

Superantigen-driven polyfunctional CD4⁺ T follicular regulatory cells impair tonsillar plasmablast function and class-switched antibody production

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Article

Keywords: superantigens, TSST-1, T follicular regulatory cells, T follicular helper cells, Staphylococcus aureus

Posted Date: June 5th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-9858212/v1>

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Additional Declarations: There is **NO** Competing Interest.

Abstract

Invasive *Staphylococcus aureus* infections have high mortality and reinfection rates, in part due to impaired development of enduring antibody-mediated immunity. The generation of pathogen-specific antibodies is typically orchestrated and counter-regulated by CD4⁺ T follicular helper (T_{FH}) and T follicular regulatory (T_{FR}) cells respectively. Given their central roles in mediating adaptive immunity, these T cell subsets may form targets for *S. aureus* virulence factors, including superantigens (SAGs). SAGs force excessive production of proinflammatory cytokines which can trigger pathological cytokine ‘storms’, yet their impact on CD4⁺ T_{FH} and T_{FR} cells, and their contribution to impaired humoral immunity during *S. aureus* infections, remains poorly characterised. In this study, we characterised the phenotype and behaviour of CD4⁺ T_{FH} and T_{FR} cells in response to SAG stimulation, and the corresponding impact on antibody production. We found that SAGs elicited an *in vitro* expansion of circulating and tonsillar CD4⁺ T_{FR} cells, which were polyfunctional with co-inhibitory signatures consistent with enhanced suppressive capacity. This expansion correlated with impaired plasmablast function and class-switched immunoglobulin production in human tonsil organoids which was rescued by interleukin-21 supplementation. Furthermore, acute expansions of CD4⁺ T_{FR} cells were replicated during systemic *S. aureus* infection *in vivo*. Together, these findings establish CD4⁺ T_{FR} cells as a target for SAG-mediated immune evasion and a novel target for vaccine development.

INTRODUCTION

Staphylococcus aureus represents a leading cause of death related to bacterial infections worldwide (1) despite asymptomatically colonising up to 30% of healthy humans (2). Breach of epithelial barriers, particularly in immunocompromised individuals, can progress local infection and colonisation to invasive disease, with bacterial entry into the bloodstream enabling systemic dissemination (2–5). The incidence of *S. aureus* bloodstream infection (bacteraemia) is increasing worldwide, which is associated with mortality rates of up to 50% (6, 7) and recurrent invasive infection in 5–10% of cases (8). The devastating clinical impact of *S. aureus* is exacerbated by the continuing rise of multidrug-resistant strains (9–11). Despite the clinical burden of invasive *S. aureus* infections, no licensed vaccines currently exist (12). Understanding the immune mechanisms that determine infection outcome is therefore critical to guide effective vaccine development.

Antibody production is a correlate of long-lasting protective immunity elicited by vaccination or previous infection by many pathogens. The high recurrence rates following *S. aureus* infections suggest impaired development of enduring humoral immunity, and it is now emerging that prior natural exposure to *S. aureus* may “imprint” non-protective antibody and CD4⁺ T cell responses that can impair vaccine efficacy (13–15). Whilst the underlying regulatory mechanisms are not clear, virulence factors produced by *S. aureus* to enhance pathogenicity and aid immune evasion may have a key role (3, 16). In this regard, approximately 80% of clinical *S. aureus* isolates encode genes for a family of potent exotoxins known as superantigens (SAGs) that interfere with adaptive immune responses by specifically targeting B cells or T

cells (17, 18). T cell-targeting SAGs force T cells to overproduce pro-inflammatory cytokines such as interferon gamma (IFN- γ) and tumour necrosis factor (TNF), which can trigger pathological, and sometimes fatal, cytokine ‘storms’ marked by dysregulated immune cell recruitment and function (19–21). Persistent stimulation of T cells can result in their subsequent anergy, rendering them hyporesponsive to further stimuli. Excessive activation is achieved through the unique binding of SAGs to T cell receptors (TCRs) indiscriminately of their antigen specificity (22, 23). To achieve this, SAGs bind to $\alpha\beta$ TCRs at germline-encoded residues within the variable segment of either TCR chain, and create a cross-link to MHC class II molecules on antigen presenting cells at regions outside of the peptide-binding grooves (22, 23). To date, at least 26 different T cell-targeting SAGs have been described for *S. aureus*, including staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1) (24, 25), which is a causative agent of menstrual toxic shock syndrome (TSS) (26). Of note, SAG production is not limited to *S. aureus*, with scarlet fever and streptococcal TSS linked to the production of streptococcal pyrogenic exotoxin A (SpeA) by *Streptococcus pyogenes* (27). Given the lack of protective *S. aureus* vaccines, SAGs may provide a promising target for vaccine development, where strategies utilising mutated versions of SAGs have already proven effective at reducing *S. aureus* burden (28, 29) and achieving seroconversion in clinical trials (30, 31). However, due to the capacity of individual *S. aureus* strains to encode multiple distinct SAGs, it is important that we comprehensively understand the mechanisms of SAG-mediated immune modulation in order to design of broadly protective vaccine strategies.

The generation of pathogen-specific antibodies in the germinal centres (GCs) of secondary lymphoid organs (32) is orchestrated by the interactions between antibody-producing B cells and specialised CD4⁺ T follicular helper (T_{FH}) cells, and counter-regulated by a subset of CXCR5⁺ CD4⁺ T regulatory (T_{REG}) cells known as CD4⁺ T follicular regulatory (T_{FR}) cells (33–35). Given the central role of follicular T cells in mediating adaptive immunity, they are potential targets for bacterial virulence factors, including T cell-targeting SAGs, aimed at evading the host immune response. Consistent with this, streptococcal SAGs have been reported to alter CD4⁺ T_{FH} cell behaviour *in vitro*, resulting in reduced class-switched antibody production (36, 37). Furthermore, tonsillar CD4⁺ T_{FH} cells were shown to adopt an SpeA-induced ‘killer’ phenotype in children with recurrent streptococcal tonsillitis, characterised by the production of granzyme B and perforin, which correlated with low SAG-specific neutralising antibody titres (37). Whether staphylococcal SAGs induce similar phenotypic changes in CD4⁺ T_{FH} cells, and their subsequent contribution to impaired adaptive immunity during *S. aureus* bacteraemia, remains unknown. Additionally, although multiple studies have reported increases in the frequency of functionally suppressive CD4⁺ T_{REG} cells in response to staphylococcal and streptococcal SAG stimulation (38–43), the impact of SAGs on circulating and tonsillar CD4⁺ T_{FR} cells remains largely uncharacterised.

To address this knowledge gap, we characterised the phenotype and functional behaviour of circulating and tonsillar CD4⁺ T_{FH} and T_{FR} cells in response to staphylococcal and streptococcal SAG stimulation. While CD4⁺ T_{FH} cells were more responsive to SAGs than non-follicular effector memory CD4⁺ T cells *in*

vitro, SAg stimulation drove a disproportionate increase in CD4⁺ T_{FR} cells over time. The expanded CD4⁺ T_{FR} cells were polyfunctional and expressed a co-inhibitory receptor (co-IR) signature consistent with enhanced suppressive capabilities. These functional and phenotypic changes were dependent on TNF receptor 2 (TNFR2) and correlated with a decline in plasmablasts and suppression of class-switched immunoglobulin production in a human tonsil organoid model. In support of the impact of SAGs on CD4⁺ T_{FR} cells *in vitro*, we observed an early expansion of splenic CD4⁺ T_{FR} cells in a murine model of systemic *S. aureus* infection, providing preliminary *in vivo* evidence of infection-driven modulation of CD4⁺ T_{FR} cells. These findings are consistent with a potent immune evasion strategy by which SAg-releasing pathogens such as *S. aureus* evade antibody-mediated host immunity through a T cell-targeted approach, revealing a novel target for the development of vaccines.

RESULTS

Staphylococcal and streptococcal superantigens disproportionately expand CD4⁺ cT_{FR} cells

Circulating CD4⁺ T_{FH} cells represent a memory counterpart of germinal centre CD4⁺ T_{FH} cells that have retained the capacity to differentiate into effector cells upon re-activation (44). Studying circulating CD4⁺ T_{FH} and T_{FR} cells (hereon termed T_{FH} and T_{FR} cells) can thus serve as a surrogate for germinal centre cells, without requiring access to secondary lymphoid tissues. We examined whether the frequency of circulating T_{FH} and T_{FR} cells (cT_{FH} and cT_{FR} cells respectively) were altered by increasing durations of SAg stimulation in *in vitro* cell culture assays. Peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated for 24h, 48h and 72h with recombinant forms of TSST-1 and SEB (produced by *S. aureus*) as well as SpeC (produced by *S. pyogenes*) to examine both intra- and inter-species differences in SAg response profiles. Multi-parameter flow cytometry was used to identify cT_{FH} (defined here as CD4⁺CD45RA⁻CXCR5⁺PD-1⁺FOXP3⁻ T cells) and cT_{FR} (CD4⁺ CD45RA⁻CXCR5⁺PD-1⁺FOXP3⁺) cells (Figure S1). Non-follicular T regulatory cells (CD4⁺CD45RA⁻CXCR5⁻ FOXP3⁺; hereon termed classical T_{REG} cells), and non-follicular, non-regulatory T cells (CD4⁺CD45RA⁻CXCR5⁻FOXP3⁻), which represented the majority of circulating memory CD4⁺ T cells, served as controls. SAg stimulation drove a significant increase in the frequency of both follicular CD4⁺ T cell subsets compared to unstimulated controls, peaking at 24h for cT_{FH} cells before declining with continued stimulation (Fig. 1A-C). In contrast, the frequency of cT_{FR} cells increased further with duration of SAg stimulation, constituting ~ 21% of all memory CD4⁺ T cells after 72h (Fig. 1C). This rise reflected a mean 76.8-fold increase in frequency compared to control conditions and resulted in a significant decline in the ratio of cT_{FH}:cT_{FR} cells from ~ 18:1 in unstimulated cells to ~ 1:2 after 72h stimulation with each SAg (Fig. 1D). We also observed a significant increase in the frequency of classical T_{REG} cells after stimulation with all three SAGs compared to unstimulated controls (Figs. 1A and S2A), in agreement with previous studies (38–43).

When compared to cT_{FR} cells, however, this rise represented a lower, mean 2.4-fold-change in frequency compared to control conditions. In contrast, the frequency of CXCR5⁺FOXP3⁺ T cells declined over time with SAg stimulation (Figs. 1A and S2B).

Together, these data demonstrate that while circulating CXCR5⁺ T cell subsets are more responsive to SAg stimulation than their CXCR5⁺ counterparts, SAg preferentially expand regulatory FOXP3⁺ CD4⁺ T cells, most notably cT_{FR} cells, leading to a decline in the cT_{FR}:cT_{FR} cell ratio over time.

SAg-expanded CD4 cT cells are highly activated and cytotoxic T cells

Following TCR engagement, OX40 and CD40L are upregulated on activated CD4⁺ T cells, and their co-expression has been used in activation-induced marker assays to identify antigen-specific CD4⁺ T cells, notably within CXCR5⁺ CD4⁺ T cell subsets following vaccination or viral infection (45–47). To investigate the activation status of SAg-expanded cT_{FR} cells, we first assessed the co-expression of OX40 and CD40L on cT_{FR} cells in comparison to cT_{FR}, classical T_{REG} and CXCR5⁺FOXP3⁺ T cells. In response to SAg stimulation, all CD4⁺ memory T cell subsets showed an increase in OX40⁺CD40L⁺ cells compared to unstimulated controls (Figures S2A, S2B, S3A and S3B). For cT_{FR} cells, peak responses were observed after 48h of stimulation (Figs. 2A, 2B, S3A and S3B). Indeed, the proportion of OX40⁺CD40L⁺ cT_{FR} cells was significantly greater than all other CD4⁺ T cell subsets analysed at this timepoint for stimulation with TSST-1 and SEB, including cT_{FR} cells and classical T_{REG} cells (Figs. 2A, 2B and S3A). While cT_{FR} cells responded comparably to classical T_{REG} cells, the proportion of OX40⁺CD40L⁺ CXCR5⁺FOXP3⁺ T cells was lower at all timepoints (Figs. 2B, S3A and S3B).

To further examine the function of each memory CD4⁺ T cell subset, the proportion of cells producing the pro-inflammatory cytokine IFN- γ or the cytotoxic protease granzyme B (GzmB) were quantified following SAg stimulation. GzmB is typically associated with the cytolysis of infected or cancerous cells by cytotoxic T cells but can also be produced by regulatory CD4⁺ T cells as a mechanism for suppressing cellular activity through apoptosis of immune cells (48–50). Aberrant production of GzmB by tonsillar T_{FR} cells in response to SpeA was reported in children with recurrent streptococcal tonsillitis, resulting in increased B cell apoptosis (37). In the present study, whilst a similar proportion of each memory CD4⁺ T cell subset produced IFN- γ after 24h of stimulation, the frequency of IFN- γ ⁺ cT_{FR} and classical T_{REG} cells increased further at 48h or 72h post-stimulation, which was not observed for cT_{FR} or CXCR5⁺FOXP3⁺ T cells (Figs. 2C, S4A and S4B). In correlation with data on OX40⁺CD40L⁺ cells, the greatest frequency of IFN- γ ⁺ cells was amongst the expanding cT_{FR} cell population, most notably at 72h post-stimulation (Fig. 2C, S4A and S4B). Increasing durations of SAg stimulation drove significant increases in the percentage of GzmB⁺ cells across all memory CD4⁺ T cell subsets, yet cT_{FR} cells displayed the greatest frequency of GzmB⁺ cells (~ 89.8%) after 72h of stimulation (Fig. 2D, S4C and S4D). This was

significantly greater than for cT_{FH} or $CXCR5^-FOXP3^-$ T cells following TSST-1 stimulation (Fig. 2D), although classical T_{REG} cells also showed a large increase in the frequency of $GzmB^+$ cells (Figs. 2D, S4C and S4D). To explore correlations between T cell activation (i.e. OX40 and CD40L expression) and production of IFN- γ , and/or $GzmB$ in each memory $CD4^+$ T cell subset, we used Boolean gating and Simplified Presentation of Incredibly Complex Evaluations (51) (SPICE)-based analysis to define polyfunctionality by observing cells expressing OX40, CD40L, IFN- γ or $GzmB$ in any combination following 72h of TSST-1 stimulation. Using data concatenated from six donors, cT_{FR} cells exhibited the greatest percentage of polyfunctional cells with 86% of cells co-expressing two or more of these proteins, and the highest proportion of $OX40^+CD40L^+IFN-\gamma^+GzmB^+$ cells across all $CD4^+$ T cell subsets analysed (Fig. 2E).

Collectively, these data show that in response to SAg stimulation, expanded $CD4^+$ cT_{FR} cells adopt greater polyfunctional capacity, demonstrating a higher degree of activation and cytotoxicity than other memory $CD4^+$ T cell subsets, including classical T_{REG} cells.

SAg-expanded populations of $CD4$ cT cells co-express multiple co-IRs

SAgs are known to induce the expression of co-IRs on T cells, such as cytotoxic T lymphocyte-associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), T cell immunoreceptor with Ig and ITIM domains (TIGIT) and T cell immunoglobulin and mucin-domain containing-3 (TIM-3) (20, 52). In regulatory $CD4^+$ T cell subsets, the expression of these co-IRs has been suggested as a mechanism for promoting their function, enabling suppression of other cell types through interference with co-stimulatory signaling (53–55). Following our observations of increased activation and cytotoxicity in SAg-expanded cT_{FR} cells, we next explored whether this population displayed a phenotype consistent with regulatory function, by comparing the expression of CTLA-4, LAG-3, TIGIT and TIM-3 on cT_{FR} cells to classical T_{REG} cells. For these experiments, TSST-1 was used as a candidate SAg given the comparable response profiles of all three SAg on cT_{FR} cell frequency and activation. Under resting conditions, a similar proportion of both cT_{FR} and classical T_{REG} cells expressed at least one co-IR, which was predominantly CTLA-4 for both cell types (Figs. 3A, S5A and S5B). For classical T_{REG} cells, CTLA-4 expression was rarely seen in combination with other co-IRs (< 2%) (Fig. 3A). In contrast, unstimulated cT_{FR} cells displayed more diversity in co-IR expression, with cells expressing TIGIT (9%), LAG-3 (7%) and/or TIM-3 (4%) in addition to CTLA-4 (24%). Of these, 11% of cells expressed combinations of two or more co-IRs (Fig. 3A). For comparison, unstimulated $CXCR5^-FOXP3^-$ T cells exhibited almost no co-IR expression, suggesting an association between co-IR expression and a regulatory phenotype under resting conditions (Figs. 3A and S5C). In response to TSST-1 stimulation, a significant rise in the proportion of CTLA-4 $^+$, LAG-3 $^+$ and TIM-3 $^+$ cells was seen for both the cT_{FR} and classical T_{REG} cells, which increased over time (Figure S5A and S5B). However, cT_{FR} cells exhibited more CTLA-4 $^+$ LAG-3 $^+$ TIM-

3⁺ cells compared to classical T_{REG} cells and a greater percentage of cells co-expressing at least two co-IRs (Fig. 3A). Although TSST-1 stimulation increased the expression of CTLA-4, LAG-3 and TIM-3 on CXCR5⁺FOXP3⁺ T cells (Figs. 3A and S5C), this was much lower in comparison to the regulatory populations, likely reflecting a dysfunctional or possibly 'exhausted' state amongst effector CD4⁺ T cells (20, 56).

SAGs have been found to upregulate the expression of the T_H1-associated chemokine receptor, CXCR3, on T cells (57, 58). Its expression on classical CD4⁺ T_{REG} cells has been linked to increased migration and prolonged localisation at sites of inflammation, leading to a stronger suppressive capacity (59, 60). As an indicator of their suppressive potency, we therefore measured the expression of CXCR3 on cT_{FR} and classical T_{REG} cells after TSST-1 stimulation. The proportion of cells expressing CXCR3 peaked at 72h for both regulatory T cell populations, but was consistently greater for cT_{FR} cells, reaching 89.8% of cells at this timepoint (Fig. 3B). As with the expression of co-IRs, CXCR3 expression was low on CXCR5⁺FOXP3⁺ T cells and did not increase with TSST-1 stimulation over time (Fig. 3B).

Taken together, these results suggest that the SAg-expanded CD4⁺ cT_{FR} cell population represents cells with potentially enhanced suppressive capacity, based on CXCR3 and co-IR expression profiles, compared to classical T_{REG} cells.

The induction of polyfunctional CD4 cT cells following SAg stimulation is TNFR2-dependent

The induction of highly activated, polyfunctional CD4⁺ cT_{FR} cells by three different SAGs from two distinct bacterial species, despite different TCR binding-specificities (19), suggested that TCR-independent routes of cellular activation may be responsible for driving SAg-biased responses in cT_{FR} cells. In line with this concept, SAg-driven CD4⁺ T_{REG} cell expansions have been found to be dependent on the TNF/TNFR2 axis (43), and like classical T_{REG} cells, cT_{FR} cells have been shown to express higher levels of TNFR2 compared to non-regulatory CD4⁺ T cells (61–63). Consistent with this, we found that 20% of cT_{FR} cells constitutively expressed TNFR2, in contrast to cT_{FH} cells, which displayed no TNFR2 expression (Fig. 4A). Following TSST-1 stimulation (24-72h), the proportion of TNFR2⁺ cells among both CXCR5⁺ subsets increased but remained significantly higher for cT_{FR} cells than cT_{FH} cells at all timepoints (Fig. 4A). We therefore used a TNFR2 neutralising antibody to block TNFR2 activity and investigated whether a TNFR2-dependent mechanism was important for driving the SAg-mediated expansion and phenotypic rewiring of cT_{FR} cells.

In line with earlier findings (Fig. 1), TSST-1 stimulation increased the frequency of cT_{FR} cells, yet TNFR2 blockade significantly reduced this expansion (Fig. 4B). In contrast to this effect on cT_{FR} cells, TNFR2 blockade produced a significant increase in cT_{FH} cells after TSST-1 stimulation (Fig. 4B), resulting in a

less skewed $cT_{FH}:cT_{FR}$ cell ratio after 72h compared to control conditions (Figure S6). In addition to frequency changes, blocking TNFR2 significantly reduced the percentage of $OX40^+CD40L^+$ cT_{FR} cells, and a similar inhibitory effect was observed for cT_{FH} cells (Fig. 4C). While the proportion of $OX40^+CD40L^+$ cT_{FR} cells was significantly greater than that seen in cT_{FH} cells in the presence of an isotype control antibody, specific TNFR2 blockade eliminated this difference, resulting in comparable activation of the two CXCR5⁺ T cell subsets (Fig. 4C). TNFR2 blockade also significantly reduced the proportion of $IFN-\gamma^+$ $GzmB^+$ cT_{FR} cells, but not $IFN-\gamma^+$ $GzmB^+$ cT_{FH} cells, in response to TSST-1 stimulation (Fig. 4D). As with the expression of CD40L and OX40, TNFR2 blockade eliminated the difference between the two populations after 72h of stimulation (Fig. 4D).

Collectively, these results demonstrate a role for TNFR2 in the response of follicular CD4⁺ T cells to TSST-1 stimulation, particularly cT_{FR} cells, which upregulate TNFR2 expression upon stimulation. Blocking TNFR2 eliminated the disproportionate activation of cT_{FR} cells compared to cT_{FH} cells, which increased in frequency under the same conditions.

Tonsillar CD4 T cells with enhanced co-IR-mediated suppressive capabilities expand upon SAg stimulation

Streptococcal SAg (i.e. SpeA) have been shown to phenotypically and functionally alter tonsillar T_{FH} cells in children with recurrent tonsillitis, resulting in B cell death and a lack of class-switched antibody production (36, 37). However, no such studies have been conducted on the effect of SAg on tonsillar T_{FR} cell responses. Accordingly, we next explored if SAg could promote disproportionate expansions of polyfunctional tonsillar T_{FR} cells expressing multiple co-IRs, phenocopying our findings in peripheral CD4⁺ cT_{FR} cells. To do this, tonsillar mononuclear cells (TMNCs) collected from adult donors undergoing tonsillectomy for sleep apnea and/or recurrent infection (n = 4) were stimulated for up to 72h with TSST-1 (as a candidate SAg) before assessing the frequency, phenotype and functional changes in tonsillar T_{FR} cells, compared to tonsillar T_{FH} cells. SAg stimulation drove a significant expansion of tonsillar T_{FR} cells which peaked at 72h (Fig. 5A), mirroring the expansion of cT_{FR} cells (Fig. 1A-B). In contrast, tonsillar T_{FH} cells showed minimal changes in frequency following TSST-1 stimulation (Fig. 5B), resulting in a decline in the $T_{FH}:T_{FR}$ ratio (Fig. 5C), recapitulating that seen in circulating populations (Fig. 1D). When assessing OX40 and CD40L expression after TSST-1 stimulation, tonsillar T_{FR} cells had the highest percentage of $OX40^+CD40L^+$ cells at all timepoints, which was significantly higher than that seen for tonsillar T_{FH} cells after 72h stimulation (Fig. 5D). Using SPICE-based analysis of T cell function, we found that SAg-stimulated tonsillar T_{FR} cells were highly polyfunctional, with ~ 99% of cells expressing at least one marker of cell activation after 72h, and a small proportion of cells being $OX40^+CD40L^+IFN-\gamma^+GzmB^+$ (Fig. 5E). However, fewer tonsillar T_{FR} cells produced $IFN-\gamma$ and/or $GzmB$ in comparison to cT_{FR} cells (Figs. 5E and 2E). In contrast, resting tonsillar T_{FR} cells expressed considerably more co-IRs than their

circulating counterparts, in agreement with published data on tonsillar T_{REG} cells (64), with ~ 98% expressing at least one co-IR (Figs. 3A & 5F). The co-expression of distinct co-IRs increased in response to TSST-1 stimulation, with 13% of cells expressing a CTLA-4⁺LAG-3⁺TIGIT⁺TIM-3⁺ phenotype after 72h of stimulation. Echoing observations in circulating cells, this was greater than that seen for the tonsillar classical T_{REG} cell population (Fig. 5F). Compared to cT_{FR} cells, a higher proportion of resting tonsillar T_{FR} cells expressed CXCR3, which remained high following 72h of stimulation with TSST-1 (Fig. 5G). Although CXCR3 was upregulated on classical T_{REG} cells in response to stimulation with TSST-1, the proportion of CXCR3⁺ tonsillar T_{FR} cells was significantly higher than that for classical T_{REG} cells at each timepoint (Fig. 5G).

Collectively, these results show that SAg stimulation induces a population of polyfunctional tonsillar CD4⁺ T_{FR} cells that express multiple co-IRs and expand in a time-dependent manner, mirroring our observations in peripheral cT_{FR} cells.

SAg stimulation impairs plasmablast function and antibody production in tonsil organoids

During invasive *S. aureus* infection, low production of immunoglobulin (Ig) has been associated with disease severity and risk of re-infection (65, 66). Following our observations in circulating and tonsillar CD4⁺ follicular T cells, we questioned whether the expansion of a polyfunctional CD4⁺ T_{FR} cell population could impact the development of antibody-producing plasmablasts (PB) and the subsequent production of different antibody types (e.g. IgM, IgG). To investigate this, we established tonsil organoids (67) from separate donors (n = 3) that were cultured for up to 21 days either in the presence of B cell-activating factor (BAFF) alone (control), or with TSST-1. In control organoids, the tonsillar PB population (CD38^{hi}CD27^{hi}, Figure S7) significantly increased in frequency with time, to 8.4% of all B cells after 14 days of culture (Fig. 6A and 6B). The addition of TSST-1 on day 0 significantly disrupted this process, resulting in a complete lack of tonsillar PBs on day 3, which only minimally recovered by day 14 (Fig. 6A and 6B). To determine if this effect was driven by TSST-1-mediated apoptosis of tonsillar PBs, we used a fixable viability dye and Annexin V to define viable (alive), apoptotic (early or late) and non-viable (dead) tonsillar PB cells. We found that stimulation with TSST-1 led to an increased proportion of early and late apoptotic tonsillar PBs at day 3 post-stimulation, concurrent with a reduction in viable tonsillar PBs (Figure S8A). The proportion of late apoptotic tonsillar PBs then increased further on day 14 in TSST-1-stimulated organoids compared to controls (Figure S8A). Consistent with this effect, we found there was a strong correlation between the presence of TSST-1 and reduced production of IgG and IgM. For control organoids, IgG and IgM production increased with time, mirroring the growing tonsillar PB population (Fig. 6C and 6D). For tonsil organoids stimulated with TSST-1, the production of IgG and IgM was ablated, barely increasing above baseline levels until day 18, when the concentration of TSST-1 in the culture media had decreased ~ 64-fold as determined by serial media dilution (Fig. 6C and 6D). These changes were in conjunction with an early expansion of T_{FR} cells and decline in T_{FH} cells (Figure S8B and

S8C). Moreover, the TSST-1-expanded T_{FR} cell population appeared highly polyfunctional compared to control organoids after 72h of stimulation (Figure S8D), with enhanced expression of co-IRs (Figure S8E), consistent with earlier observations (Fig. 5E and 5F). By day 14 of culture, the frequency of T_{FR} and T_{FH} cells in TSST-1-stimulated organoids matched levels seen in controls (Figures S8B and S8C), but the TSST-1-stimulated T_{FR} cells retained an elevated co-IR profile (Figure S8E).

Production of interleukin-21 (IL-21) by T_{FH} cells is critical for T cell-mediated antibody production in lymphoid organs and is known to be suppressed by T_{FR} cells (68). We therefore investigated whether supplementation of exogenous IL-21 could rescue the inhibitory effect of TSST-1 on tonsillar PBs and IgG production. Strikingly, IL-21 prevented the depletion of PBs in tonsil organoids stimulated with TSST-1, matching frequencies observed in control organoids on day 14 (Fig. 6B). Furthermore, whilst control tonsil organoids cultured with IL-21 displayed enhanced IgG production at all timepoints, TSST-1 had less impact on IgG levels in the presence of IL-21 (Fig. 6E). When exploring the phenotype of T_{FR} and T_{FH} cells, addition of IL-21 reduced the TSST-1-induced upregulation of co-IR expression on T_{FR} cells, particularly LAG-3, suggesting reduced suppressive capacity (Fig. 6F). Similarly, IL-21 reduced the upregulation of co-IRs on T_{FH} cells (Fig. 6F), which could indicate a protective effect against TSST-1-mediated exhaustion of effector T cells.

Collectively, these data demonstrate that the actions of TSST-1 disrupt tonsillar PB formation and the production of class-switched antibodies. Exogenous IL-21 helped prevent the suppressive effects of TSST-1, indicative of impaired T_{FH} cell function in the presence of SAg, which may result from T_{FR} cell-mediated expansions.

Systemic *S. aureus* infection induces an early expansion of CD4⁺ T_{FR} cells in vivo

Our observations so far suggested that staphylococcal SAgS disrupt the development of humoral immunity and equally expand suppressive circulating and tonsillar CD4⁺ T_{FR} cells. To investigate whether the expansion of T_{FR} cells *in vitro* were a true reflection of *in vivo* responses to *S. aureus* infection, we used an established murine model of systemic *S. aureus* infection (69, 70) using the LAC USA300 methicillin-resistant *S. aureus* strain which encodes the SAgS SEK, SEQ and SEI-X (71). C57BL/6 mice were challenged via intraperitoneal injection and lymphoid-resident follicular CD4⁺ T cells were examined at days 3 and 7 post-infection. These timepoints were chosen to observe early CD4⁺ T cell responses, comparable to timepoints used during SAg stimulations of human cells. We found that *S. aureus* infection induced an increased frequency of splenic CXCR5⁺ follicular CD4⁺ T cells, which was detectable at 3 days post-infection, and significantly greater at 7 days post-infection (Fig. 7A and 7B). Within this population, the frequency of T_{FR} cells was increased at day 3, which was significantly greater than the frequency of T_{FR} cells observed at 7 days post-infection (Fig. 7B and 7C). Importantly, a significantly higher proportion of the expanded T_{FR} cell population co-expressed CTLA-4 and CD40L in *S. aureus*-infected mice at 3 days-post infection compared to naïve mice (Fig. 7D).

These data suggest that systemic *S. aureus* infection induces an early expansion of CD4⁺ T_{FR} cells *in vivo* as a proof-of-concept, providing preliminary evidence of infection-driven regulation of CD4⁺ T_{FR} cells.

DISCUSSION

The mechanisms underpinning impaired humoral immunity during invasive *S. aureus* infections remain poorly defined, particularly regarding the association between SAg production and antibody formation. Here, we investigated how SAg modulate follicular CD4⁺ T cells and uncovered a previously uncharacterised immune evasion strategy whereby SAg induce the expansion of T_{FR} cells. The expanded T_{FR} cell population acquired an enhanced immunosuppressive phenotype characterised by the production of granzyme B and co-expression of multiple co-IRs, which correlated with impaired antibody production in human tonsil organoids. Exogenous IL-21 restored B cell function, implicating the involvement of CD4⁺ T_{FR} cell-mediated suppression of T_{FH} cells and B cells. Furthermore, *S. aureus* induced an early expansion of T_{FR} cells during acute infection *in vivo*, implicating T_{FR} cell expansion as a possible feature of an infection-mediated immune response. These findings establish T_{FR} cells as a target of SAg-mediated immune evasion, identifying a novel mechanism for the suppression of antibody responses during invasive infection.

SAg have been primarily implicated in driving excessive activation and cytokine release by T cells (72, 73), however more targeted effects on certain T cell subsets have been described. These include T_{FH} cells, where streptococcal SAg have been shown to induce an expansion of dysfunctional T_{FH} cells, linked to reduced GC function (36). In this study, although we observed a modest increase in the frequency of cT_{FH} cells in response to SAg stimulation, we observed a more substantial increase in the percentage of FOXP3⁺ T_{FR} cells in both circulatory and tissue-resident compartments, reaching peak differences after 72h of stimulation *in vitro*. These findings were observed with different staphylococcal SAg (TSST-1 and SEB) but also across bacterial species (SpeC), suggesting a conserved mechanism utilised by SAg-releasing bacteria. In addition to the expansion of T_{FR} cells, we saw an expansion of classical T_{REG} cells which was previously reported in both the blood and tissues (38, 40, 41, 43, 74). It has also been shown that low-dose stimulation with either staphylococcal or streptococcal SAg can induce CD8⁺FOXP3⁺ T cells with suppressive capacities as potent as classical CD4⁺ T_{REG} cells (75, 76). These studies suggest that the expansion of regulatory populations represents a common immune evasion mechanism among SAg-releasing bacteria. Our findings extend this paradigm further by demonstrating a disproportionate expansion in the CXCR5⁺ follicular CD4⁺ T cell compartment, suggesting a more targeted strategy aimed to specifically interfere with antibody-mediated protection.

Our experiments demonstrated that SAg-expanded T_{FR} cell populations exhibited a polyfunctional phenotype in both peripheral blood and tonsils, with production of IFN- γ , GzmB and expression of co-IRs. While the mechanisms of T_{FR} cell-mediated regulation are incompletely defined, their protein expression profiles suggest that they may employ similar methods to classical T_{REG} cells. Cytolytic populations of

classical T_{REG} cells have been described which induce apoptosis of both effector T cells and antigen-presenting B cells through production of GzmB to suppress immune responses (77–79). This has additionally been described as a key mechanism of CD8⁺ T_{REG} function (76). Indeed, knockout of GZMB from tonsillar CD8⁺ T cells resulted in an increase in CD4⁺ T_{FH} cells and plasmablasts in tonsil-derived organoid models (80). In contrast to apoptosis-mediated suppression, regulatory CD4⁺ T cells also express co-IRs to interfere with co-stimulatory signals on effector cells (53–55). In this study, expression of CTLA-4, LAG-3 and TIM-3 on T_{FR} cells increased with the duration of SAg stimulation up to 72h, with more co-expression on T_{FR} cells than classical T_{REG} cells. These data suggest that the expanded T_{FR} cell population represents a true regulatory subset with potent suppressive capacity. Overall, the proportion of cells producing GzmB was greater for circulating T_{FR} cells than their tonsillar equivalents, with co-IR expression greater amongst tonsillar T_{FR} cells. These differences suggest that SAg could drive alternative methods of regulation dependent on the immune context to maximise suppressive function. For example, apoptosis-induced suppression in the blood, mediated by soluble factors, versus co-IR-mediated regulation in the tonsils, supported by the proximity of cells in the lymphoid microenvironment. Given this observed feature of SAg stimulation, the increased expression of co-IRs, as well as CXCR3, on tonsillar T_{FR} cells under resting conditions compared to both tonsillar T_{REG} cells but also their circulating counterparts could be partly driven by continual exposure to *S. aureus* at mucosal sites. Whilst information on the colonisation status of each donor would be required to investigate this further, it could implicate SAg activity in colonised individuals as a mechanism by which *S. aureus* may “imprint” non-protective CD4⁺ T cell responses, by driving an enhanced immunosuppressive profile on T_{FR} cells (13–15).

The production of GzmB in response to SAg stimulation has been reported for other cell types, including T_{FH} cells. In children with recurrent tonsillitis, it was shown that GzmB production by tonsillar T_{FH} cells in response to SpeA stimulation resulted in B cell apoptosis and diminished immunological memory (37). Although we observed GzmB production by SAg-stimulated T_{FH} cells, this was low in comparison to the T_{FR} cell population in our study. These discrepancies may be context-dependent and could reflect an infection-specific effect in GC T_{FH} cells or differences between how T_{FR} cell populations respond to SAg in children and adults, akin to that across memory CD4⁺ T cell populations (81). It is likely that SAg-producing bacteria may employ multiple, potentially redundant immune evasion strategies that are preferentially engaged in different contexts. Further investigation into the responsiveness of follicular CD4⁺ T cells across the life course would be valuable to explore this relationship.

In human tonsil organoid cultures, addition of TSST-1 abrogated the development of tonsillar CD38^{hi}CD27^{hi} PBs. In TSST-1 stimulated organoids, the frequency of the tonsillar PB population remained low for the duration of organoid culture and correlated with reduced IgG and IgM production. Stimulation with streptococcal SAg has been previously shown to diminish B cells and antibody production in tonsil cell suspensions and histocultures (36). These data were in association with an overall increase in tonsillar T cells but reduced CD4⁺ T_{FH} cell function. Our findings corroborate and build

on these data in the tonsil organoid model, demonstrating the impact of SAg stimulation on IgG and IgM production over longer time periods, and phenotyping both effector and regulatory follicular CD4⁺ T cell subsets. Adding exogenous IL-21 to tonsillar organoids counteracted the effect of TSST-1 in our study, retaining the PB compartment and IgG production at the same level as unstimulated tonsil organoids. IL-21 is a critical cytokine for CD4⁺ T_{FH} cell-mediated B cell support and is known to be suppressed by CD4⁺ T_{FR} cells (82). It has previously been demonstrated that the addition of IL-21 to cultures containing suppressive CD4⁺ T_{FR} cells and B cells rescued class-switch recombination and IgG secretion, rendering B cells immune to CD4⁺ T_{FR} cell-mediated suppression (82). These data implicate an immunosuppressive mechanism driven by SAg-expanded CD4⁺ T_{FR} cell populations, rather than direct SAg-mediated destruction of B cells, even though we detected enhanced levels of apoptotic tonsillar PBs in organoid cultures following stimulation with TSST-1.

CD4⁺ T_{FR} cells express transmembrane TNF (tmTNF) in response to TNFR2 stimulation, which binds TNFR2 through cell-cell contact, leading to the suggestion that CD4⁺ T_{FR} cells can further promote their own proliferation via a tmTNF/TNFR2 loop (83). In line with previous findings for classical CD4⁺ T_{REG} cells (43), we demonstrated a role for TNFR2 in the disproportionate response of T_{FR} cells to stimulation with SAg. Blocking TNFR2 reduced T_{FR} cell expansion and the SAg-induced polyfunctional phenotype in these cells but did not completely inhibit responses to stimulation. These data suggest a role for TNFR2 in the response of T_{FR} cells to SAg stimulation, but the mechanism is likely multifaceted and may include differentiation of other cells, such as classical T_{REG} or T_{FH} cells, towards a CD4⁺ T_{FR} cell phenotype (84).

While the tonsil organoid model used in this study provides useful insight into the interactions between follicular CD4⁺ T and B cells and the functional consequences of stimulation with SAg, we could not explicitly define the CD4⁺ T_{FR} cell expansion as the central causative agent of disrupted tonsillar PB maintenance and antibody production. The fact that IL-21 could rescue the suppressive effects of TSST-1 on tonsillar plasmablasts and antibody production implicated a role for CD4⁺ T_{FR} cell-mediated immunosuppression. However, depletion of T_{FR} cells from tonsil organoid cultures would provide more direct evidence in future studies. While tonsil organoid models lack systemic immune context, preliminary evidence from small-scale murine systemic *S. aureus* infection experiments provided initial support for this mechanism, with an early expansion of CD4⁺ T_{FR} cells observed in the spleens of infected mice. Following the initial expansion, the frequency of T_{FR} cells dropped. This observation mirrors findings from tonsil organoids, of early SAg-driven increases in T_{FR} cells being transient, returning to resting frequencies by day 14. In the tonsil organoids, this could be explained by a decreasing concentration of TSST-1 with each media change. This could be relevant in the *in vivo* setting, with mice typically clearing infection by day 7, and could also explain the observed increase in splenic T_{FH} cells at this timepoint, if infection is no longer driving expansions of suppressive T_{FR} cells. Alternative infection doses could be useful to determine whether increased *S. aureus* exposure affects the duration of T_{FR} cell expansion. Indeed, while these experiments provide tentative proof of infection-

mediated regulation of CD4⁺ T_{FR} cells, further investigations using SAg-knockout strains are necessary to fully establish the *in vivo* relevance and clinical significance of these findings. Despite these limitations, our findings establish a framework for understanding SAg-mediated immune evasion and identify hypotheses to be tested in more complex systems.

In summary, we have provided evidence for a mode of immune evasion elicited by *S. aureus*, whereby SAg drive an expansion of CD4⁺ T_{FR} cells to suppress T cell-mediated antibody responses. This work provides mechanistic insight into the poor immunological memory and high reinfection rates observed during *S. aureus* infection, identifying CD4⁺ T_{FR} cell-mediated regulation of antibody production as a novel target for therapeutic interventions and vaccines designed to enhance protective immunity and improve clinical outcomes.

METHODS

Human subjects and ethical approval

Buffy coats obtained from peripheral blood venous samples were purchased from the Welsh Blood Service. The use of venous blood samples from healthy volunteers was approved by the Cardiff University School of Medicine Research Ethics Committee (18/4). All donors provided written informed consent for the collection of peripheral blood samples and subsequent analyses in accordance with the principles of the Declaration of Helsinki. Tonsils from adults undergoing routine tonsillectomy at University Hospitals Bristol and Western NHS Foundation Trust for sleep apnea and/or recurrent infection were obtained following informed consent and under approval of the Bristol BioBank (NHS REC 20/WA/0325).

Mice

C57BL/6J mice were bred by the Comparative Medicine Unit, Trinity College Dublin, and were housed under specific-pathogen-free conditions. Mice were maintained under the guidelines and regulations of the Health Products Regulatory Authority and experiments were carried out under license with the approval of Trinity College Dublin Animal Research Ethics Committee. Experiments were conducted with either male or female 10–12 week old age-matched mice. Mice were infected intraperitoneally with *S. aureus* strain USA300 (2.5×10^8 CFU) and splenocytes were harvested and analysed on days 3 and 7 post infection. Naïve control mice were injected with PBS and splenocytes were harvested and analysed at equivalent timepoints.

Human PBMC and TMNC isolation

PBMCs were isolated from buffy coats via density gradient centrifugation using Histopaque-1077 (Sigma-Aldrich) and cryopreserved in foetal bovine serum (FBS) containing 10% dimethyl sulfoxide (Sigma-Aldrich). Tonsil tissue was processed as previously described. Briefly, palatine tonsils were collected in Hanks' balanced salt solution (HBSS) immediately following surgery. Penicillin-Streptomycin

(Merck) was added for 30 mins to ensure decontamination of the tissue. Tonsils were mechanically dissociated and passed through a 70µM strainer to obtain single-cell suspensions. TMNCs were obtained via density gradient centrifugation using Ficoll-Paque Plus (Merck) before cryopreservation in foetal bovine serum (FBS) containing 10% dimethyl sulfoxide (Merck) and stored in liquid nitrogen until use.

Ex vivo SAg stimulation of human PBMCs and TMNCs

PBMCs and TMNCs were thawed at 37°C and then rested overnight at 37°C, 5% CO₂, in pre-warmed R10 media (RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all Thermo Fisher Scientific)). Cells were then seeded in 24-well tissue culture plates (Thermo Fisher Scientific) for subsequent stimulations. Recombinant forms of TSST-1 and SEB, from *S. aureus*, or SpeC from *S. pyogenes* (Toxin Technology) were added to cells for 24h, 48h or 72h at a dose of 100 ng/mL in accordance with previously published work (85). Unstimulated cells were included for all donors as negative controls. In mechanistic experiments, PBMCs were incubated with 2 µg/mL recombinant anti-human TNFR2 (clone 22221, BioTechne) or mouse IgG2A (clone MG2a-53, BioLegend) for 1h prior to SAg stimulation.

Mouse splenocyte isolation

Spleens were removed and placed into cold R10 before straining (70µM) to achieve single-cell suspensions. Red blood cells were lysed using ammonium-chloride-potassium lysing buffer (Thermo Fisher Scientific) and cells were cryopreserved in Bambanker media (GeneFlow). For analysis, splenocytes were either stained fresh or thawed rapidly at 37°C then stained immediately for analysis by flow cytometry.

Human tonsil organoids

Tonsil organoids were set up in triplicate using previously published methods (67) and cultured for up to 21 days. TMNCs were thawed as described above, strained (100µm filter), and plated in complete media (R10 media supplemented with 1% non-essential amino acids, 1% sodium pyruvate, 1% insulin transferrin selenium (all Thermo Fisher Scientific), 0.5 µg/ml BAFF (BioLegend)) at a density of 1.5x10⁶ cells per well across 96-well ultra-low binding plates. For organoids receiving stimulation, recombinant TSST-1 was added at a dose of 100ng/mL on day 0. Plates were incubated at 37°C, 5% CO₂ for the duration. 100 µL of supernatant was collected every 2–3 days for analysis of Ig secretion, and volumes were replaced with fresh complete media. Where relevant, 50 ng/mL recombinant human IL-21 (BioLegend) was added with each media change.

Flow cytometry

PBMCs, TMNCs and tonsil organoids were analysed by flow cytometry using the same methodology. For examining expression of CXCR5 and CXCR3, the following directly conjugated antibodies were added directly to cell culture wells for 24h prior to the end of the stimulation: anti-CXCR5-BV421 (clone

J252D4), anti-CXCR3-BV605 (clone G025H7), (both BioLegend). In functional experiments assessing the production of cytokines and cytotoxic molecules, GolgiPlug (1:1,000) and GolgiStop (1:1500; both BD Biosciences) were added for the final 6h of stimulation to block protein transport. Cells were washed in Dulbecco's phosphate-buffered saline (PBS) (Thermo Fisher Scientific) before being labelled with Zombie Aqua fixable viability dye (BioLegend) to allow exclusion of dead cells. Fc receptors were blocked using Human TruStain FcX (BioLegend) to reduce non-specific binding, before surface staining with combinations of the following directly conjugated monoclonal antibodies: anti-CD3-PerCP (clone SK7), anti-CD4-APC/Fire 750 (clone SK3), anti-CD45RA-FITC (clone HI100), anti-CD45RA-BV711 (clone HI100), anti-PD-1-PE (clone EH12.2H7), anti-OX40-PE/Dazzle 594 (clone ACT35), anti-TIM-3-PE/Cyanine7 (clone F38-2E2), anti-TIGIT-BV605 (clone A15153G), anti-CD19-FITC (clone SJ25C1), anti-CD27-APC (clone LG.3A10), anti-CD38-PE/Cyanine7 (clone HIT2), anti-CD38-BV421 (clone HIT2), anti-IgD-PE (clone IA6-2), anti-IgM-PE/Dazzle 594 (clone MHM-88), and anti-HLA-DR-BV605 (clone L243) (all BioLegend). Cells were fixed and permeabilised using the FOXP3 Transcription Factor Staining Buffer Kit (Thermo Fisher Scientific) according to manufacturer instruction's to enable intra-cytoplasmic and intra-nuclear staining, using combinations of directly conjugated monoclonal antibodies: anti-FOXP3-APC (clone PCH101, Thermo Fisher Scientific), anti-CD40L-BV711 (clone 24-31), anti-LAG-3-FITC (clone 11C3C65), anti-CTLA-4-PE/Dazzle 594 (clone L3D10), anti-IFN- γ -PE/Cyanine7 (clone B27) and anti-Granzyme B-PE/Cyanine7 (clone QA16A02) (all BioLegend). Single-stained controls were set up for all fluorophores using anti-mouse Ig, κ /Negative Control Particles (BD Biosciences) to validate antibody panels.

For identifying apoptotic cells, cells were resuspended following surface staining in Annexin V Binding Buffer at a concentration of 1×10^6 cells/mL and stained with Annexin V-PE/Cyanine7 (both BioLegend) for 15 mins before analysing immediately by flow cytometry without fixation.

Murine splenocytes were analysed by flow cytometry using the same methods with combinations of the following directly conjugated anti-mouse monoclonal antibodies: anti-CD3-PE/Cyanine5 (clone 17A2), anti-CD4-FITC (clone GK1.5), anti-CXCR5-biotin (clone L138D7), streptavidin-BV421, anti-PD-1-PE/Dazzle 594 (clone 29F.1A12), anti-FOXP3-Alexa Fluor 647 (clone MF-14), anti-CD40L-PE/Cyanine7 (clone MR1), anti-CTLA-4-BV605 (all BioLegend). Anti-CXCR5-biotin was added to cells for 30 min before washing and continuing with surface antibody staining. Fc receptors were blocked using Mouse TruStain FcX (BioLegend).

ELISA

High protein-binding MaxiSorp 96-well plates (Nunc, BioLegend) were coated with 2 μ g/mL capture antibody (Mabtech) diluted in PBS at 4°C overnight. Plates were washed with wash buffer (PBS supplemented with 0.05% tween-20) before blocking with incubation buffer (PBS, 0.05% tween-20, 0.1% bovine serum albumin) for 1h at room temperature (RT). Purified protein standards (Mabtech) and supernatant samples diluted in incubation buffer (1 in 160 for IgG, 1 in 50 for IgM) were added to plates and incubated for 2h at RT. ALP-conjugated detection antibodies (Mabtech) diluted 1 in 1000 in incubation buffer were added to samples and incubated for 1h at RT. Plates were washed 3 times using wash buffer between each step. Assays were developed with the substrate para-nitrophenylphosphate

(p-NPP) (Mabtech) for 1h before reading optical densities at 405nm using the ClarioSTAR microplate reader (BMG Labtech).

Data acquisition and statistical analyses

Flow cytometric data were acquired using the Attune NxT flow cytometer (Thermo Fisher Scientific) or BD LSRFortessa (BD Biosciences). Analyses were conducted using FlowJo software v10.10 (FlowJo LLC). Differences between two groups were analysed using an unpaired nonparametric T test, while three or more groups were analysed using either a mixed-effects analysis with Dunnett's multiple comparisons test, or an ANOVA (one-way or two-way) with Tukey's multiple comparisons test/ uncorrected Fisher's LSD on Prism (GraphPad). Statistical significance was assigned at $p < 0.05$, depicted by asterisks where applicable. Concatenated polyfunctionality and co-IR expression profiles were assessed using the permutation test over 10,000 iterations on Simplified Presentation of Incredibly Complex Evaluations (SPICE) software version 6.1. For data collected by ELISA, standard curves were plotted using a non-linear regression model on Prism, and concentrations were interpolated with reference to the standard curve.

Declarations

ACKNOWLEDGEMENTS

We would like to acknowledge the role of Dr Alexandra Kondratiuk, Dr Conor O'Donovan, Dr Rachael Barr and Mr Julian Gaskin at University Hospitals Bristol and Weston NHS Foundation Trust for their help in tonsil sample collection. We would like to thank the sample donors and Bristol Biobank for facilitating access to samples for this project.

FUNDING

This work was funded by grants from the Royal Society (RGS\R2\222186) and Spencer Dayman Meningitis Research (charity number: 1202202), project title The AVIATOR strep A study (Vaccine development using a tonsil organoid system). RMcL was supported by a Research Ireland FFP Award (23/FFP-A/12244). KD was supported by a Medical Research Council GW4 BioMed2 Doctoral Training Partnership studentship and a Taith Research Mobility Award. LM, EO, AH and AG acknowledge support from the NIHR Health Protection Research Focus Award in Vaccines & Immunisation at University of Bristol (NIHR207421).

AUTHOR CONTRIBUTIONS

K.D: Conceptualisation, Investigation, Formal Analysis, Methodology, Resources, Validation, Visualisation, Funding Acquisition, Writing - Original Draft, Writing – Review & Editing

L.M: Investigation, Resources, Validation, Methodology, Writing – Review & Editing

E.O: Investigation, Resources, Validation, Methodology, Writing – Review & Editing

C.M.L: Investigation, Formal Analysis, Resources, Validation, Methodology, Writing – Review & Editing

S.C: Investigation, Formal Analysis, Resources, Validation, Methodology, Writing – Review & Editing

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COMPETING INTERESTS

The authors declare no competing interests. Funders and employers had no role in the conception, content, delivery and analysis of the event, decision to publish or preparation of this manuscript.

DATA AVAILABILITY

The raw data use to generate graphs in this article are publicly available via the Cardiff University Research Data Repository

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Figures

Figure 1

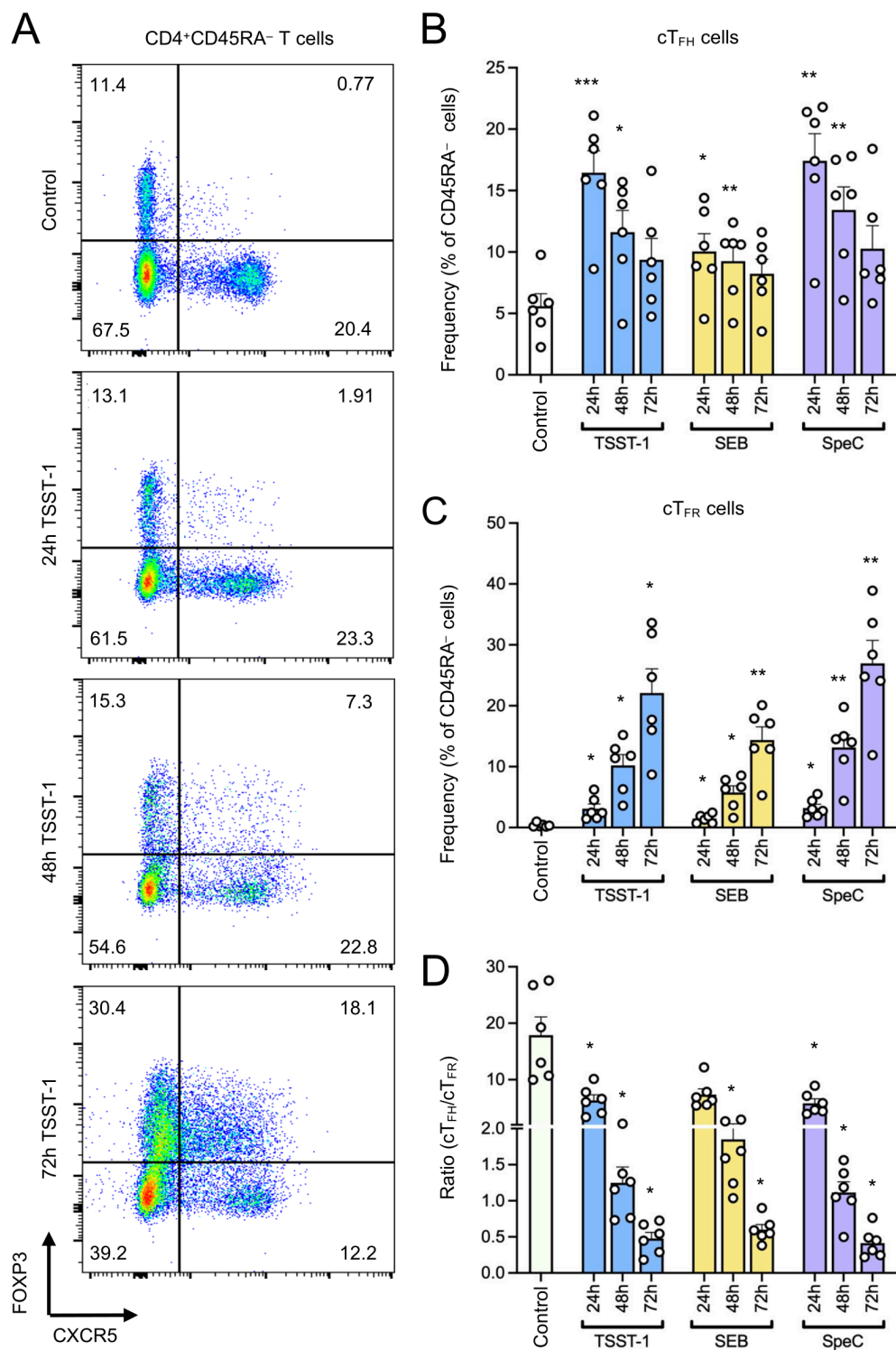


Figure 1

Staphylococcal and streptococcal SAgS disproportionately expand CD4⁺ cT_{FR} cells

Representative flow cytometry plots showing the expression of CXCR5 and FOXP3 on live CD4⁺CD45RA⁻ T cells from human PBMCs that were either cultured in media alone (control) or stimulated with TSST-1 for 24h, 48h or 72h (A). Frequency of cT_{FH} (B), cT_{FR} (C), and the ratio of cT_{FH}:cT_{FR} cells (D) as a

Figure 2



Figure 2

TSST-1-expanded cT_{FR} cells are polyfunctional with enhanced production of IFN-g and granzyme B

Representative flow cytometry plots showing the expression of OX40 and CD40L on $CD4^+ cT_{FH}$ ($CXCR5^+FOXP3^-PD-1^+$) and cT_{FR} ($CXCR5^+FOXP3^+PD-1^+$) cells from human PBMCs collected from healthy donors either cultured in media alone (control) or stimulated with TSST-1 for 72h (A). Frequency of $OX40^+CD40L^+$ (B), IFN-g $^+$ (C) and granzyme B $^+$ (D) cT_{FH} , cT_{FR} , classical T_{REG} and $CXCR5^-FOXP3^-$ T cells in response to 24h, 48h or 72h TSST-1 stimulation. Each dot represents one donor. Data are shown as mean $\pm$ SEM. For each length of stimulation, cellular subsets are compared using a one-way ANOVA with Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (E) Polyfunctional profiles of cT_{FH} , cT_{FR} , classical T_{REG} and $CXCR5^-FOXP3^-$ T cells from human PBMCs either cultured in media alone (control) or stimulated with TSST-1 for 72h. Pie chart segments from concatenated data ($n=6$ donors) represent the proportion of cells expressing the indicated number of cell surface or intracellular markers (functions). Arcs describe the individual markers found on expressing cells including OX40, IFN-g, GzmB and CD40L (arc legend).

Figure 3

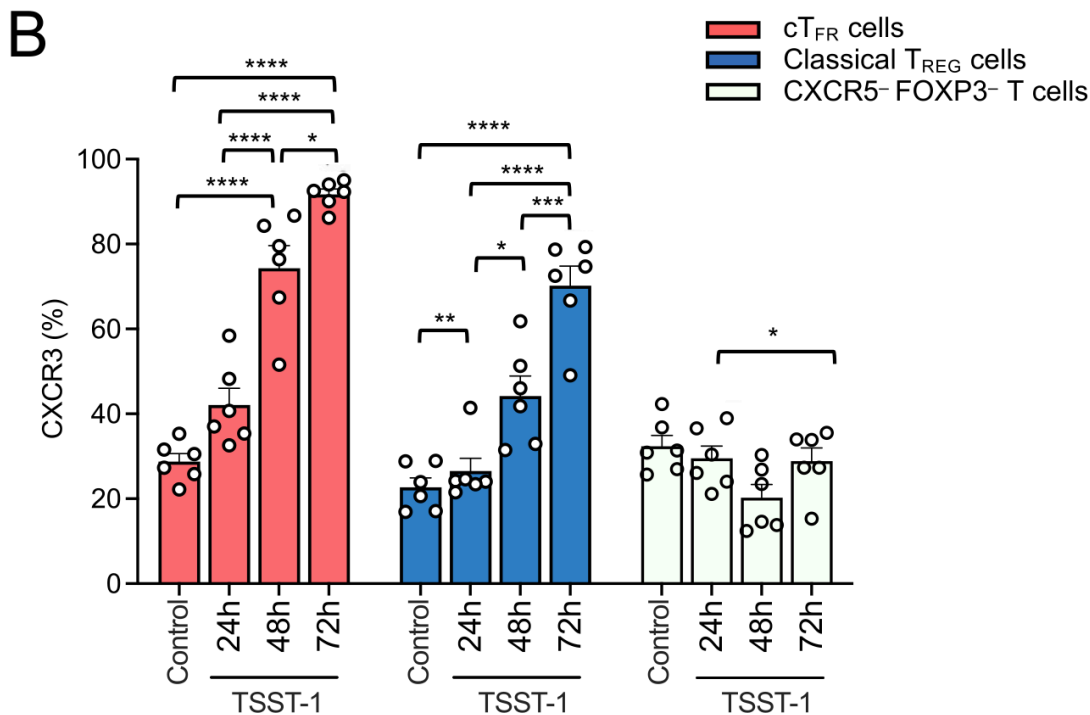
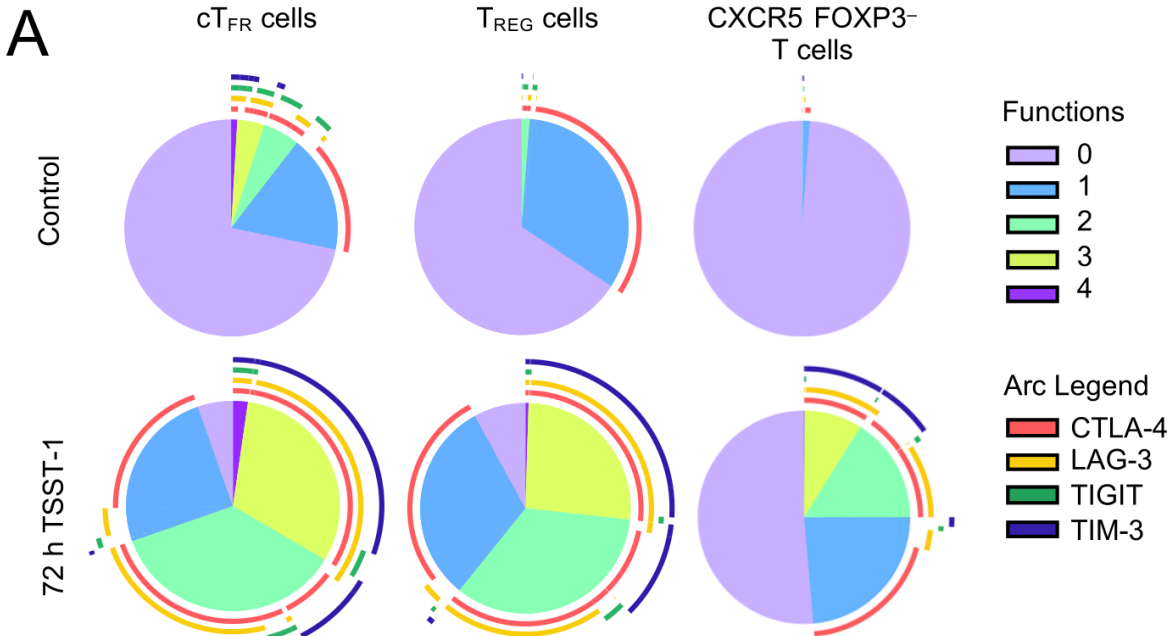


Figure 3

TSST-1-expanded cT_{FR} cells display an immunosuppressive phenotype, dictated by co-expression of multiple co-IRs

Co-IR profiles of cT_{FR}, classical T_{REG} and CXCR5⁺FOXP3⁺ T cells from human PBMCs collected from healthy donors either cultured in media alone (control) or stimulated with TSST-1 for 72h (A). Pie chart

segments from concatenated data (n=6 donors) represent the proportion of cells expressing the indicated number of cell surface or intracellular markers (functions). Arcs describe the individual markers found on expressing cells including CTLA-4, LAG-3, TIGIT and TIM-3 (arc legend). Frequency of CXCR3⁺ cT_{FR}, classical T_{REG} and CXCR5⁺FOXP3⁺ T cells from human PBMCs either cultured in media alone (control) or stimulated with TSST-1 for 24h, 48h or 72h (B). Each dot represents one donor. Data are shown as mean ± SEM. For each cellular subset, data are compared using a one-way ANOVA with Tukey's multiple comparisons test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figure 4

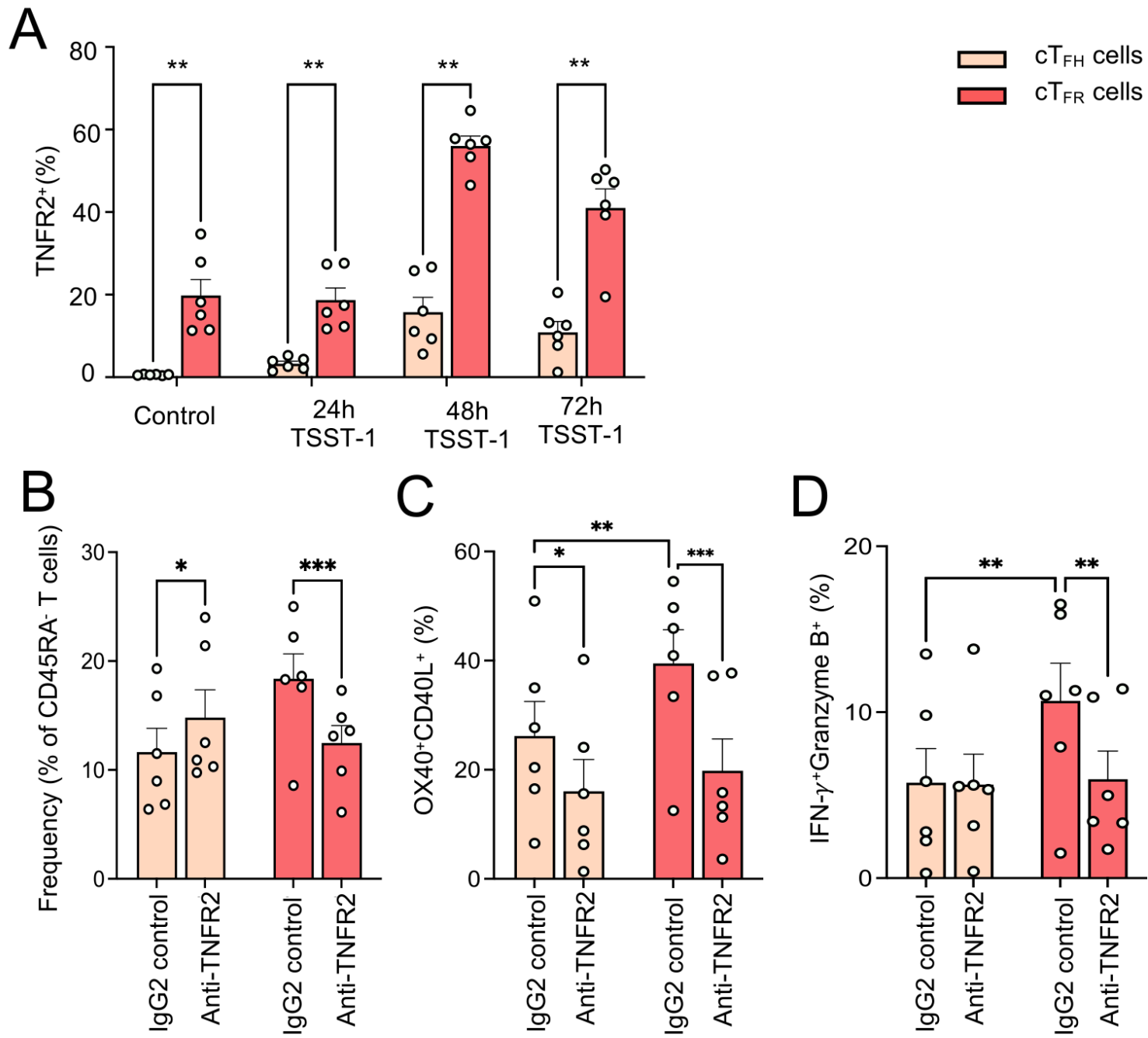


Figure 4

The induction of polyfunctional CD4⁺ cT_{FR} cells by TSST-1 stimulation is TNFR2-dependent

Frequency of TNFR2⁺ cells amongst cT_{FH} and cT_{FR} cells from human PBMCs collected from healthy donors either cultured in media alone (control) or stimulated with TSST-1 for 24h, 48h or 72h (A). Each dot represents one donor. Data are shown as mean $\pm$ SEM. For each timepoint, cT_{FH} and cT_{FR} cells are compared using a two-tailed nonparametric T test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Frequency of cT_{FH} and cT_{FR} cells as a proportion of CD4⁺CD45RA⁻ T cells from human PBMCs in response to 72h TSST-1 stimulation, either pre-incubated with an IgG2 isotype control, or a TNFR2 blocking antibody (B). Frequency of OX40⁺CD40L⁺ (C), and IFN- γ ⁺granzyme B⁺ (D) cT_{FH} or cT_{FR} cells under the same conditions described in (B). Each dot represents one donor. Data are shown as mean $\pm$ SEM. Data are compared using a two-way ANOVA with uncorrected Fisher's LSD: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 5

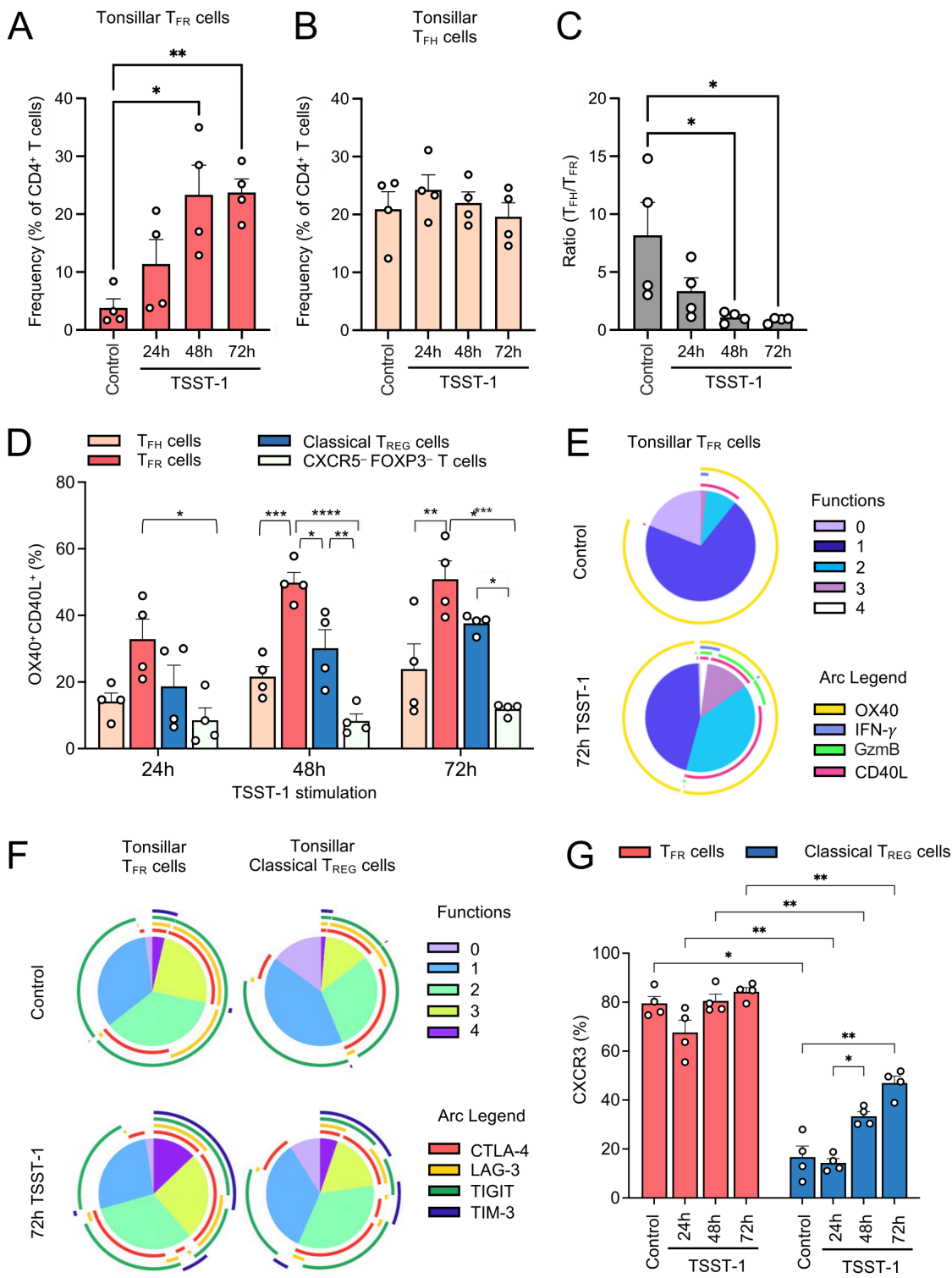


Figure 5

Tonsillar T_{FR} cells with enhanced co-IR-mediated suppressive capabilities expand upon TSST-1 stimulation

Frequency of tonsillar T_{FR} (A), T_{FH} (B) and the ratio of T_{FR}:T_{FH} cells (C) as a proportion of CD4⁺ T cells from human TMNCs cultured in media alone (control) or stimulated with TSST-1 for 24h, 48h or 72h.

Frequency of OX40⁺CD40L⁺ tonsillar T_{FH}, T_{FR}, classical T_{REG} and CXCR5⁺FOXP3⁺ T cells from human TMNCs in response to 24h, 48h or 72h TSST-1 stimulation (D). Each dot represents one donor. Data are shown as mean ± SEM. Data are compared using a one-way ANOVA with Tukey's multiple comparisons test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Polyfunctionality profiles of tonsillar T_{FR} cells from human TMNCs either cultured in media alone (control) or stimulated with TSST-1 for 72h (E). Pie chart segments from concatenated data (n=4 donors) represent the proportion of cells expressing the indicated number of cell surface or intracellular markers (functions). Arcs describe the individual markers found on expressing cells including OX40, IFN-γ, GzmB and CD40L (arc legend). Co-IR profiles of tonsillar T_{FR} and classical T_{REG} cells from human TMNCs either cultured in media alone (control) or stimulated with TSST-1 for 72h (F). Pie chart segments from concatenated data (n=4 donors) represent the proportion of cells expressing the indicated number of cell surface or intracellular markers (functions). Arcs describe the individual markers found on expressing cells including CTLA-4, LAG-3, TIGIT and TIM-3 (arc legend). Frequency of CXCR3⁺ tonsillar T_{FR} and classical T_{REG} cells from human TMNCs either cultured in media alone (control) or stimulated with TSST-1 for 24h, 48h or 72h (G). Each dot represents one donor. Data are shown as mean ± SEM. Data are compared using a two-way ANOVA with Tukey's multiple comparisons test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6

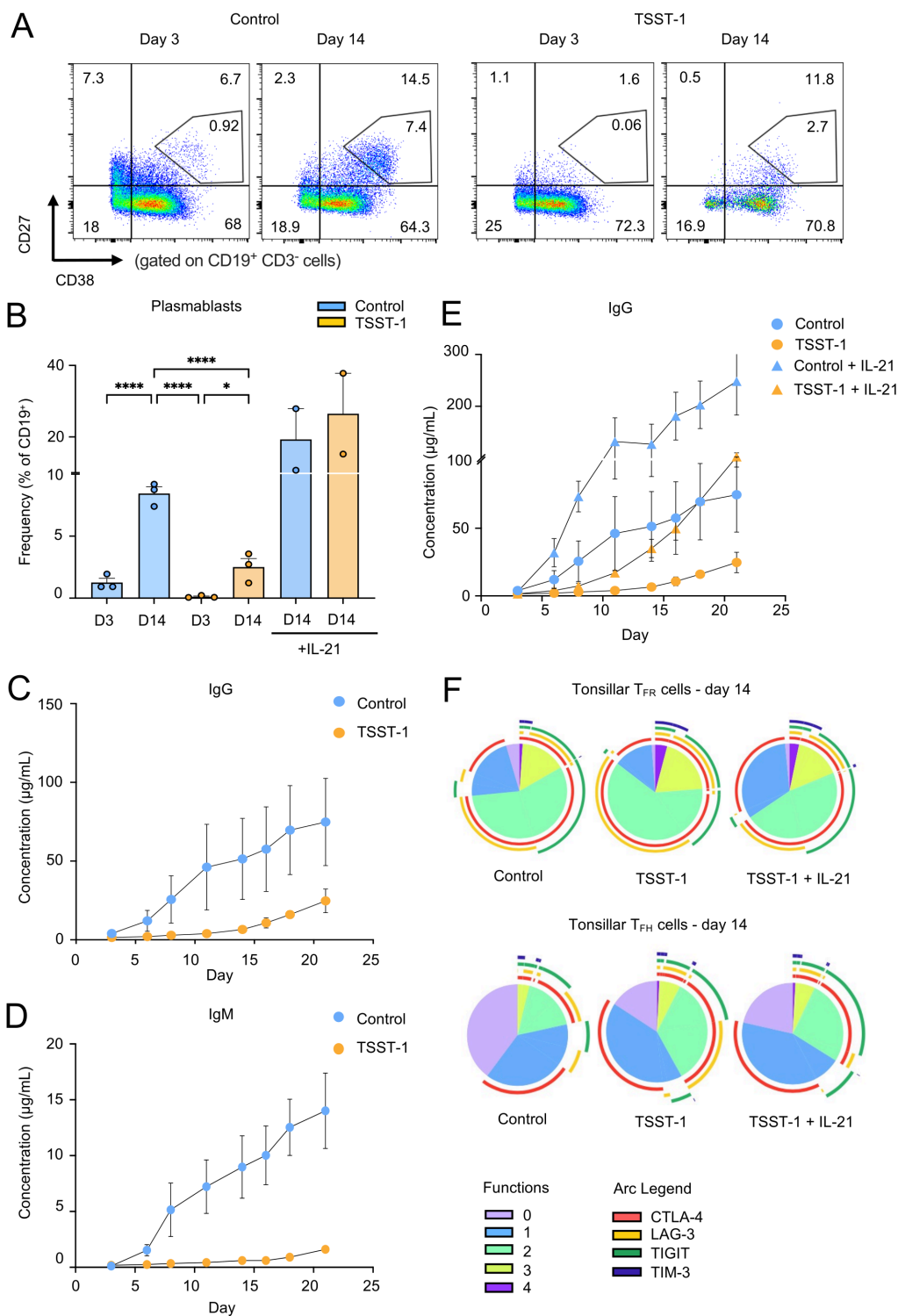


Figure 6

TSST-1 disrupts plasmablast function and class-switched antibody production in human tonsil organoids

Representative flow cytometry plots showing the expression of CD38 and CD27 on CD19⁺ B cells from human tonsil organoids either cultured in media with BAFF alone (control) or cultured in media with

BAFF and stimulated with TSST-1 for 3 or 14 days (A). Plasmablast populations are defined as CD38^{hi}CD27^{hi} CD19⁺ B cells. Frequency of plasmablasts as a proportion of all CD19⁺ B cells from human tonsil organoids either cultured in media alone (control) or cultured in media with BAFF and stimulated with TSST-1 for 3 or 14 days, with or without exogenous IL-21 (B). Each dot represents one donor. Data are shown as mean $\pm$ SEM. Data are compared using a one-way ANOVA with Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Concentration of IgG (C) and IgM (D) measured in the supernatant of human tonsil organoids either cultured in media with BAFF alone (control, blue dots) or stimulated with TSST-1 (orange dots) for 21 days. Concentration of IgG measured in the supernatant of human tonsil organoids either cultured in media with BAFF alone (control, blue dots), BAFF + IL-21 (control, blue triangles), or cultured in media with BAFF and stimulated with TSST-1 (orange dots), or TSST-1 + IL-21 (orange triangles) for 21 days (E). Organoids were cultured in triplicate and a mean concentration per timepoint and condition were calculated for each donor. Data are shown as mean $\pm$ SEM across donors ($n=3$). Co-IR profiles of T_{FR} and T_{FH} cells from human tonsil organoids either cultured in media with BAFF alone (control) or cultured in media with BAFF and stimulated with TSST-1, or TSST-1 + IL-21 for 14 days (F). Pie chart segments from concatenated data ($n=3$ donors) represent the proportion of cells expressing the indicated number of surface or intracellular markers (functions). Arcs describe the individual markers including CTLA-4, LAG-3, TIGIT and TIM-3 (arc legend).

Figure 7

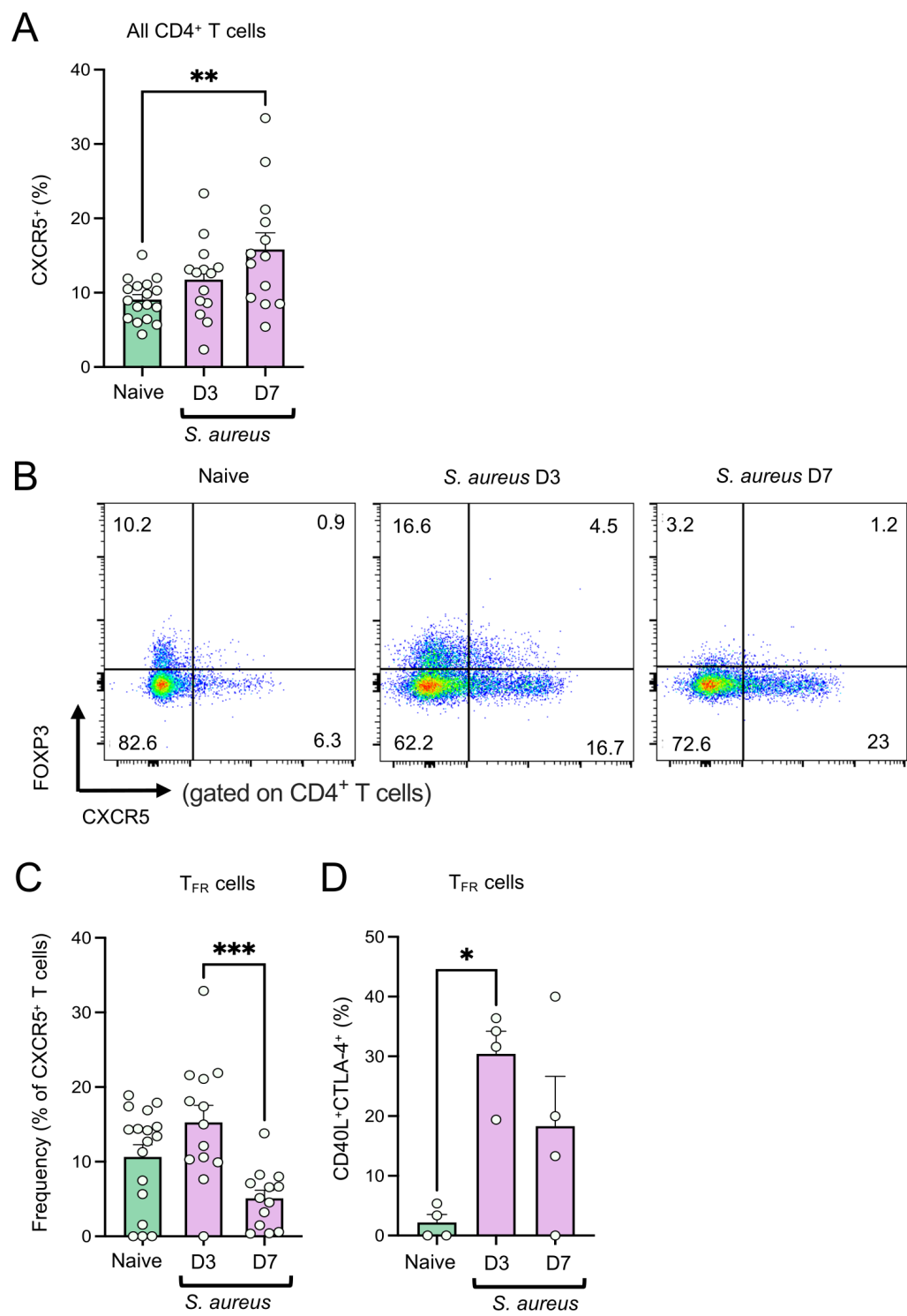


Figure 7

Systemic *S. aureus* infection induces an early expansion of CD4⁺ T_{FR} cells *in vivo*

Groups of C57BL/6J mice were challenged with USA300 LAC (2.5 x10⁸ CFU) or PBS (naïve) via intraperitoneal injection. On day 3 and day 7 post challenge, spleenocytes were isolated and stained for analysis by flow cytometry. Frequency of CXCR5⁺ cells as a proportion of splenic CD4⁺ T cells from naïve,

uninfected mice, or *S. aureus*-infected mice at 3 and 7 days post-infection (A). Each dot represents one mouse. Data are shown as mean $\pm$ SEM. Data are compared using a one-way ANOVA with Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Representative flow cytometry plots showing the expression of CXCR5 and FOXP3 on splenic CD4⁺ T cells from naïve, uninfected mice or *S. aureus*-infected mice at 3 and 7 days post-infection (B). Frequency of T_{FR} cells as a proportion of all splenic CXCR5⁺CD4⁺ T cells (C) and proportion of CD40L⁺CTLA-4⁺ T_{FR} cells (D) from naïve, uninfected mice or *S. aureus*-infected mice at 3 and 7 days post-infection. Each dot represents one mouse. Data are shown as mean $\pm$ SEM. Data are compared using a one-way ANOVA with Tukey's multiple comparisons test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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