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Antibody-suppressor CD8+ T cells require CXCR5

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Abstract

Background—We previously reported the novel activity of alloprimed $CD8^+$ T cells that suppress post transplant alloantibody production. The purpose of the current study is to investigate the expression and role of CXCR5 on antibody-suppressor $CD8^+$ T cell function.

Methods—C57BL/6 mice were transplanted with FVB/N hepatocytes. Alloprimed $CD8^+$ T cells were retrieved on day 7 from hepatocyte transplant recipients. Unsorted or flow-sorted ($CXCR5^+CXCR3^-$ and $CXCR3^+CXCR5^-$) alloprimed $CD8^+$ T cell subsets were analyzed for in vitro cytotoxicity and capacity to inhibit *in vivo* alloantibody production following adoptive transfer into C57BL/6 or high alloantibody-producing CD8 KO hepatocyte transplant recipients. Alloantibody titer was assessed in CD8 KO mice reconstituted with naïve $CD8^+$ T cells retrieved from C57BL/6, CXCR5 KO or CXCR3 KO mice. Antibody suppression by OVA-primed monoclonal OT-I $CXCR5^+$ or $CXCR3^+$ $CD8^+$ T cell subsets was also investigated.

Results—Alloprimed $CXCR5^+CXCR3^-CD8^+$ T cells mediated in vitro cytotoxicity of alloprimed "self" B cells while $CXCR3^+CXCR5^-CD8^+$ T cells did not. Only flow-sorted alloprimed $CXCR5^+CXCR3^-CD8^+$ T cells (not flow-sorted alloprimed $CXCR3^+CXCR5^-CD8^+$ T cells) suppressed alloantibody production and enhanced graft survival when transferred into transplant recipients. Unlike $CD8^+$ T cells from wild-type or CXCR3 KO mice, $CD8^+$ T cells from CXCR5 KO mice do not develop alloantibody-suppressor function. Similarly, only flow-sorted

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$CXCR5^+CXCR3^-$ (and not $CXCR3^+CXCR5^-$) OVA-primed OT-I $CD8^+$ T cells mediated in vivo suppression of anti-OVA antibody production.

Conclusion—These data support the conclusion that expression of CXCR5 by antigen-primed $CD8^+$ T cells is critical for the function of antibody-suppressor $CD8^+$ T cells.

Introduction

A key challenge in the field of transplantation is the lack of definitive approaches to suppress the development of alloantibody production or to treat antibody-mediated rejection (AMR). Clinical and experimental data indicate that de novo production of MHC-directed alloantibodies after transplant has pathologic and clinical consequences contributing to acute and chronic rejection of solid-organ (reviewed in[1]) and cellular transplants.[2,3] A successful therapeutic approach to suppress the production of post transplant alloantibody would not only prevent AMR but also enhance long-term graft survival. New immunotherapies to suppress post transplant humoral alloimmunity require enhanced understanding of the immune mechanisms that regulate alloantibody production.

Conventional approach to modulating post transplant humoral alloimmunity has focused on the suppression of $CD4^+$ T cells,[4] which "help" B cells produce antibody.[5,6] However, despite the use of T cell depletion induction immunotherapies and conventional maintenance immunosuppressive agents which target $CD4^+$ T cells, the development of de novo donor-specific antibody (DSA) occurs in ~20%−40% of solid organ(reviewed in[7]) and also after hepatocyte[2] or islet cell[3] transplant. Promising results with co-stimulatory blockade therapies, which suppressed alloantibody production and rejection in experimental transplant models,[8–13] paved the way for clinical trials testing the efficacy of costimulatory blockade in humans. Unfortunately, clinical trials testing the efficacy of recombinant humanized monoclonal antibody targeting CD154 in humans were associated with thromboembolic complications which resulted in the early suspension of these trials.[14,15] More recently clinical trials testing the efficacy of humanized fusion protein targeting CTLA-4 (Belatacept) reported an acceptable safety profile with improved allograft function, allograft survival, and significant reduction in the incidence of alloantibody production compared to cyclosporine-based immunosuppression. However, an unexpectedly higher rate and severity of early acute rejection occurred in Belatacept-treated recipients.[16] Thus, new immunotherapeutic approaches which suppress the development of humoral alloimmunity and prevent AMR are needed. Our group has focused on a novel CD8-dependent immunoregulatory mechanism which downregulates post transplant alloantibody production.[17] We reported that these antibody-suppressor $CD8^+$ T cells ($CD8^+$ $T_{Ab\text{-}supp}$ cells) mediate alloantigen-specific suppression of post transplant alloantibody by an IFN-γ-dependent mechanism, which involves cytotoxic killing of alloprimed B cells[18] and inhibition of $IL\text{-}4^+CD4^+$ T cells.[17]

Since we previously noted that the suppression of alloantibodies occurs, in part, due to CD8-dependent killing of host MHC I^+ alloprimed IgG^+ B cells[18] and that host alloprimed $CD8^+$ T cells and alloprimed IgG^+ B cells co-localize in lymphoid depots, we reasoned that antibody-suppressor $CD8^+$ T cells might migrate to lymphoid tissue via expression of the lymphoid-homing chemokine receptor, CXCR5, to mediate their effector functions. The

current studies were designed to investigate the expression and role of CXCR5 for antibody-suppressor $CD8^+$ T cell function.

Materials and Methods

Experimental animals

FVB/N ($H\text{-}2^q$ MHC haplotype, Taconic), C57BL/6 (wild-type; WT), CD8 KO, mOVA Tg, OT-I Tg, CXCR5 KO, and CXCR3 KO mice (all $H\text{-}2^b$) and B10.BR ($H\text{-}2^k$) mouse strains (all 6–10 weeks of age, Jackson Labs) were used in this study. Transgenic FVB/N mice expressing human α−1 antitrypsin (hA1AT) were the source of "donor" hepatocytes, as previously described.[19] Male and female mice of 6–10 weeks of age were used in these studies. All experiments were performed in compliance with the guidelines of the IACUC of The Ohio State University (Protocol 2008A0068-R2).

Hepatocyte isolation, purification, and transplantation

Hepatocyte isolation and purification was completed, as previously described.[19] Hepatocyte viability and purity was >95%. Donor FVB/N hepatocytes (2×10^6) were transplanted by intrasplenic injection with circulation of donor hepatocytes to the host liver.[19] Graft survival was determined by detection of secreted hA1AT in serial recipient serum samples by ELISA.[19,20]

$CD8^+$ T cell isolation

Isolation of $CD8^+$ T cells from naïve or primed hosts was performed using negative selection columns as per the manufacturer's recommendations (R&D Systems, Minneapolis, MN; purity routinely >90%). In some cases, primed $CD8^+$ T cells were sorted into $CXCR5^+CXCR3^-$ and $CXCR3^+CXCR5^-$ $CD8^+$ T cell populations by FACS Aria flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using anti-CXCR5 (clone 2G8) and anti-CXCR3 mAbs (clone CXCR3–173; both Becton Dickinson).

Preparation of Primed $CD8^+$ T cells

Alloprimed $CD8^+$ T cells were isolated from spleens of FVB/N hepatocyte recipients (day 7). To retrieve OVA-primed OT-I $CD8^+$ T cells, naïve OT-I $CD8^+$ T cells were adoptively transferred into OVA-primed CD8 KO hosts [i.p. injection of hepatocyte lysate from mOVA Tg mice as a source of OVA peptide (1 μg OVA)]. mOVA Tg hepatocytes (2×10^6) underwent 5 freeze/thaw cycles to create cell-free lysate.

B cell isolation

B cells (B220) and primed IgG^+ B cells were purified from splenocytes using anti-mouse B220 or anti-IgG magnetic beads following the manufacturer's instructions (Miltenyi Biotech, Auburn, CA; purity routinely >95%).

Donor-reactive alloantibody titer

To quantitate alloantibody titer, we analyzed the recipient serum using published methods.[21] Briefly, serum was serially diluted and incubated with allogeneic target splenocytes.

Splenocytes were then stained with FITC-conjugated goat anti-mouse IgG Fc (Organon Teknika, Durham, NC). Background staining using anti-mouse IgG Fc with allogeneic splenocytes (alone or with naïve serum) is negligible. 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 naïve serum was divided by two and recorded as the titer.

Detection of serum anti-OVA IgG

Anti-OVA IgG levels in mOVA hepatocyte-lysate treated mice were detected using the mouse anti-OVA IgG antibody assay kit following the manufacturer's instructions (Chondrex, Redmond, WA).

In vitro cytotoxicity assay

Cytotoxicity was measured using a LIVE/DEAD cell-mediated cytotoxicity kit (Invitrogen, Eugene, OR) and performed according to the manufacturer's instructions. In brief, target B cells were stained with CFSE. $CD8^+$ T cells and B cells were co-cultured at a 10:1 ratio for 4 hours. Propidium iodide (PI) was added to the co-cultures to assess cell death and PI uptake in $CFSE^+$ B cells was immediately analyzed by flow cytometry, as previously described.[22]

Flow cytometric lymphocyte subset analysis and intracellular cytokine staining

Splenocytes were isolated from FVB/N transplant or FVB/N hepatocyte lysate recipients (day 7) and incubated for 4 hours with Leukocyte Activation Cocktail (PMA, ionomycin, and Brefeldin A; Becton Dickinson). Next, splenocytes were stained with Live/Dead Fixable Aqua Dead Cell Stain Kit following manufacture's recommendations (Thermo Fisher, Grand Island, NY). Following Live/Dead cell staining, splenocytes were treated with anti-FcγR mAb [supernatant from 2.4G2 hybridoma (ATCC; Manassas, VA)]. Splenocyte T (CD4: clone GK1.5, CD8: clone 53–6.7) and B (B220: clone RA3-GB2) cell subsets were subsequently stained and analyzed for expression of markers of activation (CD44: clone IM7, CD62L: clone MEL-14), co-inhibition (PD-1: clone RMPI-30), chemokine receptors (CXCR5: clone 2G8, CXCR3: clone CXCR3–173), and germinal center markers (GL-7). Intracellular staining was performed using FIX&PERM cell permeabilization kit (Thermo Fisher). Analysis for intracellular cytokines IFN-γ (clone XMG1.2), IL-21 (clone RM0268–6G53) and IL-4 (clone 11B11) was performed following Becton Dickinson's recommendations. Flow cytometric analysis was performed by gating on lymphocyte populations (viable, single cells) of $CD8^+$ T cells, or $CXCR5^+PD\text{-}1^+CD4^+$ T cells. Fluorescence-minus-one (FMO) was utilized as negative controls to set the positive/negative boundary for protein expression.[23]

Statistical analysis

General linear models were fit for each continuous outcome and contrasts used to compare relevant groups to test the primary hypothesis/hypotheses in each experiment. Model assumptions were assessed and violations to the normality assumption were addressed by transforming the data to the natural log scale. Log rank tests were used to compare time to graft rejection between groups. Multiple comparisons between groups were adjusted using

Tukey-Kramer method (analysis of in vitro cytotoxicity), Bonferroni's method (analysis of transplant survival), or Dunnett's method (all other figures) to maintain the overall type 1 error rate at 5% for each outcome respectively. All analyses were conducted using SAS statistical Software Version 9.4 (SAS Institute, Inc., Cary, NC). To demonstrate the distribution of the data, results are listed as the mean ± standard error.

Results

Alloprimed CD8$^+$ T cells that express CXCR5 mediate in vitro killing of self IgG1$^+$ B cells

CXCR5 is a chemokine receptor important for homing to the germinal center in lymphoid tissue where B cell maturation occurs.[24–26] CXCR3 is a chemokine receptor important for homing to sites of tissue inflammation (including allografts)[27–32] and for cell-mediated rejection.[33,34] In order to investigate the expression of chemokine receptors CXCR5 and CXCR3 by alloprimed CD8$^+$ T cells, C57BL/6 mice underwent FVB/N hepatocyte transplantation. On day 7 post transplant, alloprimed CD8$^+$ T cells were retrieved, analyzed for CXCR5 and CXCR3 expression by flow cytometry and flow-sorted populations were tested for in vitro killing of self IgG1$^+$ B cells, an assay which correlates with antibody-suppressor CD8$^+$ T cell function.[18]

We found that bulk, unsorted CD8$^+$ T cells from alloprimed recipients expressed CXCR5 or CXCR3 (very few double positive cells) (Figure 1A). Alloprimed CXCR5$^+$CXCR3$^-$ (and CXCR3$^+$CXCR5$^-$) CD8$^+$ T cells were predominantly CD44$^+$ (>80%) (Figure S1). Alloprimed CD8$^+$ T cells (from day 7 C57BL/6 hepatocyte transplant recipients) were purified and flow-sorted into CXCR5$^+$CXCR3$^-$CD8$^+$ and CXCR3$^+$CXCR5$^-$CD8$^+$ T cell subsets. Flow-sorted CD8$^+$ T cells were co-cultured with alloprimed "self" IgG1$^+$ B cells (H-2^q primed C57BL/6 B cells) or allogeneic B cells (FVB/N, H-2^q) in an in vitro cytotoxicity assay. Third-party primed "self" IgG1$^+$ B cells (H-2^k primed C57BL/6 B cells) and B cells expressing third-party alloantigens (H-2^k, B10BR) were used as control target cells. Unsorted alloprimed CD8$^+$ T cells and naïve CD8$^+$ T cells served as positive and negative control effector cells, respectively. Positive control unsorted alloprimed CD8$^+$ T cells induced significant cytotoxicity to both alloprimed self IgG1$^+$ B cells (9.1±3.8%; $p<0.0001$) and allogeneic B cells (12.0±5.5%; $p=0.002$) compared to naïve control CD8$^+$ T cells (0.9±.04% and 0.8±0.7%, respectively; Figure 1B). Alloprimed CD8$^+$ T cells sorted for the CXCR5$^+$CXCR3$^-$CD8$^+$ T cell subset also mediated significant cytotoxicity to alloprimed self IgG1$^+$ B cells (12.7±1.8%; $p<0.0001$) compared to naïve control CD8$^+$ T cells but not to allogeneic B cells (1.8±1.8%; p=ns). In contrast, sorted CXCR3$^+$CXCR5$^-$CD8$^+$ T cells mediated cytotoxicity to allogeneic B cells (15.6±8.8%; $p<0.0001$) but did not mediate cytotoxicity to alloprimed self IgG1$^+$ B cells (1.9±1.9%; p=ns) compared to co-cultures with naïve control CD8$^+$ T cells. No significant cytotoxicity was observed for any CD8$^+$ T cell group against control third-party primed "self" IgG1$^+$ B cells or third-party party B cells. These results suggested that antibody-suppressor CD8$^+$ T cells express CXCR5 but not CXCR3. Next, we investigated the in vivo function of CXCR5$^+$ and CXCR3$^+$ alloprimed CD8$^+$ T cell subsets.

CXCR5 is critically important for in vivo CD8+ T cell alloantibody-suppressor function

To determine the functional relevance of chemokine receptor expression by activated $CD8^+$ T cells, we adoptively transferred CD8 KO hepatocyte recipients on day 0 (relative to transplant) with naïve $CD8^+$ T cells isolated from WT C57BL/6, CXCR5 KO, or CXCR3 KO mice. Recipients were assayed for alloantibody production on day 14 post transplant. As in previous studies, we found that adoptive transfer of WT $CD8^+$ T cells significantly inhibits alloantibody production by more than three-fold ($p=0.004$ compared to no $CD8^+$ T cell transfer; Figure 2A).[17,18] Adoptive transfer of CXCR3 KO $CD8^+$ T cells also significantly suppressed day 14 alloantibody production ($p=0.001$ compared to no $CD8^+$ T cell transfer). In contrast, adoptive transfer of CXCR5 KO $CD8^+$ T cells had no effect on the amount of alloantibody produced by day 14 post transplant (p=ns compared to no $CD8^+$ T cell transfer). The failure to suppress alloantibody production by CXCR5 KO $CD8^+$ T cells was not due to a failure to become activated as demonstrated by the upregulation of IFN-γ expression by $CD8^+$ T cells from all groups (WT, CXCR3 KO and CXCR5 KO) (Figure 2B). Furthermore, the quantity of $IFN\gamma^+CD44^+CD8^+$ T cells retrieved on day 7 from recipient spleen was equivalent between WT (3,700±500 cells per million splenocytes), CXCR3 KO (3,800±700 cells per million splenocytes) and CXCR5 KO (4,400±600 cells per million splenocytes) groups suggesting equivalent $CD8^+$ T cell reconstitution. Thus, the absence of CXCR5 (but not CXCR3) expression abrogates the capacity for alloprimed $CD8^+$ T cells to suppress in vivo alloantibody production.

To avoid any potential experimental biases associated with CD8 KO hosts, we repeated these studies using WT hepatocyte transplant recipients. Alloantibody production in WT hepatocyte recipients is also CD8-regulated and a substantial increase in alloantibody titer is observed in $CD8^+$ T cell-depleted recipients.[17] Low titer alloantibody (25±5) is detected by day 5 in WT recipients and peaks on day 14 after transplant.[17] For these studies, we retrieved alloprimed $CD8^+$ T cells from C57BL/6 hepatocyte transplant hosts on day 7 post transplant and flow-sorted alloprimed $CD8^+$ T cells into $CXCR5^+CXCR3^-$ and $CXCR3^+CXCR5^-$ subsets. These $CD8^+$ T cell populations were adoptively transferred (1×10^6 cells) into WT hepatocyte transplant recipients (day 5 post transplant). Recipient mice were assayed for alloantibody titer on day 14 post transplant (9 days after adoptive transfer of alloprimed $CD8^+$ T cells). Only the $CXCR5^+CXCR3^-CD8^+$ T cell population downregulated alloantibody production ($p=0.02$ compared to WT recipients with no $CD8^+$ T cell transfer; Figure 2C).

CXCR5+CD8+ T cell-mediated suppression of alloantibody enhances hepatocyte allograft survival

In order to investigate the consequence of CD8-mediated alloantibody suppression upon hepatocyte allograft survival in a model where rejection is antibody-mediated, $CXCR5^+CXCR3^-CD8^+$ T cells were adoptively transferred into high alloantibody producing CD8 KO hepatocyte recipients.[17] Rejection in CD8 KO hepatocyte recipients is alloantibody-dependent and macrophage-mediated.[35,36] On day 5 post transplant, cohorts of CD8 KO hepatocyte recipients were adoptively transferred with 2×10^6 flow-sorted alloprimed $CXCR5^+CXCR3^-$ or $CXCR3^+CXCR5^-CD8^+$ T cells. CD8 KO recipients that received $CXCR5^+CXCR3^-CD8^+$ T cells had significantly decreased quantity of

alloantibody by day 14 post transplant compared to the control group that did not receive adoptive transfer of $CD8^+$ T cells (titer=90±40 versus titer=1,300±500 respectively; $p<0.0001$, Figure 3A). This suppression of alloantibody was accompanied by significantly enhanced graft survival post transplant (MST=32 days versus MST=14 days; p=0.002, Figure 3B). CD8 KO recipients that received $CXCR3^+CXCR5^-CD8^+$ T cells produced quantities of alloantibody (1,400±600, p=ns) similar to the control group and had similar allograft survival (MST=14 days) compared to CD8 KO recipients that did not receive $CD8^+$ T cells (alloantibody titer=1,300±500; MST=14 days, p=ns). Additionally, we find that unlike bulk alloprimed $CD8^+$ T cells, alloprimed flow-sorted $CXCR5^+CD8^+$ T cells do not initiate acute rejection when transferred into immunoincompetent RAG1 KO hepatocyte recipients (not shown). These data demonstrate that alloprimed $CXCR5^+CXCR3^-CD8^+$ T cells (but not $CXCR3^+CXCR5^-CD8^+$ T cells) significantly downregulate alloantibody production which is accompanied by prolonged allograft survival. We next evaluated the potential impact of antibody-suppressor $CXCR5^+CXCR3^-CD8^+$ T cells on quantity of alloprimed B cells and $CD4^+$ T_{FH} cells in the germinal center.

Suppression of alloantibody production by alloprimed $CXCR5^+CD8^+$ T cells is accompanied by a reduction in the quantity of Germinal Center B cells and $CD4^+$ T_{FH} cells

Germinal centers are the primary site for T follicular helper ($CD4^+$ T_{FH}) cell and primed B cell interactions, which give rise to long-lived plasma cells and memory B cells (reviewed in[37]). Since germinal center trafficking relies on CXCR5[25,26] and we previously reported that $CD8^+$ T cells kill alloprimed B cells[18], we investigated the consequence of alloprimed $CXCR5^+CD8^+$ T cells adoptive transfer upon the quantity of germinal center (GC) B cells and $CD4^+$ T_{FH} cells in WT hepatocyte transplant recipients. We analyzed the number of GC B cells ($GL\text{-}7^+B220^+$) as well as the number of IL-4 or IL-21 expressing $CD4^+$ T_{FH} cells ($CXCR5^+PD\text{-}1^+CD4^+$) on day 14 post transplant after adoptive transfer of flow-sorted $CXCR5^+CXCR3^-$ or $CXCR3^+CXCR5^-CD8^+$ T cells into WT hepatocyte transplant recipients, as described above (as in Figure 2C). We find that while adoptive transfer of $CXCR5^+CXCR3^-$ $CD8^+$ T cells significantly inhibits the quantity of GC B cells (p=0.001), $IL\text{-}4^+CD4^+$ T_{FH} cells (p=0.04), and dual $IL\text{-}4^+IL\text{-}21^+$ $CD4^+$ T_{FH} cells (p=0.003) that are detected post transplant, adoptive transfer of $CXCR3^+CXCR5^-CD8^+$ T cells does not (Figure 4A–D).

We have previously reported that in vivo $CD8^+$ T cell-mediated inhibition of alloantibody production and in vitro cytotoxicity to alloprimed self $IgG1^+$ B cell is allospecific.[18] Likewise, in these studies alloprimed $CD8^+$ T cell mediated inhibition of $CD4^+$ T_{FH} cells is allospecific as third-party primed (B10BR; $H\text{-}2^k$) $CD8^+$ T cells do not reduce the quantity of $IL\text{-}4^+$ or $IL\text{-}4^+IL\text{-}21^+$ $CD4^+$ T_{FH} cells detected after allogeneic hepatocyte transplant (FVB/N; $H\text{-}2^q$) into CD8 KO hosts ($H\text{-}2^b$; Figure S2). Further, in prior co-culture work we found that bulk alloprimed $CD8^+$ T cells do not mediate cytotoxic killing of $CD4^+$ T_{FH} cells (Figure S3A) but rather mediate IFN-γ-dependent suppression of IL-4 and IL-21 expression by alloprimed $CD4^+$ T_{FH} cells (Figure S3B). Together, these data suggest that $CXCR5^+CXCR3^-CD8^+$ T cells downregulate humoral alloimmunity by trafficking to germinal centers where they kill and/or impair GC B cells and $CD4^+$ T_{FH} cells. In order to

study monoclonal antigen-specific antibody-suppressor $CD8^+$ T cells, we performed studies using OVA-peptide-specific OT-I $CD8^+$ T cells.

Dose-dependent downregulation of antibody to OVA-peptide by OT-I antibody-suppressor $CD8^+$ T cells

Monoclonal TCR transgenic (Tg) $CD8^+$ T cells from OT-I Tg mice have specificity for OVA peptide $SIINFEKL_{257–264}$. To stimulate the development of primed OT-I $CD8^+$ T cells, CD8 KO mice were adoptively transferred with OT-I $CD8^+$ T cells and primed with mOVA lysate (membrane-bound OVA) derived from mOVA Tg mice (mOVA.B6, H-2^b). To investigate the suppressive function and dose response of unsorted OVA-primed OT-I $CD8^+$ T cells on anti-OVA antibody production, we adoptively transferred increasing numbers of OVA-primed OT-1 cells (0.5, 1, or 5 $\times 10^6$ cells) on day 0 into cohorts of WT (C57BL/6) mice immunized with mOVA.B6 lysate (day 0) and measured serum anti-OVA antibody levels on day 14. Adoptive transfer of OVA-primed OT-I $CD8^+$ T cells significantly suppressed the production of OVA-specific antibody in WT hosts ($p<0.0004$ for all quantities of $CD8^+$ T cells transferred; Figure 5A). In addition, the adoptive transfer of 5×10^6 OVA-primed OT-I $CD8^+$ T cells inhibited anti-OVA antibody production to a greater extent than 0.5 or 1.0×10^6 cells ($p<0.04$ for both). These data indicate that antigen-specific, antibody-suppressor $CD8^+$ T cells mediate downregulation of anti-OVA antibody production in a dose-dependent fashion. For reference, 5×10^6 OVA-primed WT (C57BL/6) $CD8^+$ T cells also inhibited anti-OVA antibody production following adoptive transfer into mOVA-primed WT hosts ($p=0.04$, data not shown).

Primed OT-I $CD8^+$ T cells (isolated on day 7 following mOVA lysate stimulation) were co-cultured with OVA-primed $IgG1^+$ B cells and in vitro cytotoxicity was assayed. OVA-primed OT-I $CD8^+$ T cells mediated significant cytotoxicity to OVA-primed $IgG1^+$ B cells (17±1.4%; n=6) compared to negative control cultures with no $CD8^+$ T cells (5.7±0.3%) or cultures with naïve OT-I $CD8^+$ T cells (6.3±0.3%) ($p<0.001$ for both; Figure 5B). OVA-primed OT-I $CD8^+$ T cells did not mediate cytotoxicity to FVB/N alloprimed $IgG1^+$ B cells (5.1±0.6%, p=ns compared to controls). These results suggest that primed OT-I $CD8^+$ T cells suppress OVA antibody production, in part, by mediating cytotoxic killing of OVA-primed $IgG1^+$ B cells. Next, we investigated the expression of CXCR5 and CXCR3 on OVA-primed OT-I cells and the capacity of flow-sorted subsets to mediate suppression of OVA antibody production.

OVA-primed OT-I $CXCR5^+CD8^+$ T cells inhibit anti-OVA antibody production and reduce the quantity of GC B cells, and IL-21^+CD4^+ T_{FH} cells

OT-I $CD8^+$ T cells were adoptively transferred into OVA-primed CD8 KO hosts (mOVA lysate). OVA-primed OT-I cells were retrieved on day 7 post transfer and analyzed by flow cytometry for CXCR5 and CXCR3 chemokine receptor expression. OVA-primed OT-I $CD8^+$ T cells upregulated expression of CXCR5 (11.7±1.0% of OT-I cells expressed $CXCR5^+CXCR3^-$ phenotype) and CXCR3 (17.2±0.7% of OT-I cells expressed $CXCR3^+CXCR5^-$ phenotype; Figure 6A). In order to further analyze the role of CXCR5 expression on antigen-specific antibody-suppressor $CD8^+$ T cells, OVA-primed OT-I cells were flow-sorted for $CXCR5^+CXCR3^-CD8^+$ or $CXCR3^+CXCR5^-CD8^+$ subsets (day 7 post

stimulation). These primed OT-I cell subsets (1×10^6) were adoptively transferred into OVA-primed CD8 KO hosts (day 5 post stimulation). Adoptive transfer of control unsorted, primed OT-I cells into OVA-primed CD8 KO hosts significantly inhibited anti-OVA antibody production (p=0.007). Similar to what was observed using polyclonal alloprimed T cells (Figure 2B), monoclonal OT-I $CXCR5^+CXCR3^-CD8^+$ T cells downregulated anti-OVA antibody production (p<0.0001; tested on day 14) while OT-I $CXCR3^+CXCR5^-CD8^+$ T cells did not (p=ns, Figure 6B). As a control, primed OT-I $CD8^+$ T cells do not inhibit alloantibody production in hepatocyte transplant recipients (data not shown).

When we analyzed the quantity of GC B cells and IL-21^+CD4^+ T_{FH} cells on day 14 following mOVA lysate stimulation we found that adoptive transfer of primed OT-I $CXCR5^+CXCR3^-CD8^+$ T cells significantly reduced the quantity of GC B cells (p<0.0001) and IL-21^+CD4^+ T_{FH} cells (p<0.0001) detected post transplant while adoptive transfer of primed OT-I $CXCR3^+CXCR5^-CD8^+$ T cells did not (p=ns for both; Figure 6C, D). Adoptive transfer of unsorted primed OT-I cells also inhibited the quantity of GC B cells and IL-21^+CD4^+ T_{FH} cells (p<0.008 for both). Taken together, these results indicate that OVA-primed monoclonal OT-I $CXCR5^+CD8^+$ T cells and alloprimed polyclonal WT $CXCR5^+CD8^+$ T cells manifest similar phenotype and in vivo antibody-suppressor function.

Discussion

Our group is the first to report that humoral alloimmunity is CD8-regulated and that suppression of alloantibody production occurs, in part, by killing of alloprimed self $IgG1^+$ B cells (MHC I-restricted alloantigen presentation) which is detected both in vitro and in vivo.[18] The current study demonstrates that only antigen-primed $CXCR5^+CXCR3^-CD8^+$ T cells manifest antibody-suppressor function. Furthermore, expression of CXCR5 is critical to the function of antibody-suppressor $CD8^+$ T cells as alloprimed CXCR5 KO or flow-sorted WT $CXCR5^-CD8^+$ T cells do not downregulate alloantibody production. In previous studies we found that IFN-γ is also critical to antibody-suppressor $CD8^+$ T cell function since adoptive transfer of $CD8^+$ T cells from IFN-γ KO mice (unlike IL-4 KO and WT mice) did not suppress alloantibody production.[17] Flow cytometric studies demonstrate that the majority (>70%) of alloprimed $CD44^+CXCR5^+CD8^+$ T cells express IFN-γ (Figure S1). Based on these collective findings, antibody-suppressor $CD8^+$ T cell phenotype can be refined to $CXCR5^+IFN\gamma^+CD8^+$ T cells. Our data also show that alloantibody suppression by $CXCR5^+IFN\gamma^+CD8^+$ $T_{Ab\text{-}supp}$ cells is accompanied by a reduction in the number of GC B cells and cytokine-expressing $CD4^+$ T_{FH} cells. This supports the hypothesis that $CXCR5^+IFN\gamma^+CD8^+$ $T_{Ab\text{-}supp}$ cells have a functional niche in the germinal center. This hypothesis is strengthened by the detection of human $CXCR5^+CD8^+$ T cells in lymphoid tissue as in Chu et al.'s publication which reports that human $CXCR5^+CD8^+$ T cells are present in the tonsil germinal center (and correlates with *decreased* T_{FH} and plasma cell differentiation in an in vitro co-culture).[38] $CD8^+$ $T_{Ab\text{-}supp}$ cells only mediate suppression of humoral immunity and are distinguished from $CD4^+$ T follicular regulatory (Tfr) cells which appear to both limit GC responses,[39–41] and in other circumstances augment antibody responses.[42,43] However, it is not understood what controls Tfr suppressor versus helper functions (reviewed in[44]). Furthermore, unlike $CD4^+$ T_{FR} cells, $CD8^+$ $T_{Ab\text{-}supp}$ cells do not express FoxP3 or PD-1 (Figure S1).

We observed a two- to three-fold reduction in antibody production by monoclonal and polyclonal $CD8^+$ $T_{Ab\text{-}supp}$ cells. It has been reported in human kidney transplant recipients that a two-fold reduction in donor specific antibody (by mean fluorescence intensity) correlates with a lower risk for developing AMR.[45] In our murine studies, we show that $CD8^+$ $T_{Ab\text{-}supp}$ cell mediated suppression of alloantibody production is biologically significant since it is accompanied by prolongation of allograft survival. Furthermore, the capacity for $CD8^+$ $T_{Ab\text{-}supp}$ cells to downregulate a primed humoral response is indicated by the reduction of peak alloantibody production even after adoptive transfer on day 5 after transplant.

Comparison of the alloprimed $CXCR5^+IFN\gamma^+CD8^+$ $T_{Ab\text{-}supp}$ cells reported in the current study with regulatory $CD8^+$ T cell ($CD8^+$ T_{reg}) subsets reported in the literature (reviewed in[45]) supports the conclusion that these cells are a unique subset of $CD8^+$ T cells. $CD8^+$ $T_{Ab\text{-}supp}$ cells are IFN-γ-dependent; they do not express FoxP3, IL-10, CD103, ICOSL, or PD-1 (Figure S1) and are IL-15 and IL-10 *in*dependent (data not shown). Thus, $CD8^+$ $T_{Ab\text{-}supp}$ cells are distinguished from all reported $CD8^+$ regulatory T cells including $ICOSL^+$ IL-15-producing Qa-1-restricted $CD8^+$ T cells which regulate autoreactive Qa-1^+CD4^+ T cells,[46,47] IFN-γ-*in*dependent $CD8^+$ T_{reg} cells which suppress IgE in allergy models,[48,49] and $FoxP3^+CD8^+$ T_{reg} cells which inhibit proliferation[50,51] or cytokine production[52,53] of $CD4^+$ T cells. They do not resemble $CD8^+$ T_{reg} cells that are reported to be IL-10-dependent[54–57] or $CD103^+CD8^+$ T_{reg} cells associated with spontaneous liver tolerance[58]; T_{reg} cells express FoxP3 and TGF-β but not IFN-γ.[58–61] Importantly, none of these other $CD8^+$ T cell subsets have been reported to kill B cells or directly suppress B cell function.

Anti-viral $CD8^+$ T cells that develop during acute infection do not express CXCR5[62,63] and instead require CXCR3 to recruit to the infection site.[27–30] In chronic viral infections, "exhausted" $CXCR5^+CD8^+$ T cells have been detected in the T cell (not B cell) zones of lymph nodes, express high levels of the inhibitory molecule PD-1 and exhibit stem cell like properties proliferating and differentiating from $CXCR5^+CD8^+$ T cells into $CXCR5^-CD8^+$ T cells.[64–67] However, in the current studies $CD8^+$ $T_{Ab\text{-}supp}$ cells develop in response to acute antigen stimulation and these cells do not express PD-1. Recently an $IL\text{-}21^+CXCR5^+ICOS1^+PD\text{-}1^+$ $CD8^+$ T cell subset was reported to acquire $CD4^+$ T_{FH} functions and weakly enhance production of autoantibody; however these cells are not similar to $CD8^+$ $T_{Ab\text{-}supp}$ cells since they *enhance* rather than suppress antibody production and are only detected in mice with $CD4^+$ T_{reg} deficits (such as IL-2 KO and scurfy mice) and are not detected in WT mice.[68] Thus the $CXCR5^+IFN\gamma^+CD8^+$ $T_{Ab\text{-}supp}$ cells reported in the current studies differ from other $CXCR5^+CD8^+$ T cell subsets by phenotype ($PD\text{-}1^-$) and function (B cell killing and antibody suppression).[69–71] Overall, our work to date indicates that $CD8^+$ $T_{Ab\text{-}supp}$ cells are Ag-specific,[18] IFN-γ-dependent,[17] self-MHC Class I-restricted,[18] $CXCR5^+CXCR3^-IFN\text{-}\gamma^+$ and are phenotypically and functionally distinguished from all other $CD8^+$ T cell subsets reported in the literature.

While our published studies report the activity of this novel $CD8^+$ $T_{Ab\text{-}supp}$ cell subset after hepatocyte transplant,[17,18,22] we have subsequently determined that their activity is also observed following islet and skin transplantation (not shown). These $CD8^+$ $T_{Ab\text{-}supp}$ cells are likely also important for regulation of humoral alloimmunity after kidney, heart, and aorta

transplant since others have reported increased levels of alloantibodies in these recipients when they are CD8-deficient.[72–74] Similarly, CD8-deficient mice exhibit increased antigen-specific antibody production in mouse models of allergy, bacterial infection, viral infection, and platelet transfusion.[18,73–81] In the current studies we extended our investigations with polyclonal alloreactive $CD8^+$ T cells to monoclonal OT-I TCR transgenic $CD8^+$ T cells. Our results demonstrate that antigen-specific OT-I $CXCR5^+CD8^+$ T cells suppress humoral immunity and similar to studies with polyclonal alloprimed $CD8^+$ $T_{Ab\text{-}supp}$ cells this is accompanied by a reduction in GC B cells and $CD4^+$ T_{FH} cells. Thus, activity of $CD8^+$ $T_{Ab\text{-}supp}$ cells is not limited to alloimmune responses and likely these cells play a critical role in regulation of humoral immunity more broadly.

In conclusion, these studies for the first time clarify the critical role of CXCR5 and the distinct $CXCR5^+IFN\gamma^+$ ($FoxP3^-IL\text{-}10^-CD103^-ICOSL^-PD\text{-}1^-$) phenotype of this novel $CD8^+$ $T_{Ab\text{-}supp}$ cell subset that mediates antigen-specific suppression of humoral immunity. We extend prior studies reporting that these cells mediate cytotoxic killing of self $IgG1^+$ B cells[18] by correlating the in vivo suppression of antibody production with a concomitant reduction in germinal center B cells and $CD4^+$ T_{FH} cells. Interestingly, we have reported that $CD8^+$ T cell antibody-suppressor function is impaired by calcineurin inhibition but not mTOR inhibition[22] both commonly used for maintenance immunosuppressive therapy in clinical transplantation. The implication is that suppression of de novo post transplant alloantibody production may be optimized with mTOR inhibition, in part, by preservation of antibody-suppressor $CD8^+$ T cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations Page

AT	adoptive transfer
AMR	antibody-mediated rejection
DSA	donor specific antibody
hA1AT	human alpha-1 antitrypsin
IFN	interferon
IL	interleukin
I.P.	intraperitoneal
KO	knockout

MHC	major histocompatibility complex
MFI	mean fluorescence intensity
MST	median survival time
PI	propidium iodide
T_{FH}	T follicular helper
Tg	transgenic
WT	wild-type

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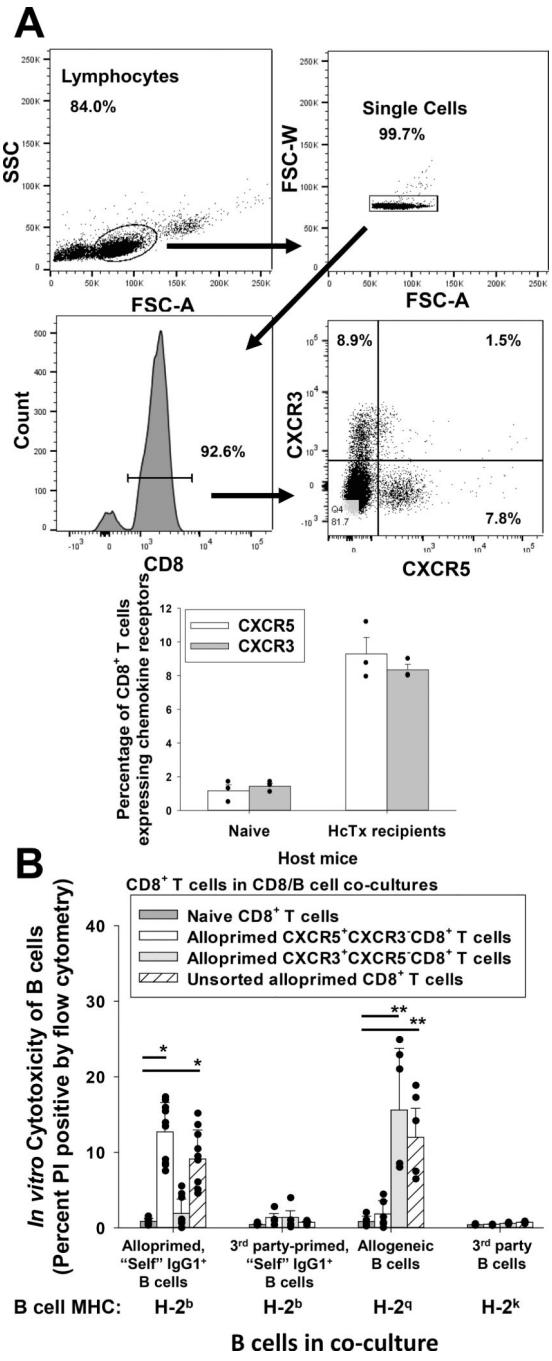

Figure 1. CXCR5$^+$CD8$^+$ T cells mediate in vitro cytotoxicity of alloprimed self IgG$^+$ B cells.
C57BL/6 (wild-type, WT; H-2^b) mice were transplanted with FVB/N (H-2^q) hepatocytes. On day 7 post transplant, splenic CD8$^+$ T cells were retrieved and purified. **A)** Flow cytometric analysis of purified CD8$^+$ T cells shows that CXCR5$^+$CXCR3$^-$ (9.3±1.0%) and CXCR3$^+$CXCR5$^-$ (8.4±0.3%) CD8$^+$ T cell subsets are detected (CD8$^+$ T cells pooled from 10–15 mice) for cell sorting; data for n= 3 sorts are shown in the bar graph. **B)** In an in vitro cytotoxicity assay, flow-sorted alloprimed CXCR5$^+$CXCR3$^-$ or CXCR3$^+$CXCR5$^-$ CD8$^+$ T cell populations and B cell targets were co-cultured at a 10:1 ratio for 4 hours and analyzed

for cytotoxicity (propidium iodide (PI) uptake). Naïve $CD8^+$ T cells and unsorted alloprimed $CD8^+$ T cells were utilized as negative and positive controls (effector cells), respectively. Significant cytotoxicity of alloprimed, self $IgG1^+$ B cells was observed in co-cultures with $CXCR5^+CXCR3^-CD8^+$ T cells (12.7±1.8%, n=10) or with unsorted alloprimed $CD8^+$ T cells (9.1±3.8, $p<0.0001$ for both signified by "*"; n=10) in comparison to co-cultures with naïve $CD8^+$ T cells (0.9±0.4%, n=9) but no significant cytotoxicity was detected in co-cultures with $CXCR3^+CXCR5^-CD8^+$ T cells (1.9±1.9%, p=ns, n=10). Significant cytotoxicity of allogeneic B cells was observed in co-cultures with $CXCR3^+CXCR5^-CD8^+$ T cells (15.6±8.8%; n=6) or with unsorted alloprimed $CD8^+$ T cells (12.0±5.5%; n=6, $p<0.002$ for both signified by "**"), but not with $CXCR5^+CXCR3^-CD8^+$ T cells (1.8±1.8%; n=6, p=ns) in comparison to control co-cultures with naïve $CD8^+$ T cells (0.8±0.7%; n=5). No significant cytotoxicity was detected against third-party (3^{rd}) party primed "self" $IgG1^+$ B cells (H-2^b targets) or 3^{rd} party B cells (H-2^k targets) in any co-cultures. Error bars indicate standard error from triplicate experiments.

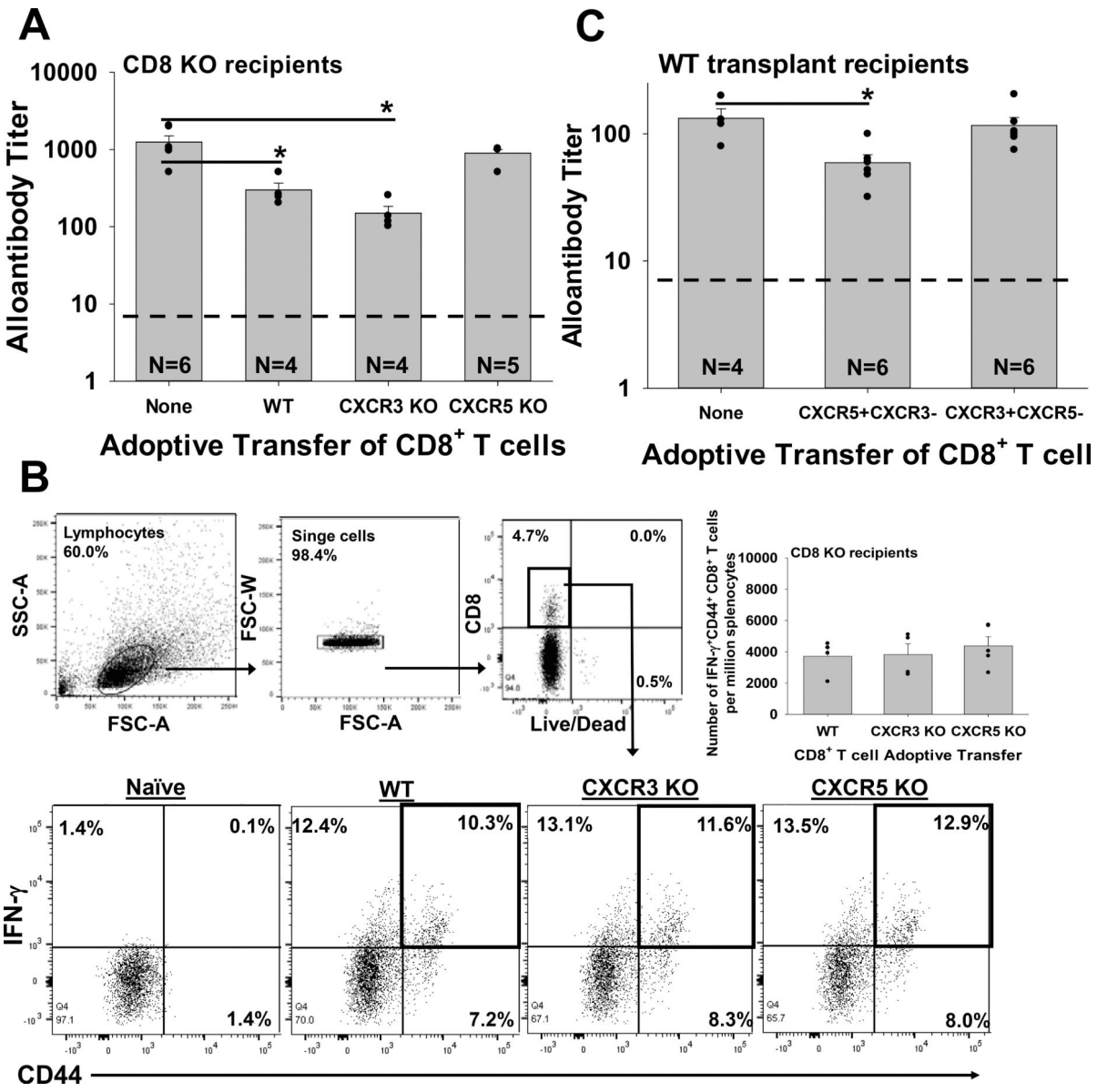

Figure 2. CXCR5 is critical to CD8$^+$ T cell-mediated suppression of antibody production.

A) CD8 KO mice were transplanted with FVB/N hepatocytes. On day 0, recipients were adoptively transferred (AT) with 10×10^6 naïve CD8$^+$ T cells (WT, CXCR5 KO or CXCR3 KO). Recipient serum was analyzed for alloantibody titer on day 14 post transplant. Significantly reduced alloantibody titer was observed in transplant mice that received AT of WT (300±70; n=4) or CXCR3 KO (150±30; n=4) CD8$^+$ T cells ($p<0.004$ for both signified by "*") compared to recipients that received AT with CXCR5 KO CD8$^+$ T cells (900±100; n=5) or no AT (1,250±250; n=6). **B)** All groups of recipients that underwent transplant and adoptive transfer of WT, CXCR3 KO or CXCR5 KO CD8$^+$ T cells developed alloprimed CD8$^+$ T cells that were activated. Flow cytometric analysis gating on total leukocytes and CD8$^+$ T revealed that by day 7 post transplant, approximately 10–13% of CD8$^+$ T cells from all three groups (WT, CXCR3 KO, CXCR5 KO) of AT mice were IFN-γ^+CD44$^+$CD8$^+$ T cells; similarly the quantity of IFN-γ^+CD44$^+$CD8$^+$ T cells per million splenocytes was

similar in all three groups. **C)** WT mice were transplanted with FVB/N hepatocytes and analyzed for production of alloantibody on day 14 post transplant. Transplant recipients that received AT (on day 5 post transplant) of flow-sorted (day 7) alloprimed $CXCR5^{+}CXCR3^{-}$ WT $CD8^{+}$ T cells exhibited significant reduction of alloantibody titer (60±10; n=5) compared to recipients which received AT of flow-sorted (day 7) alloprimed $CXCR3^{+}CXCR5^{-}CD8^{+}$ T cells (120±20; n=6) or no AT (130±20; n=4, $p<0.02$ for both signified by "*"). The dashed line on Figures 2A and 2C represents negative control sera from naïve mice. Error bars indicate standard error from duplicate experiments.

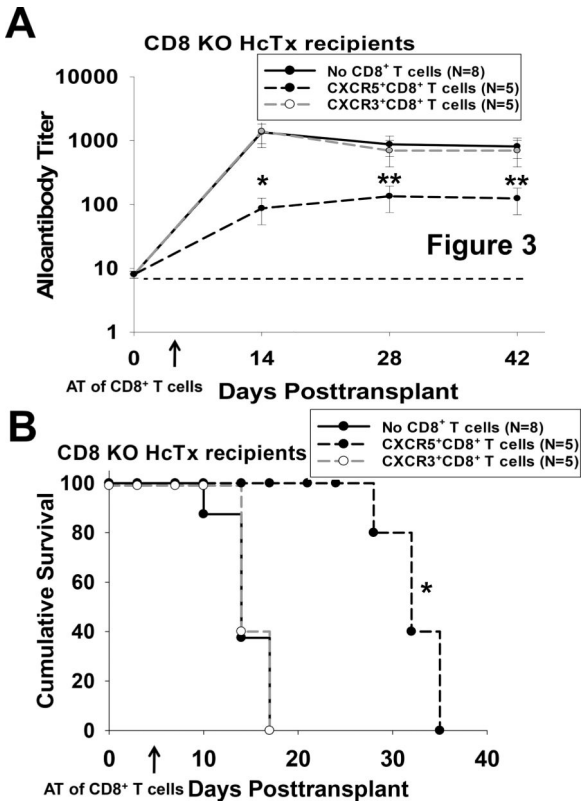

Figure 3. CXCR5$^+$CD8$^+$ T cell-mediated suppression of alloantibody enhances hepatocyte allograft survival.

CD8 KO mice were transplanted with FVB/N hepatocytes. On day 5, cohorts of transplant recipients received adoptive transfer of flow-sorted (day 7) alloprimed CXCR5$^+$CXCR3$^-$CD8$^+$ T cells, CXCR3$^+$CXCR5$^-$CD8$^+$ T cells, or no CD8$^+$ T cells. Mice were observed for alloantibody production and hepatocyte survival. **A)** Adoptive transfer of CXCR5$^+$CXCR3$^-$CD8$^+$ T cells into CD8 KO recipients significantly inhibited alloantibody titer on day 14 (90±40; n=5, $p<0.0001$ signified by "*") compared to no AT (1,300±500; n=8) while adoptive transfer of CXCR3$^+$CXCR5$^-$CD8$^+$ T cells did not (1,400±600; n=5, p=ns). Alloantibody titer remained suppressed after day 14 in CD8 KO mice that received AT of CXCR5$^+$CXCR3$^-$CD8$^+$ T cells ($p<0.005$ for days 28 and 42 post transplant, "**"). The dashed line represents negative control sera from naïve mice. Error bars indicate standard error. **B)** CD8 KO hepatocyte recipients that received adoptive transfer of CXCR5$^+$CXCR3$^-$CD8$^+$ T cells had prolonged allograft survival (MST=day 32; p=0.002, signified by "*") compared to those that received AT of CXCR3$^+$CXCR5$^-$CD8$^+$ T cells (MST= day 14) or those with no AT (MST= day 14).

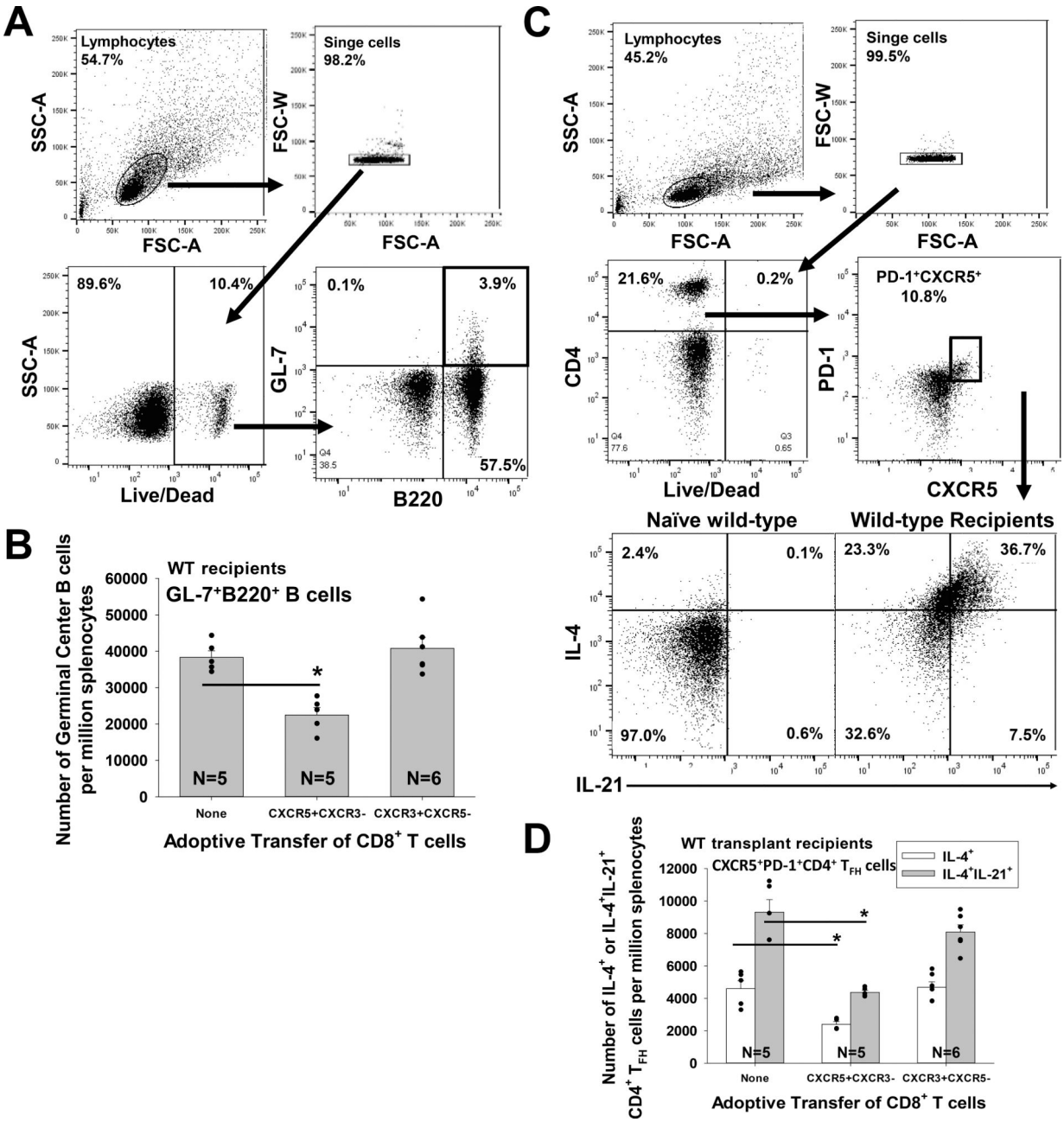

Figure 4. Suppression of alloantibody production by alloprimed CXCR5$^+$CD8$^+$ T cells is accompanied by a reduction in the quantity of Germinal Center B cells and CD4$^+$ T$_{FH}$ cells.

WT mice were transplanted with FVB/N hepatocytes (day 0) and on day 5 received adoptive transfer (AT) of sorted alloprimed CXCR5$^+$CXCR3$^-$CD8$^+$ or CXCR3$^+$CXCR5$^-$CD8$^+$ T cells. On day 14 post transplant, recipient splenocytes were analyzed for the number of germinal center (GC) B cells and CD4$^+$ T$_{FH}$ by flow cytometry. **A)** GC B cells (GL-7$^+$B220$^+$) were analyzed by gating on lymphocytes. **B)** Recipients which received AT of CXCR5$^+$CXCR3$^-$CD8$^+$ T cells exhibited significantly reduced number of GC B cells (22,000±2,000 per million splenocytes; n=5) compared to WT recipients without AT (38,000±2,000 per million splenocytes; n=5, p=0.001 signified by "*"). In contrast, the

number of GC B cells was not significantly altered in recipients which received AT of $CXCR3^{+}CXCR5^{-}CD8^{+}$ T cells (41,000±3,000 per million splenocytes; n=6, p=ns) compared to control recipients without $CD8^{+}$ T cell transfer. **C)** $CD4^{+}$ T_{FH} cells were analyzed by gating on lymphocytes, $CD4^{+}$ T cells, and $PD\text{-}1^{+}CXCR5^{+}$ cells. **D)** Recipients which received AT of $CXCR5^{+}CXCR3^{-}CD8^{+}$ T cells exhibited significantly reduced number of $IL\text{-}4^{+}IL\text{-}21^{+}$ $CD4^{+}$ T_{FH} cells (4,400±100 per million splenocytes; n=5, p=0.003 signified by "*") compared to WT recipients without AT (9,300±800 per million splenocytes; n=5). AT of $CXCR5^{+}CXCR3^{-}CD8^{+}$ T cells was associated with a reduced number of $IL\text{-}4^{+}$ $CD4^{+}$ T_{FH} cells (2,400±100 per million splenocytes; n=5, p=0.04) compared to control recipients without $CD8^{+}$ T cell transfer (4,600±500 per million splenocytes). In contrast, the number of $IL\text{-}4^{+}$ (4,600±300 per million splenocytes; n=6) and $IL\text{-}4^{+}IL\text{-}21^{+}$ $CD4^{+}$ T_{FH} cells (8,100±500 per million splenocytes; n=6) was not significantly altered after AT of $CXCR3^{+}CXCR5^{-}CD8^{+}$ T cells compared to the control group with no $CD8^{+}$ T cell transfer (p=ns for both $IL\text{-}4^{+}$ and $IL\text{-}4^{+}IL\text{-}21^{+}$ $CD4^{+}$ T_{FH} cells). Error bars indicate standard error from duplicate experiments.

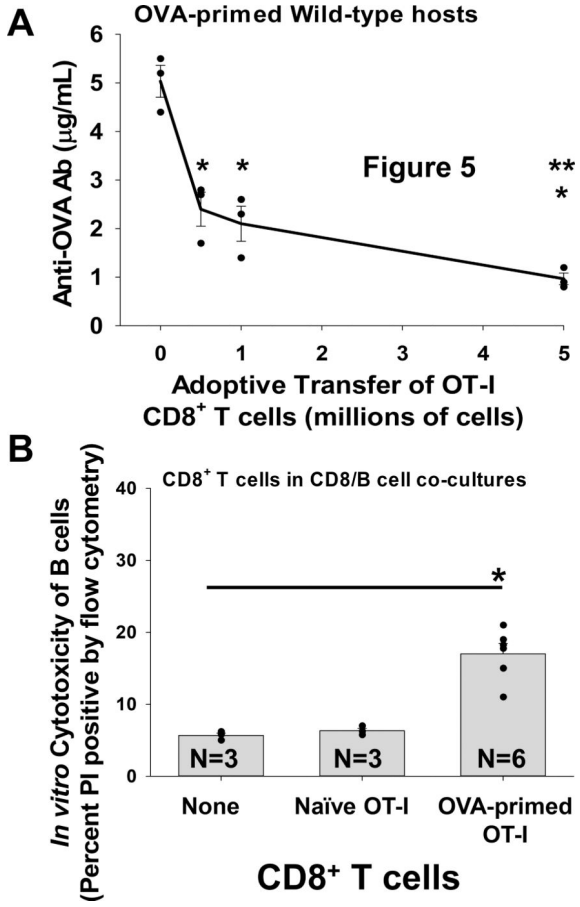

Figure 5. OVA-primed OT-I CD8$^+$ T cells suppress anti-OVA antibody production in a dose-dependent manner and mediate in vitro cytotoxicity of OVA-primed B cells.

A) WT mice were primed with mOVA lysate (i.p. injection) on day 0. Cohorts of WT mice received adoptive transfer (AT) on day 0 of increasing quantities of OVA-primed OT-I TCR transgenic CD8$^+$ T cells (0, 0.5, 1, 5×10^6 cells i.v.). Mouse serum was assayed for anti-OVA antibodies (Ab) by ELISA on day 14 following antigen stimulation. WT mice primed with mOVA lysate exhibit maximal levels of serum anti-OVA antibodies on day 14 following antigen stimulation (5.0±0.3 μg/mL; n=3). AT of 0.5, 1, or 5×10^6 OVA-primed OT-I CD8$^+$ T cells on day 0 inhibited anti-OVA antibody production in these mice (2.4±0.4, 2.1±0.4, and 1.0±0.1 μg/mL respectively; n=3 and $p<0.0004$ for all groups signified by "*"). The AT of 5×10^6 OVA-primed OT-I CD8$^+$ T cells inhibited anti-OVA antibody production significantly more than 0.5 or 1×10^6 OVA-primed OT-I CD8$^+$ T cells ($p<0.04$ for both signified by "**"). **B)** OVA-primed OT-I CD8$^+$ T cells or naïve OT-I CD8$^+$ T cells were co-cultured with OVA-primed IgG1$^+$ B cell targets. CD8$^+$ T cells and B cells were co-cultured at a 10:1 ratio for 4 hours and analyzed for cytotoxicity. Significant cytotoxicity of OVA-primed IgG1$^+$ B cells was observed in co-cultures with OVA-primed OT-I CD8$^+$ T cells (17±1.4%; n=6, $p<0.001$ signified by "*") compared to negative control cultures with no CD8$^+$ T cells (5.7±0.3%) or with naïve OT-I CD8$^+$ T cells (6.3±0.3%). Error bars indicate standard error.

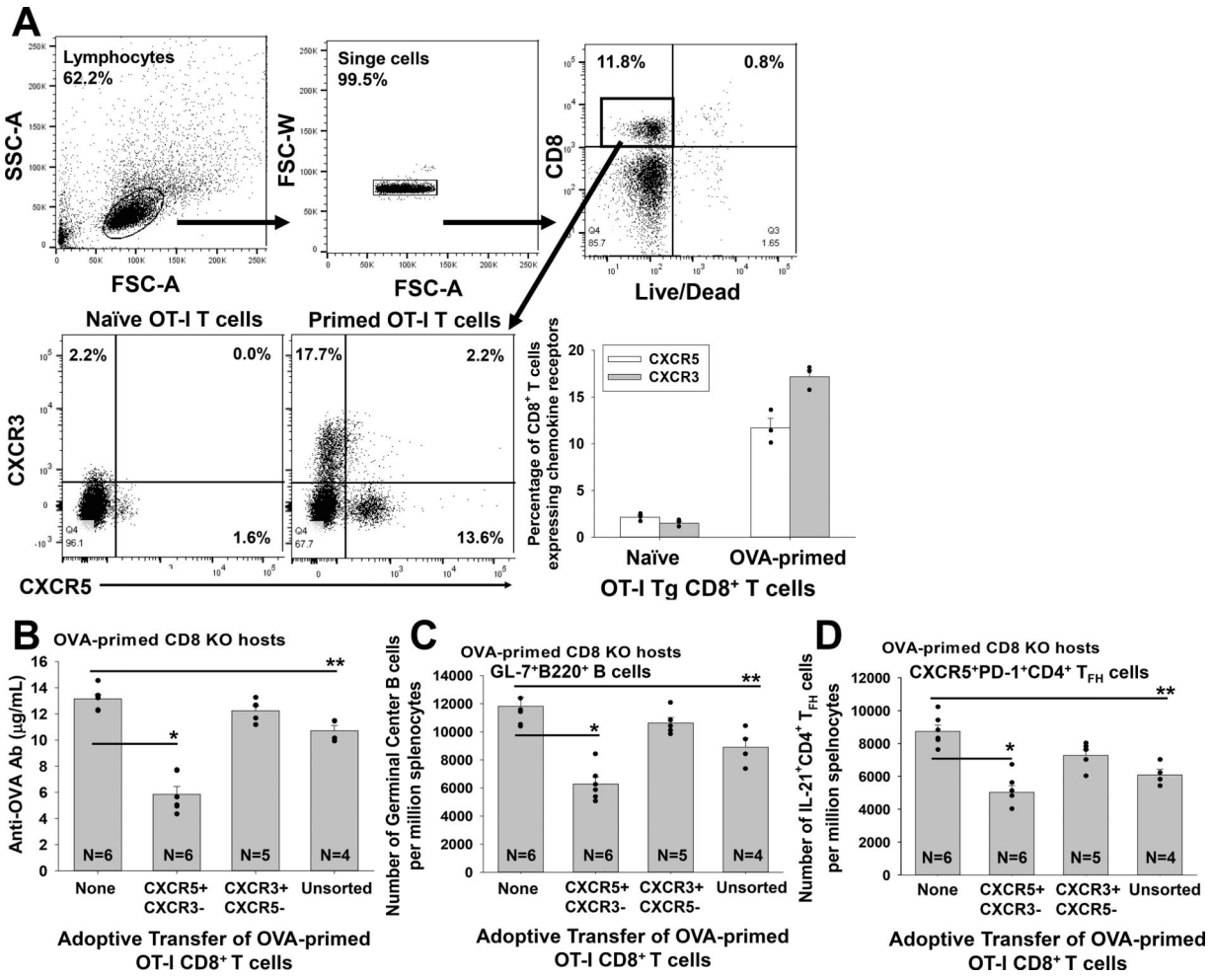

Figure 6. OVA-primed OT-I CXCR5$^+$CD8$^+$ T cells mediate suppression of anti-OVA antibody production accompanied by reduced quantity of Germinal Center B cells and IL-21$^+$CD4$^+$ T$_{FH}$ cells.

A) Naïve OT-I CD8$^+$ T cells were adoptively transferred (AT) into OVA-primed CD8 KO recipients. 7 days later, OT-I CD8$^+$ T cells were isolated and analyzed by flow cytometry for expression of CXCR5 and CXCR3. A representative flow plot shows OVA-primed OT-I CD8$^+$ T cells include CXCR5$^+$CXCR3$^-$ (11.7±1.0%) and CXCR3$^+$CXCR5$^-$ (17.2±0.7%) OT-I CD8$^+$ T cell subsets. **B)** Unsorted or flow-sorted CXCR5$^+$CXCR3$^-$ or CXCR3$^+$CXCR5$^-$ OVA-primed OT-I CD8$^+$ T cells (1×10^6) were adoptively transferred into OVA-primed CD8 KO mice (day 5 post mOVA lysate stimulation). The amount of anti-OVA Ab produced in mice that received AT of CXCR5$^+$CXCR3$^-$ OT-I CD8$^+$ T cells (5.8±0.6 µg/mL; n=6, p<0.0001 signified by "*") or unsorted OT-I CD8$^+$ T cells (10.7±0.4 µg/mL, n=4, p=0.008 signified by "**") was significantly less than the amount produced in CD8 KO recipients without AT of CD8$^+$ T cells (13.4±0.3 µg/mL, n=6). AT of CXCR3$^+$CXCR5$^-$ OT-I CD8$^+$ T cells did not suppress anti-OVA antibody production (12.2±0.4 µg/mL; n=5, p=ns). **C)** The quantity of recipient spleen germinal center (GC) B cells (GL-7$^+$B220$^+$) and IL-21$^+$CD4$^+$ T$_{FH}$ cells (IL-21$^+$CXCR5$^+$PD-1$^+$CD4$^+$) was analyzed in OVA-primed CD8 KO mice (day 14 following mOVA lysate stimulation). The quantity of GC B cells in mice that received AT of CXCR5$^+$CXCR3$^-$ OT-I CD8$^+$ T cells (6,200±500 per million splenocytes;

n=6, p<0.0001 signified by "*") or with unsorted OT-I $CD8^+$ T cells (8,900±700; n=4, p=0.008 signified by "**") was significantly less than the quantity in CD8 KO recipients without AT (11,800±700 per million splenocytes; n=6). In contrast, AT of $CXCR3^+CXCR5^-$ OT-I $CD8^+$ T cells was not associated with reduction in the quantity of GC B cells (10,600±400 per million splenocytes; n=6, p=ns). **D)** The quantity of IL-21^+ $CD4^+$ T_{FH} cells in mice that received AT of $CXCR5^+CXCR3^-$ OT-I $CD8^+$ T cells (5,000±400 per million splenocytes; n=6, p<0.0001 signified by "*") or unsorted OT-I $CD8^+$ T cells (6,100±300, n=4, p=0.001 signified by "**") was significantly reduced compared to the quantity in CD8 KO recipients without AT (8,800±400 per million splenocytes; n=6). In contrast, AT with $CXCR3^+CXCR5^-$ OT-I $CD8^+$ T cells was not associated with a reduction in the quantity of IL-21^+ $CD4^+$ T_{FH} cells (7,200±400 per million splenocytes; n=6, p=ns). Error bars indicate standard error from duplicate experiments.