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Germinal Center B Cells are Uniquely Targeted by Antibody-Suppressor CXCR5+CD8+ T Cells

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Background. Alloprimed antibody-suppressor CXCR5+CD8+ T cells (CD8+ $T_{Ab\text{-}supp}$ cells) downregulate alloantibody production, mediate cytotoxicity of IgG+ B cells, and prolong allograft survival. The purpose of this investigation was to determine which immune-cell subsets are susceptible to CD8+ $T_{Ab\text{-}supp}$ cell–mediated cytotoxicity or noncytotoxic suppression. **Methods.** Alloprimed immune-cell subsets were evaluated for susceptibility to CD8+ $T_{Ab\text{-}supp}$ cell–mediated in vitro cytotoxicity and/or suppression of intracellular cytokine expression. In vivo CD8-mediated cytotoxicity to wild-type germinal center (GC) B cells or wild-type CD4+ T follicular helper cells (T_{FH} cells) was assessed in RAG1 knockout mice. The impact of in vivo adoptive transfer of CD8+ $T_{Ab\text{-}supp}$ cells into hepatocyte or kidney transplant recipients on the quantity of lymphoid immune-cell subsets was assessed. **Results.** CD8+ $T_{Ab\text{-}supp}$ cells mediated allospecific cytotoxicity to alloprimed GC B cells but not alloprimed extrafollicular plasmablasts, marginal zone B cells, follicular B cells, or plasma cells. CD8+ $T_{Ab\text{-}supp}$ cells did not mediate cytotoxicity to alloprimed dendritic cells, macrophages, CD4+ T_{FH} cells, CD4+ T follicular regulatory cells, or CD4+ regulatory T cell. CD8+ $T_{Ab\text{-}supp}$ cells did not suppress CD4+ T_{FH} cell, T follicular regulatory cell, or regulatory T-cell cytokine expression. Adoptive transfer of CD8+ $T_{Ab\text{-}supp}$ cells into hepatocyte or kidney transplant recipients reduced alloantibody production and the quantity of GC B cells, T_{FH} cells, and plasma cells (but not other B-cell, T-cell, or antigen-presenting cell subsets). The reduction of T_{FH}-cell quantity was dependent on CD8+ $T_{Ab\text{-}supp}$ cell–mediated major histocompatibility c omplex-I-dependent cytotoxic killing of GC B cells. **Conclusions.** The primary targets of CD8+ $T_{Ab\text{-}supp}$ cells are GC B cells with downstream reduction of T_{FH} and plasma cells.

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Transplantation is the definitive treatment for patients with end-stage kidney, heart, lung, or liver failure. Advances in medical and surgical care during the past 2 decades have gradually improved transplant outcomes with increased graft and patient survival.[1] Conventional immunosuppressive agents used to prevent transplant rejection by targeting T cells, including CD4+ T cells that provide "help" for antibody production by B cells,[2,3] have successfully reduced the incidence of acute rejection. However, this reduction in early acute cellular rejection has not been accompanied by a commensurate reduction in de novo donor-specific antibodies, and antibody-mediated rejection (AMR) remains a significant challenge for both short- and long-term allograft function.[4-9] Donor-specific humoral alloimmunity develops in a substantial proportion of transplant recipients regardless of transplant organ[9-21] and despite therapeutic immunosuppressive drug levels.[22] For example, donor-specific antibodies (DSAs) develop in 13%–27% of kidney transplant recipients within

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10 y posttransplant.[7,23-26] Approximately 50% of kidney transplant recipients lose their grafts within 10 y after posttransplant, and half of these allograft losses are due to AMR.[5,7,9,27-30] These data suggest that immunotherapeutic agents developed to suppress acute cell-mediated rejection are not as effective for suppression of humoral alloimmunity. Current therapeutic approaches for the treatment of acute AMR are limited by lack of specificity, impairment of protective immunity, association with significant side effects, and even paradoxical increase in acute cellular rejection.[31-33] Enhanced understanding of the mechanisms regulating humoral immunity is needed to develop novel immunotherapeutic approaches.

We have previously reported that transplant recipient mice lacking CD8+ T cells develop significantly higher quantities of alloantibody after transplant compared with wild-type (WT) recipients, providing the first evidence of CD8-mediated regulation of alloantibody production.[34] Since this initial observation, our group has reported that a novel subset of cytotoxic CD8+ T cells, termed antibody-suppressor CD8+ T cells (CD8+ $T_{Ab\text{-}supp}$ cells), downregulates humoral immune responses.[30,35-37] These cytotoxic CD8+ $T_{Ab\text{-}supp}$ cells express the chemokine receptor CXCR5 are allospecific, mediate major histocompatibility complex (MHC) class I-dependent in vivo and in vitro cytotoxic killing of target IgG+ B cells, and suppress in vivo alloantibody production by FasL- and perforin-dependent killing of alloantibody-producing IgG+ B cells.[30,35-38] Adoptive cell therapy with alloprimed CXCR5+CD8+ T cells into high alloantibody-producing transplant recipients results in the reduction of alloantibody titer and significant enhancement of allograft survival.[30,35-37]

CXCR5 is a chemokine receptor that guides CD4+ T cells and B cells to germinal centers (GCs).[39] GCs are defined areas within lymphatic tissue (ie, lymph nodes, spleen), where antigen-primed B cells develop into very early memory B cells or upregulate Bcl-6 transcription factor and undergo cell division, somatic hypermutation, affinity maturation, and class switch recombination eventually exiting the GC and giving rise to antibody-producing plasma cells.[39-41] GCs contain a diverse repertoire of lymphocytes, including GC B cells, CD4+ T follicular helper (T_{FH}) cells, CD4+ T follicular regulatory (T_{FR}) cells, and follicular dendritic cells (FDCs). Furthermore, antibody suppression by CD8+ $T_{Ab\text{-}supp}$ cells requires expression of CXCR5,[30] implicating GCs as the site of effector function. We previously reported that alloprimed CD8+ $T_{Ab\text{-}supp}$ cells mediate in vitro and in vivo cytotoxic killing of alloantibody-producing IgG+ B cells.[30,35-37] However, the breadth of immune-cell populations affecting humoral immune responses vulnerable to CD8+ $T_{Ab\text{-}supp}$ cytotoxicity and immunoregulation is not known. In the current study, we investigated the susceptibility of B-cell populations (marginal zone [MZ] B cells, follicular [FO] B cells, extrafollicular plasmablasts, GC B cells, plasma cells), CD4+ T follicular-cell populations (CD4+ T_{FH} cells and CD4+ T_{FR} cells), and dendritic cell (DC) populations (including FDCs) to CD8+ $T_{Ab\text{-}supp}$ cell–mediated cytotoxicity and/or immunosuppression in the highly immunogenic murine hepatocyte transplantation model[42-44] and vascularized solid organ murine kidney transplant model.[35-37,45]

MATERIALS AND METHODS

Experimental Animals

Male and female mice, 6–12 wk of age, from C57BL/6 (WT), CCR5 knockout (KO), μMT KO, β2M KO, RAG1 KO, green fluorescent protein (GFP) transgenic, CD8 KO (all H-2^b), A/J (H-2^a; Jackson Laboratories), and transgenic human alpha-1-antitrypsin FVB/N (H-2^q MHC haplotype; derived as previously described[46]) were used in these studies. All experiments were performed in compliance with the guidelines of The Institutional Animal Care and Use Committee of The Ohio State University (Protocol 2019A00000124).

Allolysate Preparation

Allolysate was prepared from murine livers as a source of alloantigen, as previously published.[30,47] See **Supplemental Materials and Methods** (**SDC,** http://links.lww.com/TXD/A725) for additional information.

T-cell Subset and Antigen-presenting Cell Isolation

CD8+ T cells were pooled and isolated from the spleens of euthanized naive or primed WT hosts. Alloprimed CD8-depleted WT (200 μg anti-CD8 monoclonal antibody, clone 53.6.7; BioXCell, Lebanon, NH) or CD8 KO mice are high alloantibody producers[34] and served as a source of pooled CD4+ T cells, B cells, macrophages, and DCs to evaluate immune-cell susceptibility to CD8-mediated regulation and for enhanced yield of alloprimed immune-cell subsets. T-cell and B-cell subsets were further purified by flow sorting. See **Supplemental Materials and Methods** (**SDC,** http://links.lww.com/TXD/A725) for additional information.

In Vitro Cytotoxicity Assay

Cytotoxicity was measured using a LIVE/DEAD cell-mediated cytotoxicity kit (Invitrogen, Eugene, OR) or Annexin V and 7AA-D (ThermoFisher, Waltham, MA) and performed according to the manufacturer's instructions. Briefly, CD8+ T-cell populations were co-cultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained (0.2 μM) target-cell populations (B-cell subsets, CD4+ T-cell subsets, macrophages, or DCs) at a 10:1 ratio for 4 h. Propidium iodide (PI) was added to the cultures before the 4-h incubation to analyze cell death. PI uptake by CFSE+ cellular targets was immediately analyzed by flow cytometry. Using an alternative cell death assay, the samples were stained with Annexin V and 7AA-D after the co-culture and CFSE+ target cells were immediately analyzed by flow cytometry.

In Vivo Cytotoxicity

Detection of CD8+ T cell–mediated in vivo cytotoxic clearance of alloprimed CD4+ T_{FH} cells or GC B cells was performed using a modification of previously described methods.[38,48] Briefly, RAG1 KO mice were AT with CellTrace Violet (CTV)-labeled naive control target cells (naive CD4+ T cells or B220+ B cells) or GFP+ alloprimed experimental target cells (CD4+ T_{FH} cells or GC B cells, respectively) pooled from multiple alloprimed mice. Experimental groups of mice were also AT with alloprimed CTV-labeled GFP+CD8+ T cytotoxic effector cells. Target cells were retrieved after 18 h, and percent cytotoxicity toward alloprimed target cells was calculated, as previously described.[48] See **Supplemental Materials and Methods** (**SDC,** http://links.lww.com/TXD/A725) for additional information.

Hepatocyte Isolation, Purification, and Transplantation

Hepatocyte isolation and purification were performed, as previously described.[46] See **Supplemental Materials and**

Methods (**SDC,** http://links.lww.com/TXD/A725) for additional information.

Kidney Isolation and Transplantation

Murine kidney transplantation with ureteral reconstruction was performed, as previously described.[35,49] See **Supplemental Materials and Methods** (**SDC,** http://links.lww.com/TXD/A725) for additional information.

Flow Cytometric Analysis of GC B Cells, CD4+ T Cells, and APCs

Splenocytes and bone marrow cells were isolated from FVB/N hepatocyte and A/J kidney transplant recipients on day 14 and immunostained with monoclonal antibodies to identify B-cell subsets, $CD4^+$ T-cell subsets, macrophages, and DC subsets (see **Supplemental Materials and Methods, SDC,** http://links.lww.com/TXD/A725 for additional details). B-cell subsets were identified as $Fas^+GL\text{-}7^+B220^+$ (GC B cells),[50,51] $IgG^+CD138^+B220^+$ (extrafollicular plasmablasts),[52] bone marrow $IgG^-CD138^+B220^-$ (plasma cells),[53] $CD21^+CD23^-IgM^+B220^+$ (MZ B cells),[54] or $CD23^+IgD^+B220^+$ (FO B cells).[55] $CD4^+$ T-cell subsets were identified as $CXCR5^+PD1^+CD4^+$ ($CD4^+$ T_{FH}),[56-58] $CD25^+CXCR5^+PD1^+FoxP3^+CD4^+$ ($CD4^+$ T_{FR}),[59] or $CD25^{high}FoxP3^+CD4^+$ ($CD4^+$ regulatory T cell [T_{REG}]).[60] T cells were stimulated for 4 h with Leukocyte Activation Cocktail (phorbol 12-myristate-13-acetate, ionomycin, and brefeldin A; Becton Dickinson). Macrophages were identified as $F4/80^+$ cells.[61] DC subsets were identified as $CD8^+Ly6c^-CD11c^+MHCII^+$ (cDC1), $CD4^+CD8^-CD11b^+CD11c^+MHCII^+$ (cDC2),[62] $CD157^+CD11c^+$ (FDC),[63] $CD11b^{high}CD11c^+MHCII^-$ (lymphoid DC),[64] and $CD8^+Lyc6^+CD11c^+MHCII^+$ (plasmacytoid DC).[65,66] Fluorescence-minus-one was used as a negative control to set the positive/negative boundaries for marker expression.[67] Samples were acquired on a Becton Dickinson LSRFortessa cytometer, and data were analyzed using FlowJo. Flow cytometric analysis was performed by gating on single-cell, lymphocyte populations of B cells and $CD4^+$ T cells.

Donor-reactive Alloantibody Titer

Alloantibody titer from recipient sera was quantitated using published methods.[45] Briefly, serum was serially diluted and incubated with allogeneic target splenocytes. Splenocytes were then stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG Fc (Organon Teknika, Durham, NC). The mean fluorescence intensity (MFI) was measured for each sample, and the dilution that returned the MFI observed when splenocytes were incubated with a 1:4 dilution of naive serum was divided by 2 and recorded as the titer.

Statistical Analysis

Paired T tests were used to analyze comparisons between in vitro cytotoxicity results for target and effector cell co-cultures. Hypothesis testing was conducted with a 5% type I error rate, and a P value of <0.05 was considered statistically significant. All analyses were conducted using SAS version 9.4 (SAS Institute, Cary, NC). Summary statistics are listed as the mean ± SE. Detailed statistical analysis results are included in the internal statistical report.

RESULTS

Alloprimed CXCR5+CD8+ T Cells Mediate Allospecific In Vitro Killing of GC B Cells (But Not Other B-cell Subsets)

We have previously reported that $CD8^+$ $T_{Ab\text{-}supp}$ cells mediate both in vitro and in vivo cytotoxicity toward IgG^+ B cells.[30,35-37] To determine which B-cell subsets are susceptible to $CXCR5^+CD8^+$ T-cell cytotoxicity, we tested MZ B cells, FO B cells, extrafollicular plasmablasts, GC B cells, and mature plasma cells retrieved from alloprimed mice in in vitro cytotoxicity assays.

WT mice and CD8-depleted WT mice were stimulated with FVB/N allolysate (2 mg protein; ip injection). CD8-depleted WT mice produce high-titer alloantibody, and the yield of alloprimed immune-cell subsets is high.[30,34] Alloprimed $CXCR5^+CD8^+$ T cells (from WT recipients) and alloprimed B-cell subsets (from CD8-depleted recipients) were isolated from splenocytes 7 d after alloantigen priming (see **Figure S1, SDC,** http://links.lww.com/TXD/A725 for flow sorting results for alloprimed splenic GC B cells, extrafollicular plasmablasts, MZ B cells, FO B cells, and bone marrow plasma cells). Flow-sorted alloprimed $CXCR5^+CD8^+$ T cells were co-cultured with each flow-sorted B-cell subset. We found that $CXCR5^+CD8^+$ T cells mediated in vitro cytotoxicity toward alloprimed GC B cells (Annexin V^+/7AA-D^+; $P < 0.0001$; Figure 1) but not alloprimed extrafollicular plasmablasts, plasma cells, MZ B cells, or FO B cells (P = NS for all). Third party–primed ($H\text{-}2^a$ primed) $CXCR5^+CD8^+$ T cells, alloprimed $CXCR5^-CD8^+$ T cells, or naive $CD8^+$ T cells did not mediate cytotoxicity to alloprimed GC B cells or any other B-cell subset (Figure 1).

Similar results are found with using B-cell targets from CD8 KO mice and PI to assess cytotoxicity (not shown). These results indicate that alloprimed $CD8^+$ $T_{Ab\text{-}supp}$ cells target GC B cells but not other B-cell subsets. To corroborate these in vitro results with in vivo studies, we analyzed the quantity of B-cell subsets in transplant recipients after adoptive cell transfer of alloprimed $CXCR5^+CD8^+$ T cells.

CD8 KO mice were transplanted with allogeneic FVB/N hepatocytes, and CCR5 KO mice were transplanted with an allogeneic A/J kidney. Groups of recipient mice were adoptively transferred (AT) with alloprimed $CXCR5^+CD8^+$ T cells on day 5 posttransplant. On day 14 posttransplant, we analyzed the quantity of MZ B cells, FO B cells, extrafollicular plasmablasts, GC B cells, and plasma cells after AT with $CXCR5^+CD8^+$ T cells compared with no AT. We found that the quantity of GC B cells was significantly reduced (2-fold) after AT of $CXCR5^+CD8^+$ T cells compared with untreated control recipients in both transplant models ($P < 0.02$; Figure 2A). Plasma cells were also reduced by 3-fold in recipients who received AT of alloprimed $CXCR5^+CD8^+$ T cells ($P < 0.001$ for both transplant models). However, AT of alloprimed $CXCR5^+CD8^+$ T cells did not reduce the quantity of MZ B cells, FO B cells, or extrafollicular plasmablasts (Figure 2B; P = NS for all). Representative flow plots are shown in Figure 2C and **Figure S2** (**SDC,** http://links.lww.com/TXD/A725). The frequencies of splenic GC B cells as a percentage of splenic lymphocytes and plasma cells as a percentage of bone marrow lymphocytes are also reduced after AT of $CXCR5^+CD8^+$ T cells compared with untreated control recipients in both transplant models (Figure 2D). As in previous studies, AT with alloprimed $CXCR5^+CD8^+$ T cells significantly reduced alloantibody titer after transplant in both

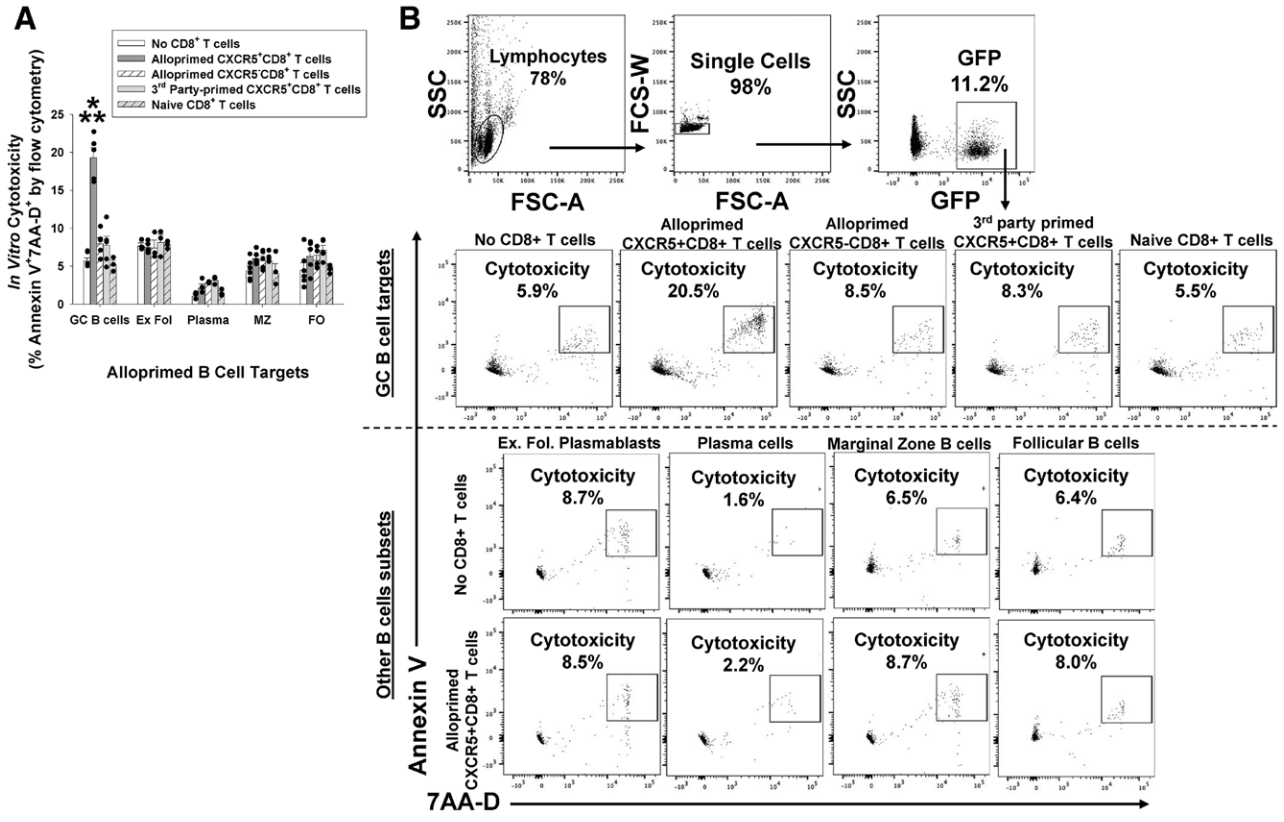

FIGURE 1. Alloprimed CXCR5+CD8+ T cells mediate in vitro cytotoxic killing of GC B cells, but not extrafollicular plasmablasts, plasma cells, MZ, or FO B cells. C57BL/6 (WT) and CD8-depleted GFP Tg (H-2^b) mice were stimulated with FVB/N (H-2^q) allolysate (2 mg protein). Seven days after stimulation, splenic CD8+ T cells (WT) and splenic and bone marrow B cells (CD8-depleted GFP Tg; B-cell enriched) were retrieved, enriched, and flow-sorted for immune subsets. B-cell target cells included splenic GC B cells (IgG+GL-7+Fas+B220+), splenic extrafollicular plasmablasts (IgG+CD138+B220+), bone marrow plasma cells (IgG-CD138+B220-), splenic MZ cells (IgM+IgD-CD23-CD21+B220+), and splenic follicular cells (IgD+IgM-CD23+B220+). A, In an in vitro cytotoxicity assay, alloprimed CXCR5+CD8+ cells were co-cultured with each B-cell population at a 10:1 ratio for 4 h and analyzed for cytotoxicity by Annexin V and 7AA-D. CXCR5+CD8+ T cells exhibited significantly higher in vitro cytotoxicity (Annexin V+7AA-D+) toward GC B cells (19.3 ± 1.3%; n = 6) compared with cytotoxicity toward extrafollicular plasmablasts (7.6 ± 0.4%; n = 4), plasma cells (2.2 ± 0.5%; n = 4), MZ B cells (6.1 ± 0.4%; n = 5), or FO B cells (6.4 ± 0.9%; n = 5; *P < 0.0001 for all). In control cytotoxicity assays, naive CD8+ T cells, alloprimed CXCR5-CD8+ T cells, or third party (A/J; H-2^a)–primed CXCR5+CD8+ T cells were co-cultured with B-cell targets. Higher in vitro cytotoxicity of GC B cells was observed in co-cultures with alloprimed CXCR5+CD8+ T effector cells compared with GC B cells alone (5.7 ± 0.4%; n = 6) or co-cultures with alloprimed CXCR5-CD8+ T cells (8.0 ± 0.7%; n = 5), third party–primed CXCR5+CD8+ T cells (7.98 ± 1.1%; n = 5), or naive CD8+ T cells (5.3 ± 0.5%; n = 3; **P < 0.0001 for all). Control CD8+ T cells did not exhibit in vitro cytotoxicity toward other B-cell subsets (P = NS for all, n = 3–5 for all). B, Representative flow plots show gating on target cells, single cells, and GFP+ target B cells. Error bars indicate SE from duplicate experiments. 7AA-D, 7-Aminoactinomycin D; FO, follicular B cell; FSC-A, foward scatter area; FSC-W, foward scatter width; GC, germinal center; GFP, green fluorescent protein; MZ, marginal zone B cell; SSC, side scatter; Tg, transgenic; WT, wild type.

transplant models (P < 0.0001; Figure 2E) and was associated with enhanced hepatocellular allograft survival (not shown). Similarly, we have reported that reduction in alloantibody titer by AT with alloprimed CXCR5+CD8+ T cells significantly enhances kidney allograft survival.[35]

Alloprimed CXCR5+CD8+ T Cells Do Not Mediate Cytotoxicity Toward Alloprimed Macrophages or DCs

Macrophages and DCs are APCs that engulf, process, and present antigens to T cells via MHC molecules (class I and/or class II). APCs support antibody production by activating CD4+ T cells.[68,69] To investigate whether alloprimed CD8+ $T_{Ab\text{-}supp}$ cells mediate cytotoxicity to APCs, WT mice were stimulated with FVB/N allogeneic lysate. On day 7 poststimulation, alloprimed CXCR5+CD8+ T cells, macrophages (peritoneal exudate; F4/80+), and DCs (splenic; CD11c+; **Figure S3, SDC,** http://links.lww.com/TXD/A725) were isolated. Alloprimed CXCR5+CD8+ T cells were co-cultured with macrophages or DCs. In comparison with control naive CD8+ T cells, alloprimed CXCR5+CD8+ T cells did not mediate significant cytotoxicity to macrophages or DCs in co-culture (P = NS for both; **Figure S3, SDC,** http://links.lww.com/TXD/A725). Similarly, after the AT of alloprimed CXCR5+CD8+ T cells into CD8 KO transplant recipients or CCR5 KO kidney transplant recipients, no change was observed in the number of splenic macrophages or DC subsets compared with untreated controls on day 14 posttransplant (**Figure S4, SDC,** http://links.lww.com/TXD/A725).

Alloprimed CXCR5+CD8+ T Cells Do Not Mediate Cytotoxicity Toward or Inhibit the Cytokine Production of CD4+ TFH Cells, CD4+ TFR Cells, or CD4+ TREG

CD4+ T_{FH} cells are required for GC formation and guide GC B-cell differentiation into long-lived plasma cells or memory B cells.[70-73] T_{FH} cells are critical for the development of alloantibody production in mice,[74,75] and in human kidney transplant recipients, the quantity of circulating T_{FH}

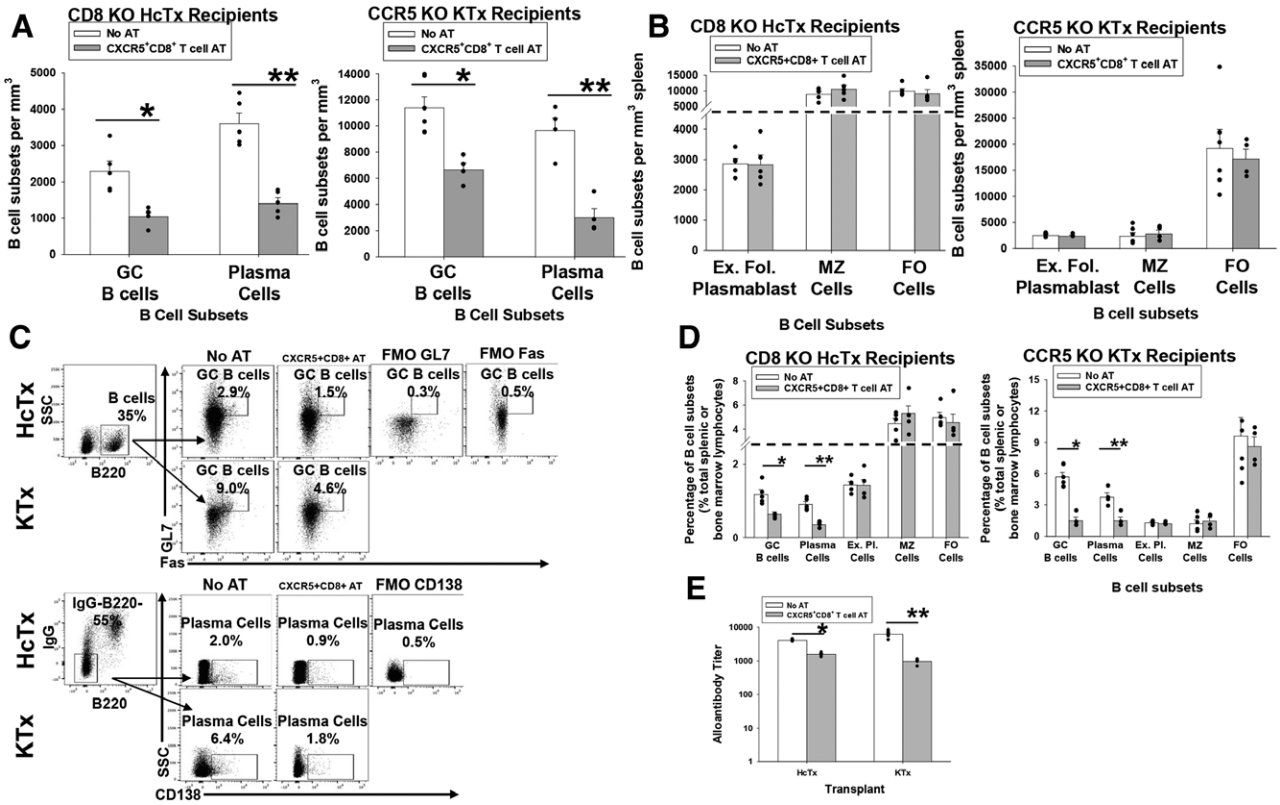

FIGURE 2. AT of alloprimed CXCR5⁺CD8⁺ T cells suppresses alloantibody titer, quantities of GC B cells, and plasma cells and enhances allograft survival. CD8 KO mice were transplanted with hA1AT-FVB/N hepatocytes (HcTx). CCR5 KO mice were transplanted with A/J kidney (KTx). On day 5 posttransplant, groups of CD8 KO and CCR5 KO recipients were AT with 2×10^6 alloprimed CXCR5⁺CD8⁺ T cells (n = 4–6 for both no AT and AT of alloprimed CXCR5⁺CD8⁺ T cells). On day 14, spleen and bone marrow were retrieved to quantify GC B cells, plasma cells, extrafollicular plasmablasts, MZ B cells, and FO B cells (plasma cells retrieved from bone marrow, all other cells retrieved from the spleen). A, The quantity of splenic GC B cells was significantly reduced after AT of CXCR5⁺CD8⁺ T cells (HcTx: 1050 ± 108; KTx: 6675 ± 507 cells per mm³) compared with untreated control (HcTx: 2301 ± 276, *$P = 0.02$; KTx: 11 404 ± 828 cells per mm³, *$P = 0.003$). Bone marrow plasma cells were also reduced in recipients of alloprimed CXCR5⁺CD8⁺ T cells (HcTx: 1409 ± 148 vs 3610 ± 288, *$P = 0.0007$; KTx: 3022 ± 622 vs 9676 ± 931 cells per mm³; **$P = 0.001$). B, However, AT of alloprimed CXCR5⁺CD8⁺ T cells did not suppress the quantity of extrafollicular plasmablasts (HcTx: 2838 ± 308 vs 2864 ± 172; KTx: 2462 ± 140 vs 2606 ± 193 cells per mm³), MZ B cells (HcTx: 10 581 ± 1271 vs 8950 ± 871; KTx: 2946 ± 663 vs 2466 ± 268 cells per mm³), or FO B cells (HcTx: 9136 ± 1327 vs 9975 ± 799; KTx: 17 247 ± 1786 vs 19 206 ± 3599 cells per mm³; P = NS for all). C, Representative flow plots for splenic GC and bone marrow plasma cells are shown for both HcTx and KTx recipients with gating on single-cell lymphocytes. FMO was used as a negative control. D, Frequencies of all B-cell subsets as percentages of total splenic or bone marrow lymphocytes are also shown for HcTx and KTx recipients. The frequency of splenic GC B cells was significantly reduced after AT of CXCR5⁺CD8⁺ T cells (HcTx: 0.6 ± 0.1%; KTx: 1.5 ± 0.3%) compared with untreated controls (HcTx: 1.2 ± 0.11%, *$P = 0.01$; KTx: 5.7 ± 0.4%; *$P < 0.0001$). Bone marrow plasma cells were also reduced in recipients of alloprimed CXCR5⁺CD8⁺ T cells (HcTx: 1.1 ± 0.1% vs 2.7 ± 0.2%, *$P = 0.0001$; KTx: 5.0 ± 0.4% vs 7.9 ± 0.5%; **$P = 0.006$). Frequencies of extrafollicular plasmablasts, MZ cells, and FO cells were unchanged following CXCR5⁺CD8⁺ T-cell AT compared with controls without AT. E, AT with alloprimed CXCR5⁺CD8⁺ T cells significantly reduced alloantibody titer (HcTx: 1602 ± 80 vs 4108 ± 136; KTx: 967 ± 96 vs 6325 ± 599, *$P < 0.0001$ for both) in HcTx and KTx recipients. Error bars indicate SE. AT, adoptive transfer; FMO, fluorescent minus one; FO, follicular B cell; GC, germinal center; hA1AT, human alpha-1-antitrypsin; HcTx, hepatocyte transplant; KO, knockout; KTx, kidney transplant; MZ, marginal zone B cell.

cells correlates with DSAs directly.[76,77] In contrast, CD4⁺ T_{FR} cells (CXCR5⁺PD1⁺Foxp3⁺CD25⁺CD4⁺) have a controversial role in antibody formation (reviewed in[78]) as they have been reported to downregulate humoral immunity[79,80] or optimize GC B cells response.[81] In clinical transplantation, although low quantities of circulating T_{FR} cells have been associated with DSAs,[82,83] mechanistic studies suggest that T_{FR} cells do not disrupt alloantibody production after transplant.[74] A disruption in T_{FH} cells, T_{FR} cells, or T_{REG} abundance or cytokine production has the potential to alter GC B-cell differentiation and alloantibody production posttransplant. Thus, we were interested in investigating whether CD8⁺ $T_{Ab\text{-}supp}$ cells mediate the immunoregulation of these CD4⁺ T-cell subsets.

First, we analyzed whether CD8⁺ $T_{Ab\text{-}supp}$ cells mediate in vitro cytotoxic killing of T_{FH} cells, T_{FR} cells, and T_{REG}. WT mice were stimulated with FVB/N allolysate. Alloprimed CXCR5⁺CD8⁺ T cells were retrieved and co-cultured with flow-sorted, alloprimed T_{FH} cells, T_{FR} cells, or T_{REG} (**Figure S5, SDC,** http://links.lww.com/TXD/A725). We found that alloprimed CXCR5⁺CD8⁺ T cells did not mediate cytotoxicity toward T_{FH} cells, T_{FR} cells, or T_{REG} when compared with the negative control co-cultures with naive CD8⁺ T cells (P = NS for all; Figure 3A). Additional controls of third party–primed CXCR5⁺CD8⁺ T cells or alloprimed CXCR5⁻CD8⁺ T cells did not mediate in vitro cytotoxicity directed toward T_{FH} cells, T_{FR} cells, or T_{REG} (Figure 3A).

We have previously reported that AT of alloprimed CXCR5⁺CD8⁺ T cells into CD8 KO recipients results in reduced proportions of T_{FH} and interleukin (IL)-21⁺ T_{FH}-cell subsets.[30,36,37] In the current studies, we confirm and extend these previous findings by reporting that AT of alloprimed CXCR5⁺CD8⁺ T cells reduced the quantity of T_{FH} cells but

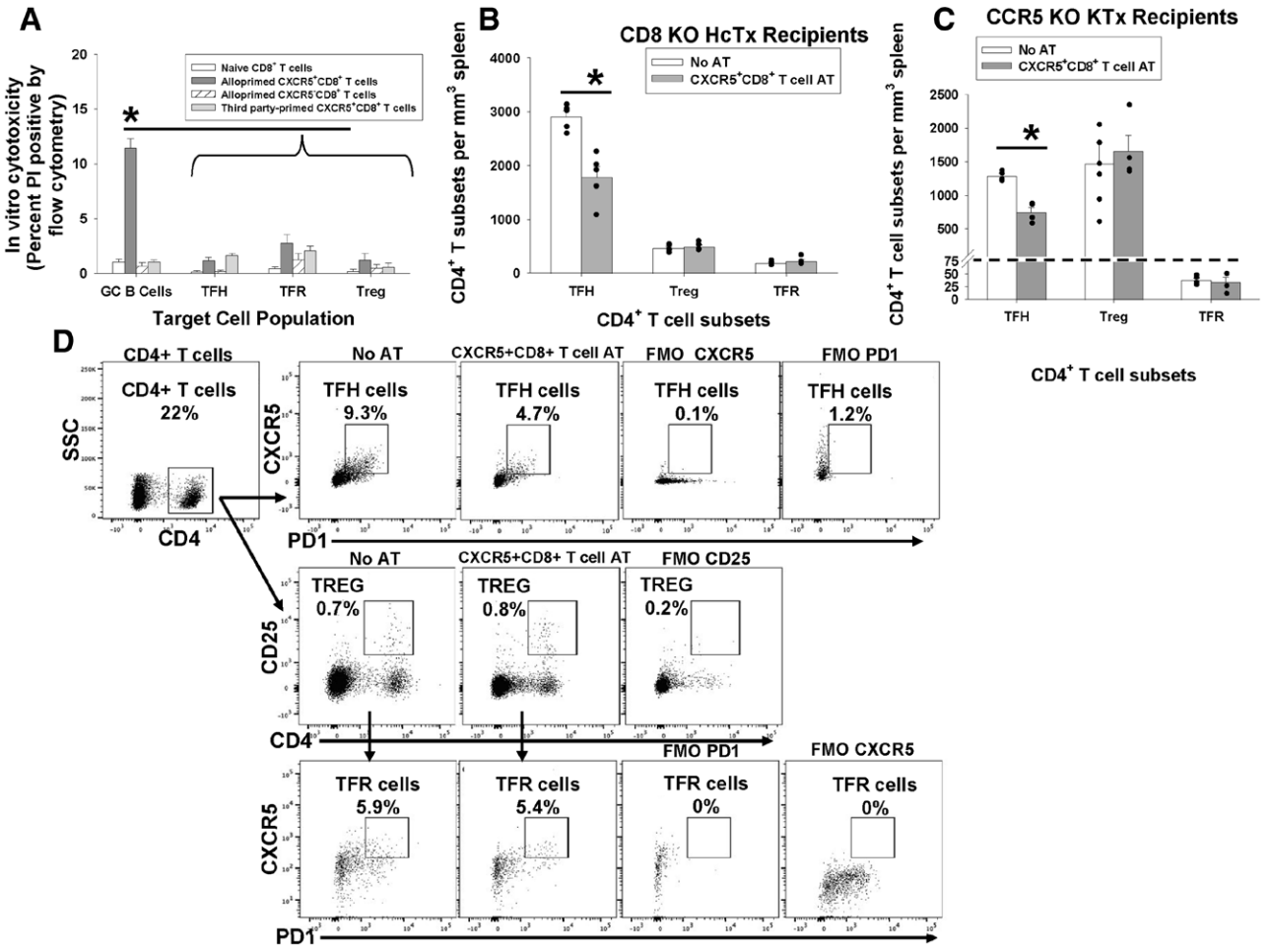

FIGURE 3. Alloprimed CXCR5+CD8+ T cells do not mediate cytotoxicity toward or reduce in vivo quantities of CD4+ T_{FH} cells, CD4+ T_{FR} cells, or CD4+ T_{REG} cells. C57BL/6 (WT) and CD8 KO (H-2^b) mice were stimulated with FVB/N (H-2^q) allolysate (2 mg protein). Seven days after stimulation, CD8+ T cells (WT), IgG+ B cells (CD8 KO), CD4+ T cells (CD8 KO), and $CD25^{high}$CD4+ T cells (CD8 KO) were isolated from splenocytes and purified. Alloprimed CXCR5+CD8+ T cells, CXCR5+PD1+CD4+ T_{FH} cells, $CD25^{int}$CXCR5+PD1+CD4+ T_{FR} cells, $CD25^{high}$CD4+ T_{REG} cells, and IgG+ GC B cells (GL-7+Fas+) were purified by flow cytometric sorting. A, In an in vitro cytotoxicity assay, CXCR5+CD8+ or CXCR5-CD8+ T-cell (or naive control CD8+ T cell) populations were co-cultured with each target-cell population at a 10:1 ratio for 4 h and analyzed for cytotoxicity (Annexin V staining and 7AA-D uptake). Alloprimed CXCR5+CD8+ T cells mediated significantly higher in vitro cytotoxicity toward GC B cells (11.6 ± 1.6%; n = 9) compared with alloprimed CD4+ T_{FH} cells (1.2 ± 0.3%; n = 9), CD4+ T_{FR} cells (2.7 ± 0.7%; n = 5), and CD4+ T_{REG} cells (1.2 ± 0.6%; n = 5), and compared with the cytotoxicity by naive CD8+ T cells (1.2 ± 0.3%; n = 15; *$P < 0.0001$ for all). No significant cytotoxicity was mediated by CXCR5-CD8+ T cells to GC B cells (0.8 ± 0.4%; n = 9), CD4+ T_{FH} cells (0.2 ± 0.1%; n = 9), CD4+ T_{FR} cells (1.6 ± 0.6%; n = 5), or to CD4+ T_{REG} cells (0.8 ± 0.5%; n = 3) when compared with the target-cell cytotoxicity in co-cultures with naive CD8+ T cells (P = NS). B and C, CD8 KO mice were transplanted with FVB/N hepatocytes (HcTx). CCR5 KO mice were transplanted with A/J kidney (KTx). On day 5 posttransplant, groups of transplant recipients were AT with 2 × 10^6 alloprimed CXCR5+CD8+ T cells (n = 4–6 for both no AT and AT of alloprimed CXCR5+CD8+ T cells). On day 14, spleens were retrieved from transplant recipients and evaluated for proportions of T_{FH}, T_{FR}, and CD4+ T_{REG} cells. Following CXCR5+CD8+ T-cell AT, the quantity of T_{FH} cells was significantly reduced compared with untreated recipients (HcTx: 1782 ± 202 vs 2909 ± 105, *$P = 0.008$; KTx: 747 ± 74 vs 1290 ± 36 per mm^3, *$P = 0.0006$, n = 4–6 for all). In contrast, no changes were observed in quantity of T_{FR} (HcTx: 223 ± 30 vs 187 ± 15; KTx: 35 ± 10 vs 38 ± 4 per mm^3; P = NS) and T_{REG} subsets (HcTx: 494 ± 31 vs 464 ± 30; KTx: 1662 ± 233 vs 1477 ± 315 per mm^3; P = NS). D, Representative flow panels of splenic of T_{FH}, T_{FR}, and T_{REG} cells are shown with gating on single-cell lymphocytes and CD4+ T cells. FMO was used as a negative control. Error bars indicate SE. 7AA-D, 7-Aminoactinomycin D; AT, adoptive transfer; FMO, fluorescent minus one; FO, follicular B cell; GC, germinal center; HcTx, hepatocyte transplant; KO, knockout; KTx, kidney transplant; MZ, marginal zone B cell; T_{FH}, CD4+ T follicular helper cell; T_{FR}, CD4+ T follicular regulatory cell; Treg, CD4+ regulatory T cell; WT, wild type.

does not influence the quantity of T_{FR} cells or T_{REG} in vivo after hepatocyte transplant (Figure 3B) or after kidney transplant (Figure 3C).

To further investigate whether CD8+ $T_{Ab\text{-}supp}$ cells mediate noncytotoxic suppression of CD4+ T-cell subsets, we analyzed the cytokine expression profiles of CD4+ T_{FH} cells, T_{FR} cells, and CD4+ T_{REG} when co-cultured with alloprimed CXCR5+CD8+ T cells. The expression of IL-4, IL-21, and interferon-γ cytokines by CD4+ T_{FH} cells and the expression of IL-10 by T_{FR} cells and T_{REG} were assessed by flow cytometry. We found that co-culture with alloprimed CXCR5+CD8+ T cells does not alter the cytokine production of CD4+ T_{FH} cells (P = NS for both proportions of cells expressing cytokine and MFI for cytokine expression; **Figure S6, SDC,** http://links.lww.com/TXD/A725). Similarly, the proportion of CD4+ T_{FR} cells and T_{REG} that express IL-10 and the mean fluorescent intensity of IL-10 expression were not different in co-cultures with or without alloprimed CXCR5+CD8+ T cells (P = NS). These data indicate that alloprimed CXCR5+CD8+ T cells do not directly kill or alter the cytokine production of T_{FH} cells, T_{FR} cells, and T_{REG}.

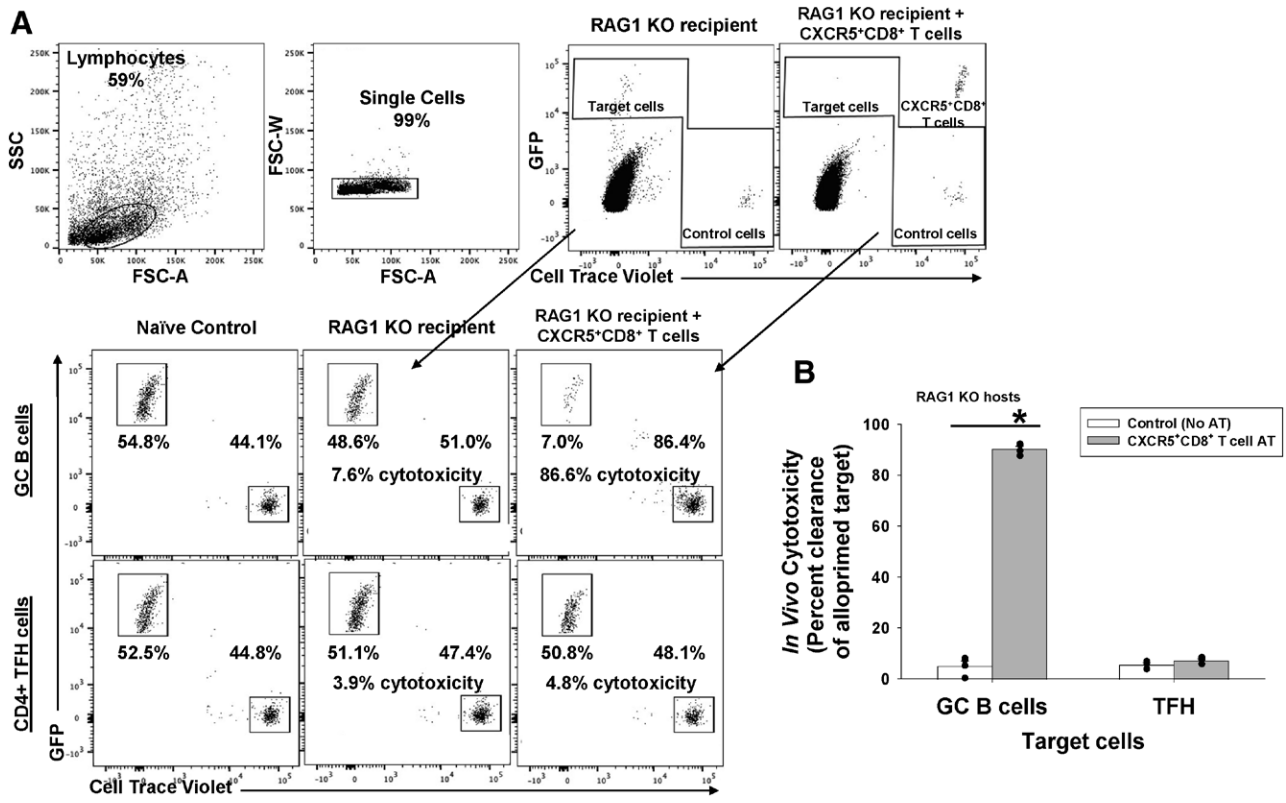

FIGURE 4. Alloprimed CXCR5$^+$CD8$^+$ T cells mediate in vivo cytotoxicity to GC B cells, not T_{FH} cells. GFP and CD8-depleted GFP mice (H-2^b) were primed with FVB/N (H-2^q) allolysate (2 mg protein). Seven days after priming, splenic CD8$^+$ T cells (from GFP hosts), splenic CD4$^+$ T cells, and IgG$^+$ B cells (from CD8-depleted GFP hosts) were pooled from multiple alloprimed mice and purified. Alloprimed CXCR5$^+$CD8$^+$ T cells, GC B cells (GL-7$^+$Fas$^+$B220$^+$), and CD4$^+$ T_{FH} cells (CXCR5$^+$PD1$^+$CD4$^+$) were further purified by flow cytometric sorting. Naive WT B220$^+$ B cells and naive WT CD4$^+$ T cells were stained with CTV (1 μM). Alloprimed flow-sorted GFP$^+$CXCR5$^+$CD8$^+$ T cells were also CTV-stained to allow for differentiation from target and control cells. Next, alloprimed RAG1 KO mice (H-2^b) were AT with equal numbers (2 × 10^6) of flow-sorted, alloprimed GFP$^+$ target cells (GC B cells or CD4$^+$ T_{FH} cells) and CTV-stained, naive controls (naive B cells or naive CD4$^+$ T cells). Some groups of RAG1 KO mice also were AT with 2 × 10^6 alloprimed CTV$^+$GFP$^+$CXCR5$^+$CD8$^+$ T cells. A, Representative flow plots show gating on lymphocytes, single cells, and GFP$^+$ (target cells; GC B cells or CD4$^+$ T_{FH} cells) and CTV$^+$ (control cells; naive B cells or naive CD4$^+$ T cells) that were used to determine in vivo cytotoxicity. B, The AT of CXCR5$^+$CD8$^+$ T cells results in significant in vivo cytotoxicity toward GC B cells (90.3 ± 1.1%, $^*P < 0.0001$) but not to T_{FH} cells (7.3 ± 0.6%, P = NS) compared with untreated RAG1 KO controls (GC B cells: 5.1 ± 1.8%, T_{FH} cells: 5.7 ± 0.7%, n = 4 for all). Error bars indicate SE. 7AA-D, 7-Aminoactinomycin D; AT, adoptive transfer; CTV, Cell Trace Violet; FSC-A, foward scatter area; FSC-W, foward scatter width; GC, germinal center; GFP, green fluorescent protein; KO, knockout; SSC, side scatter; T_{FH}, CD4$^+$ T follicular helper cell; WT, wild type.

Next, we investigated the in vivo cytotoxic clearance of alloprimed CD4$^+$ T_{FH} cells in alloprimed RAG1 KO mice. RAG1 KO mice were used as hosts to avoid CD4$^+$ T_{FH} cell interaction with naive GC B cells (or other naive B cells) that could confound results. Groups of RAG1 KO mice received AT of alloprimed GFP$^+$CD4$^+$ T_{FH} target cells (pooled from multiple alloprimed mice) and CTV-stained naive CD4$^+$ T control target cells. Experimental RAG1 KO mice were also AT with CTV-stained GFP$^+$CXCR5$^+$CD8$^+$ T cells on day 5 posttransplant. Eighteen hours after AT of alloprimed CD4$^+$ T_{FH} (and control naive) target cells, the cytotoxic clearance of CD4$^+$ T_{FH} target cells was assessed by flow cytometric analysis for relative quantity of GFP$^+$CD4$^+$$T_{FH}$ versus naive CTV$^+$CD4$^+$ T-cell subsets. We found no in vivo cytotoxicity directed toward CD4$^+$ T_{FH} target cells in alloprimed RAG1 KO mice with or without alloprimed CXCR5$^+$CD8$^+$ T cells (P = NS; Figure 4). As a positive control, in vivo cytotoxicity against alloprimed GC B cells (pooled from multiple alloprimed mice) compared with naive B cells was assessed in alloprimed RAG1 KO mice with or without AT of alloprimed CXCR5$^+$CD8$^+$ T cells. Significant CXCR5$^+$CD8$^+$ T cell–mediated in vivo cytotoxicity toward GC B cells was observed compared with RAG1 KO mice without AT of alloprimed CXCR5$^+$CD8$^+$ T cells ($P < 0.0001$; Figure 4). No homeostatic proliferation of CTV-stained cells was observed. Collectively, these data indicate that alloprimed CXCR5$^+$CD8$^+$ T cell–mediated suppression of alloantibody production is associated with in vitro and in vivo cytotoxicity to GC B cells and in vivo reduction in the quantity of GC B cells, plasma cells, and CD4$^+$ T_{FH} cells in transplant recipient mice. In contrast, alloprimed CXCR5$^+$CD8$^+$ T cells do not mediate in vitro or in vivo cytotoxic killing of plasma cells or CD4$^+$ T_{FH} cells. However, these subsets are decreased in transplant recipients who receive alloprimed CXCR5$^+$CD8$^+$ T-cell AT.[30,36,37] We performed further studies to determine whether alloprimed CXCR5$^+$CD8$^+$ T cells indirectly impact the quantity of CD4$^+$ T_{FH} cells through the reduction in the quantity of GC B cells.

CXCR5+CD8+ T Cells Indirectly Suppress CD4+ TFH-cell Quantities by Cytotoxic Reduction of GC B Cells

We have previously reported that AT of alloprimed CXCR5$^+$CD8$^+$ T cells reduces alloantibody as well as quantities of both GC B cells and cytokine-producing CD4$^+$

T_{FH} cells.[30,36,37] Since we found in the current studies that CXCR5$^+$CD8$^+$ T cells do not mediate in vitro or in vivo cytotoxic killing or inhibit in vitro cytokine production of CD4$^+$ T_{FH} cells, we hypothesized that the in vivo reduction of CD4$^+$ T_{FH} cells occurs secondary to the cytotoxic killing and overall reduction of GC B cells necessary to sustain CD4$^+$ T_{FH}-cell population. Yusuf et al[84] and Baumjohann et al[85] independently published that CD4$^+$ T_{FH}-cell quantity depends on GC B cells. To investigate whether alloprimed CXCR5$^+$CD8$^+$ T cells directly or indirectly inhibit CD4$^+$ T_{FH}-cell quantities, B cell–deficient μMT KO mice and RAG1 KO mice were transplanted with FVB/N hepatocytes and received AT of naive B cells and naive CD4$^+$ T cells (day 0). By day 14, GC B cells and CD4$^+$ T_{FH} cells were detected in μMT KO and RAG1 KO recipient spleens, and alloantibody was also detected in the serum (Figure 5). Experimental μMT KO and RAG1 KO mice received AT of alloprimed CXCR5$^+$CD8$^+$ T cells on day 5 after AT of CD4$^+$ T cells and B cells. We found that by day 14 after AT of alloprimed CXCR5$^+$CD8$^+$ T cells, alloantibody titer was reduced in both μMT KO ($P < 0.0001$) and RAG1 KO recipients ($P = 0.006$) and proportions of GC B cells and CD4$^+$ T_{FH} cells were significantly reduced in both μMT KO ($P < 0.03$) and RAG1 KO recipients ($P < 0.0001$ for both cell types; Figure 5) similar to results in immunocompetent mice.[38] Higher alloantibody titer, quantity of GC B cells, and T_{FH} cells were observed in RAG1 recipients compared with μMT KO (after AT of naive CD4$^+$ T cells and B cells; $P < 0.0005$ for all). This may reflect the activity of endogenous alloprimed antibody-suppressor CXCR5$^+$CD8$^+$ T cells in μMT KO recipients. To directly assess whether the observed reduction in

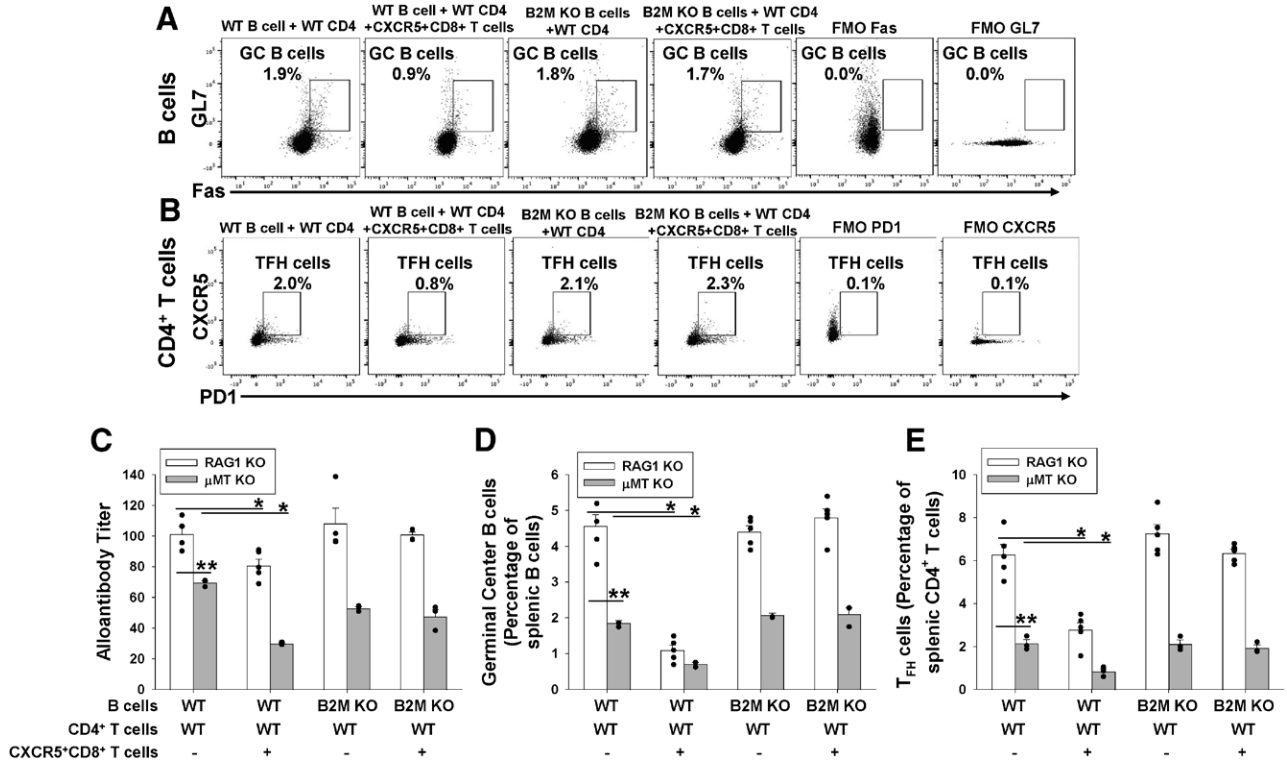

FIGURE 5. Alloprimed CXCR5$^+$CD8$^+$ T-cell suppression of T_{FH} cell quantity correlates with MHC class I-dependent cytotoxic reduction of GC B cells. μMT KO and RAG1 KO mice were transplanted with FVB/N hepatocytes. On day 0, recipient mice were AT with 10×10^6 naive B cells (WT or β2M KO) and 10×10^6 naive WT CD4$^+$ T cells. Then, on day 5 posttransplant, groups of KO recipients received AT of 2×10^6 alloprimed CXCR5$^+$CD8$^+$ T cells. On day 14, splenocytes were analyzed for quantity of GC B cells and CD4$^+$ T_{FH} by flow cytometry. A, GC B cells (GL-7$^+$Fas$^+$B220$^+$) were analyzed by gating on single-cell lymphocytes and B cells. FMO was used as a negative control. B, T_{FH} cells (CXCR5$^+$PD1$^+$CD4$^+$) were analyzed by gating on single-cell lymphocytes and CD4$^+$ T cells. C, CXCR5$^+$CD8$^+$ T-cell AT into KO transplant recipient mice that received WT B cells and WT CD4$^+$ T cells resulted in reduced alloantibody titer (μMT KO: 29.9 ± 0.5, n = 3; RAG1 KO: 76 ± 4, n = 5) compared with no AT (μMT KO: 69.5 ± 1.3, n = 3; RAG1 KO: 101 ± 5, n = 5; $^*P < 0.006$ for both). However, in recipient mice that received β2M KO B cells and WT CD4$^+$ T cells, alloantibody titer was similar with or without CXCR5$^+$CD8$^+$ T-cell AT (μMT KO: 47.5 ± 4.7 vs 52.9 ± 1.1, n = 3; RAG1 KO: 101 ± 2 vs 108 ± 10, n = 4 for both; P = NS for both). RAG1 KO recipients produce more alloantibody compared with CD8-sufficient μMT KO recipients ($^{**}P = 0.0005$). D, CXCR5$^+$CD8$^+$ T-cell AT into transplant recipient mice that received WT B cells and WT CD4$^+$ T cells resulted in significantly reduced proportion of GC B cells (μMT KO: 0.7 ± 0.05%, n = 3; RAG1 KO: 1.1 ± 0.1%; n = 5) compared with no AT (μMT KO: 1.8 ± 0.1%, n = 3; RAG1 KO: 4.6 ± 0.3%; n = 5, $^*P < 0.004$ for both). However, in μMT KO and RAG1 KO mice that received β2M KO B cells (and WT CD4$^+$ T cells), the proportion of GC B cells was similar between recipients who received CXCR5$^+$CD8$^+$ T-cell AT (μMT KO: 2.1 ± 0.2%, n = 3; RAG1 KO: 4.8 ± 0.2%; n = 5) or no AT (μMT KO: 2.1 ± 0.1%, n = 3; RAG1 KO: 4.4 ± 0.2%; n = 5, P = NS). RAG1 KO recipients have more GC B cells compared with CD8-sufficient μMT KO recipients ($^{**}P < 0.0001$). E, CXCR5$^+$CD8$^+$ T-cell AT in μMT KO and RAG1 KO mice that received WT B cells and WT CD4$^+$ T cells resulted in reduced proportion of T_{FH} cells (μMT KO: 0.8 ± 0.1%, n = 3; RAG1 KO: 2.8 ± 0.3%, n = 5) compared with no AT (μMT KO: 2.1 ± 0.2%, n = 3; RAG1 KO: 6.2 ± 0.5%, n = 5; $^*P < 0.03$ for both). However, in μMT KO and RAG1 KO recipient mice that received β2M KO B cells (and WT CD4$^+$ T cells), the proportion of T_{FH} cells was similar between mice that received CXCR5$^+$CD8$^+$ T-cell AT (μMT KO: 1.9 ± 0.1%, n = 3; RAG1 KO: 6.3 ± 0.2%, n = 5) or no AT (μMT KO: 2.1 ± 0.2%, n = 3; RAG1 KO: 7.2 ± 0.4%, n = 5; P = NS). RAG1 KO recipients have more T_{FH} cells compared with CD8-sufficient μMT KO recipients ($^{**}P < 0.0001$). Error bars indicate SE from duplicate experiments. AT, adoptive transfer; FMO, fluorescent minus one; GC, germinal center; KO, knockout; MHC, major histocompatibility complex; T_{FH}, CD4$^+$ T follicular helper cell; WT, wild type.

CD4+ T_{FH}-cell proportion was dependent on CXCR5+CD8+ T cell–mediated GC B-cell killing, we repeated the μMT KO and RAG1 KO studies with AT of naive B cells from β2M KO mice (in place of WT B cells) and naive CD4+ T cells from WT mice. Alloantibody production readily occurs (and alloantibody titer is comparable) in μMT KO and RAG1 KO hepatocyte transplant mice reconstituted with naive B cells from β2M KO and naive CD4+ T cells from WT mice. B cells from β2M KO mice lack MHC class I expression and are not susceptible to CXCR5+CD8+ T cell–mediated in vitro cytotoxicity.[38] We found that, unlike the results with AT of MHC class I sufficient WT B cells, alloantibody titer and proportions of MHC class I–deficient (β2M KO) GC B cells were not reduced by the AT of alloprimed CXCR5+CD8+ T cells in both μMT KO and RAG1 KO recipients (P = NS for both; Figure 5C and D). Furthermore, AT of alloprimed CXCR5+CD8+ T cells did not impact the proportions of CD4+ T_{FH} cells that develop in μMT KO and RAG1 KO mice that were reconstituted with naive β2M KO B cells and WT CD4+ T cells (P = NS for both; Figure 5E). Since CXCR5+CD8+ T cells do not mediate cytotoxicity toward CD4+ T_{FH} cells and, in the absence of T cell receptor/MHC I cognate interaction, cannot kill IgG+ B cells,[38] these data suggest that alloprimed CXCR5+CD8+ T-cell cytotoxic reduction of GC B cells secondarily results in reduced in vivo quantities of CD4+ T_{FH} cells and an overall dampening of the humoral immune response.

DISCUSSION

Our group is the first to report the discovery of a novel subset of antibody-suppressor CD8+ T cells that express the chemokine receptor CXCR5, mediate in vitro and in vivo cytotoxic killing of IgG+ B cells, suppress in vivo alloantibody production, and prolong allograft survival after cell (hepatocyte) and vascularized solid organ (kidney) transplant in mice.[30,34,35,38] The current study expands upon previous findings by providing evidence that alloprimed CXCR5+CD8+ T cells inhibit alloantibody production by direct cytotoxic killing of GC B cells but no other IgG+ or IgG- B-cell subsets or plasma cells. CD4+ T follicular-cell subsets and APCs (macrophages or DCs) contribute to the development of humoral immunity; however, alloprimed CXCR5+CD8+ cytotoxic T cells do not target these other cell populations. Consistent with the functional capacity of CD8+ $T_{Ab\text{-}supp}$ cells to mediate cytotoxic killing of GC B cells, we found that AT of alloprimed CD8+ $T_{Ab\text{-}supp}$ cells into transplant recipient mice is associated with reduced quantities of GC B cells and plasma cells but has no effect on the quantity of other B-cell subsets, CD4+T_{FR}, or T_{REG} subsets, macrophages, or DCs. These results support an antibody-suppressor mechanism in which CD8+ $T_{Ab\text{-}supp}$ cells traffic to GC within lymphoid tissues and downregulate alloantibody production by preferentially killing GC B cells, which leads to downstream reduced quantity of antibody-producing plasma cells and T_{FH} cells.

To limit investigation to CXCR5+CD8+ T-cell in vivo cytotoxic effector function, we examined the co-transfer of CXCR5+CD8+ T cells and various target cells into CD8-deficient transplant recipient mice (CD8 KO or RAG1 KO mice). These models allow for an evaluation of CXCR5+CD8+ T-cell effector function in the absence of endogenous CXCR5+CD8+ T cells. A potential limitation of using the CD8 KO recipient model is the theoretical concern that B-cell development might not be normal in these recipients. However, we found that CXCR5+CD8+ T cell–mediated in vitro cytotoxicity toward GC B cells (and other B-cell targets) is similar whether using B-cell targets from alloprimed CD8-depleted WT or alloprimed CD8 KO mice, suggesting that the development of GC B cells in CD8 KO mice is not appreciably different from in WT mice.

We also used the RAG1 KO mouse model as another approach to study the isolated effector function of CXCR5+CD8+ T cells when RAG1 KO mice were transferred with both B cells and CD4+ T cells. The absence of T and B cells in the RAG1 KO host allows for the AT of a specified quantity of naive B cells and CD4+ T cells that can develop into GC B cells and T_{FH} cells and be correlated directly with the alloantibody titer, that is produced in vivo without the confounding effects of endogenous cell subset function. No evidence of homeostatic proliferation was observed in these RAG1 KO studies (data not shown). These studies revealed that when CXCR5+CD8+ T-cell cytotoxic effector function is impaired by the absence of MHC class I expression on target B cells, no suppression of humoral alloimmunity is observed, and the quantity of both GC B cells and T_{FH} cells is unaltered compared with controls without CXCR5+CD8+ T-cell transfer.

We repeated the same co-transfer studies in μMT KO recipients to exclude any limitations of the RAG1 KO model because these mice have normal quantities of endogenous CD4+ T cells and CD8+ T cells. We found similar results as in the RAG1 KO recipients, suggesting that alloprimed CXCR5+CD8+ T cell–mediated GC B-cell cytotoxicity results in an indirect reduction of T_{FH}-cell quantity.

The immunologic basis for CD8+ $T_{Ab\text{-}supp}$ cells selective targeting of GC B cells, beyond MHC I/T cell receptor interactions, remains to be determined. CXCR5 directed trafficking of the cells to the GC position CD8+ $T_{Ab\text{-}supp}$ cells in proximity to GC B cells but theoretically also in proximity to T_{FH} and follicular DCs. Interestingly, DC and macrophage expression of CD80 has been reported to stimulate T-cell expression of Cytotoxic T lymphocyte associated protein 4, which decreases T-cell contact time and increases T-cell motility.[86,87] GC B cells have low expression of CD80,[88-91] and perhaps this increases contact time between CD8+ $T_{Ab\text{-}supp}$ cells and GC B cells compared with other potential target cells. CD8+ $T_{Ab\text{-}supp}$ cell FasL-dependent cytotoxic mechanisms[38] may be biased toward killing GC B cells, which are known to highly express Fas and prone to Fas-mediated cytotoxicity.[92,93] In contrast, DC and macrophages are resistant to Fas-mediated killing.[94-96] CD8+ $T_{Ab\text{-}supp}$ cells also mediate perforin-dependent cytotoxicity.[38] However, DC and macrophages (but not FO B cells) express the protease serpin (or Sip6), which inhibits granzyme activity.[97-99] Future investigation is warranted to determine the molecular interactions that initiate or sustain CD8+ $T_{Ab\text{-}supp}$ cell–mediated cytotoxic killing of GC B cells and spare cytotoxic killing of alloprimed DC and macrophages.

CD4+ T_{FH} cells promote the differentiation of centrocytes into plasma cells via the secretion of cytokines IL-4 and IL-21.[100,101] We have previously reported that AT of CXCR5+CD8+ T cells into transplant recipients is associated with decreased quantities of CD4+ T_{FH} cells.[30,35] In the current study, we found that reductions in CD4+ T_{FH}-cell quantity (unlike reduction in GC B-cell quantity) are not mediated by direct cytotoxic killing by CD8+ $T_{Ab\text{-}supp}$ cells. Furthermore, CD8+ $T_{Ab\text{-}supp}$ cells do not mediate direct suppression of

$CD4^+$ T_{FH}-cell function as we observed that IL-4, IL-21, and interferon-γ cytokine expression by $CD4^+$ T_{FH} cells were not affected by co-culture with $CD8^+$ $T_{Ab\text{-}supp}$ cells. In these studies, IL-21 cytokine expression by $CD4^+$ T_{FH} cells in co-cultures was high, likely due to the use of brefeldin and phorbol 12-myristate-13-acetate, known to increase T_{FH}-cell IL-21 expression.[102] We found that $CD4^+$ T_{FH}-cell quantity correlates with GC B-cell quantity. Our results are consistent with reports by others that quantities of $CD4^+$ T_{FH} cells are reduced when the quantity of GC B cells is reduced.[84,85] GC B cell–mediated support of $CD4^+$ T_{FH} cells critically requires persistent antigen presentation by GC B cells as well as continuous Inducible T Cell Costimulator (ICOSL)/ICOS ligand (ICOSL) and CD40/CD40L interactions.[84,85] Thus, the reduction in the quantity of $CD4^+$ T_{FH} cells associated with AT of $CD8^+$ $T_{Ab\text{-}supp}$ cells appears to be an indirect effect of $CD8^+$ $T_{Ab\text{-}supp}$ cell–mediated GC B-cell cytotoxic clearance. The regulatory role of primed $CXCR5^+CD8^+$ cytotoxic T cells is consistent with a homeostatic mechanism to dampen humoral immune responses.

We have found in a murine kidney transplant model that a deficit of $CXCR5^+CD8^+$ T cells corresponds with extraordinarily high alloantibody production. CCR5 KO transplant recipients develop high alloantibody titers by day 14 posttransplant that are 4–10 times above those observed in WT kidney transplant recipients.[35,45,103-106] We published that this dysregulation of humoral alloimmunity in CCR5 KO kidney transplant recipient mice is associated with a relative deficit of (failure to expand) alloprimed $CXCR5^+CD8^+$ T cells (day 7 posttransplant[35,36]) that can be rescued by adoptive cell therapy. Akin to these murine studies, we found in a prospective human study that the quantity of $CXCR5^+CD8^+$ T cells in first-time kidney transplant recipients inversely correlates with the development of DSAs in the first year posttransplant, which is consistent with a potential antibody-suppressor function.[22] These data suggest that immune assessment of the quantity of $CXCR5^+CD8^+$ T cells could be developed as a biomarker for DSA risk posttransplant.

Current therapies to treat transplant recipients who develop AMR include plasmapheresis, IVIG, proteasome inhibitors, B-cell depletion, and/or complement inhibitors. Newer agents tested for treatment of AMR include drugs that target IL-6 receptor blockade, B lymphocyte stimulator, CD38-expressing plasma cells, and cysteine protease-mediated cleavage of IgG antibodies (reviewed in[107]). However, these therapeutic approaches are not supported by high-level evidence, produce variable results, and carry the risk of broad and nonspecific immunosuppression that concurrently compromises the protective immunity of transplant recipients.[32,107] In contrast, $CD8^+$ $T_{Ab\text{-}supp}$-cell effector function is allospecific since alloprimed but not third party–primed $CD8^+$ T cells mediate cytotoxic killing of GC B cells and alloantibody suppression. Furthermore, in published studies, we have reported that AT of $CD8^+$ $T_{Ab\text{-}supp}$ cells successfully interrupts active in vivo antibody production by alloprimed B cells, thus holding promise not only as a strategy to prevent or reduce DSA development but also as a novel therapeutic approach to treat acute AMR. Our findings that $CD8^+$ $T_{Ab\text{-}supp}$ cells target GC B cells specifically (not other T, B, or APC subsets) enhances their translational potential as novel cell therapy and reduces the risk of off-target effects. Altogether, our results support research directed toward the development of $CD8^+$ $T_{Ab\text{-}supp}$ cell–based immunotherapies to increase their quantity by exogenous cell therapy or treatments to boost endogenous quantities. Finally, while this investigation to elucidate the mechanism by which $CD8^+$ $T_{Ab\text{-}supp}$ cells inhibit in vivo alloantibody production was performed in the setting of transplantation, the results provide impetus to study their role in modulating humoral immune responses in other conditions, such as in autoimmunity and vaccination strategies.

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