TIGIT-positive circulating follicular helper T cells display robust B-cell help functions: potential role in sickle cell alloimmunization

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ABSTRACT

T follicular helper cells are the main CD4$^+$ T cells specialized in supporting B-cell responses, but their role in driving transfusion-associated alloimmunization is not fully characterized. Reports of T follicular helper subsets displaying various markers and functional activities underscore the need for better characterization/identification of markers with defined functions. Here we show that a previously unidentified subset of human circulating T follicular helper cells expressing TIGIT, the T-cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory domains, exhibit strong B-cell help functions. Compared to the subset lacking the receptor, T follicular helper cells expressing this receptor up-regulated co-stimulatory molecules and produced higher levels of interleukins (IL-21 and IL-4) critical for promoting B-cell activation/differentiation. Furthermore, this subset was more efficient at inducing the differentiation of B cells into plasmablasts and promoting immunoglobulin G production. Blocking antibodies abrogated the B-cell help properties of receptor-expressing T follicular helper cells, consistent with the key role of this molecule in T follicular helper-associated responses. Importantly, in chronically transfused patients with sickle cell anemia, we identified functional differences of this subset between alloimmunized and non-alloimmunized patients. Altogether, these studies suggest that expression of the T-cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory domains not only represents a novel circulating T follicular helper biomarker, but is also functional and promotes strong B-cell help and ensuing immunoglobulin G production. These findings open the way to defining new diagnostic and therapeutic strategies in modulating humoral responses in alloimmunization, and possibly vaccination, autoimmunity and immune deficiencies.

Introduction

T follicular helper (T_{FH}) cells have emerged as the main effector CD4$^+$ T cells specialized in supporting B-cell responses to generate the initial wave of antibody response as well as in promoting B-cell differentiation into high affinity antibody-producing cells and long-lasting IgG antibody.[1] T_{FH} cells express chemokine (C-X-C motif) receptor 5 (CXCR5),[2-4] which allows their migration into B-cell follicles in response to its ligand CXCL13. Bcl-6 is the main lineage-associated transcription factor driving T_{FH} differentiation.[5,6] Interleukin (IL)-21 is the canonical T_{FH}-associated cytokine driving B-cell help,[5,7,8] although T_{FH} cells can also secrete additional cytokines promoting growth, differentiation and class-switching of B cells, such as IL-4.[9,10] T_{FH} cells also express several key co-stimulatory molecules specialized in providing B-cell help, including inducible T-cell co-stimulator (ICOS),[11,12] required for T-cell proliferation and T/B-cell interactions as well as CD40 ligand (CD40L), a potent activator of B cells, inducing their activation and differentiation.[1]

Transfusion therapy remains an important treatment modality for patients with sickle cell disease (SCD). Despite its therapeutic benefits, 20-60% patients with SCD develop alloantibodies with specificities against disparate antigens on transfused red blood cells, causing complications ranging from life-threatening hemolytic transfusion reactions, to logistical problems in finding compatible red cells for transfusion.[13] Given their key role in providing help to B cells and driving antibody responses, T_{FH} cells are likely to be critical in alloimmunization biology. In a recent study of a cohort of transfused patients with SCD studied by Vingert *et al.*,[14] despite a higher frequency of T_{FH} cells in non-alloimmunized patients, only the alloimmunized group displayed antigen-specific T_{FH} cells expressing IL-21, suggesting that T_{FH} cells may play a role in alloimmunization.

Exciting new studies indicate that circulating T_{FH}-related cells (cT_{FH}), ranging from 5-25% of CD4$^+$ memory cells, exist in the peripheral blood in humans and mice, and can promote antibody production.[7,12,15-20] These blood T_{FH} cells (CD45RA$^-$) express similar markers as lymphoid T_{FH} cells (including CXCR5, ICOS, CD40L and IL-21),[21] but do not express the Bcl-6 protein.[7,12,15-17,22] Importantly, cT_{FH} levels correlate with auto-antibodies and levels of protective antibodies following vaccination.[12,16,20,23,24] Several cT_{FH} subsets have been reported, each displaying drastically different functions.[25] For example, cT_{FH} subsets harboring T_H1-, T_H2- and T_H17-like effector functions have been identified in humans, with T_H2- and T_H17-like cT_{FH} being more efficient in inducing B cells to produce IgG than T_H1-like cT_{FH}.[12,21] Furthermore, recent studies have identified lymphoid Foxp3$^+$ T_{FH} cells [named follicular regulatory T (T_{FR}) cells] that can suppress germinal center reactions,[26-28] also detectable in the periphery.[29,30] In addition, similar to lym-

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phoid T_{FH}, cT_{FH} cells can express significant levels of surface programmed cell death-1 (PD-1), [31] although the function of PD-1 on T_{FH} cells remains controversial since it is associated with both promotion[16,17] and inhibition of B-cell responses.[18,32-34] Taken together, these reports underscore the need for better characterization of markers for cT_{FH} cells displaying defined functions not only in steady state but also in diseases. Remarkably, PD-1 has been described as a member of the growing family of inhibitory receptors also referred to as immune checkpoints, responsible for aborting T-cell responses.[35] Interestingly, another member of the immune checkpoint family, TIGIT (T-cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibitory domains), was reported to be over-expressed on both tonsillar and cT_{FH} cells,[17] and was shown to be involved in interactions between T cells and follicular dendritic cells to regulate B-cell responses.[36,37] However, the functional activity of TIGIT on T_{FH} cells, including cT_{FH} cells, has not been studied to date.

In this study, we took the approach of using TIGIT and PD-1 to characterize the phenotype and function of circulating T_{FH} subsets and to investigate whether expression of these molecules on cT_{FH} cells modulated their functions in healthy volunteer donors and in a group of chronically transfused SCD patients with or without alloantibodies.

Methods

Human samples

All studies were approved by the Institutional Review Boards of the New York Blood Center (NYBC). De-identified fresh leukopaks were obtained from healthy donors at the NYBC. For SCD patients' samples, blood was obtained solely from discard apheresis waste bags obtained during erythrocytapheresis procedures at the Children's Hospital of Philadelphia (see *Online Supplementary Material* for details).

T-cell studies

Freshly-sorted $CD4^+$ T-cell subsets and autologous naïve or memory B cells were used (see *Online Supplementary Material* for details). Blocking antibodies for TIGIT[38] and PD-1[34,39] were pre-incubated with sorted T cells before being co-cultured with autologous B cells.

Results

PD-1$^+$ cT_{FH} cells co-express TIGIT and represent a limited fraction of TIGIT$^+$ cT_{FH} cells

In healthy donors, a large subset of cT_{FH} cells, as defined by $CD4^+CD45RA^-CXCR5^+$ T cells in peripheral blood, express TIGIT: 6.3%±0.8 of all $CD4^+$ T cells (Figure 1A,B) or 47%±3.2 of cT_{FH} (Figure 1A,C). Strikingly, we also found that >92% PD-1-expressing cT_{FH} cells co-express TIGIT and that cT_{FH} cells expressing PD-1, but lacking TIGIT (PD-1$^+$/TIGIT$^-$) were barely detectable (<0.042%±0.008 of $CD4^+$ T cells or 2.0%±0.4 of cT_{FH}). Within the cT_{FH} subset, the PD-1$^+$/TIGIT$^+$ cT_{FH} population represented a significantly lower frequency of cT_{FH} (8.6%±0.9) as compared to PD-1$^-$/TIGIT$^+$ cT_{FH} cells (32.5%±1.6, P=0.0001; Figure 1).

Expression of ICOS, CD40L and IL-21 by TIGIT$^+$ cT_{FH} cells

We next tested whether TIGIT$^+$ cT_{FH} cells were functionally different from cT_{FH} cells lacking TIGIT (PD-1$^-$/TIGIT$^-$). To this end, PD-1$^-$/TIGIT$^+$ and PD-1$^+$/TIGIT$^+$ $CXCR5^+$ cT_{FH} populations from a small number of healthy donors (n=3 or 4) were sorted and their ability to express T_{FH}-associated co-stimulatory markers and cytokines following stimulation was compared to those of sorted autologous PD-1$^-$/TIGIT$^-$ subsets (gating strategy shown in *Online Supplementary Figure S1*). As a control, sort-purified autologous $CXCR5^-$ non-cT_{FH} cells expressing TIGIT

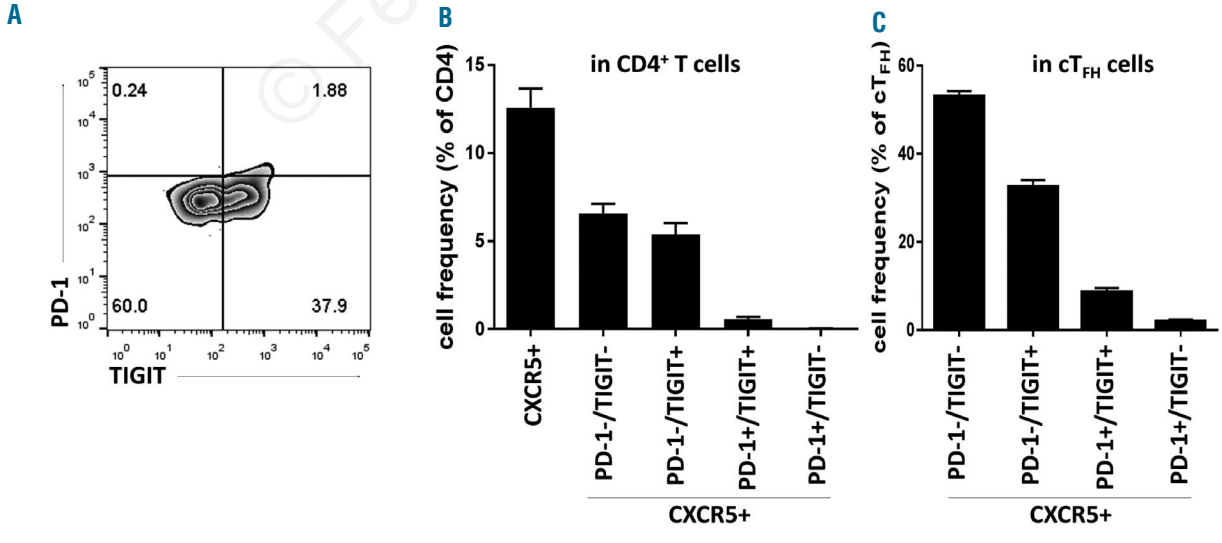

Figure 1. Frequency of circulating T_{FH} subsets according to PD-1 and TIGIT expression in healthy donors. $CD4^+/CD45RA^-/CXCR5^+$ cT_{FH} subsets were immunostained for PD-1 and TIGIT. Three cell subsets can be distinguished: PD-1$^-$/TIGIT$^-$, PD-1$^-$/TIGIT$^+$ and PD1$^+$/TIGIT$^+$. (A) Dot plot analysis of TIGIT and PD-1 expression in the $CD4^+/CD45RA^-/CXCR5^+$ cT_{FH} subset in a representative donor. (B, C) The frequency of each indicated cT_{FH} subpopulation within (B) $CD4^+$ T cells or (C) cT_{FH} cells is represented as the mean of eight donors ± sem.

("TIGIT+") or not ("TIGIT-") were also tested.

We first monitored expression of co-stimulatory molecules CD40L and ICOS, both specialized in providing B-cell help, on T-cell subsets before or after stimulation by immunostaining. TIGIT$^+$ cT$_{FH}$ cells significantly over-expressed ICOS (>2-fold) prior to stimulation i.e. at resting state (Figure 2A,C; *P*=0.001 as compared to TIGIT$^-$ cT$_{FH}$ cells or CXCR5$^-$ non-T$_{FH}$ cells; n=3). Although not statistically significant, CD40L was also over-expressed (>2-fold) by TIGIT$^+$ cT$_{FH}$ cells (Figure 2B,D). After stimulation by phorbol myristate acetate/ionomycin, the expression of both ICOS and CD40L was still significantly higher on TIGIT$^+$ cT$_{FH}$ cells than on TIGIT$^-$ cT$_{FH}$ cells (Figure 2E,F) or CXCR5$^-$ non-T$_{FH}$ cells. Altogether, these data suggest that TIGIT$^+$ cT$_{FH}$ cells are well-equipped to provide B-cell help, as they display stable intrinsic functions both at resting and activated states.

Next the production of T$_{FH}$-associated cytokine IL-21 by TIGIT$^+$ cT$_{FH}$ cells was assessed upon stimulation with phorbol myristate acetate/ionomycin. Significantly, TIGIT$^+$ cT$_{FH}$ cells expressed more IL-21 (n=4, PD1$^-$/TIGIT$^+$: 14.4%±2.4 and PD1$^+$/TIGIT$^+$: 17.8%±3.2) than TIGIT$^-$ cT$_{FH}$ cells (6.5%±2.1) or CXCR5$^-$ non-T$_{FH}$ cells (Figure 2G and *Online Supplementary Figure S2A*). We also tested expression of interferon gamma (IFNγ) and IL-4 (*Online Supplementary Figure S2B,C*, respectively). In contrast, TIGIT$^+$ cT$_{FH}$ cells produced less IFNγ than TIGIT$^-$ cT$_{FH}$ cells (*Online Supplementary Figure S2B*) and overall, fewer CXCR5$^+$ cT$_{FH}$ cells expressed IFNγ as compared to CXCR5$^-$ non-T$_{FH}$ cells. Within cT$_{FH}$ cells, IL-4 production was also measured, but no significant differences were found between subsets (*Online Supplementary Figure S2C*). Given the role of IL-21 in driving antibody responses, these data suggest that cT$_{FH}$ cells could represent an efficient subset in promoting humoral responses.

TIGIT$^+$ cT$_{FH}$ cells constitute a novel subset

Several CXCR5$^+$ cT$_{FH}$ subsets with distinct capabilities in

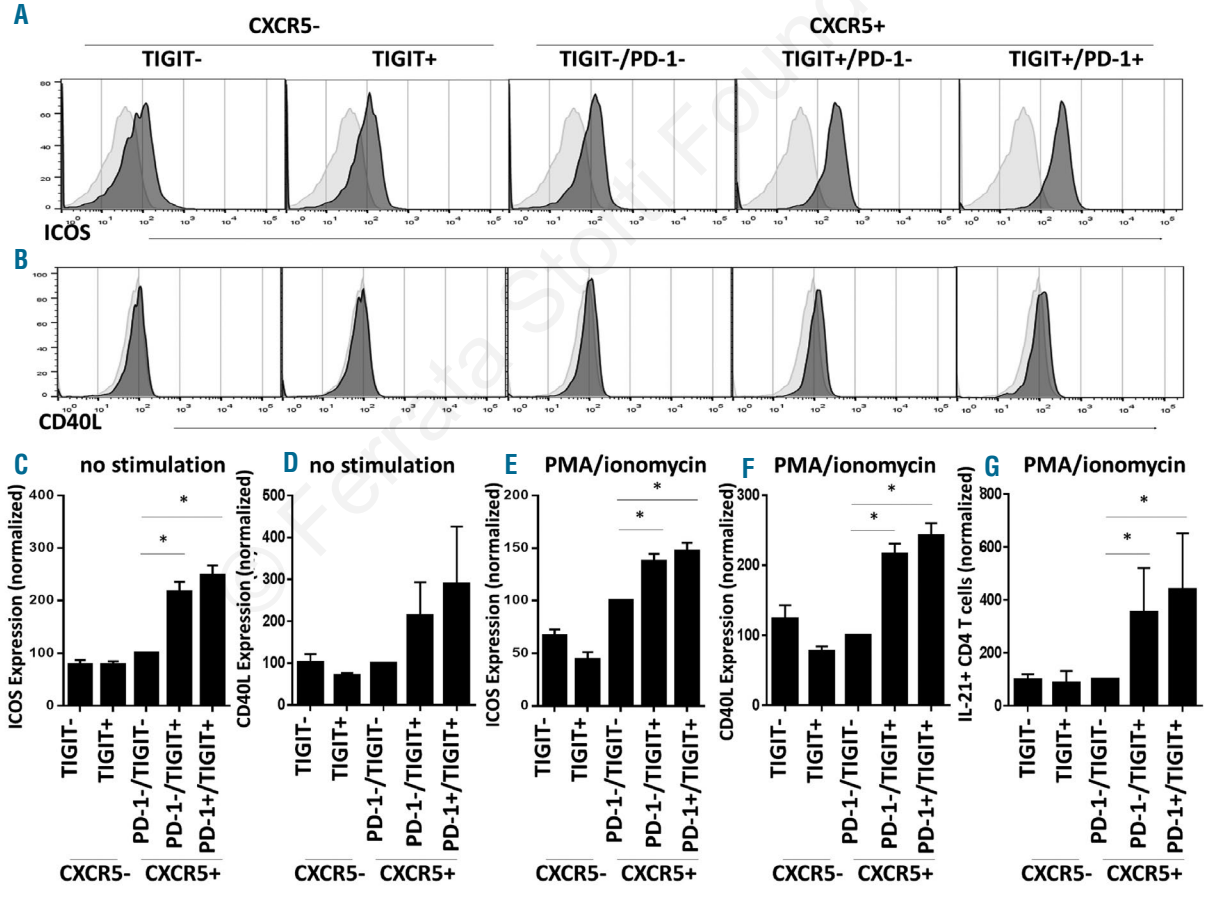

Figure 2. Expression of ICOS, CD40L and IL-21 by TIGIT$^+$ cT$_{FH}$ cells. (A-F) PD-1$^-$/TIGIT$^-$, PD-1$^-$/TIGIT$^+$ and PD1$^+$/TIGIT$^+$ as well as TIGIT$^+$ or TIGIT$^-$CXCR5- non-T$_{FH}$ control subsets were sorted from healthy donors. Histogram overlays of (A) ICOS and (B) CD40L expression for each freshly isolated subset *versus* an unstained control. Results for donors (n=3) tested are shown either (C,D) in the absence of stimulation or (E, F) 2 days after phorbol myristate acetate (PMA)/ionomycin stimulation. ICOS or CD40L expression (measured as mean fluorescence intensity, MFI) in various subsets was normalized to levels in PD-1$^-$/TIGIT$^-$ cells to highlight the role of TIGIT on cT$_{FH}$ cells. (G) Sorted T-cell subsets as indicated from healthy donors (n=4) were rested overnight and stimulated for 5 h with PMA/ionomycin in the presence of brefeldin A (BFA) before being intracellularly stained for IL-21. The percentages of CD4$^+$ T cells expressing IL-21 in various subsets were normalized to the levels expressed by PD-1$^-$/TIGIT$^-$ cells. *P* values were obtained using an unpaired two-tailed Student t-test; (error bars, sem). *P≤0.05 (statistically significant).

supporting B-cell activation and antibody production have been characterized, including three subsets distinguished based on their co-expression of T_H1, T_H2 or T_H17 features and differential expression of chemokine receptors, namely CXCR3 and CCR6.[7,12] We therefore compared TIGIT$^+$ cT_{FH} cells to CXCR3$^+$/CCR6$^-$ (type 1-like), CXCR3$^-$/CCR6$^-$ (type 2-like) and CXCR3$^-$/CCR6$^+$ (type 17-like) cT_{FH} subsets. These subsets all contained significant levels (from 24% to 54% of total cT_{FH} cells) of at least two out of the three subsets, specifically PD-1$^+$/TIGIT$^+$ ("DP") PD-1$^-$/TIGIT$^+$ ("SP") and/or PD-1$^-$/TIGIT$^-$ ("DN") cells (Figure 3A). Furthermore, PD-1$^+$/TIGIT$^+$, PD1$^-$/TIGIT$^+$ and PD-1$^-$/TIGIT$^-$ subsets each displayed type 1, 2 and 17-like cT_{FH} cells (Figure 3B). Each subset contained between 10% and 61% of every type 1, 2 and 17-like subsets within total cT_{FH} cells, demonstrating that TIGIT$^+$ cT_{FH} subset(s) is(are) separate/independent, at least phenotypically, of these three previously described subsets.

Together these results establish TIGIT$^+$ cT_{FH} cells as a phenotypically distinct subset potentially efficient at promoting humoral responses.

TIGIT functionally drives T_{FH} function/B-cell help

To further characterize TIGIT$^+$ cT_{FH} cells and determine whether TIGIT was functionally responsible for their ability to promote B-cell help, sorted PD-1$^-$/TIGIT$^-$, PD-1$^-$/TIGIT$^+$ and PD-1$^+$/TIGIT$^+$ subsets from healthy donors were co-cultured with autologous CD27$^-$ naïve or CD27$^+$ memory B cells in the presence of SEB to facilitate T/B-cell interactions. At day 7, TIGIT$^+$ cT_{FH} cells (both PD-1$^+$ and PD-1$^-$) expressed 1.5- to 3-fold higher levels of both ICOS and CD40L compared to TIGIT$^-$ cT_{FH} cells when co-cultured with CD27$^-$ naïve or CD27$^+$ memory B cells (*Online Supplementary Figure S3*).

T-cell subsets were also assessed at day 7 for cytokine expression upon re-stimulation using phorbol myristate acetate/ionomycin to assess non-specific T-cell function. Both PD-1$^+$ and PD-1$^-$ TIGIT$^+$ cT_{FH} cells expressed more IL-21 than TIGIT$^-$ cT_{FH} cells when co-cultured with naïve or memory B cells (Figure 4A). As expected, non-T_{FH} cells produced less IL-21 than cT_{FH} cells, including TIGIT$^-$ cT_{FH} cells (Figure 4A). Strikingly, TIGIT$^+$ cT_{FH} cells, independently of their PD-1 expression, also produced less IFNγ than TIGIT$^-$ cT_{FH} cells when co-cultured with naïve or memory B cells (Figure 4B). Furthermore, TIGIT$^+$ cT_{FH} cells, regardless of PD-1 expression, produced higher levels of IL-4 than TIGIT$^-$ cT^{FH} cells, although the increase was only significant in co-cultures with naïve B cells (Figure 4C). Of note, both CXCR5$^-$ and CXCR5$^+$ cells produced similar amounts of IL-4 (Figure 4C). Hence, within cT_{FH} cells, TIGIT expression increases IL-21 and IL-4 production, but inhibits IFNγ production.

The ability of sorted cT_{FH} subsets to drive B cells to differentiate into CD19^{lo}/CD38$^+$ plasmablasts was also determined on day 7 by immunostaining. Naïve and memory B cells differentiated into plasmablasts more efficiently when co-cultured with TIGIT$^+$ cT_{FH} cells than with TIGIT$^-$ cT_{FH} cells (Figure 5A,B and *Online Supplementary Figure S4*). Accordingly, more IgG was produced by both naïve (>1.3- to 1.7-fold) and memory B cells (>3-fold) co-cultured with TIGIT$^+$ cT_{FH} cells than with TIGIT$^-$ cT_{FH} cells (Figure 5C and *Online Supplementary Figure S4*).

To further confirm that TIGIT expression strengthens B-cell help function, blocking antibodies against TIGIT or PD-1 were added to T/B-cell co-cultures. Relative to isotype control, up-regulation of ICOS by TIGIT$^+$ cT_{FH} subsets expressing PD-1 or not, co-cultured with memory B cells were significantly inhibited by anti-TIGIT (22% to 30%, Figure 6A). Of note, the anti-TIGIT antibody clone used (MBSA43) has previously been reported to block TIGIT signaling,[38] further supporting the belief that this decrease results from TIGIT blockade. However, PD-1$^+$/TIGIT$^+$ cells were not inhibited by anti-PD-1 (Figure 6A), despite the known blocking function of this antibody.[34,39] As expected, PD-1 blockade did not affect PD-1$^-$/TIGIT$^+$ cell responses. To a lesser extent, TIGIT$^+$ cT_{FH} cells co-cultured with naïve B cells also displayed decreased levels of ICOS (23% to 28% inhibition com-

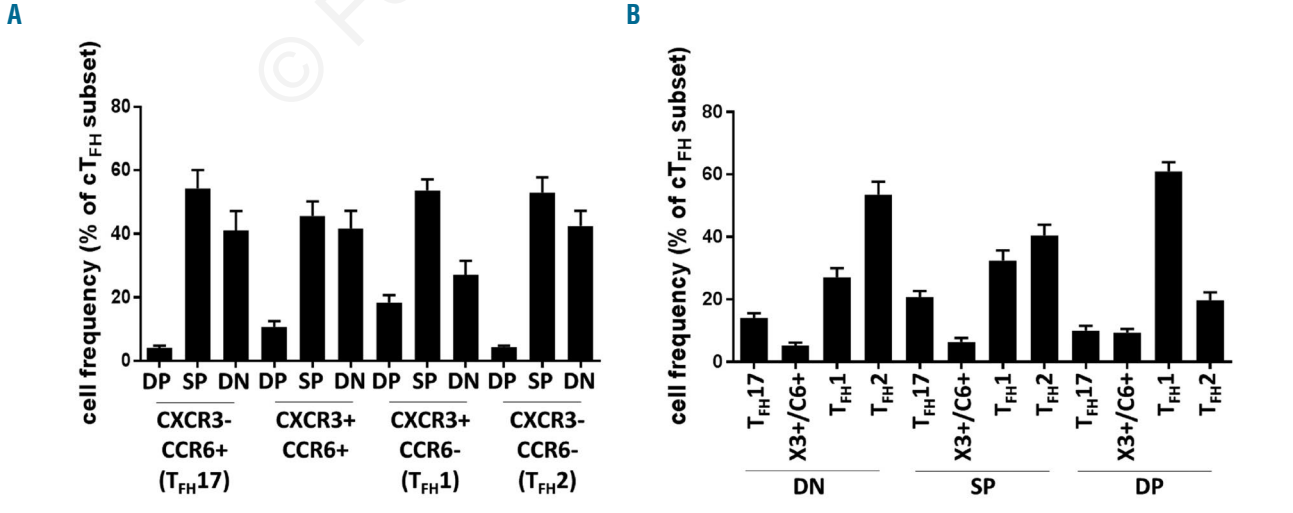

Figure 3. TIGIT$^+$ cT_{FH} cells represent a distinct subset. Fresh peripheral blood mononuclear cells from healthy donors were gated on CD4$^+$/CD45RA$^-$/CXCR5$^+$. (A) Percentages of PD-1$^-$/TIGIT$^-$ (DN), PD-1$^-$/TIGIT$^+$ (SP) and PD1$^+$/TIGIT$^+$ (DP) cT_{FH} cells within each CXCR3$^-$/CCR6$^+$, CXCR3$^+$/CCR6$^+$, CXCR3$^+$/CCR6$^-$ and CXCR3$^-$/CCR6$^-$ cT_{FH} subset are represented. (B) Percentages of CXCR3$^-$/CCR6$^+$, CXCR3$^+$/CCR6$^+$, CXCR3$^+$/CCR6$^-$ and CXCR3$^-$/CCR6$^-$ cT_{FH} cells within each PD-1$^-$/TIGIT$^-$ (DN), PD-1$^-$/TIGIT$^+$ (SP) and PD1$^+$/TIGIT$^+$ (DP) cT_{FH} subset are represented.

pared to isotype control) when TIGIT, but not PD-1, was blocked (Figure 6A). No significant inhibition of CD40L was observed using the same antibodies (*data not shown*). Furthermore, IL-21 expression by TIGIT$^+$ cT_{FH} cells was significantly inhibited by TIGIT blockade (49% to 66% inhibition compared to isotype control), but not by anti-PD-1 (Figure 6B). On the other hand, IFNγ and IL-4 expression was not affected by TIGIT blockade (*data not shown*). Regarding B-cell responses, a trend toward lower plasmablast differentiation (between 26% to 65% inhibition) was observed in co-cultures with TIGIT$^+$ cT_{FH} cells following TIGIT blockade, but not using an anti-PD-1 antibody (Figure 6C). IgG production (between 39% to 59% inhibition) was also decreased by anti-TIGIT in naïve and memory cell co-cultures with TIGIT$^+$ cT_{FH} cells (Figure 6D). There was a trend towards lower IgG responses with anti-PD-1 in co-cultures with TIGIT$^+$ cT_{FH} cells; however, the inhibition may not be specific as the same effect was also observed in co-cultures with TIGIT$^+$ cT_{FH} cells lacking PD-1 (Figure 6D).

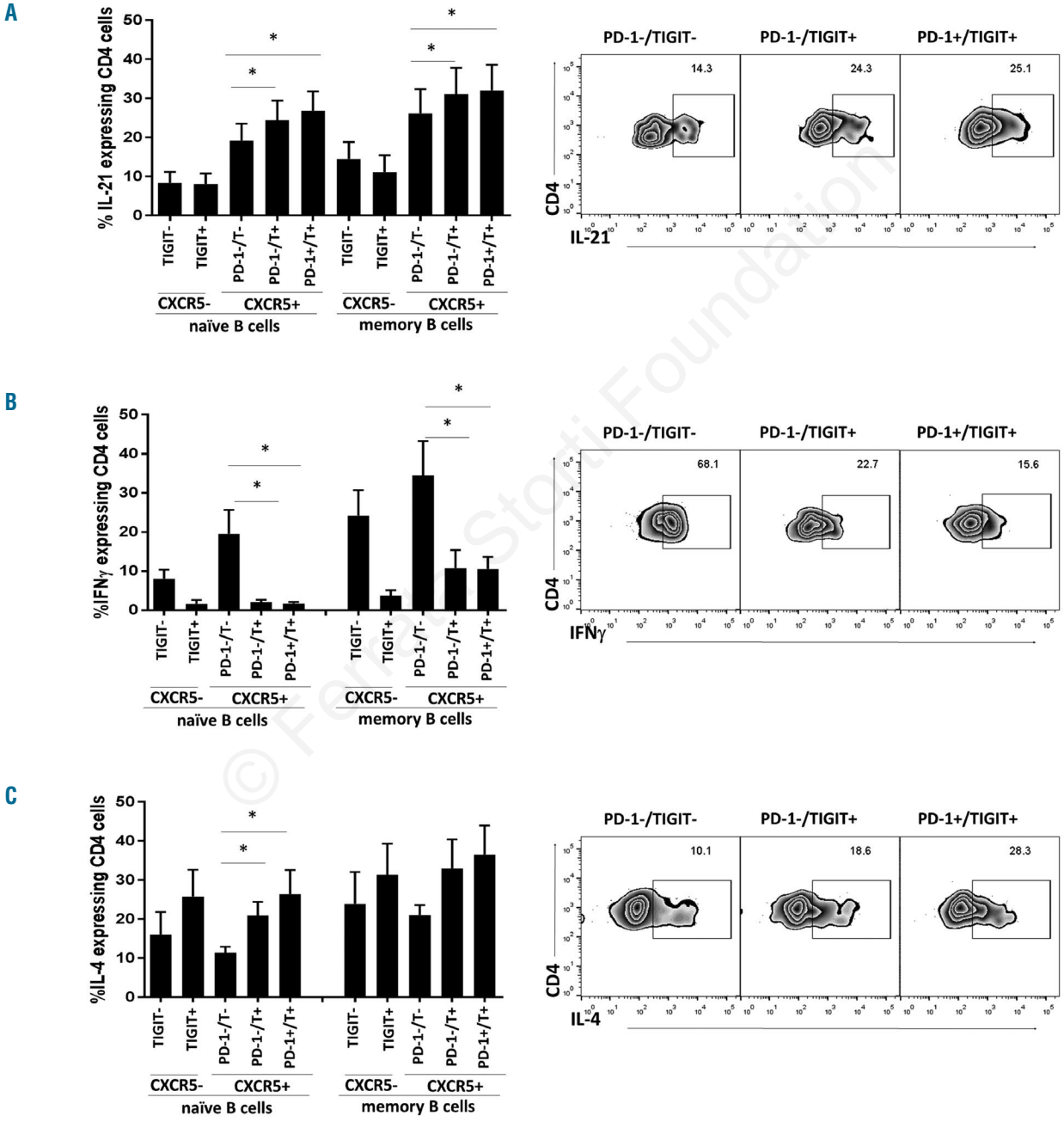

Figure 4. TIGIT$^+$ cT_{FH} cells display robust T_{FH} function. Sort-purified PD-1$^-$/TIGIT$^-$, PD-1$^-$/TIGIT$^+$ and PD-1$^+$/TIGIT$^+$ as well as TIGIT$^+$ or TIGIT$^-$ CXCR5$^-$ non-T_{FH} control subsets from healthy donors were co-cultured with sorted autologous naïve CD27$^-$/CD19$^+$ or memory CD27$^+$/CD19$^+$ B cells in the presence of 0.2 μM SEB (to facilitate T-B interactions) for 7 days. Cells were stimulated for 5 h with phorbol myristate acetate/ionomycin in the presence of brefeldin A before being intracellularly stained for (A) IL-21, (B) IFNγ and (C) IL-4. Results are shown as percentages of CD4$^+$ T cells producing cytokines. Representative zebra plots of cytokine production by each PD-1$^-$/TIGIT$^-$, PD-1$^-$/TIGIT$^+$ and PD1$^+$/TIGIT$^+$ cT_{FH} subsets are shown on the right. *P* values were obtained using a paired two-tailed Student t-test; n=6 (error bars, sem). *$P \leq 0.05$ (statistically significant).

TIGIT expression by cT_{FH} in patients with sickle cell disease

To assess whether TIGIT-expressing CXCR5$^+$ cT_{FH} cells were different in SCD patients between those who were alloimmunized (with a history and/or currently detectable red blood cell-specific allo-antibodies) and those who were not alloimmunized (had never produced any auto- or allo-antibodies), we analyzed a group of SCD patients on a chronic transfusion protocol (n=21). The frequency of CD4$^+$ cells was lower in SCD patients than in healthy donors (*data not shown*), consistent with a previous report.[40] The percentage of total cT_{FH} cells within CD4$^+$ T cells was also lower in samples from chronically transfused SCD patients (7.8%±1.1% *versus* 11.2%±1.2% in healthy donors). Since peripheral blood mononuclear cells from healthy donors were obtained from leukocyte-enriched peripheral blood whereas peripheral blood mononuclear cells from SCD patients were derived from their transfusion exchange waste bags, we restricted all subsequent analysis to comparing data from alloimmunized *versus* non-alloimmunized SCD patients in order to control for potential confounding effects related to blood collection and transfusion. No significant difference in the percentage of total cT_{FH} cells within CD4$^+$ T cells was detected between samples from non-alloimmunized (n=6) or alloimmunized patients currently expressing antibodies ("active"; n=5) or not ("non-active"; n=10) (Figure 7A). As seen in healthy donors, the majority of PD-1$^+$ cT_{FH} cells co-expressed TIGIT in this patient population, but no significant difference in the percentage of TIGIT$^+$ cT_{FH} subsets between alloimmunized or non-alloimmunized patients were detected (Figure 7B). Additionally, levels of TIGIT expression per cT_{FH} cell did not differ between patient groups or healthy donors (*data not shown*).

TIGIT-dependence of cT_{FH}-mediated allo-responses in patients with sickle cell disease

To further investigate TIGIT-dependent functions in samples from chronically transfused patients with SCD, we performed 7-day cT_{FH} subset/B-cell co-culture experiments using sorted PD-1$^+$/TIGIT$^+$, PD-1$^-$/TIGIT$^+$ or PD-1$^-$/TIGIT$^-$ cT_{FH} subsets from 16 SCD patients. For these analyses, alloimmunized patients without (n=6) or with (n=4) detectable alloantibodies were grouped as alloimmunized due to the limited number of patients studied. Relative to the PD-1$^-$/TIGIT$^-$ cT_{FH} subset, there was a trend toward higher levels of ICOS (Figure 7C) and CD40L (Figure 7D) on TIGIT$^+$ cT_{FH} cells co-cultured with autologous B cells from alloimmunized as compared to non-alloimmunized patients. Strikingly, and consistent with the observed co-stimulatory molecule expression pattern, TIGIT$^+$ cT_{FH} cells relative to the PD-1$^-$/TIGIT$^-$ cT_{FH} subset from alloimmunized patients also produced significantly more IL-21 (>2.5-

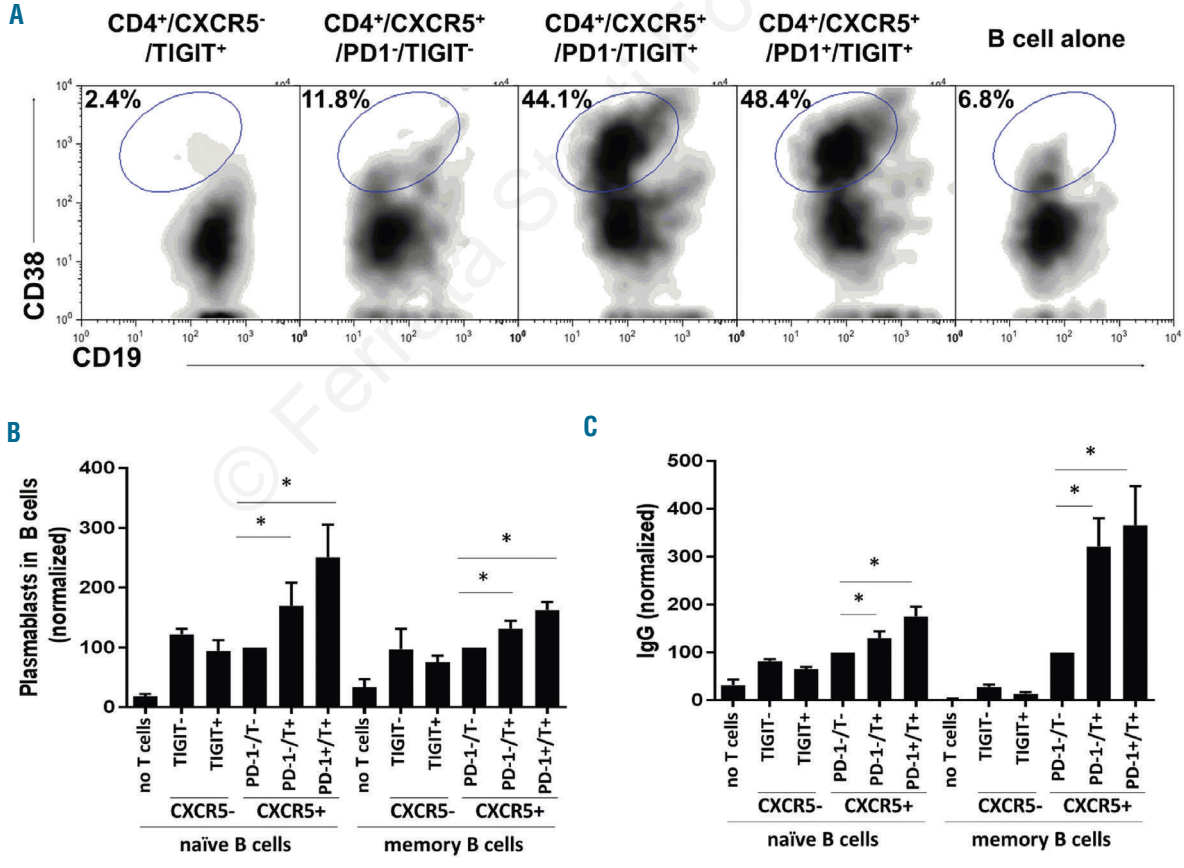

Figure 5. TIGIT$^+$ cT_{FH} cells drive B-cell differentiation. Indicated sort-purified subsets were co-cultured with sorted autologous naïve CD27$^-$/CD19$^+$ or memory CD27$^+$/CD19$^+$ B cells in the presence of 0.2 μM SEB for 7 days. (A) Plasmablast differentiation was detected in the CD4$^-$/CD19$^+$/CD38$^+$ gate as shown in this representative series of density plots from T-cell-memory B-cell co-cultures from a donor. (B). Percentages of plasmablasts within total CD19$^+$ B cells for each subset were normalized to that of PD-1$^-$/TIGIT$^-$ cT_{FH} cells. *P* values were obtained using an unpaired two-tailed Student t-test; n=6 (error bars, sem). (C) IgG levels in co-culture supernatants were measured at day 7 by enzyme linked immunosorbent assay and normalized to those of PD-1$^-$/TIGIT$^-$ cT_{FH} cells. *P* values were obtained using an unpaired two-tailed Student t-test; n=7 (error bars, sem). **P*≤0.05 (statistically significant).

fold increase) upon re-stimulation (Figure 7E) as compared to non-alloimmunized patients. As in healthy donors, IFNγ production by TIGIT$^+$ cT_{FH} cells, relative to PD-1$^-$/TIGIT$^-$ cT_{FH}, was also lower, but no significant difference was detected between the two groups of patients (Figure 7F).

B-cell differentiation into CD19^{lo}/CD38$^+$ plasmablasts was determined as for healthy donors at day 7. Poor plasmablast differentiation, particularly from naïve B cells, was observed for several patients. For patients whose memory B cells differentiated into plasmablasts, their global IgG production was determined by enzyme-linked immunosorbent assay. A trend toward higher IgG levels was detected

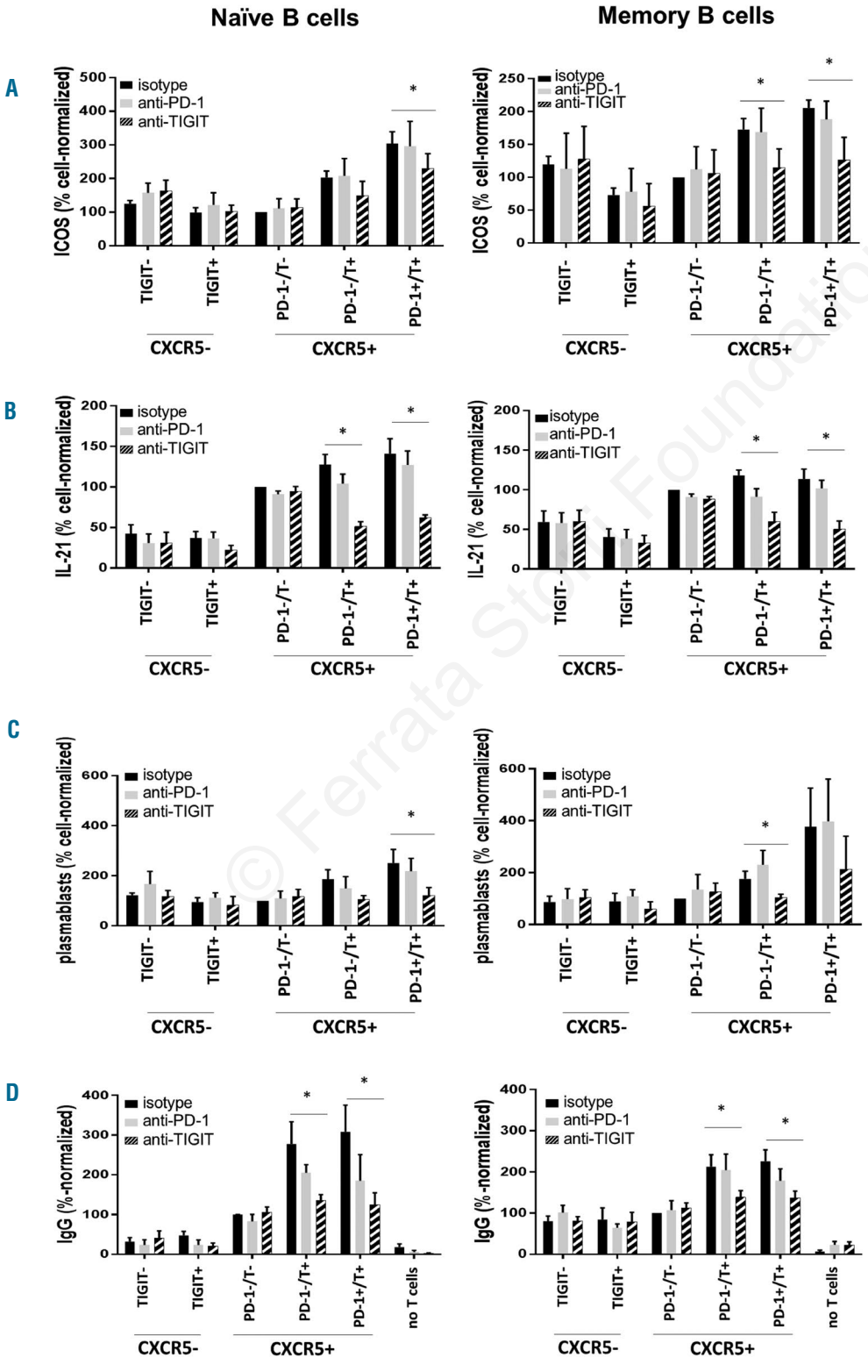

Figure 6. TIGIT functionally drives T_{FH} functions and B-cell differentiation. (A-E) Indicated sort-purified subsets from healthy donors were pre-incubated with anti-PD-1 or anti-TIGIT blocking antibodies or an isotype control and co-cultured with autologous naïve CD27$^-$/CD19$^+$ (left graph) or memory CD27$^+$/CD19$^+$ (right graph) B cells in the presence of 0.2 μM SEB for 7 days. Not all co-cultures were analyzed for every functional output. Results for (A) ICOS expression (n=12), (B) IL-21 expression upon phorbol myristate acetate/ionomycin stimulation (n=6), (C) plasmablast differentiation (n=6) as well as (D) IgG production (n=14) are represented after normalization to values obtained with PD-1$^-$/TIGIT$^-$ cT_{FH} cells co-cultured with isotype control. *P* values were obtained using an unpaired two-tailed Student t-test; (error bars, sem). *$P \leq 0.05$ (statistically significant).

in TIGIT$^+$ cT$_{FH}$ co-cultures from alloimmunized *versus* non-alloimmunized patients (Figure 7G), although significance was not reached, likely due to the limited numbers analyzed. Consistent with their red cell alloimmunization state, red blood cell-specific IgG was detected in the B-cell-TIGIT$^+$ cT$_{FH}$ co-culture supernatants in four of ten alloimmunized patients [adjusted mean fluorescence intensity (MFI): 7, 14, 122, 193] and in one of six of non-alloimmunized patients, although the latter was only weakly reactive (adjusted MFI: 5, *Online Supplementary Figure S5*).

Altogether these results suggest that TIGIT$^+$ cT$_{FH}$ cells from chronically transfused alloimmunized patients with

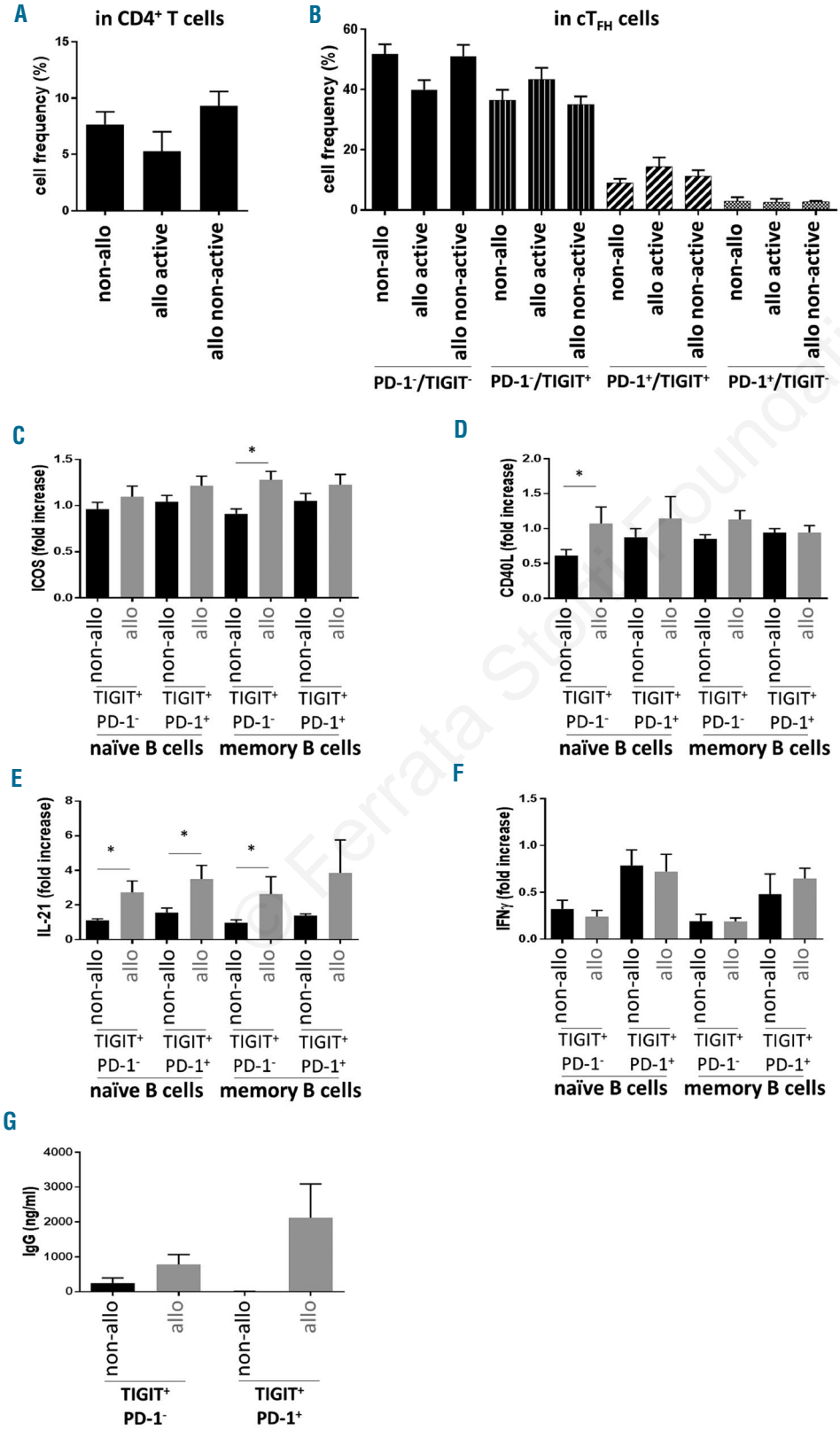

Figure 7. Role of TIGIT$^+$ cT$_{FH}$ cells in alloimmunization. Frequency of (A) CD4$^+$/CD45RA$^-$/CXCR5$^+$ cT$_{FH}$ cell subsets (PD-1$^-$/TIGIT$^-$, PD-1$^-$/TIGIT$^+$ and PD1$^+$/TIGIT$^+$ and PD-1$^+$/TIGIT$^-$ cells) within CD4$^+$ T cells and (B) within CXCR5$^+$ cT$_{FH}$ cells for groups of SCD patients. (C-F) Sort-purified PD-1$^-$/TIGIT$^-$, PD-1$^-$/TIGIT$^+$ and PD-1$^+$/TIGIT$^+$ cT$_{FH}$ cell subsets from SCD patients (n=16) were co-cultured with sorted autologous naïve CD27$^-$/CD19$^+$ or memory CD27$^+$/CD19$^+$ B cells in the presence of 0.2 μM SEB. At day 7, (C) ICOS, (D) CD40L, (E) IL-21 and (F) IFNγ production upon phorbol myristate acetate/ionomycin stimulation was determined. Results are shown for both alloimmunized (gray bars, n=10) and non-alloimmunized (black bars, n=6) patients and are represented as a ratio determined from PD-1$^-$/TIGIT$^-$ cT$_{FH}$ cells. (G) IgG production was measured by enzyme-linked immunosorbent assay in co-cultures of TIGIT$^+$ cT$_{FH}$ cell subsets and memory B cells which exhibited plasmablast differentiation (10 alloimmunized and 6 non-alloimmunized). *P* values were obtained using a Mann-Whitney test (error bars, sem). **P*≤0.05 (statistically significant).

SCD are more potent in providing B-cell help than those from non-alloimmunized patients.

Discussion

The present study identifies TIGIT-expressing circulating T_{FH} cells as a novel subset displaying heightened B-cell help functions. Specifically, we found that TIGIT$^+$ cT_{FH} cells expressed higher levels of ICOS and CD40L co-stimulatory molecules associated with B-cell help, as compared to TIGIT$^-$ cT_{FH} cells at steady state as well as following activation (Figure 2A-F and *Online Supplementary Figure S3*). Upon stimulation, they also secrete substantially more IL-21, and to some extent IL-4, than TIGIT$^-$ cT_{FH} cells (Figures 2G and 4A,C). Both cytokines, especially IL-21, play major roles in promoting B-cell activation/differentiation and antibody production. Accordingly, TIGIT$^+$ cT_{FH} cells induced enhanced differentiation into plasmablasts from both naïve and memory B cells relative to the TIGIT$^-$ cT_{FH} subset (Figure 5A,B and *Online Supplementary Figure S4*). B-cells co-cultured with TIGIT$^+$ cT_{FH} cells also produced significantly higher levels of IgG than when co-cultured with other T-cell subsets (Figure 5C and *Online Supplementary Figure S4*), further emphasizing the key role of TIGIT$^+$ cT_{FH} cells in supporting B-cell responses and antibody production. Of note, the higher levels of IgG produced by memory as compared to naïve B cells in TIGIT$^+$ cT_{FH} cell co-cultures may reflect differences in interactions between TIGIT$^+$ cT_{FH} cells and naïve *versus* memory B cells. Indeed, TIGIT binds its high affinity receptor CD155 on target cells.[36,41] CD155 is expressed by both memory and naïve B cells, albeit at slightly higher levels on memory B cells (*Online Supplementary Figure S6*). This may explain the differential IgG production of the B-cell subsets upon co-culture with identical TIGIT-expressing cT_{FH} cells, but needs further investigation.

Anti-TIGIT blocking experiments indicated a functional role for TIGIT on cT_{FH} cells. Indeed, TIGIT blockade in co-cultures, using naïve or memory B cells, decreased TIGIT$^+$ cT_{FH} cells' ability to up-regulate ICOS (Figure 6A) and to produce IL-21 (Figure 6B) and also prevented B cells from secreting IgG (Figure 6D). It remains to be determined whether TIGIT signaling improves cT_{FH} cells' ability to stimulate B cells (e.g. through IL-21 secretion) or whether TIGIT co-stimulation to B cells directly induces B-cell maturation/differentiation.

CXCR5$^+$ cT_{FH} subsets exhibiting distinct functions have been characterized, including three subsets based on co-expression of type 1, 2 or 17 features and differential expression of CXCR3 and/or CCR6.[7,12] None of the cT_{FH} subsets defined based on TIGIT (and PD-1) expression entirely falls into one of the type 1, 2 or 17 cT_{FH} subsets or *vice versa* (Figure 3), demonstrating that TIGIT$^+$ cT_{FH} subsets are distinct from these three previously described subsets. Surprisingly, PD-1$^+$/TIGIT$^+$ cT_{FH} cells contained around 50% of CXCR3$^+$/CCR6$^-$ type 1 T_{FH} cells, formerly reported to secrete IFNγ.[12] In our assays, and consistent with data obtained in non-T_{FH} cells,[42-45] TIGIT$^+$/PD-1$^+$ cT_{FH} cells produced very low amounts of IFNγ, reinforcing the idea that these subsets are substantially different. Nonetheless, whether TIGIT/PD1 cT_{FH} subsets can differentially modulate isotype switching as described for type 1, 2 and 17-like cT_{FH} cells, remains unknown. Taken together, these results show that TIGIT$^+$ cT_{FH} cells represent a phenotypically and potentially functionally distinct subset from those described previously, although a direct comparison of the activities of TIGIT$^+$ cT_{FH} and these three cT_{FH} subsets or other previously-described T_{FH} subsets[7,12,15-20] remains to be performed.

In our study, PD-1$^+$ cells represented about 10% of cT_{FH} cells; others have found this population to be about 30% of cT_{FH} in humans.[16,17] This discrepancy could be due to different anti-PD-1 clones used for detection and isolation of the PD-1$^+$ cell population [MIH4 (us) *versus* eBioJ105 (others)]. PD-1$^+$ human cT_{FH} cells were previously shown to drive heightened B-cell help responses,[16,17] although PD-1 expression on T_{FH} cells has also been associated with inhibition of B-cell responses.[18,32-34] Remarkably, we found no difference in B-cell help function between TIGIT$^+$ cT_{FH} cells expressing PD-1 or not (Figures 2, 4, and 5 and *Online Supplementary Figure S2*), and virtually all PD-1$^+$ cT_{FH} cells co-expressed TIGIT (Figure 1). Furthermore, unlike other experimental systems,[18,32-34] no notable effects of PD-1 blockade on T_{FH} cells were detected in our system using purified cell populations. Together, these results raise the possibility that PD-1 expression on human cT_{FH} cells might represent a non-functional biomarker and that the function of these cells might be due to their concomitant TIGIT expression. This hypothesis is also compatible with the results obtained with blocking antibodies: while TIGIT blockade affected B-cell help, PD-1 blockade had no specific effect (Figure 6). To date, TIGIT has mainly been studied for its role in inhibiting type 1 responses, e.g. IFNγ and IL-2 production or cytotoxicity.[37,41,46-48] Our data indicate that TIGIT also drives T_{FH} responses. This raises the possibility that other immune "checkpoints", especially those over-expressed by T_{FH} cells such as BTLA[49] may also strengthen T_{FH} responses. While representing a provocative hypothesis, this could reflect an evolutionary mechanism by which TIGIT and potentially other immune checkpoints are not only involved in reducing chronic inflammation, but also in switching lymphocyte responses from cellular toward potentially more adaptive humoral responses.

Our study of chronically transfused SCD patients suggests that alloimmunized patients have TIGIT$^+$ cT_{FH} cells with more robust B-cell help functions than non-alloimmunized patients (Figure 7C-G, *Online Supplementary Figure S5*). We should note that patients' samples were from apheresis waste bags whereas leukocyte-enriched preparations, rather than whole blood, were used for the healthy donor studies. Direct comparison of the TIGIT$^+$ cT_{FH} compartment between SCD patients and healthy donors was not, therefore, possible; such studies would require whole blood sampling, a challenging undertaking given the anemic state of the patients being studied. Instead, we compared the functional characteristics of TIGIT$^+$ cT_{FH} populations in alloimmunized *versus* non-alloimmunized SCD patients. Since all the samples from patients with SCD were taken from teenagers or young adults (15-30 years of age) who had been on a chronic red cell exchange program for 2 or more years and had, therefore, been heavily exposed to allogeneic transfusions, the comparison of alloimmunized *versus* non-alloimmunized patients' cells is especially interesting. Relative to TIGIT$^-$ cT_{FH} cells, IL-21 and CD40L expression by TIGIT$^+$ cT_{FH} cells from non-alloimmunized patients was lower (whereas it was higher on TIGIT$^+$ cT_{FH} cells from alloimmunized patients). Defective TIGIT signaling in non-alloimmunized patients and/or an exacerbated TIGIT-mediated responses in

alloimmunized patients may be responsible for such functional differences and needs to be further investigated. Additionally, the presence of regulatory Foxp3$^+$/CXCR5$^+$ follicular T_{FR}[26-30] may also participate in modulating cT_{FH} functions. Our preliminary data suggest that alloimmunized patients with currently detectable alloantibodies have lower frequencies of circulating T_{FR} than alloimmunized patients whose alloantibodies were undetectable at the time of drawing blood or healthy donors (manuscript in preparation). Unraveling mechanisms responsible for different functional activities of TIGIT$^+$ cT_{FH} cells between groups of SCD patients without or with alloantibodies would represent a key step in identifying predictive biomarkers as well as developing therapeutic strategies to limit SCD alloimmunization.

In conclusion, we have identified TIGIT as a novel marker of cT_{FH} with ability to drive potent B-cell help responses. These findings have the potential to influence therapeutic strategies, not only regarding alloimmunization, but also in vaccination as well as in autoimmunity and immune deficiencies in which the role of T_{FH} cells, particularly cT_{FH} cells, is being increasingly appreciated.[21]

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Authorship and Disclosures

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