Antibody-suppressor $CD8^+$ T cells require IFN-γ and $CD4^+$ T cells for optimal development and effector function

Undergraduate Honors Research Thesis

Presented in Partial Fulfillment of the Requirements of Graduation with Honors Research Distinction in Biomedical Engineering at The Ohio State University

By

Madison Hart

The Ohio State University

April 2021

Project Advisors: Dr. Ginny Bumgardner, Department of Surgery, and Dr. Keith Gooch, Department of Biomedical Engineering

Abstract

Antibody-mediated rejection is a significant contributor to organ transplant failure. The Bumgardner Lab has recently discovered a novel subset of $CD8^+$ T cells ($CD8^+$ $T_{Ab\text{-}supp}$ cells) that express CXCR5 and suppress transplant-specific antibody (alloantibody), which results in prolonged transplant survival in mouse models. To further understand these cells, we set out to investigate the requirements for $CXCR5^+CD8^+$ $T_{Ab\text{-}supp}$ cell development. Previous reports suggest that IFN-γ cytokine may be critical for the development of $CD8^+$ $T_{Ab\text{-}supp}$ cells, and I further hypothesized that $CD4^+$ T cells are also important for their development. To investigate these hypotheses, wild-type mice or mice lacking IFN-γ, IFN-γ receptor (IFN-γR), and/or $CD4^+$ T cells were stimulated with allogeneic antigen. In some cohorts, naïve $CD8^+$ T cells were adoptively transferred (AT) into stimulated mice. One week following stimulation, cells were isolated from the spleens of stimulated mice and analyzed for the $CD8^+$ $T_{Ab\text{-}supp}$ phenotype (CXCR5), activation (CD44), proliferation, and cytotoxic effector molecules. To date, we have found that the expression of CXCR5 on $CD8^+$ T cells (wild-type, 13.5%) was downregulated in mice that lacked $CD4^+$ T cells (6.8%; 2-fold) or IFN-γ (8.5%; 1.5-fold). While activation was not affected by IFN-γ or $CD4^+$ T cells, proliferation of wild-type $CXCR5^+CD8^+$ T cells is dependent on host expression of IFN-γ (wild-type=33.5% versus IFN-γ KO=19.7%). In addition, the cytotoxic effector phenotype of activated $CXCR5^+CD8^+$ T cells is nearly abrogated when hosts are IFN-γ deficient (Lamp1: WT=70.0% versus IFN-γ KO=6.7%; FasL: WT=36.5% versus IFN-γ KO=6.7%).

To date, experiments using mutant $CD8^+$ T cells adoptively transferred into wild-type hosts show no variance in CXCR5 expression, activation, or effector molecule expression. Thus, our current data, despite relatively small sample sizes, suggests a critical role for both host IFN-γ expression and $CD4^+$ T cells in maximizing CXCR5 expression on $CD8^+$ T cells as well as

proliferation and the expression of cytotoxic effector molecules on $CXCR5^{+}CD8^{+}$ T cells. Our current hypothesis is that IFN-$\gamma^{+}CD4^{+}$ T cells are critically important for the development of $CXCR5^{+}CD8^{+}$ $T_{Ab\text{-}supp}$ cells, and ongoing studies will continue to investigate the influence these factors have on the novel cell subset.

Acknowledgements

I would like to thank Dr. Ginny Bumgardner, my research advisor, for the opportunity to pursue this thesis project and to work in her laboratory during my undergraduate career at The Ohio State University. Her mentorship and encouragement throughout my independent research endeavor and over the last two years has been invaluable and provided me with a strong foundation to make significant contributions to science as I enter the medical field. I would also like to thank Dr. Jason Zimmerer for his guidance on this project and throughout my time in the Bumgardner Lab. His dedication and patience to teach me the necessary skills to be a successful undergraduate researcher were essential to my growth as a student and a scientist, and I am incredibly grateful to have learned from him every day in the lab. Furthermore, I would like to express my gratitude to Dr. Keith Gooch for serving as a member on my Thesis Defense committee and as an engineering mentor throughout this project, as well as the National Institute of Health and the College of Engineering for funding this research project.

Additionally, I would like to thank the current members of the Bumgardner Lab, Sachi Chaudhari, Chelsea Peterson, and Jing Han, for their assistance during these experiments, as well as Bryce Ringwald for his feedback during the writing process. These individuals made this project and the duration of my time in the lab an enjoyable and gratifying experience while challenging me to become a better researcher. Finally, I would like to thank my family as well as Sakhi Patel, Avery Scholl, and Kelly Tobin for their steadfast support beyond the lab space. Their encouragement and patience helped make this thesis possible, and I am incredibly grateful for their faith in me and this project throughout the past year.

Table of Contents

Background

Organ transplantation is an efficacious therapeutic strategy for people suffering from organ damage or end-stage organ failure (1). The replacement of damaged tissue with healthy donor tissue has prolonged survival and improved the quality of life for these patients, but rejection, namely antibody-mediated rejection (AMR), of the transplanted tissue by the recipient's immune system continues to be an obstacle in the long-term success of transplantation (2-4). The immune system is stimulated by foreign proteins from the transplanted tissue, namely major histocompatibility complex molecules, or MHC, and the immune-mediated responses can occur through cellular-mediated rejection or AMR (5). To date, cellular immune responses are largely inhibited by immunosuppressive therapy. However, despite improvements, recipients can still produce antibodies that target the transplant (also known as "alloantibodies") (6). Antibody-mediated rejection occurs when a recipient's B cells secrete alloantibodies that recruit cytotoxic complexes (complement) and other immune cells expressing cytotoxic molecules to kill allogeneic cells, compromising the transplanted organ (7). This mechanism is a major contributor to graft rejection, and therefore a deeper understanding of the AMR pathway is necessary to develop new and effective immunosuppressive therapies.

The focus of our research is to gain a clearer understanding of the immune mechanisms contributing to alloantibody production and AMR, and to find highly specific interactions that can interrupt AMR without compromising protective immunity. Current therapies for AMR prevention consist of immunosuppressive drugs that weaken all immune system mechanisms, which can leave patients vulnerable to additional infections and cardiovascular risks (8, 9). An alternative to this approach is finding therapies that specifically target and suppress AMR-related immune responses, namely alloantibody production (10). Our lab has previously observed that mice deficient in $CD8^+$

T cells have a significant increase in alloantibody compared to wild-type recipients, which indicated that $CD8^+$ T cells play a critical role in the suppression of alloantibody (11). We further found that $CD8^+$ T cells activated against transplanted cells (alloprimed) used cytotoxic mechanisms to kill alloantibody producing B cells, which led to decreased alloantibody (12). Subsequent experiments led to the discovery of a novel subset of $CD8^+$ T cells that express $CXCR5^+$, which is a receptor required for the recruitment of cells to the germinal centers of lymphoid tissue. In the germinal center (GC), GC B cells are major contributors to the production of antibody (13). Our lab has recently found that $CXCR5^+CD8^+$ T cells are critical to the reduction of alloantibody (14) and specifically kill antibody-producing GC B cells (unpublished observations). Our lab refers to this cell subset as antibody-suppressor $CD8^+$ T cells ($CD8^+$ $T_{Ab\text{-}supp}$ cells). The role of these cells may be clinically relevant as $CXCR5^+CD8^+$ T cells are also detectable in the peripheral blood of human kidney transplant recipients, and the quantity of these cells was shown to be inversely associated with the incidence of alloantibody production (15). Additionally, it has been previously reported that in the absence of $CD8^+$ T cells, antibody production is enhanced in a variety of models including viral infection (16), bacterial infection (17), allergy (18, 19), transplant (20-23), and platelet transfusion (24), which suggests that the role of antibody-suppressive $CD8^+$ T cells has a wide impact on immune responses. Thus, further investigation of these cells is warranted in both mouse models and human patients.

One area that requires further investigation is how $CXCR5^+CD8^+$ T cells develop following antigen stimulation. We have previously reported that $CD8^+$ T cells from an interferon-gamma (IFN-γ)-deficient host did not inhibit alloantibody in hepatocyte or kidney transplanted mice (11). We also considered the potential involvement of $CD4^+$ T cells in the developmental process since we have previously reported that rejector $CD8^+$ T cells (mediators of cellular rejection) require

$CD4^+$ T cells for optimal activity (25), but it is currently unknown whether the development of $CD8^+$ $T_{Ab\text{-}supp}$ cells requires $CD4^+$ T cell-mediated help. Based on this knowledge, we hypothesized that $CD4^+$ T cells and IFN-γ are both critical for the development, activation, proliferation, and effector function of $CD8^+$ $T_{Ab\text{-}supp}$ cells. To address these developmental questions, we utilized mice from different strains, including wild-type (normal) and mice lacking IFN-γ (IFN-γ knock out, KO), the receptor to IFN-γ (IFN-γR KO) or $CD4^+$ T cells (CD4 KO or CD4-depletion). Following allogeneic protein stimulation, mice were tracked for the development of $CXCR5^+CD8^+$ T cells.

Methods

Mouse strains

C57BL/6 (wild-type; WT), CD4 knockout (KO), IFN-γ KO, IFN-γR KO, Jα18 KO, GFP transgenic (Tg), OT-I Tg x GFP Tg, mOVA Tg (all H-2^b, Jackson, Bar Harbor, ME), and FVB/N (H-2^q, Taconic, Hudson, NY) mouse strains were used in these studies. FVB/N and mOVA mice were the source of lysate used to stimulate the immune response in other mouse strains. Mice that were used as experimental hosts or as a source of donor cells were at least 6 weeks old, and all experiments were performed in accordance with the Institutional Animal Care and Use Committee of the Ohio State University (Protocol 201900000124).

T cell isolation for adoptive transfer

Splenocytes were isolated from mice, and $CD8^+$ or $CD4^+$ T cells were isolated from the bulk splenocytes with a negative selection column per manufacturer instructions (STEMCELL Technologies, Cambridge, MA; purity routinely >95%). A cohort of $CD8^+$ T cells were stained with CellTrace Violet (Thermo Fisher, Walther, MA) at a dose half of the manufacturer's recommendation (2.5 μM, mean fluorescent intensity (MFI) > 100-fold versus unstained).

In vitro cytotoxicity assay

Splenic $CD8^+$ T cells were co-cultured with CFSE-stained target B cells at a 10:1 ratio for 4 hours at 37°C / 5% CO_2. Propidium iodide (PI) was added to the cultures to measure cell death, and PI uptake by $CFSE^+$ B cells was analyzed with flow cytometry. A LIVE/DEAD cell-mediated cytotoxicity kit (Invitrogen, Eugene, OR) was used to measure cytotoxicity according to the manufacturer's instructions.

Lysate preparation

Livers were isolated from FVB/N (H-2^q) and mOVA (H-2^b) mice and placed in PBS (1 liver/4 mL PBS). The livers were diced and freeze/thawed for five cycles (-80°C freezing; room temperature thaw). The final solution was spun down to remove debris from the supernatant. Protein concentration was analyzed by the Bradford Coomassie brilliant blue assay and quantitated by spectrometry (Thermo Scientific Multiskan GO).

Allogeneic stimulation of host mice and adoptive transfer

Recipient mice were primed with allogeneic, FVB/N liver lysate or syngeneic, mOVA liver lysate through intraperitoneal injection (2 mg) on day 0. Several cohorts of mice were adoptively transferred with naïve $CD8^+$ T cells stained with CellTrace Violet or naïve $CD4^+$ T cells through a tail vein injection alongside the lysate stimulation. Additional cohorts of mice were depleted of $CD4^+$ T cells with anti-CD4 monoclonal antibody (GK1.5; Bioexpress Cell Culture Services, West Lebanon, New Hampshire) through intraperitoneal injection (250 μg, day -3, -1).

Flow cytometric analysis of lymphocytes and intracellular molecule staining

Splenocytes were isolated from the recipient mice (day 7 post-FVB/N or mOVA lysate treatment) and underwent a four-hour incubation (37°C, 5% CO2) with Leukocyte Activation Cocktail (2 μL/mL; PMA, ionomycin, and Brefeldin A; Becton Dickinson) or monesin (Thermo Fisher). Splenocytes were then stained for CD8 (Clone #53-6.7) and extracellular markers for chemokine receptors (CXCR5, clones #FAB6198G, 2G8, MU5UBEE, and L138D7), activation (CD44, clone #IM7; CD62L, clone #MEL-14), and effector molecules (Lamp1, clone #1D4B; FasL, clone #MFL3). The FIX&PERM cell permeabilization kit (Thermo Fisher) was added to the splenocytes,

and intracellular staining was performed for IFN-γ (clone #XMG1.2), Perforin (clone #eBio0MAK-D), and Granzyme B (clone #NGZB). Splenocytes were formalin-fixed after intracellular staining and analyzed with an LSR Fortessa flow cytometer. The cells were gated on lymphocytes, single-cell, and $CD8^+$ T cells. Additional analysis gated on CXCR5 and/or CD44 before analyzing additional markers.

Statistical analysis

The distribution of data is represented by average ± standard error. A student t-test was used as a preliminary statistical analysis of the data (Microsoft Excel).

Results

CXCR5 expression and activation peaks at day 7 post-transplant on $CD8^+$ T cells. Our lab has previously performed time course studies on the expression of CXCR5 and CD44 on $CD8^+$ T cells in hepatocyte transplant models to determine the optimal time of the development and activation of $CXCR5^+CD8^+$ T cells. A cohort of wild-type mice were transplanted with FVB/N hepatocytes and splenocytes were isolated from the mice at days 0, 2, 5, 7, 10, and 14 for flow cytometric analysis. The number of cells expressing CXCR5 on $CD8^+$ T cells on day 7 (11.1± 1.1%; **Figure 1A**) was 8-fold greater than the number of $CXCR5^+CD8^+$ T cells on day 0 (1.4± 0.1%; p=0.0005). Similarly, the expression of CD44 on day 7 (30.4±1.8%; **Figure 1B**) was significantly elevated compared to CD44 expression on day 0 (3.0±0.5%; p=0.0001). Since day 7 post-transplant had maximal development and activation of $CXCR5^+CD8^+$ T cells, I utilized this time point for the investigation of developmental requirements of the $CD8^+$ $T_{Ab\text{-}supp}$ cell subset. In addition, we have previously reported that $CD8^+$ $T_{Ab\text{-}supp}$ cells are highly functional (inhibit alloantibody and kill antibody-producing B cells) on day 7 post-lysate treatment (14).

CXCR5 expression on $CD8^+$ T cells is dependent on $CD4^+$ T cells, IFN-γ, and IFN-γR in the host. To analyze the role of $CD4^+$ T cells and IFN-γ on $CD8^+$ $T_{Ab\text{-}supp}$ cell development, our early studies focused on how these components affected host $CD8^+$ T cells. Cohorts of C57BL/6, CD4 KO, IFN-γ KO, and IFN-γR KO mice were stimulated with allogeneic FVB/N liver lysate. An additional cohort of C57BL/6, IFN-γ KO, and IFN-γR KO were treated with anti-CD4 mAb, and the splenocytes from all cohorts were isolated on day 7 post-lysate treatment for flow cytometric analysis. The quantity of $CXCR5^+CD8^+$ T cells in wild-type mice (10.0±2.5%; **Figure 2A**) was

significantly higher than the quantity of $CXCR5^+CD8^+$ T cells in IFN-γ KO mice (3.3±0.8%; p=0.021) or in IFN-γR KO mice (3.7±0.6%; p=0.024). A similar reduction in the quantity of $CXCR5^+CD8^+$ T cells was observed in CD4-depleted wild-type mice compared to wild-type mice (6.1±1.1%; p=0.167; **Figure 2B**) as well as in CD4 KO mice (2.0±0.3%; p=0.033). Interestingly, $CXCR5^+CD8^+$ T cells in IFN-γ KO mice (3.3±0.8%; **Figure 2C**) were elevated compared to $CXCR5^+CD8^+$ T cells in IFN-γ KO mice that were CD4-depleted (4.9±0.4%; p=0.152), but this change in expression was not observed between IFN-γR KO mice (3.7±0.6%) versus CD4-depleted, IFN-γR KO mice (3.1±0.3%; p=0.294). These significant reductions in $CXCR5^+CD8^+$ T cells in each of the genetically modified hosts indicated that host $CD4^+$ T cells, IFN-γ, and IFN-γR all have a potential role in the development of functional $CD8^+$ $T_{Ab\text{-}supp}$ cells.

Maximal quantity of $CXCR5^+CD8^+$ T cells is dependent on host expression of IFN-γ and the presence of $CD4^+$ T cells. In order to investigate the role of $CD4^+$ T cells, IFN-γ, and IFN-γR on $CXCR5^+CD8^+$ T cell development, we employed two separate strategies for analyzing the impact of genetically modified hosts and donor cells. The first strategy adoptively transferred naïve wild-type $CD8^+$ T cells into genetically modified hosts, and the second strategy adoptively transferred genetically modified $CD8^+$ T cells into wild-type hosts. To better understand the role of host IFN-γ and $CD4^+$ T cells on CXCR5 expression, C57BL/6, CD4-depleted C57BL/6, IFN-γ KO, and CD4-depleted IFN-γ KO mice were adoptively transferred with naïve OT-I x GFP $CD8^+$ T cells and treated with mOVA liver lysate. Splenocytes were isolated from the host mice on day 7 post-lysate treatment and stained for CXCR5 and CD8 expression. When naïve wild-type $CD8^+$ T cells were adoptively transferred into wild-type hosts, the quantity of $CXCR5^+CD8^+$ T cells (13.5±1.2%; **Figure 3A**) was significantly enhanced compared to CD4-depleted wild-type hosts

(6.8±0.0%; p=0.020) and hosts lacking IFN-γ cytokine (8.5±0.6%; p=0.020). The number of $CXCR5^+CD8^+$ T cells was further inhibited in IFN-γ KO mice when $CD4^+$ cells were depleted (5.0±0.4%; p=0.011) compared to wild-type mice. The roles of host IFN-γ and IFN-γR on CXCR5 expression were further analyzed by adoptive transfer of naïve GFP^+CD8^+ T cells into C57BL/6, IFN-γ KO, and IFN-γR KO mice alongside FVB/N lysate stimulation. The quantity of adoptively transferred wild-type $CD8^+$ T cells that expressed CXCR5 in the lysate stimulated wild-type hosts (9.0±0.4%; **Figure 3B**) was two-folds greater than the quantity of adoptively transferred $CXCR5^+CD8^+$ T cells in IFN-γR KO hosts (5.0±0.9%; p=0.017), but the quantity of adoptively transferred $CXCR5^+CD8^+$ T cells in IFN-γ KO hosts remained unchanged compared to the wild-type hosts (7.1±1.7%; p=0.214). After determining that IFN-γ, IFN-γR, and $CD4^+$ T cells impacted CXCR5 expression on $CD8^+$ T cells, we next analyzed how these components influenced the activation of the $CXCR5^+CD8^+$ T cells.

Proportion of $CXCR5^+CD8^+$ T cells that are activated does not depend on host IFN-γ or $CD4^+$ T cells. After host $CD4^+$ T cells and IFN-γ cytokine were shown to influence the quantity of $CXCR5^+CD8^+$ T cells, further studies were performed to determine the role these components had on the activation of the $CXCR5^+CD8^+$ T cell subset. Cohorts of C57BL/6, CD4-depleted C57BL/6, IFN-γ KO, and CD4-depleted IFN-γ KO mice were stimulated with mOVA lysate and adoptively transferred with OT-I x GFP $CD8^+$ T cells. Splenocytes isolated from the host mice on day 7 underwent flow cytometric analysis for CD44, CXCR5, GFP, and CD8 expression. The activation of adoptively transferred $CXCR5^+CD8^+$ T cells in the wild-type cohort (45.2±3.2%, **Figure 4A**) was unchanged when compared to the activation of the same cell subset in the IFN-γ KO recipients (45.7±4.8%; p=0.465). Further, CD4-depletion had no effect on CD44 expression

as shown in CD4-depleted wild-type mice (44.5±4.6%; p=0.451) and in CD4-depleted, IFN-γ KO mice (38.8±0.2%; n=3; p=0.111) compared to the activation of a wild-type host. To further analyze the role of IFN-γ in the host, C57BL/6, IFN-γ KO, and IFN-γR KO mice were stimulated with FVB/N lysate and adoptively transferred with GFP^+ wild-type $CD8^+$ T cells. Flow cytometric analysis on day 7 showed that the activation of $GFP^+CXCR5^+CD8^+$ T cells in wild-type mice (27.5 ±2.1%, **Figure 4B**) did not differ from the activation in IFN-γ KO (29.8±2.1%; p=0.289) or IFN-γR KO (30.0±3.6%; p=0.389) mice. Three cohorts of GFP^+ C57BL/6 mice were adoptively transferred with wild-type, IFN-γ KO, or IFN-γR KO $CD8^+$ T cells to further analyze the role of IFN-γ on the adoptive transfer cells. The activation of adoptively transferred, $CXCR5^+CD8^+$ T cells in wild-type hosts (21.9±1.8%, **Figure 4C**) was slightly lower than the activation of IFN-γ KO $CD8^+$ T cells in the wild-type hosts (28.7±1.2%; p=0.031), but the activation of adoptively transferred IFN-γR KO $CXCR5^+CD8^+$ T cells showed no difference from the wild-type control (30.6±10.8%; p=0.276). Since the quantity of $CXCR5^+CD8^+$ T cells is altered in the absence of $CD4^+$ T cells or IFN-γ while activation is not, we next investigated if $CD4^+$ T cells and IFN-γ impact the proliferative ability of this cell subset.

Proliferation of developing, activated $CXCR5^+CD8^+$ T cells is dependent on host IFN-γ. Cohorts of C57BL/6, CD4-depleted C57BL/6, IFN-γ KO, and CD4-depleted IFN-γ KO mice were adoptively transferred with CellTrace Violet-stained $CD8^+$ T cells isolated from OT-I x GFP mice and treated with mOVA liver lysate to analyze the role of $CD4^+$ T cells and IFN-γ on the proliferation of activated $CD8^+$ T cells. Splenocytes from the host mice were isolated on day 7 post-lysate treatment and stained for CD44, CXCR5, and CD8. Interestingly, the proliferation of

activated (CD44+) $CXCR5^+CD8^+$ T cells was significantly higher than $CXCR5^-CD8^+$ T cells in C57BL/6, CD4-depleted C57BL/6, and CD4-depleted IFN-γ KO mice ($p<0.03$; **Figure 5A**), suggesting that $CXCR5^+CD8^+$ T cells may have a heightened proliferative capacity following antigen stimulation compared to $CD8^+$ T cells that do not express CXCR5. The proliferation of adoptively transferred activated $CXCR5^+CD8^+$ T cells in the wild-type hosts (33.5±1.5%) was also significantly higher than the proliferation of activated $CXCR5^+CD8^+$ T cells in the IFN-γ KO hosts (19.7±1.2%, $p=0.011$).

Cytotoxic effector phenotype of activated $CXCR5^+CD8^+$ T cells is critically dependent on host IFN-γ. After determining the impact of IFN-γ on CXCR5 expression and activation, we next analyzed how this cytokine affected the expression of cytotoxic effector molecules on $CD8^+$ T cells. Cohorts of wild-type, IFN-γ KO, and IFN-γR KO mice were treated with FVB/N lysate and adoptively transferred with naïve wild-type GFP^+CD8^+ T cells. On day 7 post-lysate stimulation, the splenocytes from the host mice were isolated and analyzed by flow cytometry. The expression of FasL on adoptively transferred, activated $CXCR5^+CD8^+$ T cells in wild-type mice (36.5±5.6%; **Figure 6A**) was significantly higher than the number of cells expressing FasL on the same cell subset in IFN-γ KO hosts (6.7±5.4%; $p=0.010$). Additionally, the number of activated $Lamp1^+CXCR5^+CD8^+$ T cells in the wild-type host mice (70.0±6.2%; **Figure 6A**) was 10-fold greater than the number of activated, $Lamp1^+CXCR5^+CD8^+$ T cells in IFN-γ KO mice (6.7±5.4%, $p=0.002$). To determine if IFN-γ was also important on the adoptive transfer cells, several cohorts of GFP^+ wild-type mice were adoptively transferred with naïve wild-type, IFN-γ KO, and IFN-γR KO $CD8^+$ T cells. The host mice were treated with FVB/N liver lysate, and splenocytes were isolated from the hosts 7 days after stimulation. The number of activated, wild-type $CXCR5^+CD8^+$

cells that expressed FasL (50.0±35.4%, **Figure 6B**) was greater than the percent of activated IFN-γ KO $CXCR5^+CD8^+$ T cells expressing FasL (8.3±6.8%, p=0.181). However, the percent of wild-type $CXCR5^+CD8^+$ T cells expressing Lamp1 (62.5±26.5%, **Figure 6B**) did not show a significant difference from the percent of IFN-γ KO $CXCR5^+CD8^+$ T cells expressing Lamp1 (48.3±21.1%, p=0.383).

$CD8^+$ T cells rely on host IFN-γ and $CD4^+$ T cells to effectively kill alloprimed B cells.

Our lab has previously performed an *in vitro* cytotoxicity assay to determine whether IFN-γ or $CD4^+$ T cells impact the development of cytotoxic effector function of $CD8^+$ T cells towards antibody-producing (IgG^+) B cells. Wild-type, IFN-γ KO, and CD4 KO mice were transplanted with FVB/N hepatocytes, and splenic $CD8^+$ T cells were isolated from these mice on day 7 post-transplant for a cytotoxicity co-culture. The $CD8^+$ T cells from different strains were co-cultured with naïve wild-type or primed wild-type IgG^+ B cells (day 7 posttransplant; CD8 KO recipient) at a 10:1 ratio. After a 4-hour incubation, the propidium iodide uptake was measured for each culture to determine the cytotoxicity of the $CD8^+$ T cells against the B cells. The percent cytotoxicity in the wild-type $CD8^+$ T cell and $IgG1^+$ B cell co-culture (6.5±0.7%; **Figure 7**) was 13-fold greater than the cytotoxicity of IFN-γ KO $CD8^+$ T cells (0.5±0.1%; $p<0.001$) and CD4 KO $CD8^+$ T cells (0.5±0.6%; $p<0.001$) against $IgG1^+$ B cells. This indicates that both IFN-γ and $CD4^+$ T cells are critical for the development of specific effector function against alloprimed B cells. Cytotoxicity against the naïve B cells was unchanged between the different $CD8^+$ T cell groups compared to the wild-type $CD8^+$ T cells (WT $CD8^+$: 1.5±0.7%; IFN-γ KO $CD8^+$: 2.5±0.7%; p=0.095, CD4 KO $CD8^+$: 2.0±0.1%, p=0.161). The $CD8^+$ T cells also showed no cytotoxicity against third-party B cells (data not shown).

CXCR5 expression on $CD8^+$ T cells is dependent on IFN-γ^+CD4^+ T cells and IFN-γR^+CD4^+ T cells in the host mouse. The critical role of $CD4^+$ T cells in $CD8^+$ $T_{Ab\text{-}supp}$ cell development was further supported by the quantity of $CXCR5^+CD8^+$ T cells in CD4 KO mice treated with FVB/N lysate. In the absence of $CD4^+$ T cells, $CXCR5^+CD8^+$ T cell development was low (2.9±0.5%). A cohort of CD4 KO mice received C57BL/6, IFN-γ KO, or IFN-γR KO $CD4^+$ T cells through adoptive transfer. Splenocytes isolated on day 7 showed an increase in the number of $CXCR5^+CD8^+$ T cells in CD4 KO mice that were reconstituted with wild-type $CD4^+$ T cells (5.2±1.1%, **Figure 8**) compared to CD4 KO mice without adoptive transfer (2.9±0.5%; p=0.156). However, CD4 KO mice that were adoptively transferred with IFN-γ KO $CD4^+$ T cells showed a slightly reduced quantity of $CXCR5^+CD8^+$ T cells (3.2±0.9%; p=0.212) compared to the mice adoptively transferred with wild-type $CD4^+$ T cells. Similarly, CD4 KO mice reconstituted with IFN-γR $CD4^+$ T cells showed a minimal difference in the quantity of $CXCR5^+CD8^+$ T cells compared to mice lacking $CD4^+$ T cells (2.3% versus 3.2±0.9% in control), which implies that IFN-γ and/or IFN-γR on $CD4^+$ T cells may play a critical role in the ability of $CD8^+$ T cells to express CXCR5. The impact of IFN-γ^+ and IFN-γR^+ $CD4^+$ T cells on the phenotype of the $CD8^+$ $T_{Ab\text{-}supp}$ is a promising preliminary result in further understanding the mechanism of development for this subset, and future studies will continue to analyze the role these cells have on the development of $CD8^+$ $T_{Ab\text{-}supp}$ cells.

Discussion

The Bumgardner Lab is the first group to report that a subset of $CD8^+$ T cells are antibody-suppressing T cells that inhibit alloantibody production (12, 14, 26), kill B cells (12, 14), and enhance allograft survival (14, 26). Since these studies were the first to analyze the function antibody-suppressing $CXCR5^+CD8^+$ T cells, the developmental process for these cells was still unknown. Our current study found that both host IFN-γ and $CD4^+$ T cells elevated the quantity of $CXCR5^+CD8^+$ T cells on day 7 after lysate treatment. While activation of the $CXCR5^+CD8^+$ T cells was unaffected by either IFN-γ or $CD4^+$ T cells, the proliferation of $CXCR5^+CD8^+$ T cells significantly increased when IFN-γ was present in the host. Host IFN-γ also elevated the expression of FasL and Lamp1 on $CD8^+$ T cells, which may be the cause of the abrogated cytotoxicity for $CD8^+$ T cells against IgG^+ B cells in IFN-γ KO mice. Pilot data also suggests that IFN-γ^+ and IFN-γR^+ $CD4^+$ T cells significantly increase the quantity of $CXCR5^+CD8^+$ T cells but do not cause a change in the activation of the $CD8^+$ $T_{Ab\text{-}supp}$ cell subset.

Since we show that both host IFN-γ and $CD4^+$ T cells maximize the quantity of $CXCR5^+CD8^+$ T cells, there is a potential role for Th1 $CD4^+$ T cell help in the expression of CXCR5 on $CD8^+$ T cells. Th1 $CD4^+$ T cells secrete IFN-γ cytokine (27), so the decrease in $CXCR5^+CD8^+$ T cells in IFN-γ KO mice may be caused by a skewed Th2 profile. It is also possible that other IFN-γ secreting cells, such as macrophages (28) or iNKT cells (29), impact the expression of CXCR5 on $CD8^+$ T cells as well. However, since CXCR5 expression was not completely abrogated in the IFN-γ KO mice, it is likely that other mechanisms contribute to the CXCR5 expression beyond IFN-γ signaling and additional pathways for expression will be investigated in future studies. In contrast, our studies also indicated that both IFN-γ and $CD4^+$ T cells did not impact the activation of the $CXCR5^+CD8^+$ T cells, which suggests that alternative

mechanisms contribute to the activation of the $CD8^+$ $T_{Ab\text{-}supp}$ cells. Further investigation is required to determine the role of $CD4^+$ T cells (and the precise $CD4^+$ T cell subset) in enhancing the quantity of $CXCR5^+CD8^+$ T cells as well as the role of iNKT cells on the activation of $CD8^+$ $T_{Ab\text{-}supp}$ cells since iNTK cells are reported to enhance $CD8^+$ T cells effector function following antigen stimulation (30-33). We will also consider the role of other antigen-presenting cells, such as dendritic cells and macrophages, on CXCR5 expression and the activation of the $CXCR5^+CD8^+$ T cell subset.

Additionally, our group observed that the proliferation of the $CXCR5^+CD8^+$ T cells was upregulated when IFN-γ was present in the host. Interestingly, we also found that activated $CXCR5^+CD8^+$ T cells proliferated at a higher rate than $CXCR5^-CD8^+$ T cells. This enhanced proliferation of the CXCR5-expressing subset is akin to $PD\text{-}1^+ICOS^+CXCR5^+CD8^+$ T cells that are highly proliferative, anti-viral cells with stem-cell-like characteristics (34, 35). While these anti-viral cells have no impact on antibody production, their proliferation results in $CD8^+$ T cells that lose CXCR5 expression. In our studies, proliferating alloprimed $CXCR5^+CD8^+$ T cells maintain CXCR5 expression and have been reported to be phenotypically distinct from anti-viral $CD8^+$ T cells through the lack of expression PD-1 and ICOS (14). We have also found that our $CXCR5^+CD8^+$ T cell subset is a short-lived effector cell ($CD44^+KLRG1^+IL\text{-}2Rb^+IL\text{-}7R^-$; data not shown), whereas the $PD\text{-}1^+ICOS^+CXCR5^+CD8^+$ T cells have gene signatures that indicate a memory precursor phenotype ($KLRG1^+IL\text{-}2Rb^-IL\text{-}7R^+$) (34). In contrast to the proliferation of alloprimed $CXCR5^+CD8^+$ T cells, alloprimed $CXCR5^-CD8^+$ T cells did not proliferate following lysate stimulation. Previous studies tracking the proliferation of antigen-specific rejector $CD8^+$ T cells showed that approximately 95% of alloprimed $CD8^+$ T cells proliferate following transplant in wild-type mice (36). In this study, the proliferation of rejector $CD8^+$ T cells was independent of

$CD4^+$ T cells. We have also recently indicated that $CXCR3^+CXCR5^-CD8^+$ T cell subsets ($CD8^+$ T_{rej} cells) (14) are cytotoxic to allogeneic cells and require "direct" antigen presentation with allo-MHC (from viable transplants) (37). Since rejector cells are CXCR5-negative, it is likely that the low proliferation of $CXCR5^-CD8^+$ T cells cannot effectively respond to allogeneic lysate ("indirect" antigen presentation). Despite these differences, the increased proliferative capacity of the $CD8^+$ $T_{Ab\text{-}supp}$ cells is a new characteristic that our group had not previously observed for this subset. While activated $CXCR5^+CD8^+$ T cells in host mice with IFN-γ had a higher proliferative capacity, further investigation is required to determine the role of IFN-γR on $CD8^+$ T cells for proliferation.

Finally, the expression of cytotoxic effector molecules was also upregulated by host IFN-γ. FasL and Lamp1 expression were significantly reduced on wild-type $CD8^+$ T cells that were adoptively transferred into mice without IFN-γ, but granzyme B and perforin remained unchanged on the wild-type $CD8^+$ T cells adoptively transferred into mice deficient in either IFN-γ or IFN-γR. Since Lamp1 indicates perforin release (38), the drop in Lamp1 suggests that without IFN-γ in the host, $CXCR5^+CD8^+$ T cells lack the ability to release perforin as a means for the cytotoxic killing of IgG^+ B cells. The downregulated release of perforin (indicated by reduced Lamp1 expression) in the absence of IFN-γ may cause the decrease in cytotoxic effector function for $CD8^+$ T cells from IFN-γ KO hosts. This possibility will be investigated in future *in vitro* or *in vivo* cytotoxicity assays that utilize $CXCR5^+CD8^+$ T cells rather than bulk $CD8^+$ T cells. Additionally, the reduction in FasL expression when IFN-γ is absent suggests that IFN-γ is critical to maintaining the FasL/Fas interactions. Both perforin and FasL have been shown as a key cytotoxic pathways for downregulating alloantibody in transplant recipients (12). This hypothesis is further corroborated by the lack of cytotoxicity towards IgG^+ B cells by $CD8^+$ T cells developed in mice

without IFN-γ. The expression of both perforin and granzyme B further differentiates $CXCR5^+CD8^+$ T cells from the antibody-enhancing $CXCR5^+CD8^+$ T cells that express PD-1 but lack granzyme B and perforin (39-41). Pilot studies also indicated that IFN-γ^+ and IFN-γR^+ $CD4^+$ T cells were critical for both CXCR5 expression and cytotoxic effector molecule expression on $CXCR5^+CD8^+$ T cells, but further data is needed to confirm these findings.

While these initial findings show that both $CD4^+$ T cells and IFN-γ impact the development of $CD8^+$ $T_{Ab\text{-}supp}$ cells, the relatively small sample size for each experiment requires repeat studies before the conclusions can be fully supported. Additionally, since IFN-γ KO mice are skewed towards Th2 $CD4^+$ T cell (42), it is possible that the $CD8^+$ T cells did not fully mature or develop in these host mice and skewed the observed results. Future studies will also analyze the role of $CD4^+$ T regulatory cells and IL-4 KO $CD4^+$ T cells on the development of $CD8^+$ $T_{Ab\text{-}supp}$ cells by adoptively transferring wild-type $CD4^+$ T cells, IFN-γ KO $CD4^+$ T cells, IL-4 KO $CD4^+$ T cells, or flow-sorted $CD4^+$ T_{reg} cells ($CD3^+CD25^+FoxP3^+$) (43) to determine their impact on the various development parameters. IL-4 KO mice are skewed towards a Th1 profile, so if Th1 $CD4^+$ T cells are required for $CD8^+$ $T_{Ab\text{-}supp}$ cell development, these mice would have expression patterns that contrast the observations in IFN-γ KO hosts. In addition to IFN-γ and $CD4^+$ T cells, we are also interested in the role that the microenvironment plays in $CD8^+$ $T_{Ab\text{-}supp}$ cell development. Current studies indicate that fully matured, alloprimed $CD8^+$ $T_{Ab\text{-}supp}$ cells traffic to the lymph node and spleen, but it is unclear if this location is required for development. We hypothesize that lymph nodes may be a key site for these cells to develop into the functional subset we have previously observed, and we will begin investigating this hypothesis with mice that lack functional lymph nodes (Ltα KO mice) and/or spleens (splenectomized mice). Beyond the microenvironment, it is still unknown whether $CXCR5^+CD8^+$ T cells are only generated by naïve $CXCR5^+CD8^+$ T cell

precursors, or if primed $CXCR5^{-}CD8^{+}$ T cells have the potential to develop a $CXCR5^{+}$ phenotype. If future studies find that the CXCR5-negative cells can develop CXCR5, then we will also investigate the cytokines and cellular interactions that promote this change to optimize the number of available $CXCR5^{+}CD8^{+}$ $T_{Ab\text{-}supp}$ cells.

The data presented in these studies provide a foundation to maximize $CD8^{+}$ $T_{Ab\text{-}supp}$ cells both *in vivo* and *in vitro* for clinical applications. We have previously shown that human kidney transplant recipients that develop donor-specific antibody (DSA) have significantly lower numbers of peripheral $CXCR5^{+}CD8^{+}$ T cells when compared to recipients that do not make DSA (44), which indicates a potential role for these cells in human DSA reduction. With further investigation into the developmental mechanisms, we can investigate strategies to optimize $CD8^{+}$ $T_{Ab\text{-}supp}$ in human patients receiving organ transplants. These strategies may involve therapies that can expand the $CD8^{+}$ $T_{Ab\text{-}supp}$ cell population in a test tube for future transfer into the recipient, or therapies that can expand the $CD8^{+}$ $T_{Ab\text{-}supp}$ cell population within the human transplant recipient to prolong transplant survival and prevent antibody-mediated rejection in a clinical setting.

Figures

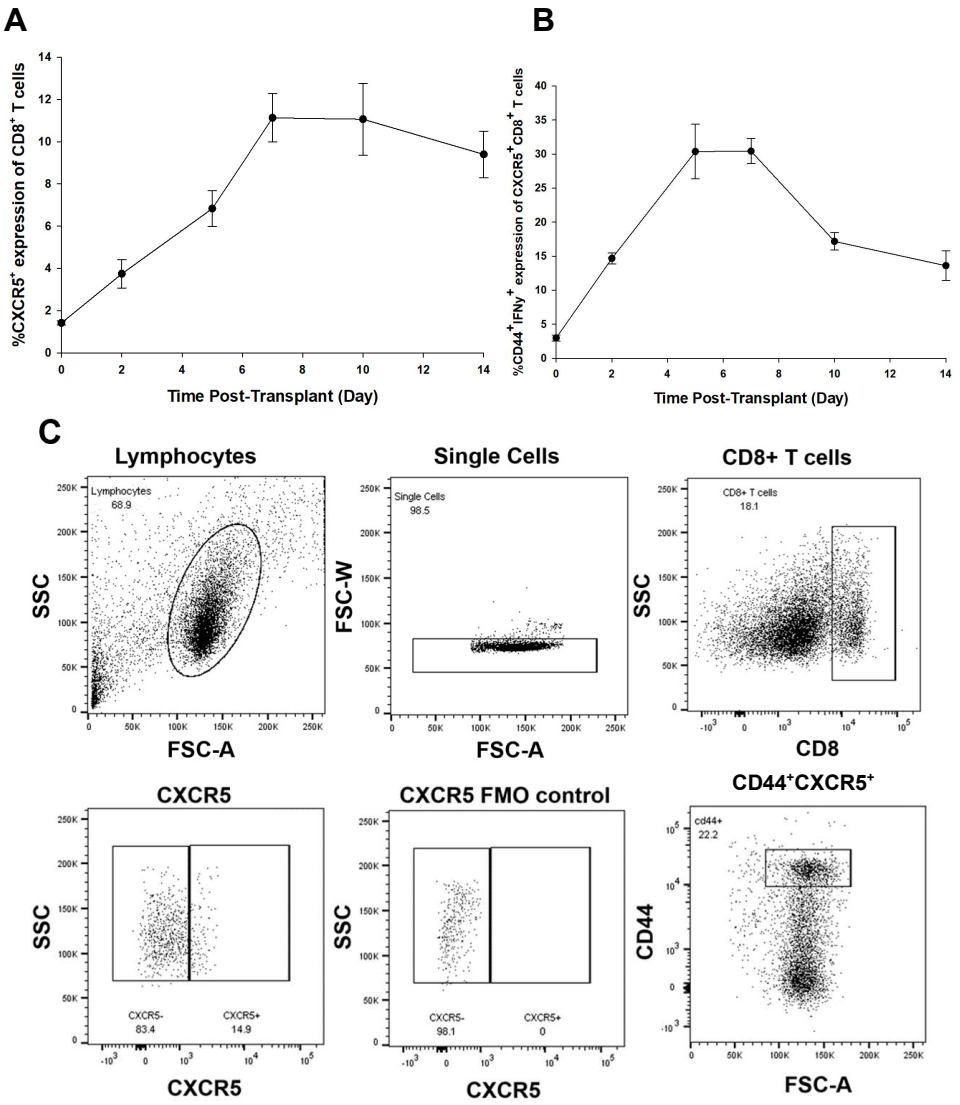

Figure 1: CXCR5 and CD44 expression on $CD8^+$ T cells peaks at day 7

C57BL/6 (wild-type, H-2^b) mice were transplanted with hepatocytes isolated from FVB/N (H-2^q) mice. Splenocytes were isolated from the transplanted mice on days 0, 2, 5, 7, 10, and 14 post-transplant and analyzed by flow cytometry. **A)** The quantity of wild-type $CXCR5^+CD8^+$ T cells on day 7 (11.1±1.1%; n=3; p=0.0005 vs. day 0) was the highest of any of the time points. **B)** CD44 expression also peaked at day 7 (30.4±1.8%; n=3; p=0.0001 vs. day 0) compared to the other time points measured. **C)** A representative flow cytometry plot for CXCR5 and CD44 expression was created to demonstrate how the cell markers were quantified. The lymphocyte population was selected with the forward and side scatter profiles of the cells. Lymphocytes were then gated on single cells to remove any cells that were conjoined when passing through the laser. Single cells were gated for $CD8^+$ expression by using an FMO (flow-minus-one) control that lacked the $CD8^+$ antibody. The location of the gate for CXCR5 expression on $CD8^+$ T cells was also found using an FMO control, and CD44 expression on $CXCR5^+CD8^+$ T cells was gated with an FMO control as well.

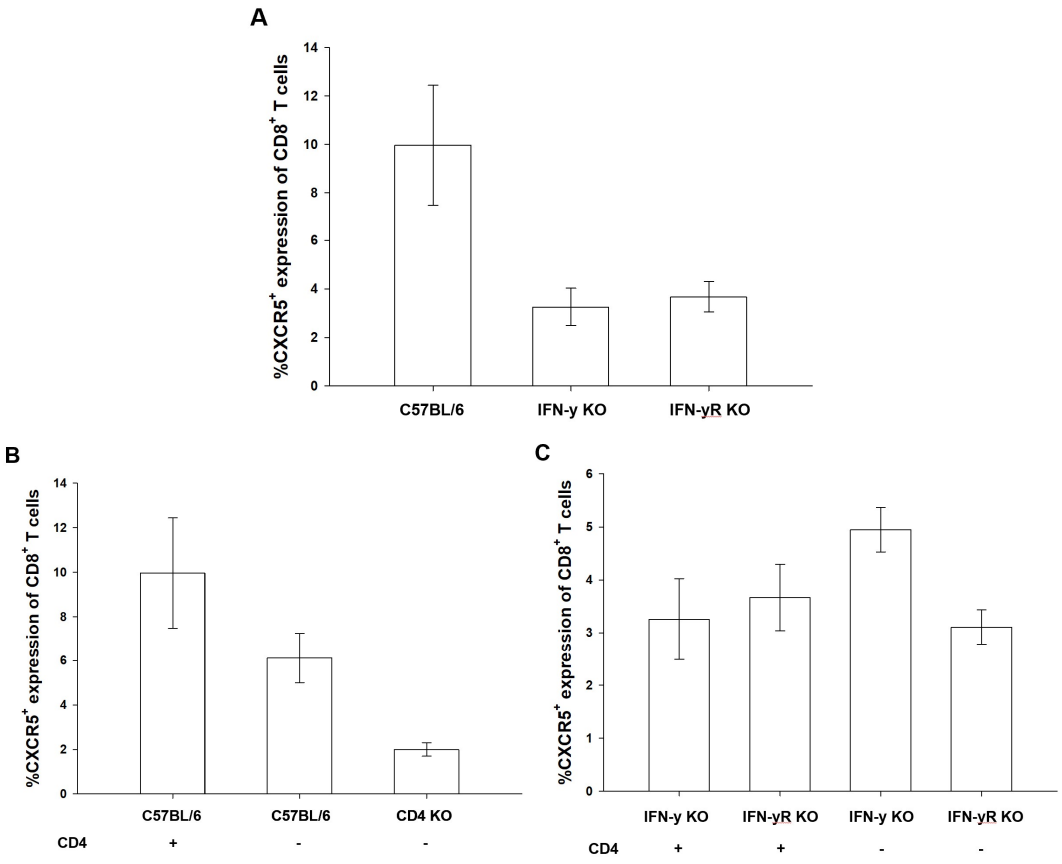

Figure 2: CD4$^+$ T cells, IFN-γ, and IFN-γR enhance CXCR5 expression of host CD8$^+$ T cells.
C57BL/6 (wild-type), CD4 KO, IFN-γ KO, and IFN-γR KO mice (all H-2^b) were stimulated with FVB/N liver lysate (H-2^q). An additional group of C57BL/6, IFN-γ KO, and IFN-γR KO mice were treated with anti-CD4 mAb on days -3 and -1 before lysate stimulation. Splenocytes were isolated from the cohorts of mice on day 7 and analyzed by flow cytometry. **A)** The number of CXCR5$^+$ wild-type CD8$^+$ T cells (10.0±2.5%; n=4) was 3-folds greater than IFN-γ KO CD8$^+$ T cells (3.3±0.8%; n=5; p=0.021) and IFN-γR KO CD8$^+$ T cells (3.7±0.6%; n=5; p=0.024). **B)** The quantity of CXCR5$^+$CD8$^+$ T cells in wild-type mice (10.0±2.5%; n=4) was also higher than the number of CXCR5$^+$CD8$^+$ T cells in CD4-depleted wild-type mice (6.1±1.1%; n=3; p=0.167) or CD4 KO mice (2.0±0.3%; n=3; p=0.033). **C)** The percent of CXCR5$^+$CD8$^+$ T cells in IFN-γ KO mice (3.3±0.8%; n=5) was lower than the percent of CXCR5$^+$CD8$^+$ T cells in CD4-depleted IFN-γ KO mice (4.9±0.4%; n=3; p=0.152). No significant difference was observed in CXCR5$^+$CD8$^+$ T cell quantity between IFN-γR KO mice (3.7±0.6%; n=5) and CD4-depleted IFN-γR KO mice (3.1±0.3%; n=3; p=0.294).

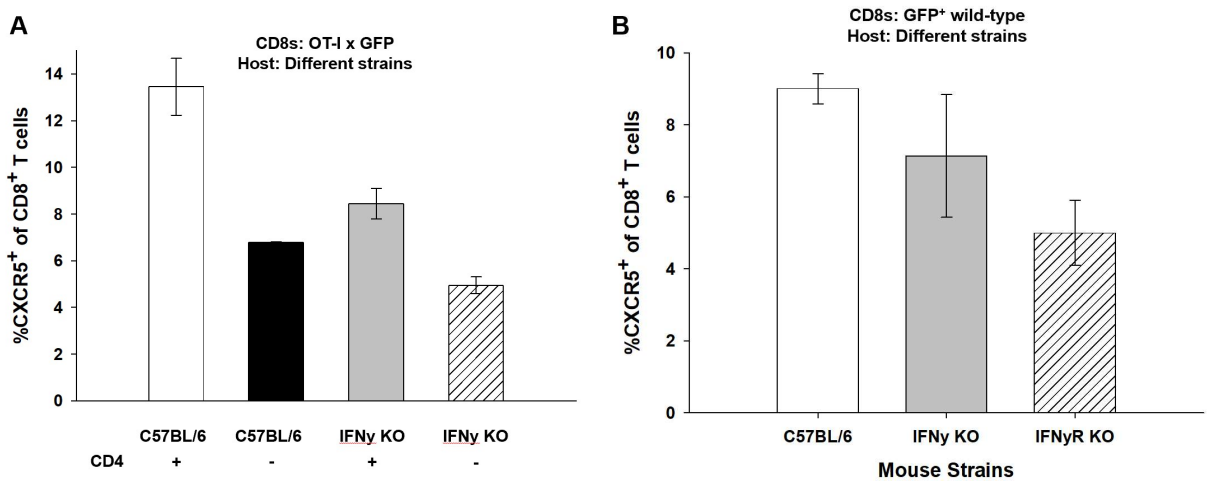

Figure 3: IFN-γ and wild-type CD4$^+$ T cells enhance the development of CXCR5$^+$CD8$^+$ T cells.
A) C57BL/6 (wild-type) and IFN-γ KO mice (both H-2^b) were treated with mOVA (H-2^b) liver lysate, and CD8$^+$ T cells from OT-I x GFP mice were adoptively transferred (AT) into the lysate-treated hosts. Cohorts of each strain were treated with anti-CD4 mAb to deplete CD4$^+$ T cells. On day 7 post-lysate treatment, splenocytes were purified from the host mice. Flow cytometric analysis of AT CD8$^+$ T cells expressing CXCR5 in the C57BL/6 hosts (13.5±1.2%; n=3) was significantly elevated compared to CD4-depleted C57BL/6 hosts (6.8±0.0%; n=3; p=0.020) and IFN-γ KO hosts (8.5±0.6%; n=3; p=0.020). The quantity of AT CXCR5$^+$CD8$^+$ T cells in IFN-γ KO hosts was further suppressed following CD4 depletion (5.0±0.4%; n=3; p=0.011) compared to the wild-type host. **B)** C57BL/6, IFN-γ KO, and IFN-γR KO (H-2 ^{b}) mice were treated with FVB/N (H-2^q) liver lysate (2mg protein) and adoptively transferred with naïve GFP$^+$ WT CD8$^+$ T cells. Flow cytometric analysis of splenocytes isolated on day 7 showed that the quantity of CXCR5$^+$CD8$^+$ T cells was (9.0±0.4%; n=3) in C57BL/6 hosts. The proportion of CXCR5$^+$CD8$^+$ T cells was unchanged on AT CD8$^+$ T cells in IFN-γ KO hosts (7.1±1.7%; n=3; p=0.214) compared to the wild-type, but the quantity of CXCR5$^+$CD8$^+$ T cells was reduced by a 2-fold reduction in IFN-γR KO hosts (5.0±0.9%; n=3; p=0.017).

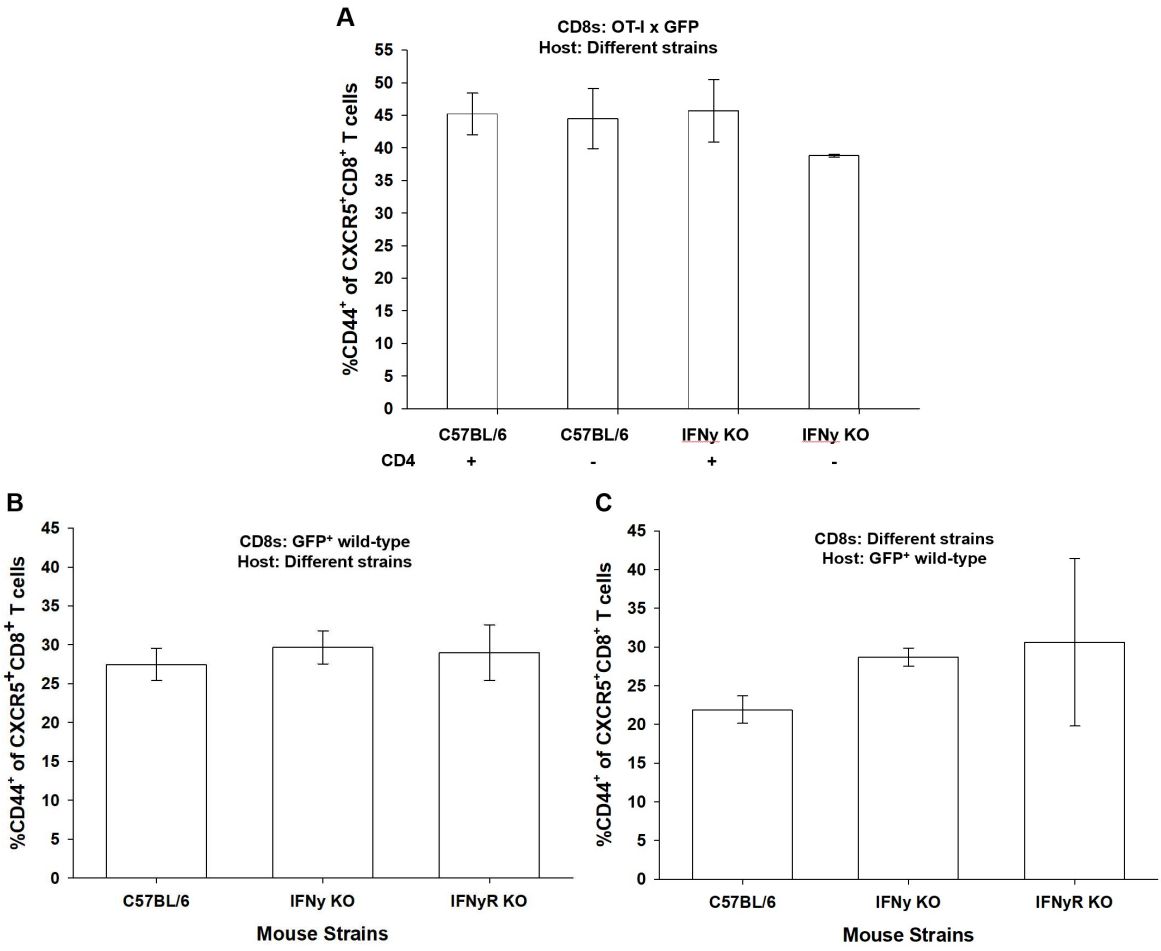

Figure 4: The proportion of activated CD8$^+$ T$_{ab\text{-}supp}$ cells is not affected by host CD4$^+$ T cells or IFN-γ.
A) C57BL/6 (wild-type) and IFN-γ KO mice (both H-2^b) were treated with mOVA (H-2^b) liver lysate (2 mg protein). CD8$^+$ T cells from OT-I x GFP mice were adoptively transferred (AT) into the mice treated with mOVA lysate, and cohorts of C57BL/6 and IFN-γ KO mice were treated with anti-CD4 mAb. Splenocytes were isolated from the host mice on day 7 post-lysate treatment, and flow cytometric analysis of CD44$^+$ on the adoptive transfer CXCR5$^+$CD8$^+$ T cells in C57BL/6 mice (45.2±3.2%; n=3) was unchanged compared to CXCR5$^+$CD8$^+$ T cells expressing CD44 in CD4-depleted C57BL/6 mice (44.5±4.6%; n=3; p=0.451). The proportion of adoptive transfer CXCR5$^+$CD8$^+$ T cells in IFN-γ KO expressing CD44 was also unchanged compared to the wild-type control (45.7±4.8%; n=3; p=0.465), and the proportion of CD44$^+$CXCR5$^+$CD8$^+$ T cells was slightly reduced in the CD4-depleted, IFN-γ KO mice (38.8±0.2%; n=3; p=0.111). **B)** C57BL/6, IFN-γ KO, and IFN-γR KO mice (H-2^b) were adoptively transferred with naïve GFP$^+$ WT CD8$^+$ T cells and treated with FVB/N liver lysate (H-2^q; 2 mg protein). Flow cytometric analysis on day 7 showed that CD44 expression on GFP$^+$CD8$^+$ T cells in wild-type mice (27.5±2.1%) was not significantly different than the proportion of CD44 expression on AT CD8$^+$ T cells in IFN-γ KO mice (29.8±2.1%; n=3; p=0.289) or IFN-γR KO mice (30.0±3.6%; n=3; p=0.389). **C)** Cohorts of GFP$^+$ C57BL/6 mice were adoptively transferred with CD8$^+$ T cells isolated from C57BL/6, IFN-γ KO, and IFN-γR KO mice (H-2^b) and lysate treated with FVB/N liver lysate (H-2^q; 2 mg protein). Splenocytes were isolated from the hosts on day 7, and flow cytometric analysis of CD44$^+$ on GFP$^-$ CXCR5$^+$CD8$^+$ T cells was performed. The wild-type control (21.9±1.8%; n=3) showed no significant difference from the cohort with IFN-γR KO CD8$^+$ T cells (30.6±10.8%; n=3; p=0.276), but the percent of CD44$^+$CXCR5$^+$CD8$^+$ T cells was slightly elevated in the IFN-γ KO CD8$^+$ T cell cohort (28.7±1.2%; n=3; p=0.031) compared to WT CD8$^+$ T cells.

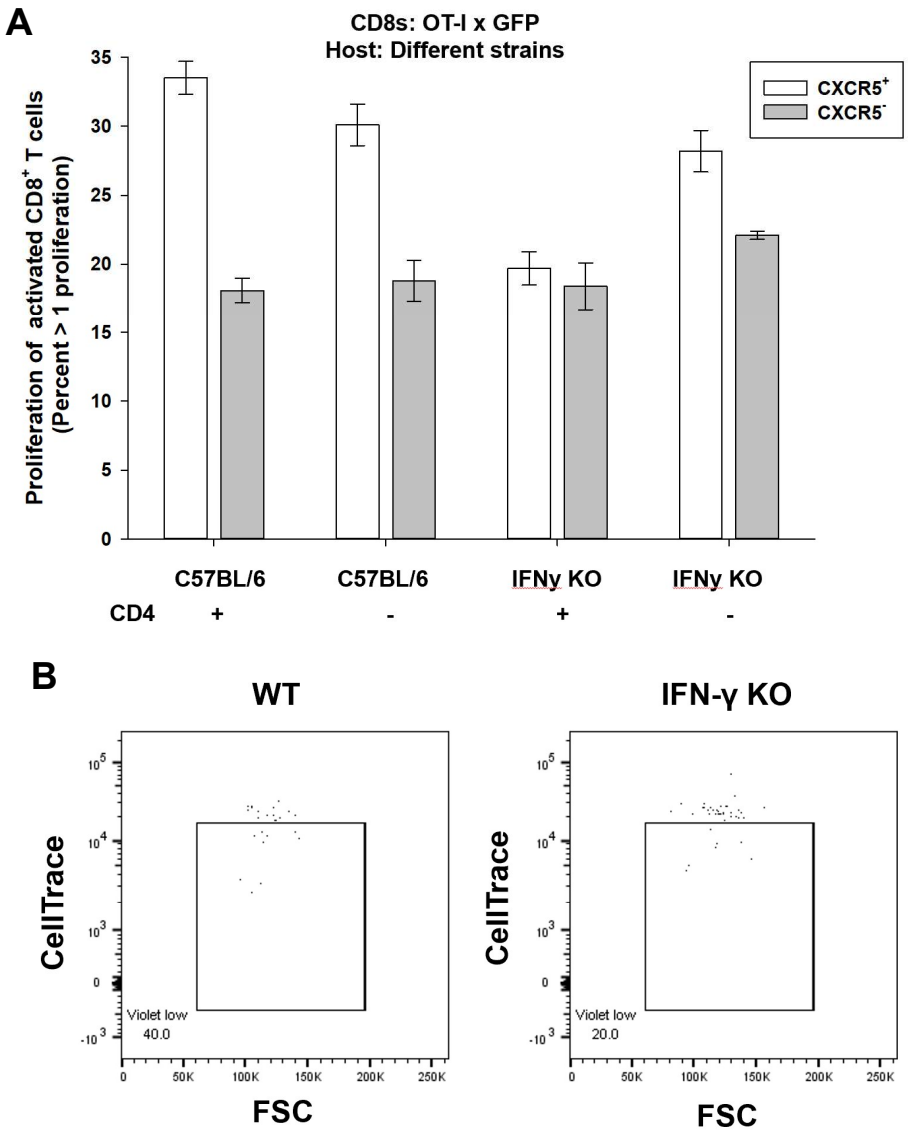

Figure 5: Host IFN-γ enhances the proliferation of CD44$^+$CXCR5$^+$ CD8$^+$ T cells

A) C57BL/6 (wild-type) and IFN-γ KO mice (both H-2^b) were stimulated with mOVA (H-2^b) liver lysate (2 mg protein) and adoptively transferred with OT-I x GFP CD8$^+$ T cells stained with CellTrace Violet (2.5 μM). Cohorts of C57BL/6 and IFN-γ KO mice were treated with anti-CD4 mAb to deplete CD4$^+$ T cells. Splenocytes were purified from the host mice on day 7 post-lysate treatment and analyzed by flow cytometry for reduced CellTrace Violet expression. In all host mice, the adoptively transferred activated CXCR5$^+$CD8$^+$ T cells proliferated significantly more than CXCR5$^-$CD8$^+$ T cells (C57BL/6 mice: CXCR5$^+$=33.5±1.5% vs. CXCR5$^-$ =18.1±0.9%, p=0.001; CD4-depleted C57BL/6 mice: CXCR5$^+$=30.1±1.5% vs. CXCR5$^-$= 18.8±1.5%, p=0.017; IFN-γ KO mice: CXCR5$^+$=19.7±1.5% vs.CXCR5$^-$ =18.4±1.7%, p=0.317; and CD4-depleted, IFN-γ KO mice: CXCR5$^+$=28.2±1.5% vs. CXCR5$^-$ =22.1±0.3%, p=0.029; n=3 for all cohorts). The proliferation of adoptively transferred, activated CXCR5$^+$CD8$^+$ T cells in C57BL/6 hosts (33.5±1.5%; n=3) was significantly elevated compared to the activated CXCR5$^+$CD8$^+$ T cells in IFN-γ KO mice (19.7±1.5%; n=3; p=0.011). **B)** A representative flow cytometry plot of proliferation gated on the number of cells that proliferated one or more times over the seven-day period was generated. The plots showed that CD44$^+$CXCR5$^+$CD8$^+$ T cells in a wild-type mouse (40.0% CellTrace Violet low) had double the percent of cells proliferating than the same cell subset in a host mouse without IFN-γ (20.0% CellTrace Violet low).

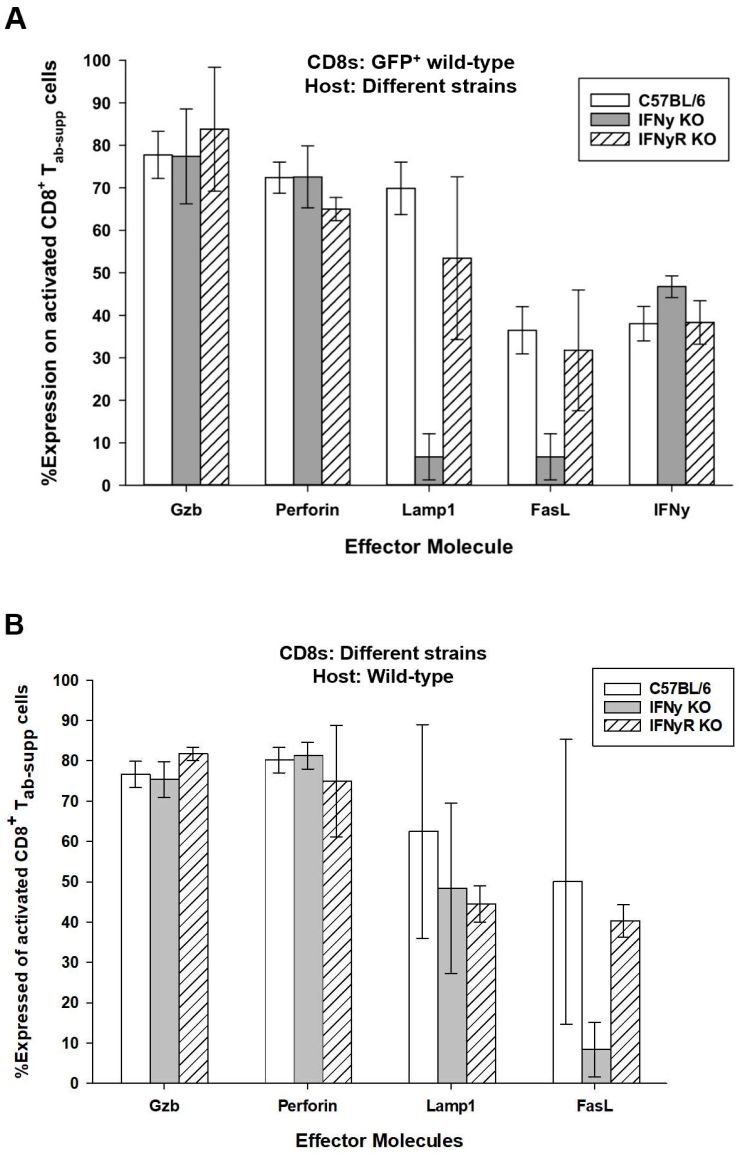

Figure 6: FasL and Lamp1 expression on CD8$^+$ $T_{Ab\text{-}supp}$ cells is elevated by host IFN-γ

A) C57BL/6 (wild-type), IFN-γ KO, and IFN-γR KO (all H-2^b) were stimulated with FVB/N (H-2^q) liver lysate and adoptively transferred with naïve GFP$^+$ wild-type CD8$^+$ T cells. Splenocytes were harvested from the hosts on day 7 for flow cytometric analysis. FasL expression on activated, adoptively transferred CD8$^+$ $T_{Ab\text{-}supp}$ cells in the wild-type hosts (36.5±5.6%; n=3) was significantly higher than the proportion of activated CXCR5$^+$CD8$^+$ T cells expressing FasL in the IFN-γ KO hosts (6.7±5.4%; n=3; p=0.010). Additionally, the proportion of Lamp1 expression on activated adoptively transferred CD8$^+$ $T_{Ab\text{-}supp}$ cells in wild-type hosts (70.0±6.2%; n=3) was significantly higher than the same cell subset in IFN-γ KO hosts (6.7±5.4%; n=3; p=0.002). **B)** Cohorts of GFP$^+$ C57BL/6 mice were adoptively transferred with naive wild-type, IFN-γ KO, or IFN-γR KO CD8$^+$ T cells (all H-2^b) and stimulated with FVB/N (H-2^q) liver lysate. Splenocytes from the host mice were purified on day 7 post-lysate treatment and stained for CD44, CXCR5, CD8, and various effector molecules. The proportion of FasL$^+$ wild-type CD8$^+$ $T_{Ab\text{-}supp}$ adoptive transfer cells (50.0±35.4%; n=3) was elevated compared to the proportion of FasL$^+$ IFN-γ KO CD8$^+$ $T_{Ab\text{-}supp}$ cells (8.3±6.8%; n=3; p=0.181). The proportion of Lamp1$^+$ wild-type CD8$^+$ $T_{Ab\text{-}supp}$ cells (62.5±26.5%; n=3) showed no difference from the proportion of Lamp1$^+$ IFN-γ KO CD8$^+$ $T_{Ab\text{-}supp}$ cells (48.3±21.1%; n=3; p=0.383).

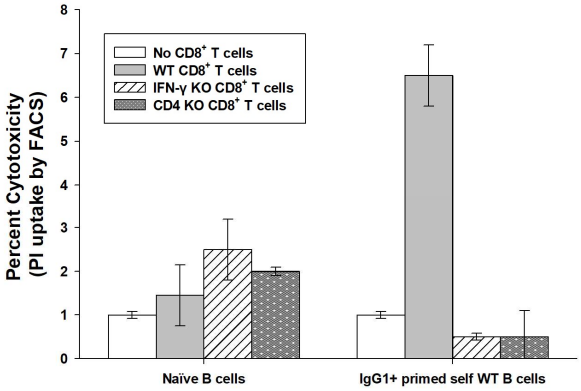

Figure 7: CD8-mediated cytotoxicity against alloantibody producing B cells is dependent on host IFN-γ and CD4$^+$ T cells. C57BL/6 (wild-type), IFN-γ KO, and CD4 KO mice (all H-2^b) were transplanted with FVB/N hepatocytes (H-2^q). CD8$^+$ T cells were isolated from the spleens of transplant mice on day 7 post-transplant. Cohorts of CD8$^+$ T cells were co-cultured with B cell targets at a 1:10 ratio for four hours at 37°C / 5% CO_2. The cytotoxicity of the CD8$^+$ T cells against the targets was measured after the 4 hours (propidium iodide (PI) uptake). The percent cytotoxicity of WT CD8$^+$ T cells against IgG1$^+$ targets (6.5±0.7%; n=3) was significantly elevated compared to IFN-γ KO CD8$^+$ T cell cytotoxicity (0.5±0.1%; n=3; p<0.001) and CD4 KO CD8$^+$ T cell cytotoxicity (0.5±0.6%; n=3; p<0.001). The percent cytotoxicity was unchanged against naïve wild-type B cells (WT CD8$^+$: 1.5±0.7%, n=3; IFN-γ KO CD8$^+$: 2.5±0.7%, n=3; p=0.095; CD4 KO CD8$^+$: 2.0±0.1%, n=3; p=0.161).

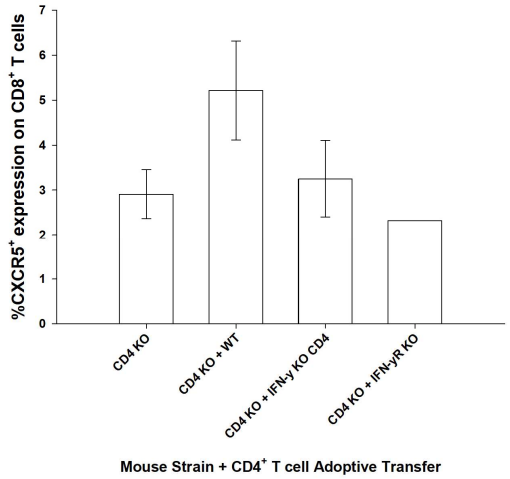

Figure 8: IFN-γ and IFN-γR are required for CD4$^+$ T cells to enhance the quantity of CXCR5$^+$CD8$^+$ T cells. CD4 KO mice (H-2^b) were adoptively transferred with CD4$^+$ T cells from C57BL/6 (wild-type, WT), IFN-γ KO, and IFN-γR KO mice (all H-2^b) and treated with FVB/N (H-2^q) liver lysate (2mg protein). Splenocytes were isolated from the host mice on day 7 and analyzed by flow cytometry for CXCR5 expression on CD8$^+$ T cells. Mice that received WT CD4$^+$ T cells (5.2±1.1%; n=2) had an elevated proportion of CXCR5$^+$CD8$^+$ T cells compared to no adoptive transfer (2.9±0.5%; n=2; p=0.156). Mice that received IFN-γ KO CD4$^+$ T cells did not show a change in CXCR5$^+$CD8$^+$ T cell quantity compared to the CD4 KO mice (3.2±0.9%; n=2; p=0.416) but were slightly reduced compared to WT CD4$^+$ T cells (p=0.212). IFN-γR KO CD4$^+$ T cells did not enhance CXCR5$^+$CD8$^+$ T cell quantity in CD4 KO hosts (2.3%; n=1).

References

1. Grinyo, J. M. 2013. Why is organ transplantation clinically important? *Cold Spring Harb Perspect Med* 3.
2. Gosset, C., C. Lefaucheur, and D. Glotz. 2014. New insights in antibody-mediated rejection. *Current opinion in nephrology and hypertension* 23: 597-604.
3. Bohmig, G. A., F. Eskandary, K. Doberer, and P. F. Halloran. 2019. The therapeutic challenge of late antibody-mediated kidney allograft rejection. *Transpl Int* 32: 775-788.
4. Valenzuela, N. M., and E. F. Reed. 2017. Antibody-mediated rejection across solid organ transplants: manifestations, mechanisms, and therapies. *The Journal of clinical investigation* 127: 2492-2504.
5. Halloran, P. F., J. P. Reeve, A. B. Pereira, L. G. Hidalgo, and K. S. Famulski. 2014. Antibody-mediated rejection, T cell-mediated rejection, and the injury-repair response: new insights from the Genome Canada studies of kidney transplant biopsies. *Kidney international* 85: 258-264.
6. O'Leary, J. G., A. J. Demetris, L. S. Friedman, H. M. Gebel, P. F. Halloran, A. D. Kirk, S. J. Knechtle, S. V. McDiarmid, A. Shaked, P. I. Terasaki, K. J. Tinckam, S. J. Tomlanovich, K. J. Wood, E. S. Woodle, A. A. Zachary, and G. B. Klintmalm. 2014. The role of donor-specific HLA alloantibodies in liver transplantation. *Am J Transplant* 14: 779-787.
7. McCaughan, J. A., and K. J. Tinckam. 2018. Donor specific HLA antibodies & allograft injury: mechanisms, methods of detection, manifestations and management. *Transpl Int*.
8. Feng, S., and J. Bucuvalas. 2017. Tolerance after liver transplantation: Where are we? *Liver Transpl* 23: 1601-1614.
9. Bamoulid, J., O. Staeck, F. Halleck, D. Khadzhynov, S. Brakemeier, M. Durr, and K. Budde. 2015. The need for minimization strategies: current problems of immunosuppression. *Transpl Int* 28: 891-900.
10. McCaughan, J., Q. Xu, and K. Tinckam. 2019. Detecting donor-specific antibodies: the importance of sorting the wheat from the chaff. *Hepatobiliary Surg Nutr* 8: 37-52.
11. Zimmerer, J. M., T. A. Pham, V. M. Sanders, and G. L. Bumgardner. 2010. CD8+ T cells negatively regulate IL-4-dependent, IgG1-dominant posttransplant alloantibody production. *J Immunol* 185: 7285-7292.
12. Zimmerer, J. M., T. A. Pham, C. L. Wright, K. J. Tobin, P. B. Sanghavi, S. M. Elzein, V. M. Sanders, and G. L. Bumgardner. 2014. Alloprimed CD8(+) T Cells Regulate Alloantibody and Eliminate Alloprimed B Cells Through Perforin- and FasL-Dependent Mechanisms. *Am J Transplant* 14: 295-304.
13. Mesin, L., J. Ersching, and G. D. Victora. 2016. Germinal Center B Cell Dynamics. *Immunity* 45: 471-482.
14. Zimmerer, J. M., B. A. Ringwald, S. M. Elzein, C. L. Avila, R. T. Warren, M. Abdel-Rasoul, and G. L. Bumgardner. 2019. Antibody-suppressor CD8+ T Cells Require CXCR5. *Transplantation* 103: 1809-1820.
15. Zimmerer, J. M., M. W. Basinger, B. A. Ringwald, M. Abdel-Rasoul, R. P. Pelletier, A. Rajab, A. El-Hinnawi, H. Parekh, K. Washburn, and G. L. Bumgardner. 2020. Inverse Association Between the Quantity of Human Peripheral Blood CXCR5+IFN-gamma+CD8+ T Cells With De Novo DSA Production in the First Year After Kidney Transplant. *Transplantation* 104: 2424-2434.
16. Coutelier, J. P. 1991. Enhancement of IgG production elicited in mice by treatment with anti-CD8 antibody. *Eur J Immunol.* 21: 2617-2620.
17. Byrom, B., A. F. Barbet, M. Obwolo, and S. M. Mahan. 2000. CD8(+) T cell knockout mice are less susceptible to Cowdria ruminantium infection than athymic, CD4(+) T cell knockout, and normal C57BL/6 mice. *Veterinary parasitology* 93: 159-172.

18. Thomas, M. J., A. Noble, E. Sawicka, P. W. Askenase, and D. M. Kemeny. 2002. CD8 T cells inhibit IgE via dendritic cell IL-12 induction that promotes Th1 T cell counter-regulation. *J Immunol* 168: 216-223.
19. Salagianni, M., K. L. Wong, M. J. Thomas, A. Noble, and D. M. Kemeny. 2007. An essential role for IL-18 in CD8 T cell-mediated suppression of IgE responses. *J Immunol* 178: 4771-4778.
20. Chan, S. Y., L. A. DeBruyne, R. E. Goodman, E. J. Eichwald, and D. K. Bishop. 1995. In Vivo Depletion of CD8+ T cells Results in Th2 Cytokine Production and Alternate Mechanisms of Allograft Rejection. *Transplantation* 59: 1155-1161.
21. Ensminger, S. M., O. Witzke, B. M. Spriewald, K. Morrison, P. J. Morris, M. L. Rose, and K. J. Wood. 2000. CD8+ T cells contribute to the development of transplant arteriosclerosis despite CD154 blockade. *Transplantation.* 69: 2609-2612.
22. Ensminger, S. M., B. M. Spriewald, H. V. Sorensen, O. Witzke, E. G. Flashman, A. Bushell, P. J. Morris, M. L. Rose, A. Rahemtulla, and K. J. Wood. 2001. Critical role for IL-4 in the development of transplant arteriosclerosis in the absence of CD40-CD154 costimulation. *J Immunol* 167: 532-541.
23. Horne, P. H., K. E. Lunsford, A. M. Eiring, Y. Wang, D. Gao, and G. L. Bumgardner. 2005. CD4+ T-cell-dependent immune damage of liver parenchymal cells is mediated by alloantibody. *Transplantation* 80: 514-521.
24. Sayeh, E., R. Aslam, E. R. Speck, H. Le-Tien, A. H. Lazarus, J. Freedman, and J. W. Semple. 2004. Immune responsiveness against allogeneic platelet transfusions is determined by the recipient's major histocompatibility complex class II phenotype. *Transfusion* 44: 1572-1578.
25. Zimmerer, J. M., P. H. Horne, M. G. Fisher, T. A. Pham, K. E. Lunsford, B. A. Ringwald, C. L. Avila, and G. L. Bumgardner. 2016. Unique CD8+ T Cell-Mediated Immune Responses Primed in the Liver. *Transplantation* 100: 1907-1915.
26. Han, J. L., J. M. Zimmerer, Q. Zeng, B. A. Ringwald, C. Cassol, R. T. Warren, M. Abdel-Rasoul, C. K. Breuer, and G. L. Bumgardner. 2020. Antibody-suppressor CD8⁺ T cells ameliorate antibody-mediated rejection following kidney transplant in mice. *The Journal of Immunology* 204: 87.16-87.16.
27. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annual review of immunology* 7: 145-173.
28. Darwich, L., G. Coma, R. Pena, R. Bellido, E. J. Blanco, J. A. Este, F. E. Borras, B. Clotet, L. Ruiz, A. Rosell, F. Andreo, R. M. Parkhouse, and M. Bofill. 2009. Secretion of interferon-gamma by human macrophages demonstrated at the single-cell level after costimulation with interleukin (IL)-12 plus IL-18. *Immunology* 126: 386-393.
29. Matsuda, J. L., O. V. Naidenko, L. Gapin, T. Nakayama, M. Taniguchi, C. R. Wang, Y. Koezuka, and M. Kronenberg. 2000. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *The Journal of experimental medicine* 192: 741-754.
30. Gorbachev, A. V., and R. L. Fairchild. 2006. Activated NKT cells increase dendritic cell migration and enhance CD8+ T cell responses in the skin. *European journal of immunology* 36: 2494-2503.
31. Hermans, I. F., J. D. Silk, U. Gileadi, M. Salio, B. Mathew, G. Ritter, R. Schmidt, A. L. Harris, L. Old, and V. Cerundolo. 2003. NKT cells enhance CD4+ and CD8+ T cell responses to soluble antigen in vivo through direct interaction with dendritic cells. *J Immunol* 171: 5140-5147.
32. Joshi, S. K., G. A. Lang, T. S. Devera, A. M. Johnson, S. Kovats, and M. L. Lang. 2012. Differential contribution of dendritic cell CD1d to NKT cell-enhanced humoral immunity and CD8+ T cell activation. *Journal of leukocyte biology* 91: 783-790.
33. Hong, S., H. Lee, K. Jung, S. M. Lee, S. J. Lee, H. J. Jun, Y. Kim, H. Song, B. Bogen, and I. Choi. 2013. Tumor cells loaded with alpha-galactosylceramide promote therapeutic NKT-dependent anti-tumor immunity in multiple myeloma. *Immunology letters* 156: 132-139.
34. Im, S. J., M. Hashimoto, M. Y. Gerner, J. Lee, H. T. Kissick, M. C. Burger, Q. Shan, J. S. Hale, J. Lee, T. H. Nasti, A. H. Sharpe, G. J. Freeman, R. N. Germain, H. I. Nakaya, H. H. Xue, and R.

Ahmed. 2016. Defining CD8+ T cells that provide the proliferative burst after PD-1 therapy. *Nature* 537: 417-421.
35. Liu, Z., S. Tugulea, R. Cortesini, and N. Suciu-Foca. 1998. Specific suppression of T helper alloreactivity by allo-MHC class I-restricted CD8+ CD28- T cells. *International immunology* 10: 775-783.
36. Horne, P. H., M. A. Koester, K. Jayashankar, K. E. Lunsford, H. L. Dziema, and G. L. Bumgardner. 2007. Disparate primary and secondary allospecific CD8+ T cell cytolytic effector function in the presence or absence of host CD4+ T cells. *J Immunol* 179: 80-88.
37. Brown, K., S. H. Sacks, and W. Wong. 2011. Coexpression of donor peptide/recipient MHC complex and intact donor MHC: evidence for a link between the direct and indirect pathways. *Am J Transplant* 11: 826-831.
38. Krzewski, K., A. Gil-Krzewska, V. Nguyen, G. Peruzzi, and J. E. Coligan. 2013. LAMP1/CD107a is required for efficient perforin delivery to lytic granules and NK-cell cytotoxicity. *Blood* 121: 4672-4683.
39. Quigley, M. F., V. D. Gonzalez, A. Granath, J. Andersson, and J. K. Sandberg. 2007. CXCR5+ CCR7- CD8 T cells are early effector memory cells that infiltrate tonsil B cell follicles. *European journal of immunology* 37: 3352-3362.
40. Le, K. S., P. Ame-Thomas, K. Tarte, F. Gondois-Rey, S. Granjeaud, F. Orlanducci, E. D. Foucher, F. Broussais, R. Bouabdallah, T. Fest, D. Leroux, S. Yadavilli, P. A. Mayes, L. Xerri, and D. Olive. 2018. CXCR5 and ICOS expression identifies a CD8 T-cell subset with TFH features in Hodgkin lymphomas. *Blood Adv* 2: 1889-1900.
41. Shen, J., X. Luo, Q. Wu, J. Huang, G. Xiao, L. Wang, B. Yang, H. Li, and C. Wu. 2018. A Subset of CXCR5(+)CD8(+) T Cells in the Germinal Centers From Human Tonsils and Lymph Nodes Help B Cells Produce Immunoglobulins. *Frontiers in immunology* 9: 2287.
42. Wang, S., Y. Fan, R. C. Brunham, and X. Yang. 1999. IFN-gamma knockout mice show Th2-associated delayed-type hypersensitivity and the inflammatory cells fail to localize and control chlamydial infection. *European journal of immunology* 29: 3782-3792.
43. Rodriguez-Perea, A. L., E. D. Arcia, C. M. Rueda, and P. A. Velilla. 2016. Phenotypical characterization of regulatory T cells in humans and rodents. *Clinical and experimental immunology* 185: 281-291.
44. Zimmerer, J. M., M. W. Basinger, B. A. Ringwald, M. Abdel-Rasoul, R. P. Pelletier, A. Rajab, A. El-Hinnawi, H. Parekh, K. Washburn, and G. L. Bumgardner. 2020. Inverse Association Between the Quantity of Human Peripheral Blood CXCR5+IFN-γ+CD8+T cells with De Novo DSA Production in the First Year After Kidney Transplant. *Transplantation* 104: 2424-2434.