SUPPLEMENTARY APPENDIX

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

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SUPPLEMENTAL FIGURES

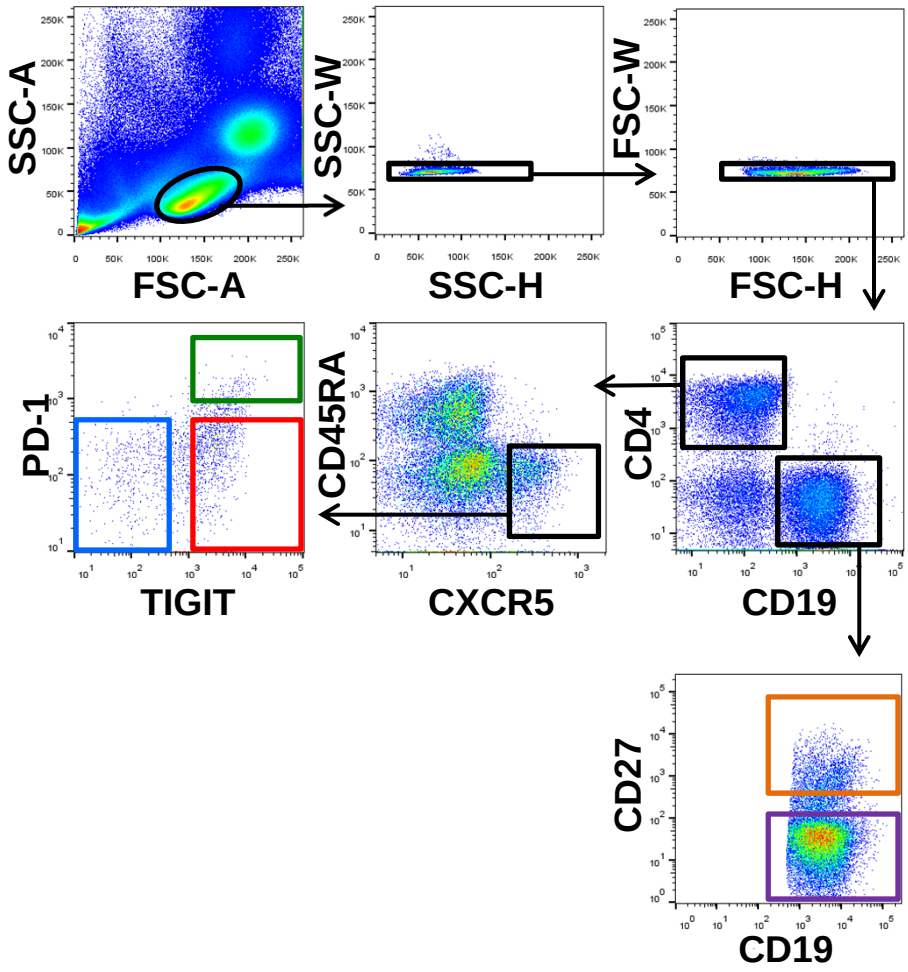

Fig.S1. Gating strategy to sort cT_{FH} subsets as well as naïve and memory B cells.
For cell sorting analysis target population was gated and cellular aggregates were excluded based on signal processing of side and forward scatter. cT_{FH} subsets, naïve and memory B cells were identified by sequential gating using the antibody selection as described under Methods and Results. Data were acquired and cells collected on a MoFlo XDP High-Speed Cell Sorter (Beckman Coulter, Miami, FL) using Summit Software (Beckman Coulter, Miami, FL).

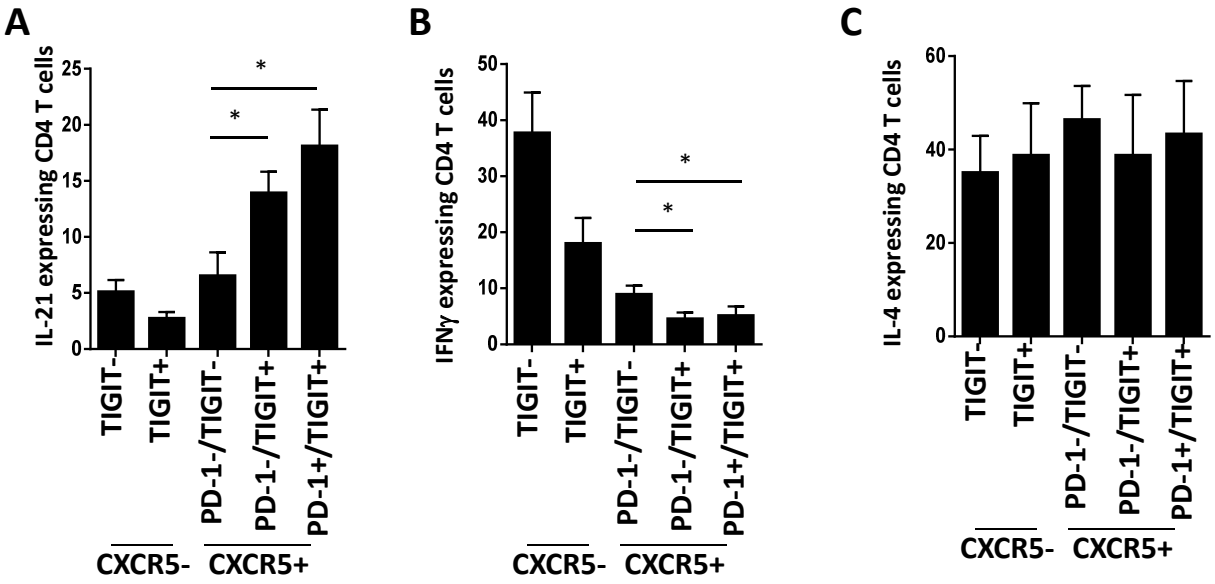

Figure S2. TIGIT$^+$ cT$_{FH}$ cells T$_{FH}$-associated cytokines. Fresh CD4$^+$ T cell subsets were sorted from the 4 healthy donors (analyzed in Fig.2G), rested overnight and stimulated for 5h with PMA/ionomycin in the presence of BFA before being intracellularly stained for IL-21 (**A**), IFNγ (**B**) and IL-4 (**C**). Results show percentages of CD4$^+$ T cells producing cytokines. p values were obtained using a paired two-tailed Student's t-test; (error bars, sem). *, p≤0.05.

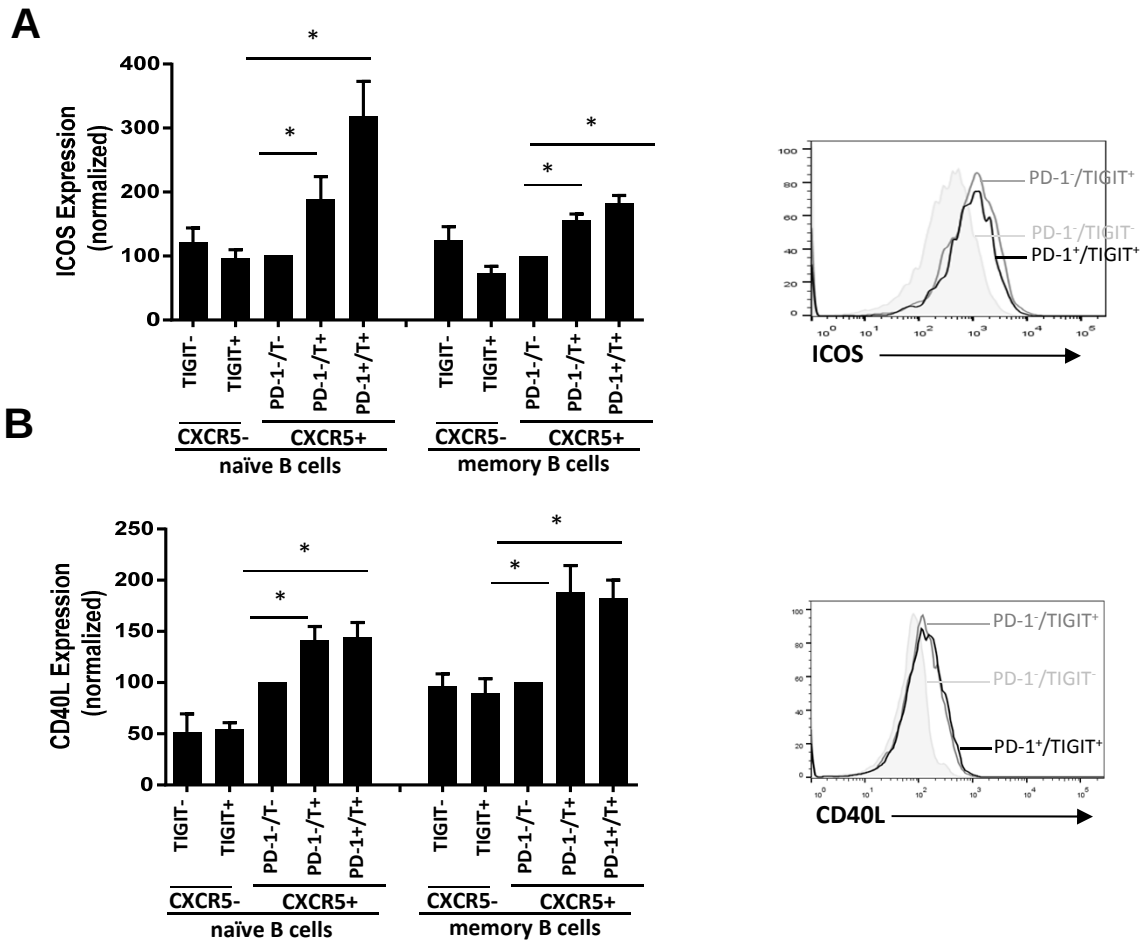

Fig.S3. TIGIT$^+$ cT$_{FH}$ cells over-express co-stimulation molecules. A,B. Indicated sort-purified CD4$^+$ subsets from healthy donors were co-cultured with autologous naïve CD27$^-$/CD19$^+$ or memory CD27$^+$/CD19$^+$ B cells in the presence of 0.2μM SEB for 7 days. Expression of ICOS (**A**) and CD40L (**B**) were normalized to that of PD-1$^-$/TIGIT$^-$ cT$_{FH}$ cells. Representative histogram plots showing ICOS and CD40L expression for PD-1$^-$/TIGIT$^-$, PD-1$^-$/TIGIT$^+$ and PD1$^+$/TIGIT$^+$ cT$_{FH}$ subsets are presented on the right. p values were obtained using a two-tailed Student's t-test; n=14-16 (error bars, sem). *, p≤0.05.

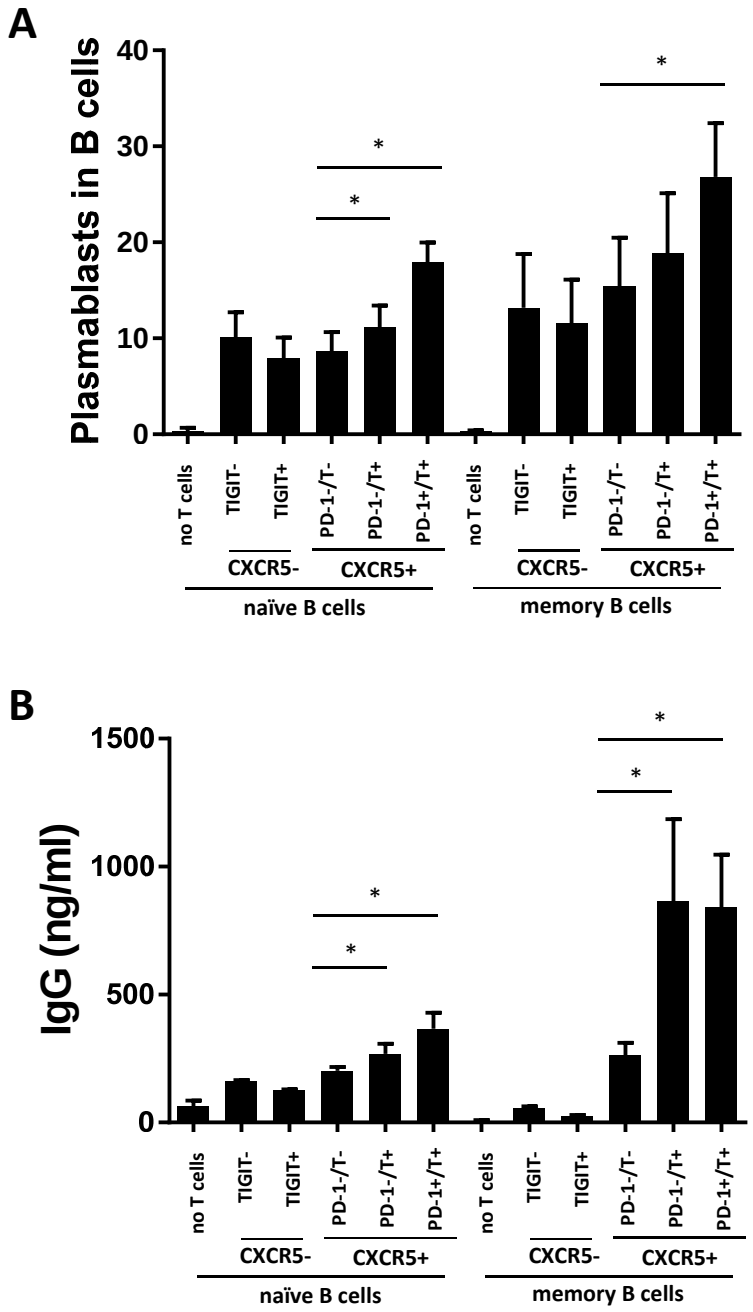

Fig.S4. TIGIT⁺ cT$_{FH}$ cells drive B cell differentiation. Indicated sort-purified subsets were co-cultured with sorted autologous naïve CD27⁻/CD19⁺ or memory CD27⁺/CD19⁺ B cells in the presence of 0.2μM SEB for 7 days. (**A**) Plasmablast differentiation was detected in the CD4⁻/CD19⁺/CD38⁺ gate. p values were obtained using an unpaired two-tailed Student's t-test; n=6 (error bars, sem). **C.** IgG levels in co-culture supernatants were measured at day 7 by ELISA. p values were obtained using an unpaired two-tailed Student's t-test; n=7 (error bars, sem). *, p≤0.05.

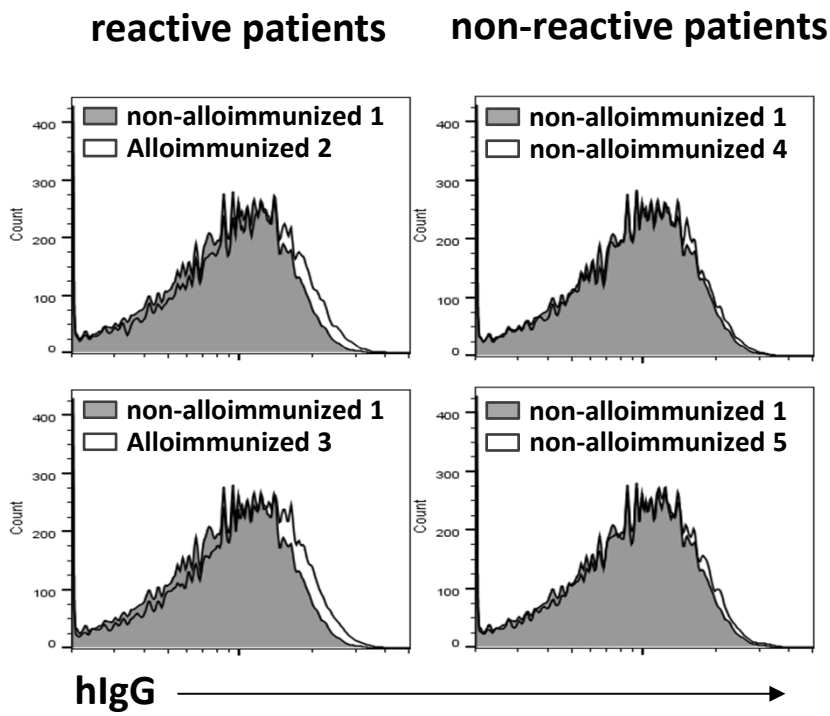

Fig.S5. Detection of RBC-specific IgG in alloimmunized SCD patients. Supernatants from day 7 TIGIT$^+$ cT_{FH}-memory B cell cocultures were incubated with reagent RBCs used for clinical serologic studies. RBC-specific IgG was detected using fluorescently labeled anti-human IgG (hIgG) by flow cytometry. After adjusting for background reactivity with supernatants from B cells alone, 1/6 supernatants from non-alloimmunized patients had detectable RBC-specific signal as compared to 4/10 from alloimmunized patients. The adjusted MFI in samples with detectable RBC-specific signal was higher in the 4 alloimmunized patients as compared to the one non-alloimmunized. Histogram overlays showing a representative RBC reactivity of a non-alloimmunized patient 1 depicted in tinted gray for all plots versus either alloimmunized patients (left panel: patients 2 and 3) or non-alloimmunized patients (right panel: patients 4 and 5). As shown, a slight shift, indicating the presence of anti-RBC IgG, can be detected with supernatants from alloimmunized (patients 2 and 3), but not non-alloimmunized (patients 4 and 5).

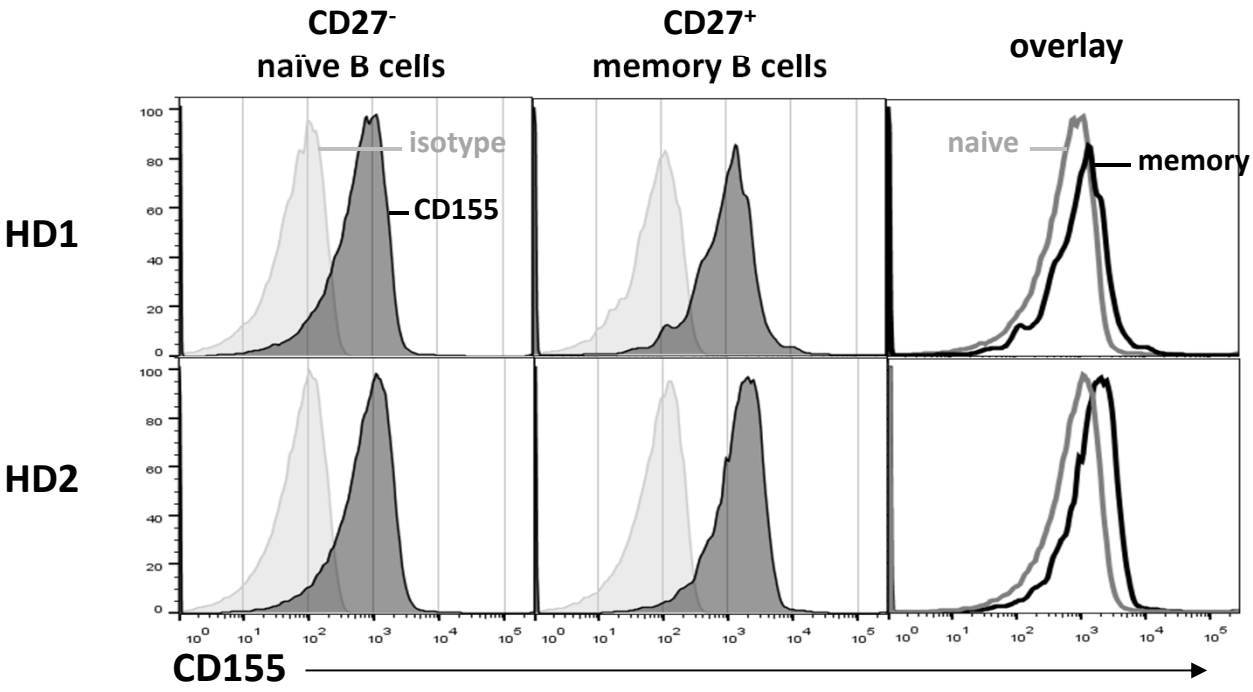

Fig.S6. Levels of CD155 expression on naïve and memory B cells. Naïve $CD27^-/CD19^+$ or memory $CD27^+/CD19^+$ B cells were assessed for CD155 expression by immunostaining. Two healthy donors (HDs) are shown. Overlay of naïve vs memory CD155 expression (far right panel) indicates higher levels of CD155 on memory B cells as compared to naïve B cells.

MATERIALS AND METHODS

Human samples.

All the studies were approved by the Institutional Review Boards of the New York Blood Center (NYBC). Fresh leukopaks containing leukocyte-enriched peripheral blood from healthy volunteer donors of the New York Blood Center were obtained without any identifiers. For patient samples, blood was obtained solely from discard apheresis waste bags from patients with SCD undergoing erythrocytapheresis procedures at Children's Hospital of Philadelphia on an outpatient basis. Waste bags were taken at the end of exchange procedures from 21 patients aged 15-30 on chronic transfusion therapy (every 3-4 weeks for at least two years). Institutional standards for donor blood selection included leukodepletion of red cells and phenotype matching for the C, E and K red cell antigens. Patients were grouped either as "non-alloimmunized" (n=6), having had no history of antibody production and those (n=15) with a history of having produced at least one alloantibody ("alloimmunized"). Of the 15 "alloimmunized" patients, 5 patients had detectable alloantibodies at the time of the blood draw. The apheresis waste bags stripped of all identifiers except the alloimmunization state were then sent to NYBC and analyzed within 18 hours of blood collection. PBMCs from either healthy donors or SCD patients were isolated by Ficoll-Paque Plus (GE Healthcare) density centrifugation and subjected for most experiments to cell sorting (purity>95%) using MoFlo XDP High Speed Cell Sorter (Beckman Coulter).

Antibodies.

The following antibodies (Abs) were used for surface staining: AF700-conjugated Ab to CD4 (clone RPA-T4), PE-conjugated Ab to CD155 (clone 2H7CD155) or to TIGIT (clone MBSA43) were from eBioscience. PECy7-conjugated Ab to CD19 (clone SJ25C1), APC-conjugated Abs to PD-1 (clone MIH4) or CD38 (clone HB7), PerCP-Cy5.5-conjugated Ab to ICOS (clone DX29), BV510-conjugated Ab to CXCR5 (clone RF8B2), BV421-conjugated Ab to CD27 (clone M-T271) or to CXCR3 (clone 1C6), BV786-conjugated Ab to CCR6 (clone 11A9) and FITC-conjugated Ab to CD45RA (clone L48) or to CD40L (clone TRAP1) were from BD Pharmingen. Abs used for intracellular staining of cytokines (IFNγ-APC, clone 4SB3 ; IL-4-PE, clone 8D4-8 ; IL-21-PE, clone eBio3A3-N2) were from eBioscience. Blocking Abs for TIGIT (eBioscience clone MBSA43; mIgG1, previously demonstrated to inhibit TIGIT triggering[1]) and PD-1 (BD Pharmingen clone EH12-1; mIgG1; reported to block interactions between PD-1 and its ligands[2;3]) were used at 10μg/ml.

Fluorescence Activated Cell Sorting and Analysis

For FACS analysis and cell sorting, target populations were gated and cellular aggregates were excluded based on signal processing of side and forward scatter. Subpopulations were identified by sequential gating using the antibody selection as listed above. FACS analysis was conducted on a LSR Fortessa SORP cell analyzer equipped with FACSDiva software (BD Bioscience, San Jose, CA). For sorting, data were acquired and cells collected on a MoFlo XDP High-Speed Cell Sorter using

Summit Software (Beckman Coulter, Miami, FL). Further data analysis was performed using FlowJo flow cytometry software (Treestar, Ashland, OR).

T cell stimulation.

Freshly-sorted $CD4^+$ T cell subsets (10 to $20x10^4$) were non-specifically stimulated using 25ng/mL phorbol myristate acetate (PMA; Sigma-Aldrich) and 0.4μg/mL ionomycin (Sigma-Aldrich) in the presence of 10μM Brefeldin A (BFA) for 5 hours. Cells were then surface stained, fixed and permeabilized using a Fixation/Permeabilization kit (eBioscience), before being intracellularly stained for indicated cytokines. For T/B co-cultures, freshly sorted $CD4^+$ T cell subsets (10 to $20x10^4$) and autologous naïve or memory B cells were incubated at a 1:1 ratio in RPMI 1640 supplemented with 2mM L-glutamine (Sigma), 100UI/mL streptomycin/100μg/mL penicillin (Sigma) and 10% fetal bovine serum (to avoid any contamination with human IgG) in the presence of 0.2μg/ml Staphylococcal enterotoxin B (SEB) superantigen to facilitate T/B interactions. At day 7, cells were harvested and the frequency of plasmablasts as well as intracellular expression of various cytokines in T cells measured. At the same time, supernatants from the co-cultures were collected and stored at -80ºC. After thawing, human total IgGs were measured in the supernatants using the Ready-Set-Go ELISA kit from eBioscience according to the manufacturer's instructions. When blocking Abs were used, T cells were pre-incubated with 10μg/ml Ab for 30min at room temperature before being co-cultured with autologous B cells and stimulated with SEB.

Detection of red blood cell (RBC)-specific IgG.

Supernatants from T-B cell cocultures of patients with SCD were incubated for 15min at 37ºC with pooled (allogeneic) reagent red blood cells (RBCs) used for clinical serological studies. After several washes, RBC-specific IgG were detected by incubating with a fluorescently-labeled anti-human IgG for 10min at 4ºC and measuring fluorescence on a LSR Fortessa cell analyzer (BD Biosciences). Specific antibody response was determined for every patient by subtracting the values from cultures with B cells alone from those with T-B cells.

Statistical Analysis.

Separate analyses were performed for each experiment individually. Data are expressed as mean values ± sem. Two-sided statistical tests were performed for all comparisons. Details for each analysis are provided in the figure legends. p values ≤0.05 were considered statistically significant. Details for each analysis are provided in the figure legends.

References

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