 4B and fig. S4B). There was no difference in the HLA DQB1*06:02 allelic frequency between non-RT children and ethnically matched SD GP (P = 0.89; Fig. 4B and fig. S4B). Overall, these data suggest that DQB1*06:02 is a protective HLA 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 and DRB1*07:01 (*28, 30*) 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 GC 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 and fig. S4, B (GC^{lo}) and C]. These children had significantly higher frequencies of HLA DRB1*01:01 compared to the GP (P = 0.03), non-RT children (P = 0.049), and the combined control groups (P = 0.03; Fig. 4C and fig. S4B). Frequencies of HLA DRB1*07:01 were also elevated compared to non-RT children and the combined control groups (P = 0.03 and P = 0.03, respectively; Fig. 4C and fig. S4B). In contrast, no differences were identified between the non-RT and GP cohorts for HLA DRB1*01:01 (P = 0.85) or HLA DRB1*07:01 (P = 0.74; Fig. 4C and fig. S4B). We additionally

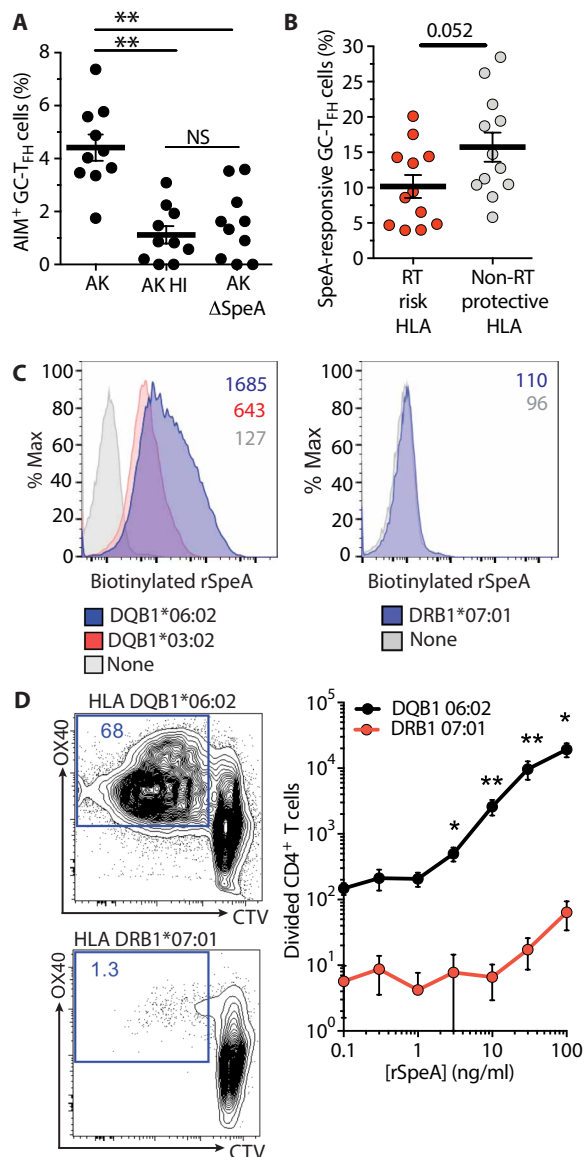Dan *et al.*, *Sci. Transl. Med.* **11**, eaau3776 (2019) 6 February 2019

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Fig. 5. HLA class II associations identified in RT and non-RT children segregate on the basis of preferential GAS superantigen SpeA binding. (**A**) Comparison of AIM$^+$ GC-T$_{FH}$ cells after stimulation with either antibiotic-killed (AK) GAS (10 μg/ml), antibiotic-killed, heat-inactivated (AK HI) GAS (10 μg/ml), or antibiotic-killed SpeA-deficient (AK ΔSpeA) GAS (10 μg/ml); $n = 10$, including RT and non-RT donors. Heat-inactivation of antibiotic-killed GAS inactivates GAS superantigens. (**B**) SpeA-responsive GC-T$_{FH}$ cells in tonsils from patients with RT with risk HLA alleles ($n = 12$) compared to non-RT patients with protective HLA alleles ($n = 12$). Tonsils were stimulated with SpeA (1 μg/ml) for 18 hours and background-subtracted as determined with unstimulated cells. (**C**) Histogram flow cytometric quantitation of SpeA binding by HLA allele using HLA cell lines. $n = 3$ experiments. (**D**) Proliferation of total CD4$^+$ T cells from peripheral blood mononuclear cells (PBMCs) of HLA DQB1*06:02$^+$ donors cocultured with recombinant SpeA (rSpeA) and a cell line expressing HLA DQB1*06:02 and of CD4$^+$ T cells from PBMCs of HLA DRB1*07:01$^+$ donors cocultured with rSpeA and a cell line expressing HLA DRB1*07:01. $n = 4$ experiments. **$P < 0.01$, *$P < 0.05$ (D). Statistical significance was determined by Mann-Whitney test. CTV, cell trace violet.

compared the lowest quartile of GC-T$_{FH}$ and B$_{GC}$ cells from non-RT children to all RT children and the GP 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 GC responses.

RT-associated HLA alleles differentially affect 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 GAS strain M1T1 5448, with or without heat inactivation, or an antibiotic-killed isogenic SpeA-deficient mutant GAS strain (ΔspeA), 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 and fig. S5A). SpeA has provided certain GAS strains with an evolutionary advantage (21, 31, 32), 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 potentially less responsive to SpeA stimulation than non-RT tonsils bearing the protective HLA DQB1*06:02 allele ($P = 0.052$; Fig. 5B and fig. S5B). Although 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 (29), but a potential role has been suggested for SpeA (26, 33, 34). 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 and fig. S5C), whereas 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 and 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. 5, C and 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

Although we observed that GC-T$_{FH}$ cell frequencies were significantly lower in RT compared to non-RT tonsils, BCL6 expression by GC-T$_{FH}$ cells was equivalent on a per-cell basis. To identify CD4$^+$ T cell factors potentially involved in SpeA superantigen–associated GC 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$_{FH}$ and immunomodulatory T helper 1 (T$_H$1), T$_H$2, or T$_H$17 cytokine mRNA expression (fig. S6). *GZMB* mRNA, encoding the cytotoxic effector protein granzyme B (GzmB), was up-regulated in RT GC-T$_{FH}$ cells ($P = 0.0079$; Fig. 6A, fig. S7A, and table S1). GzmB is typically secreted by cytotoxic CD8$^+$ T cells and natural killer cells for killing of target cells. Expression of GzmB by GC-T$_{FH}$ cells could be counterproductive to the B cell help function of GC-T$_{FH}$ cells. A potential mechanism by which GAS disrupts antibody responses could be aberrant GzmB expression by GC-T$_{FH}$ cells, converting a GC-T$_{FH}$ cell from one that helps B$_{GC}$ cells to one that kills B$_{GC}$ cells.
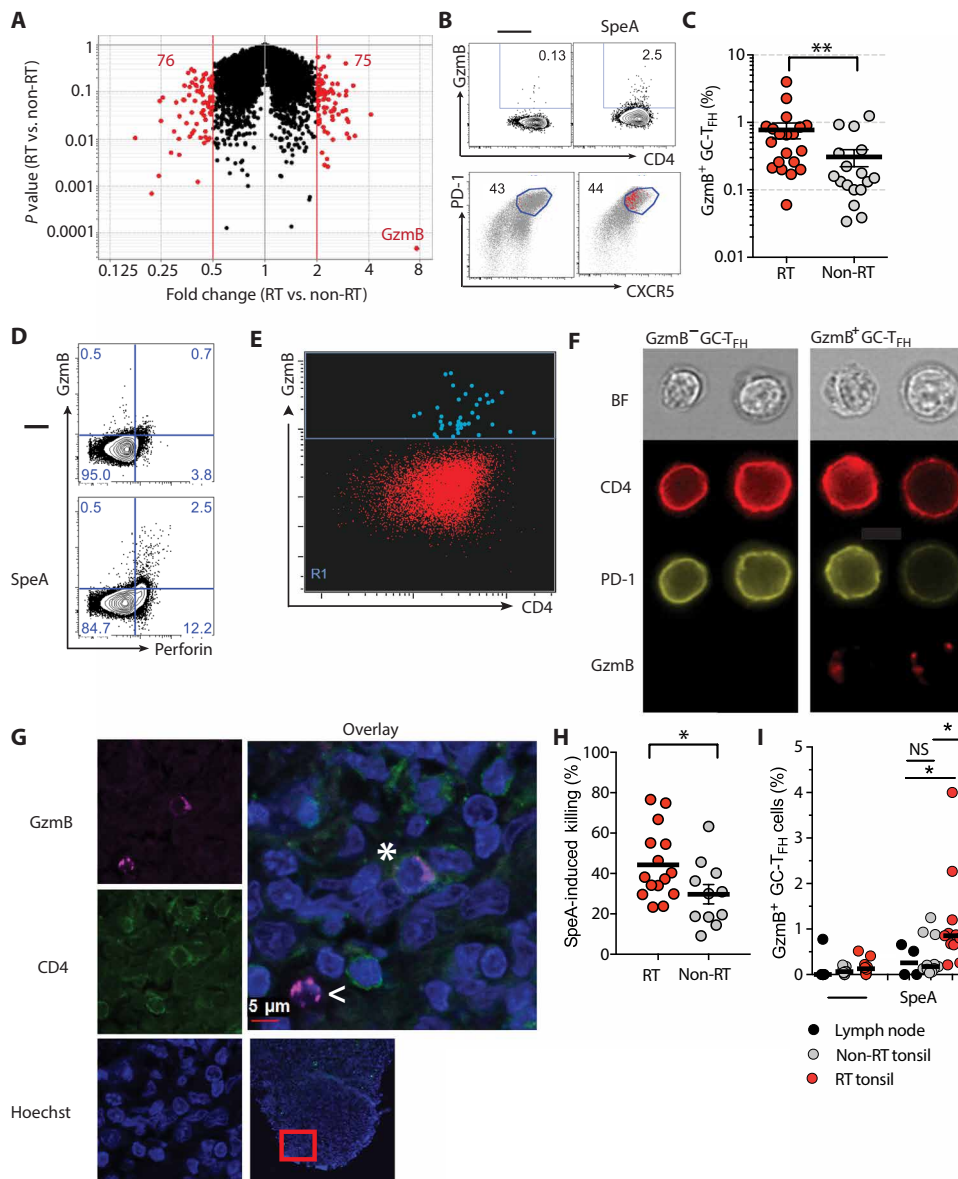
Fig. 6. SpeA stimulation of GC-T$_{FH}$ cells from RT tonsils induces GzmB. (**A**) Volcano plot showing fold change of genes in SpeA-stimulated GC-T$_{FH}$ cells from RT tonsils ($n = 5$) compared to SpeA-stimulated GC-T$_{FH}$ cells from non-RT tonsils ($n = 5$). Red dots denote genes with a fold change of <0.5 or >2. (**B**) Frequency of intracellular GzmB expression in GC-T$_{FH}$ cells by flow cytometry. Tonsil cells were stimulated with SpeA (1 μg/ml) for 24 hours (top). Backgating of the GzmB$^+$ GC-T$_{FH}$ cells among total CD45RA$^-$ CD4$^+$ T cells (bottom). (**C**) GzmB$^+$ GC-T$_{FH}$ cells in RT tonsils ($n = 20$) and non-RT tonsils ($n = 17$) after SpeA stimulation. (**D**) Fluorescence-activated cell–sorted GC-T$_{FH}$ cells and autologous B cells were cultured ± SpeA for 5 days and stained for GzmB and perforin expression. $n = 3$ donors. (**E**) ImageStream cytometry plot of GzmB$^+$ GC-T$_{FH}$ cells after SpeA stimulation. GC-T$_{FH}$ cells were gated as CXCR5^{hi}PD-1^{hi} of live CD45RA$^-$CD4$^+$ T cells. $n = 1$ donor. (**F**) ImageStream imaging of GC-T$_{FH}$ cells after SpeA stimulation, showing representative GzmB$^-$ and GzmB$^+$ cells. (**G**) Confocal microscopy of a GzmB$^+$ CD4$^+$ T cell in a GC in an RT tonsil (*). A GzmB$^+$ CD8$^+$ T cell is also shown for reference (<). m.$n = 8$ donors. (**H**) GC-T$_{FH}$ cells (CXCR5^{hi}PD-1^{hi}CD45RA$^-$CD4$^+$) were cocultured with autologous CTV-labeled B cells (CD19$^+$CD38$^-$). Killing was quantified as outlined in Materials and Methods, with controls shown in fig. S7 (J to L). $n = 15$ and 11 (RT and non-RT donors, respectively). (**I**) GzmB expression (percentage) by GC-T$_{FH}$ cells from healthy LNs and from RT and non-RT tonsils. GzmB expression after SpeA stimulation of GC-T$_{FH}$ cells from RT tonsils ($n = 11$), non-RT tonsils ($n = 11$), or healthy LNs ($n = 4$). **$P < 0.01$, *$P < 0.05$. Statistical significance was determined by Mann-Whitney test (C, H, and I).

T$_{FH}$ cells, (ii) ImageStream imaging cytometry of SpeA-stimulated GC-T$_{FH}$ cells, (iii) immunofluorescence microscopy of human tonsillar tissue, and (iv) killing of target cells. GC-T$_{FH}$ cell intracellular protein staining confirmed SpeA-induced GzmB expression ($P = 0.006$; Fig. 6, B and 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 a patient with RT by ImageStream (Fig. 6, E and F). These changes were specific to GC-T$_{FH}$ cells because there were no differences in the frequencies of GzmB$^+$ mT$_{FH}$ cells (fig. S7, D and G), non-T$_{FH}$ cells (fig. S7, E and G), or CD8$^+$ T cells (fig. S7, F and H) between RT and non-RT tonsils. These GzmB$^+$ GC-T$_{FH}$ cells were not regulatory T cells, because they did not express FoxP3 or Helios (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 typically difficult to demonstrate in vitro; nevertheless, killing of B cells by GC-T$_{FH}$ cells was observed in the presence of SpeA (Fig. 6H and fig. S7, J and 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). Phytohemagglutinin 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 Fas ligand (fig. S7, N and 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 LNs from adult 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 induced upon SpeA stimulation ($P = 0.025$; Fig. 6I). GC-T$_{FH}$ cells from non-RT tonsils and healthy LNs were comparable (Fig. 6I). Collectively, these data suggest that

To determine whether RT GC-T$_{FH}$ cells were capable of GzmB protein expression, four independent approaches were used as follows: (i) flow cytometry of intracellular-stained SpeA-stimulated GC-

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.

Table 2. Study participant demographics for cohort 2.

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

*P value determined by Fisher's exact test using *R*. †*P* value determined by Mann-Whitney *U* test.

Table 3. Study participant demographics for entire combined cohort.

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

*P value determined by Fisher's exact test using *R*. †*P* value determined by Mann-Whitney *U* test.

DISCUSSION

By integrating immune profiling and clinical data with transcriptomic and functional analyses, we revealed immunologic features of GAS RT that provide evidence that RT is an immunosusceptibility disease. We observed that (i) RT tonsils have significantly lower GC-T$_{FH}$ cell frequencies; (ii) RT children have impaired anti-SpeA antibody titers, which have been associated with protective immunity to GAS; (iii) specific HLA class II alleles were associated with RT; and (iv) SpeA can induce GzmB expression in GC-T$_{FH}$ cells. Although 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 affecting GAS superantigen binding. SpeA perturbation of GC-T$_{FH}$ cells with 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 because of 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, 35, 36*). However, previous studies have observed that RT and non-RT children have similar asymptomatic GAS carriage rates (*9, 13, 14*). 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 (*37, 38*). It is notable that the M1 serotype has a bacteriophage encoding SpeA, and the acquisition of SpeA has been implicated in the dominance of the M1 pandemic strain in the United States (*39, 40*). Here, we observed that SpeA contributed substantially 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 previously unidentified 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. 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 GC. We contend that the cytotoxicity scenario is fundamentally different for GzmB$^+$ GC-T$_{FH}$ cell disruption of GCs than it is for cytolytic control of a viral infection. B$_{GC}$ cells are probably among the most proapoptotic cells in the body. Each B$_{GC}$ cell requires stimulation by a T$_{FH}$ cell every few hours or it will die (*41–43*). In addition, unlike most cell types, the B$_{GC}$ cells are all confined to a densely packed space, the GC. GC-T$_{FH}$ cells are constantly making short (~5 min) cognate interactions with B$_{GC}$ cells (*44–46*). Thus, in a 24-hour period, 10 GC-T$_{FH}$ cells can make cognate interactions with 2880 B$_{GC}$ cells, and an average GC contains only ~1000 total B$_{GC}$ cells (*47*). In contrast, cytolytic killing of virally infected cells takes much longer cognate interactions, with more resistant cells, over a much greater three-dimensional space (*48*). Hence, we consider it a reasonable model that it may take relatively little GzmB to kill a B$_{GC}$ cell and that GzmB$^+$ T$_{FH}$ could serially poison many B$_{GC}$ 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 LNs and tonsils (*49, 50*). In this study, we observed no RNA expression of granzyme A and no differences in CD57, signal transducer and activator of transcription 3 (STAT3), or cytotoxic and regulatory T cell molecule (CRTAM) between RT and non-RT tonsils (fig. S8, A to D). However, we did observe similarities between GzmB$^+$ GC-T$_{FH}$ and recent reports of CD8$^+$ T cells acquiring T$_{FH}$ phenotypic features (*51, 52*). CXCR5$^+$ CD8$^+$ T cells have been identified in the context of HIV, simian immunodeficiency virus, and lymphocytic choriomeningitis virus chronic infections and have the capacity to migrate into B cell follicles and exhibit cytotoxicity (*53–56*). Anti-PD1 immunotherapy predominantly rescues exhausted CD8$^+$ T cells via outgrowth of CXCR5$^+$ CD8$^+$ T cells (*56*). Development of CXCR5$^+$ CD8$^+$ T cells is associated with up-regulation of key T$_{FH}$ transcription factors T cell factor 1 (TCF1) and BCL6 (*57*) and a substantial reduction in the expression of GzmB by the CXCR5$^+$ CD8$^+$ T cells (*56, 58*). In this study of RT GC-T$_{FH}$ cells, the opposite was observed; down-regulation of *TCF1* and its homolog *LEF1* occurred in SpeA-stimulated GC-T$_{FH}$ cells commensurate with GzmB up-regulation, suggesting that the TCF1/lymphoid enhancer factor (LEF1) axis may be required for separation of T$_{FH}$ and cytolytic transcriptional programs in both CD4$^+$ and CD8$^+$ T cells. Together, the data from this study suggest that conversion of GC-T$_{FH}$ cells 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 RT, 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 with 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 on the basis of history provided by the referring pediatrician or parent, with the tonsils analyzed in an unblinded fashion. There were no culture data for the RT group to determine which GAS serotype was the etiologic agent of strep throat. Tonsils were acquired at hospitals specifically in the SD area, and thus, some of the findings here may be specific to certain geographical limitations. In addition, robust statistical associations with HLA class II alleles frequently require cohorts of 1000 or more individuals. Future studies will clearly be of value to address these limitations.

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 (21, 31). 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 reexposure. 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 and as a means to reduce costly RT antibiotics treatments and surgeries per year and to reduce childhood strep throat disease burden generally.

In conclusion, we provide evidence that RT 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. Lastly, the finding of GzmB+ perforin+ GC-T_{FH} cells points to a pathological mechanism of GC control.

MATERIALS AND METHODS
Study design
The goal of the study was to understand why only some children get recurrent GAS-associated tonsillitis. On the basis of discussions with pediatricians and pediatric otolaryngologists, we restricted recruitment to children ages 5 to 18 years 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 because (i) tonsils are never removed from healthy children; (ii) partial tonsil biopsies are not feasible because of the small risk of life-threatening oropharyngeal hemorrhage; (iii) cadaveric tonsils are not adequate for GC research purposes because there are few live cells to perform functional assays, and the quality of the cells is uncertain because of the highly apoptotic nature of GCs; (iv) pediatric whole-body organ donors are extremely rare, and those with tonsils harvested are even rarer; and (v) 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. 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 criterion.

Cohort 1 (Table 1) consisted of children enrolled at the Naval Medical Center and Rady Children's Hospital. Cohort 2 (Table 2) 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 (cohorts 1 and 2) are in Table 3. Informed consent was obtained from all donors under protocols approved by the IRBs of the University of California, San Diego (UCSD; 131099), Rady Children's Hospital (RCHD 2847), Naval Medical Center (NCRADA-NMCSD-11-378), and La Jolla Institute for Immunology (LJI; VD-108-1113). Antibody panels are described in tables S2 to S8. Primary data are reported in data file S1.

Statistical analysis
All statistical analyses were performed using two-tailed Mann-Whitney test in GraphPad 7.0, unless otherwise specified. ANCOVA was performed to evaluate age or gender as a covariable. Two-tailed Fisher's exact test was used to evaluate HLA associations, using GraphPad software or R software version 3.3.1.

SUPPLEMENTARY MATERIALS
www.sciencetranslationalmedicine.org/cgi/content/full/11/478/eaau3776/DC1
Materials and Methods
Fig. S1. RT and non-RT tonsillar immunophenotyping of cohort 1.
Fig. S2. RT and non-RT tonsillar immunophenotyping of cohort 2.
Fig. S3. GAS-specific CD4+ T cells by AIM assay.
Fig. S4. HLA typing of entire tonsillar cohort.
Fig. S5. SpeA-responsive GC-T_{FH} cells.
Fig. S6. SpeA-responsive GC-T_{FH} cells by AIM assay.
Fig. S7. SpeA induced GzmB production.
Fig. S8. SpeA-responsive GC-T_{FH} cells by AIM assay.
Table S1. RNA-seq analysis.
Table S2. Flow cytometry antibodies for fresh tonsil stain.
Table S3. Flow cytometry antibodies for AIM assay.
Table S4. Flow cytometry antibodies for PBMC proliferation assay.
Table S5. Flow cytometry antibodies for GzmB detection.
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.
Table S7. Flow cytometry antibodies for GzmB detection from sorted GC-T_{FH} cells.
Table S8. Flow cytometry antibodies used for sorting for cytotoxicity assay.
Data file S1. Primary data.
References (59–70)

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Science Translational Medicine

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

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Teasing apart tonsillitis

Although exposure to group A *Streptococcus* is prevalent, only some children develop recurrent tonsillitis, which can lead to tonsillectomy. To discern why some children are susceptible and others are resistant, Dan *et al.* examined tonsil samples from two cohorts. They found that children with a history of recurrent tonsillitis had smaller germinal centers and reduced antibacterial antibodies. Moreover, the T follicular helper cells from those subjects may actually have been cytotoxic toward B cells. Class II HLA analysis also identified protective and risk alleles. Together, these results reveal that altered adaptive immune responses to group A *Streptococcus* may differentiate those at risk of recurrent infection.

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