Cellular and Molecular regulators of RBC alloimmunization

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M.S. Biological Sciences, University of Maryland, Baltimore County, 2017

A Dissertation presented to the Graduate Faculty of the
University of Virginia in Candidacy for the Degree of
Doctor of Philosophy

Department of Pathology

University of Virginia
May, 2023

Dedication and Acknowledgements

“If I have seen further, it is by standing on the shoulders of Giants.”

Sir Isaac Newton

I would like to dedicate this work to my parents who have made great sacrifices for my education and for their unwavering belief in my success.

I would like to thank my advisor, Dr. Chance John Luckey for his constant encouragement, support, and patience. He has been instrumental in my development as a scientist by always challenging me to think critically. I have greatly enjoyed our conversations about science and soccer. John was a fantastic mentor and I greatly appreciate all his help and advice over the years.

Words cannot express my gratitude for Dr. Jelena Medved and Dr. Abhinav Arneja for their friendship and mentorship in the lab. Jelena took the time to train me technically and scientifically when I first joined the lab and was always there to answer my questions. More importantly, I am grateful for her friendship and being proactive in organizing fun stuff to do outside the lab. I would like to thank Abhinav for his scientific help, for his friendship and getting me to go to the gym on a regular basis. I will always cherish our discussions on a wide range of topics including philosophy, politics, movies, food, and science. I would like to thank them both for making me feel at home in the lab.

I would like to thank Conrad Niebuhr for all his help and assistance in the lab and for making my work easier. It was great working with him, and I appreciate his contribution to this work.

I would also like to thank Juan Salazar for his friendship and work in the lab. I am also thankful to all my friends at UVA, including Ian and George.

I would also like to recognize the work done by undergraduate students, especially Alexis, Andria, Emily, Soraya, Aanika that has been very important for the completion of these experiments.

I would like to thank the members of my committee: Dr. Janet Cross, Dr. Jim Zimring, Dr. Loren Erickson and Dr. Tim Bender. I appreciate their feedback and the facilitation of important scientific discussions during our meetings which enabled the successful completion of this work.

I would also like to commend the excellent work by the Animal Care staff in the MR5 vivarium and for taking great care of our mice and supporting our experiments. I would also like to recognize the countless mice who have contributed to the advancement of our scientific understanding.

I would also like to thank the various staff members of the Pathology department: Mary White, Susan Bywaters, Michael Kidd and Mary Hall for their excellent administrative support.

I am grateful to my friends Rohit, Anuj, Abhay and Manish for their friendship over the years. I am also thankful to my brother, grandmother, and uncle for all their love and support.

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Abstract

The vast majority of RBC transfusion recipients do not generate antibodies against alloantigens expressed on the RBC surface. We therefore set out to understand why some RBC transfusions are more immunogenic than others. We hypothesized that underlying infections like cytomegalovirus (CMV) at the time of transfusion can lead to increased production of anti-RBC antibodies. Using mouse models, we found that CMV infections at the time of RBC transfusions led to enhanced production of anti-RBC antibodies. We then set out to understand the cytokine and cellular mechanism regulating enhanced antibody production. We found that type 1 IFN signaling was mostly not required for the generation of anti-RBC antibodies following CMV infection. In order to study other cytokines that might be regulating CMV-driven RBC alloimmunization, we first identified the cell types required for the generation of the anti-RBC antibody response. We found that CD4+ T cells were mostly required for generation of anti-RBC IgG. Furthermore, CMV infections led to increased expansion of anti-RBC CD4+ T cells and enhanced differentiation into helper cells. Given the importance of CD4+ T cells, we hypothesized that IL-21, a key T cell-derived cytokine might be regulating anti-RBC antibody production. Interestingly, we found that IL-21 signaling was only partially required for anti-RBC antibody generation following CMV infection, suggesting that other cytokines are regulating the antibody response. Given our mouse data, our collaborators in the Netherlands examined the association between CMV viremia and RBC alloimmunization in their patient dataset. They found that CMV positivity was associated with increased risk of RBC

alloimmunization, which supported our mouse data. Our results show that CMV infections can lead to increased anti-RBC antibody responses.

We further studied anti-RBC antibody generation in a mouse model of storage-induced RBC alloimmunization. Though mouse models allow for the mechanistic exploration of class-switching, previous studies of RBC alloimmunization in mice have focused more on the total IgG response than the relative distribution, abundance, or mechanism of IgG subclass generation. Given this major gap, we compared the IgG subclass distribution generated in response to transfused RBCs relative to protein in alum vaccination and determined the role of STAT6 in their generation. WT mice were either immunized with Alum/HEL-OVA or transfused with HOD RBCs and levels of anti-HEL IgG subtypes were measured using end-point dilution ELISAs. To study the role of STAT6 in IgG class-switching, we first generated and validated novel STAT6 KO mice using CRISPR/cas9 gene editing. STAT6 KO mice were then transfused with HOD RBCs or immunized with Alum/HEL-OVA, and IgG subclasses were quantified by ELISA. When compared to antibody responses to Alum/HEL-OVA, transfusion of HOD RBCs induced lower levels of IgG1, IgG2b and IgG2c but similar levels of IgG3. Class switching to most IgG subtypes remained largely unaffected in STAT6 deficient mice in response to HOD RBC transfusion, with the one exception being IgG2b. In contrast, STAT6 deficient mice showed altered levels of all IgG subtypes following Alum vaccination. Our results show that anti-RBC class-switching occurs via alternate mechanisms when compared to the well-studied immunogen alum vaccination.

Chapter One: Introduction

RBC Alloimmunization

RBC transfusions are given as life-saving treatment for patients who might have moderate to severe anemia and for those with acute blood loss. Most RBC transfusions are allogeneic i.e., they are “foreign” or “non-self”. Therefore, transfusion of these “foreign” RBCs has the potential of generating an immune response against molecules expressed on these RBCs, leading to adverse transfusion reactions. One such antigenic system is the ABO system, which is a group of polymorphic carbohydrate antigens that are expressed on RBCs. Antibodies against ABO are IgM and pre-existing. Transfusion of ABO-incompatible RBCs leads to an acute hemolytic reaction, followed by renal failure and in many cases, death. Therefore, typing is performed prospectively on all transfusion recipients to determine their ABO status in order to find ABO-compatible RBC units.

In addition to the ABO system, there are over 300 known alloantigens that can cause adverse transfusion reactions (Figure 1.1). Transfusion of RBCs incompatible for these non-ABO antigens leads to an IgG response that is often not a significant cause of concern during the first transfusion. However, during subsequent exposure to RBCs, these antibodies can bind to RBCs, causing delayed hemolytic transfusion reactions (DHTRs) (Figure 1.2). While the rates of alloimmunization for most alloantigens in the general population remain low (2-4%), alloantibody generation for a particular antigen, RhD, is higher (around 20%). In addition, generation of anti-RhD antibodies is particularly a problem for women during subsequent pregnancy. Due to this, RhD is also prophylactically typed

before transfusions in order to find RhD-compatible blood. In addition to ABO and RhD typing, screening is also performed to detect IgG antibodies against non-RhD alloantigens to provide compatible RBCs for transfusion. While the alloimmunization rates remain low (2-4%), there are over 4 million transfusions in the United States every year and this presents major problems.

There are two key problems associated with RBC alloimmunization:

RBC alloantibodies can make finding compatible blood difficult

The overall rates of RBC alloimmunization in the general population remain low at around 2-4%.[1] However, some patients who require chronic transfusions have higher rates of alloimmunization compared to the general population. In addition to these high rates, some patients generate antibodies to multiple surface antigens, making it challenging and causing delays in obtaining compatible RBC units for life saving transfusions.

Evanescence of RBC antibodies can lead to DHTRs

Another significant problem is that anti-RBC antibodies are evanescent. In fact, retrospective studies have shown that around 50% of newly formed alloantibodies drop below the threshold of detection within a year after initial detection.[1,2] Therefore, some patients who have been previously alloimmunized can test negative during antibody screens prior to transfusion. Subsequent transfusion with RBCs expressing a sensitized antigen can lead to an anamnestic response, leading to rapid production of secondary antibodies. These antibodies can bind to antigens on transfused RBCs and subsequently cause delayed

hemolytic transfusion reactions (DHTRs) that can cause patient morbidity and mortality.[3,4]

The mechanisms regulating RBC alloimmunization is of great importance and interest in the field of transfusion medicine and immunology

As previously mentioned, anti-RBC alloantibodies are evanescent with over 50% of all newly generated antibodies dropping below the threshold of detection within 1 year of their generation.[1,2] This is in stark contrast to antibodies generated to common infections and vaccinations that are long lived and can have half-lives of a few hundred years.[5] Furthermore, while only a small percentage of transfusion recipients generate anti-RBC antibodies, the vast majority of vaccination recipients make an antibody response. Given this disparity in antibody half-life and response rates between RBC alloimmunization and infections/vaccinations, the mechanisms regulating RBC alloimmunization are of great importance and interest in the field of transfusion medicine and immunology. Therefore, our lab and others are working to understand the underlying molecular mechanisms with the ultimate goal to prevent and manage alloantibody generation. A significant portion of this study will be dedicated to understanding the mechanistic differences in antibody generation between vaccination and RBC transfusion using mouse models.

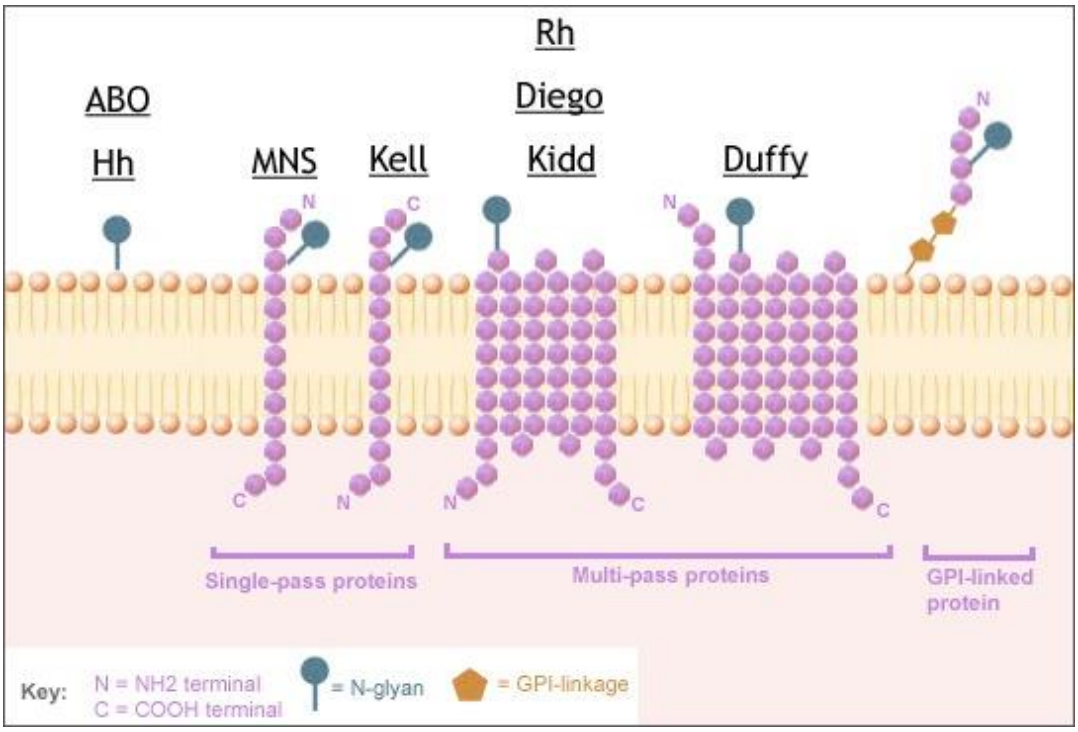


Figure 1.1: Alloantigens on RBC surface

RBC alloantigens are protein molecules expressed on the surface of RBCs. There are over 340 different RBC alloantigens that have been identified previously.[6] Some examples include Kell, Kidd and Duffy. Figure adapted from *Blood Groups and Red Cell Antigens* Chapter 2.[7]

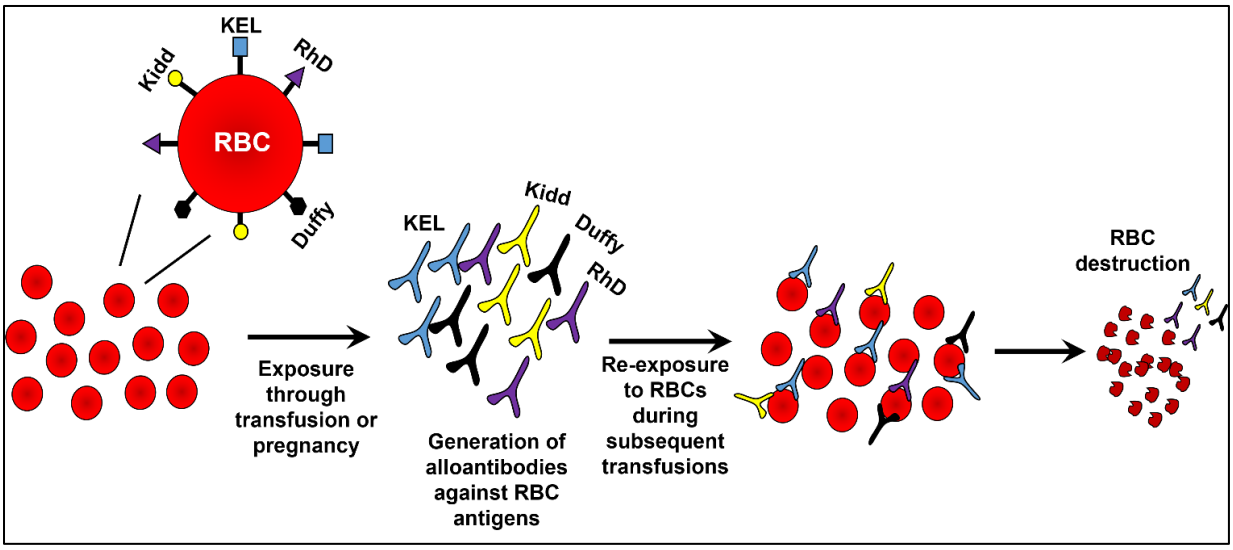


Figure 1.2: Delayed Hemolytic Transfusion Reactions (DHTRs) occur as a result of secondary antibody generation in response to transfused RBCs

Primary transfusions with RBC units can stimulate the generation of anti-RBC antibodies in some patients. These antibodies are often evanescent and fail to be detected during future screening. Re-exposure to RBCs during subsequent transfusions can induce a recall response leading to rapid increase in anti-RBC antibodies and can cause delayed hemolytic transfusion reactions (DHTRs).

Mouse models of RBC alloimmunization:

Most of our mechanistic understanding of anti-RBC antibody generation comes from work using mouse models. There are many mouse models of RBC alloimmunization that have been developed over the years such as the Duffy (Fy^b) transgenic model, Human glycophorin A (hGPA)-transgenic model, HEL-transgenic model, Kel transgenic models and many others.[8] However, the HOD transgenic model of alloimmunization remains one of the most widely used due to its experimental tractability. The HOD mouse model of RBC alloimmunization uses HOD transgenic mice that express a triple fusion protein consisting of Hen Egg Lysozyme (HEL), Ovalbumin (OVA) and human Duffy that is expressed on the RBC surface under the control of a β-globin promoter (Figure 1.3).[9] There are many advantages of the HOD mouse model over other models of alloimmunization. Existing HEL and OVA BCR and TCR transgenic mice allow us to track the fate of HOD-specific B and T cells in an alloimmune response. In addition, the anti-HOD antibody response can be easily studied by using widely used techniques such as ELISA and flow crossmatch. Finally, the HOD mouse model is also enhanced by storage and therefore can be used to study the impact of RBC storage on the alloantibody response (Figure 1.4).

Previous work in our lab and others have shown that transfusion of stored HOD RBCs into recipient mice leads to the generation of an early anti-HOD IgM response which further class-switches to IgG and is more immunogenic compared to transfusion of fresh HOD RBCs.[10–13] Given that the HOD model is enhanced by storage and can be used to study the cellular and molecular mechanisms of RBC

alloimmunization, this model will be used to study anti-RBC immune responses in this study.

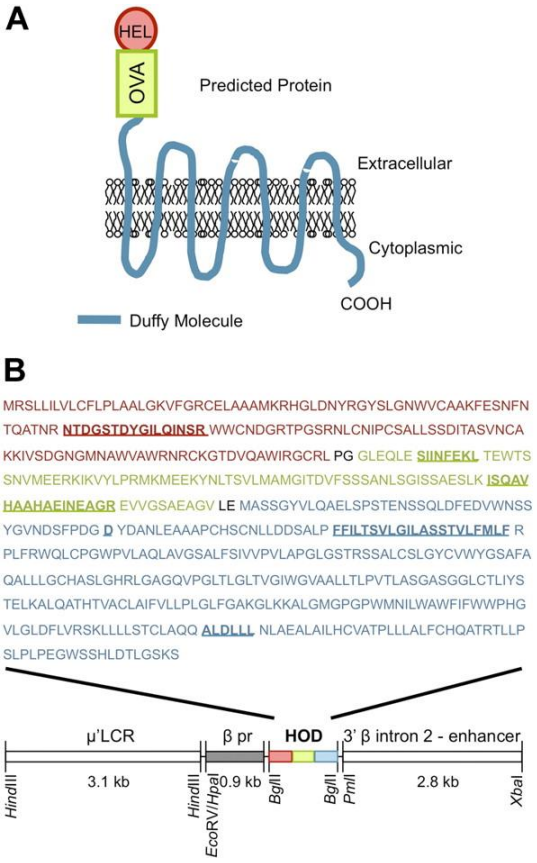


Figure 1.3: HOD model of RBC alloimmunization

HOD transgenic mice express a fusion protein consisting of hen egg lysozyme (HEL) (pink), ovalbumin (OVA) (green) and human Duffy (blue) on the surface of RBCs. The transgene is expressed in RBCs under the control of a β-globin promoter. Figure modified from *Desmarets et al., 2009*.[9]

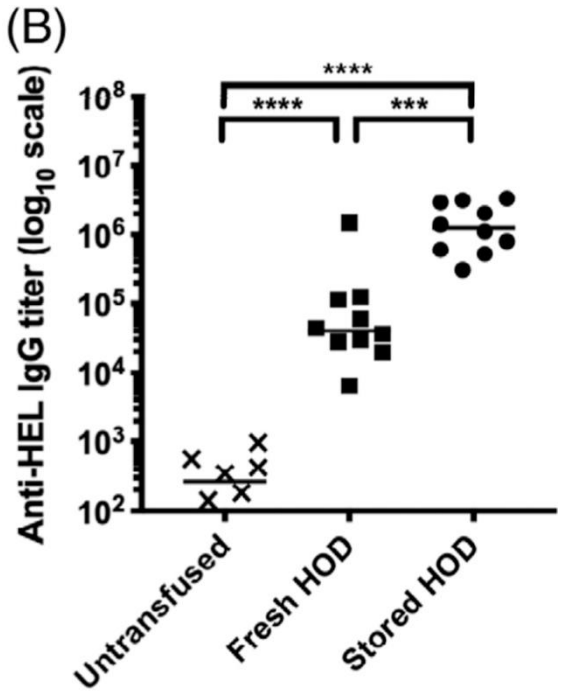


Figure 1.4: HOD model of RBC alloimmunization is storage dependent

RBCs from HOD transgenic mice were transfused into recipient C57BL/6 mice, either fresh or after storage at 4 for 12 days. Anti-HEL IgG titers were measured at 14 days post-transfusion. Figure modified from *Medved et al., 2021*.[13]

Factors affecting RBC alloimmunization:

As mentioned earlier, most transfusions do not result in an alloimmune response. Therefore, understanding why some patients generate anti-RBC antibodies compared to others is an important question in transfusion medicine. While there might be several potential risk factors regulating RBC alloimmunization, a few have been highlighted below.

Underlying inflammation/infections can impact RBC alloimmunization

Recipient inflammatory status is an important variable in RBC alloimmunization. Early work in mice has shown that viral infections such as influenza and viral mimetics such as poly IC can lead to enhancement of alloantibodies.[14,15] Conversely, the bacterial cell wall component LPS inhibited alloantibody formation in mouse models.[16] These studies suggested that recipient inflammation and infections can alter the alloantibody response. A subsequent study in humans showed that ongoing infections at the time of transfusion can have an impact on alloimmunization risk.[17] Interestingly, it was shown that while viral and fungal infections enhanced alloimmunization, certain bacterial infections suppressed alloimmune responses.[17] These mouse studies in combination with human data show that recipient infections and inflammation at the time of transfusion can alter RBC alloimmunization risk, with some enhancing while others suppress RBC alloantibody formation. Further chapters in this study will investigate the role of cytomegalovirus (CMV) infections in regulation of alloimmunization in mice and humans.

Storage of RBCs can influence RBC alloimmunization

In addition to recipient infection and inflammation, other potential risk factors might also have a significant impact on anti-RBC antibody formation.

Blood storage conditions have been explored previously as a potential variable. Collected RBCs are not transfused fresh but are stored for a maximum allowable period of 42 days.[18] The average age of transfused RBCs in the US has been estimated to be around 18 days.[18] Storage of RBCs for extended periods has been shown to cause “storage lesions” that include oxidative damage and metabolic changes.[19] Previous studies have largely shown that storage does not impact alloimmunization risk in humans[20–23], while others have shown that extremities of storage can impact alloimmunization in patients with sickle cell disease (SCD).[24] However, studies in mice show that storage of RBCs leads to an enhancement of anti-RBC alloantibodies in the HOD model of alloimmunization.[10–13] Conversely, storage of RBCs expressing the Kel antigen, show a storage-dependent decrease in anti-RBC IgG.[25] These reports suggest that the antigen expressed on RBCs might also be playing a role in storage-dependent alloimmunization. While further studies are required, storage time remains an important variable for consideration while evaluating alloimmunization risk.

Molecular and Cellular Mechanisms of RBC alloimmunization:

The spleen is an important organ for RBC alloimmunization:

Since the spleen serves as a filter for bloodborne antigens and ageing RBCs in addition to being a secondary lymphoid organ, immune responses to bloodborne antigens are initiated in the spleen. The spleen has also been shown to be an essential organ for anti-RBC antibody responses in mice.[26] Furthermore, this data was consistent with a study in human transfusion recipients, showing that splenectomies are associated with strong protection from primary RBC alloimmunization.[27]

Marginal Zone B cells regulate RBC alloimmunization in mouse models

The marginal zone (MZ) lies between the white pulp (WP) and red pulp (RP) and consists of multiple macrophage populations and MZ B cells. MZ B cells play an important role in immune responses to bloodborne antigens. MZ B cells are phenotypically distinct from follicular B cells. MZ B cells are characterized by expression of $CD21^{hi}$ and $CD23^{neg}$ while follicular B cells are characterized by $CD21^{neg}$ and $CD23^{hi}$. Following antigenic exposure, these cells can differentiate into IgM and IgG secreting cells.[28] MZ B cells have also been shown to be capable of migrating into the WP following antigenic challenge where they can activate CD4+ T cells and/or deliver antigen to follicular B cells in multiple immunization systems.[29–32] The role of MZ B cells in regulating anti-RBC immune response has been studied. Previous studies have shown that transfusion of labelled RBCs leads to co-localization of the transfused RBCs with MZ B cells.[33,34] Furthermore,

depletion of MZ B cells led to a dramatic decrease in anti-RBC IgM and IgG, suggesting their importance in anti-RBC antibody formation.[33,34]

Splenic Bridging Channel DCs (cDC2) are required for RBC alloimmunization

Splenic DC subsets also play important roles in sensing bloodborne pathogens and in activation of antigen specific CD4+ T cells. The two major types of conventional DCs (cDCs) in the spleen include cDC1 and cDC2. Splenic cDC1s express XCR1 and are localized in two regions of the spleen- migratory cDC1s are located in RP and MZ while resident cDC1s are located in the periarteriolar lymphoid sheaths (PALS) in the WP. WP-resident cDC1s access antigen from conduits or immigrating cells. cDC1s also express CD8α. Splenic cDC2s are present exclusively in the bridging channel. cDC2s are characterized by expression of DC immunoreceptor 2 (DCIR2). The role of these DC subsets has also been investigated downstream of RBC transfusions in mouse models of RBC alloimmunization. Previous work has shown that while both cDC1 and cDC2 subsets phagocytose transfused RBCs and express the co-stimulation molecule CD86, only cDC2s are required for the production of anti-RBC antibodies.[12,35] Furthermore, *ex vivo* studies have shown that cDC2s are better activators of RBC-specific CD4+ T cells compared to cDC1s.[12] These studies highlight the importance of these splenic DC subsets in RBC alloimmunization.

CD4+ T cell requirement for RBC alloimmunization seems to be antigen dependent

Primed DCs migrate to the WP where they interact with naïve CD4+ T cells in the T cell zone (TCZ). CD4+ T cells with antigen specific TCRs interact with

primed DCs, leading to their activation. Activated CD4+ T cells express the chemokine receptor CXCR5 enabling them to move towards the B cell follicle. By using antibody-mediated depletion of CD4+ T cells or MHC-II KO mice, previous reports have shown that anti-RBC IgG production required CD4+ T cells in the HOD model of alloimmunization.[12,33] However, studies using Kel expressing RBCs have shown that anti-RBC antibodies are mostly CD4+ T cell independent at early time points[36], suggesting that CD4+ T cell dependence is impacted by antigens expressed by RBCs.

Multiple cytokines regulate anti-RBC antibody production

In addition to the multiple cell types pivotal to the generation of antibody responses, there are many cytokines that play an important role in the immune response. IL-6 is an important cytokine that is important for GC development and IgG production.[37,38] IL-6 is also required for T_{fh} differentiation.[39,40] Previous work in our lab has shown that mice deficient in IL-6 signaling had significant defects in anti-RBC IgG secretion.[11] Furthermore, IL-6 signaling was shown to be T cell-intrinsic and had an impact on T_{fh} differentiation.[11] In addition to IL-6, IL-21 and IL-4 are important cytokines that are produced by T_{fh} cells in the germinal center. These cytokines play an important role in IgG class-switching and promote extrafollicular and GC responses.[39] The role of IL-4 signaling in regulating anti-RBC antibody responses will be further explored in the upcoming chapters.

While a lot of work has been done in uncovering some of the cellular and molecular mechanisms regulating RBC alloimmunization, our understanding remains limited. Our lab and many others around the world are working on

understanding the regulators of RBC alloimmunization, with the overall goal of developing intervening therapies that could prevent this condition in transfusion recipients.

Gaps in our understanding:

Previous experiments by multiple groups have advanced our understanding of mechanisms involved in the generation of anti-RBC alloantibodies. We now have a limited understanding of some of the cellular players like bridging channel DCs, CD4+ T cells and MZ B cells that are involved in alloantibody formation. We also know that cytokines such as IL-6 play an important role in anti-RBC antibody production. Additionally, we know that certain inflammatory stimuli can lead to enhancement of RBC alloimmunization while others suppress.

However, there are still large gaps in our knowledge and understanding of how alloimmunization occurs. For example, despite efforts, we still do not know why some transfusion recipients alloimmunize while others do not.

Studies of human patients have shown that most anti-RBC alloantibodies are IgG1 or IgG3 subclasses, [41–43] though it is unclear why transfused RBCs preferentially drive these subclasses over others. Though mouse models allow for the mechanistic exploration of class-switching, previous studies of RBC alloimmunization in mice have focused more on the total IgG response than the relative distribution, abundance, or mechanism of IgG subclass generation. IgG antibodies are not homogenous, as individual antibodies are expressed as a particular IgG isotype that has unique effector properties. Expression of different IgG isotypes occurs when B cells are induced to undergo class-switching, a process by which a fixed antigen binding region of the heavy chain locus is rearranged so that it is expressed along with one of four different unique IgG constant regions.[44] Since each of these constant regions interacts with different

effector molecules, class-switching serves as a mechanism to diversify the potential effector functions of a given antibody specificity. Indeed, each IgG isotype has its own unique half-life, serum abundance, affinity for specific Fc receptors, and ability to activate complement. Thus, knowing which isotypes of antibodies predominate in a given immune response can give us a better understanding of the functional outcome. Despite this, we still do not know the relative abundance and the IgG subclass distribution that is generated in response to RBC transfusions.

Given these major gaps, this study has been undertaken to answer certain key questions in the field of transfusion medicine and immunology:

1) Do clinically relevant infections such as cytomegalovirus have an impact on RBC alloimmunization?
2) What is the relative IgG subclass abundance and distribution in RBC alloimmunization?
3) What are the molecular regulators of anti-RBC IgG subclasses and how do they differ from antibody responses to vaccination?

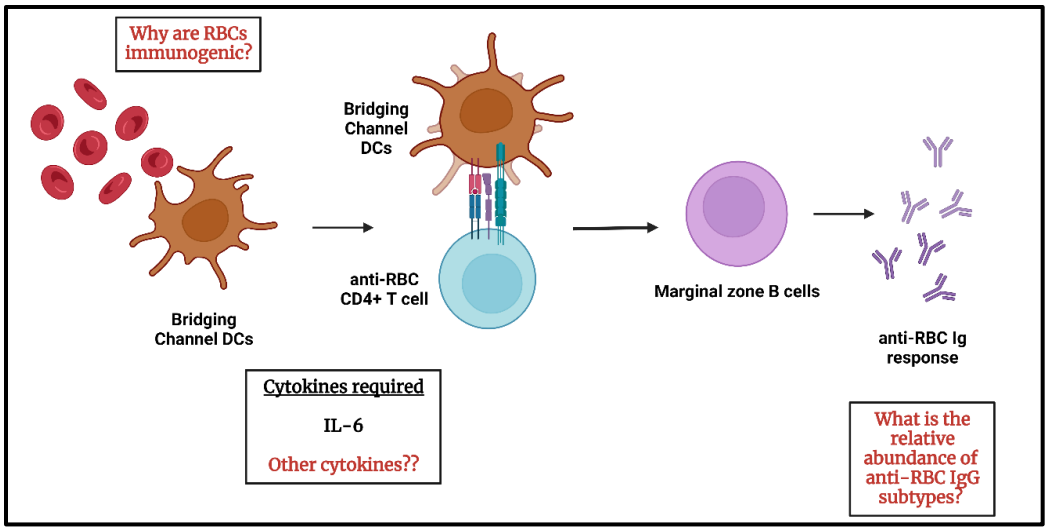


Figure 1.5: Schematic showing unanswered questions (in red) in the field of transfusion medicine and immunology that we have set out to address in this study

Chapter Two: CMV infections enhance anti-RBC antibody formation in mice and humans

Introduction:

It is interesting to note that the vast majority of transfusion recipients do not make antibodies in response to transfused RBCs. Given the low alloimmunization rates, understanding why only some transfusion recipients generate antibodies while most do not is an important question in transfusion medicine.

One potential factor that might determine whether a person makes an antibody or not is the recipient's immune status. Previous studies have shown that the inflammatory state of transfusion recipients at the time of transfusion is an important factor in the determination of alloimmunization risk. Studies using mouse models of RBC alloimmunization have shown that mice immunized with the double stranded RNA mimetic poly I:C or the single stranded RNA influenza virus prior to transfusion with RBCs showed enhanced production of anti-RBC alloantibodies.[14,15,45] In contrast, administration of the gram negative bacterial cell wall component LPS prior to transfusion led to decreased alloantibody responses.[12] A subsequent study in humans showed that patients who had disseminated viral infections and certain fungal infections during a pre-defined alloimmunization risk period had a higher risk of RBC alloimmunization.[17] Conversely, patients with gram negative bacteremia appeared to have decreased risk of RBC alloimmunization.[17] These studies in mice and humans establish the role of the inflammatory state of the transfusion recipient as a key regulator of RBC alloimmunization. These studies further show that the type of infectious stimulus and resulting recipient inflammation can differentially impact anti-RBC alloimmunization.

Different classes of infections activate the immune system through recognition by innate immune receptors, known as pattern recognition receptors (PRRs) specific for pathogen associated molecular patterns (PAMPs) expressed by the infectious agent. For instance, Influenza virus (ssRNA virus) consists of viral components that activate TLR3, TLR7, TLR8, RIG-I and NLRP-3;[46] Poly I:C is a synthetic analog of dsRNA viruses that is recognized by TLR3, RIG-I and MDA-5;[47–49] while LPS, a gram-negative bacterial cell wall component activates TLR4.[50] Differential activation of PRRs by different PAMPs drives rapid secretion of different pro-inflammatory cytokines that can support antigen-specific immune responses. While influenza virus drives secretion of type 1 IFNs, IFN-γ, IL-1, IL-6, IL-18 and IL-10 among others,[51–53] poly I:C administration leads to production of type 1 IFNs, IL-6, IL-12, TNF-α and many other cytokines.[54–56] In contrast, LPS activation of TLR4 leads to secretion of cytokines including TNF-α, IL-6, IFN-γ, IL-1β.[57,58] Therefore, different infections can lead to differential activation of downstream cytokines and can have varying impacts on immune cell activation.

Given that various infectious PAMPs activate different PRRs and generate differential cytokine milieus, they can have varying effects on anti-RBC antibody formation. While previous reports have studied the effects of dsRNA, ssRNA and gram-negative bacteria on RBC alloimmunization, our understanding of dsDNA viral infections remains unclear. Therefore, we set out to investigate the impact of a novel, clinically relevant dsDNA viral infection in the context of RBC alloimmunization.

Cytomegalovirus (CMV) is a dsDNA virus and a member of the *Betaherpesvirinae*. CMV associated PAMPs are recognized by TLR3, TLR7, TLR9 and TLR2.[59–62] In addition, CMV associated PAMPs can also activate the cGAS-STING pathway.[63,64] Activation of these innate sensing pathways lead to rapid secretion of a host of cytokines including IL-12, IFN-γ, TNF-α, IFN-α, IFN-β and IL-6.[65–69]

Furthermore, CMV is clinically relevant, especially for those patients undergoing hematopoietic stem cell transplantation (HSCT) as a treatment for hematological malignancies including acute and chronic leukemias, multiple myeloma, Hodgkin's and non-Hodgkin's lymphomas. These patients also receive multiple RBC transfusions as part of supportive care while undergoing HSCT. Given that HSCT conditioning regimens often leads to bone marrow aplasia, the rates of RBC alloimmunization rates remain low in these patients, as shown by several studies.[70–73] However, despite neutropenia in these patients, a small subset of these patients still produce anti-RBC alloantibodies. Given the increased risk of CMV viremia in HSCT recipients and the prevalence of RBC alloimmunization in some HSCT recipients, we hypothesized that ongoing CMV infection at the time of RBC transfusion might lead to enhanced RBC alloimmunization.

In order to study the effects of murine CMV infection on RBC alloimmunization in mice, we turned to the experimentally tractable HOD mouse model of RBC alloimmunization. The HOD model is a widely used system to understand mechanisms of RBC alloimmunization. HOD transgenic mice express

a fusion protein consisting of Hen Egg Lysozyme (HEL), Ovalbumin (OVA) and human Duffy on the surface of RBCs.[74] Our lab has previously shown that transfusion of freshly collected HOD RBCs into recipient C57BL/6 mice leads to generation of a small, but detectable anti-HOD IgG response.[13]

In this study, we show that ongoing CMV infection at the time of transfusion leads to enhancement of alloantibody responses in the HOD mouse model of RBC alloimmunization. We further show that murine CMV infection leads to a robust expansion of RBC-specific CD4+ T cells and increased anti-RBC CD4+ T cell activation. To validate these findings in humans, our collaborators measured the relative risk of RBC alloimmunization in CMV positive HSCT patients retrospectively and found that CMV infections significantly increased the risk of developing alloimmunization in their patient dataset.

Materials and Methods:

Mice: 8-10 weeks old C57BL/6 mice (Jackson) were housed at the University of Virginia Animal Care Facility. HOD transgenic mice were maintained on an FVB background as previously described.[9] HOD transgenic RBCs contain the triple fusion protein of Hen Egg Lysozyme, Ovalbumin and Duffy. CD45.1+ OT-II mice were generated by crossing CD45.2+OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J, Jackson) with CD45.1 B6.SJL (B6.SJL-Ptprca Pepcb/BoyJ, Jackson) for multiple generations. All mouse protocols were approved by Institutional Animal Care and Use Committees of University of Virginia, Charlottesville.

Murine blood collection and transfusion: Blood from HOD mice was aseptically collected by cardiac puncture into the anticoagulant citrate phosphate dextrose adenine (CPDA-1, Boston Bioproducts IBB-420). The final volume was adjusted to 20% CPDA-1 (v/v). Collected HOD blood was leukoreduced using whole blood cell leukoreduction filter (Pall, AP-4851). Leukoreduced blood was centrifuged at 1200 x g for 10 minutes, adjusted to a final hematocrit of 75% and transfused. Recipient mice received 100ul of 75% Hct HOD blood intravenously via retroorbital injection.

MCMV infections: Mice were injected i.p. with MCMV (Smith Strain ATCC VR1399, 5 X 10^4 PFU). Mice that were injected with MCMV and HOD RBCs received HOD RBCs 10 hours post infection.

Adoptive Transfer Experiments: OVA-specific naïve CD4+ T cells were purified from spleens of CD45.1+ OT-II mice using the CD4+ T cell Isolation Kit, mouse (Miltenyi Biotec 130-104-454), followed by a second round of purification using the

Naïve CD4 T cell Isolation Kit, mouse (Miltenyi Biotec 130-104-453), according to manufacturer's instructions. 10,000 naïve CD45.1+ OT-II cells were adoptively transferred into recipient C57BL/6 mice intravenously by retroorbital injection 2 days prior to transfusion with HOD RBCs. 2 days post adoptive transfers, recipient mice either received HOD RBCs with or without MCMV infections as described above. Mice were euthanized at days 5 and 8 post transfusion and splenocytes were stained for transferred OT-II cells.

OT-II enumeration and T cell activation staining: Following adoptive transfer with OT-II cells and infection with MCMV/HOD RBC transfusion, mice were euthanized at day 5 post-transfusion. Spleens were harvested and mechanically disrupted to obtain a suspension of splenocytes, followed by RBC lysis (eBioscience Cat: 00-4300-54). Splenocytes were first stained with Fixable Viability Dye eFluor™ 780 (eBioscience Cat: 65-0865-14) to exclude dead cells. To enumerate OT-II cells and activated T cells, splenocytes were first stained with biotinylated rabbit anti-mouse CXCR5 antibody (1:25) (BD Biosciences Cat: 551960), followed by staining with biotinylated goat anti-rabbit IgG (1:50) (BD Biosciences Cat: 550338). Finally, cells were stained with an antibody cocktail containing: Streptavidin brilliant violet 421 (1:100) (Biolegend Cat: 405226), anti-CD4 AlexaFlour488 (1:200) (Biolegend Cat:100425), anti-CD8 APC-Cy7 (1:400) (Biolegend Cat: 100713), anti-B220 APC-Cy7 (1:400) (Biolegend Cat: 103223), anti-PD-1 Brilliant Violet 711 (1:100) (Biolegend Cat: 135231), anti-CD45.1 PE-Cy7 (1:200) (Biolegend Cat: 110729) and anti-CD45.2 Brilliant Violet 510 (1:200) (Biolegend Cat: 109837). For determination of CXCR5hi BCL-6+ cells, the above protocol was used, following

which, cells were fixed and permeabilized, and stained with either anti-BCL-6 AlexaFlour647 (1:100) (BD Pharmingen Cat: 561525) or mouse IgG1k AlexaFlour647 isotype control (1:100) (BD Pharmingen Cat: 557732) using the BD Pharmingen Transcription Factor Buffer Set (BD Pharmingen Cat: 562574) according to the manufacturer's protocol. Cells were analyzed using Attune NxT flow cytometer and data were analyzed using FlowJo software.

Measurement of anti-HOD antibodies: Anti-HOD antibodies were measured using flow crossmatch. Serum was collected from experimental mice at 1 week and 2 weeks post transfusion. Serum from transfused mice or untransfused controls were diluted 1:5 in FACS buffer ((PBS + 0.5% BSA + 2% FBS + 0.1% sodium azide) and incubated with 5 x 10^6 FVB.HOD RBCs or FVB RBCs for 10 minutes at room temperature. RBCs were then washed 3 times with FACS buffer and cells were split into two plates. The first plate was incubated with a secondary antibody to detect IgG (Alexa Fluor 647 anti-mouse IgG, Invitrogen, A21236). The second plate was incubated with with the following secondary antibodies: PE anti-mouse IgG1 (Invitrogen, 31862), Alexa Fluor 647 anti-mouse IgG2b (Invitrogen, A21242), FITC anti-mouse IgG2c (SouthernBiotech, 1077-02) or PE-Cy7 anti-mouse IgG3 (SouthernBiotech, 1100-17). For IgM detection, serum was collected at 1 week post transfusion and was detected using a similar protocol as mentioned above. PE anti-mouse IgM (Invitrogen, M31504) was used to detect anti-HOD IgM following primary incubation. All antibodies were used at 1:200 dilution in FACS buffer and incubated at room temperature for 10 minutes. Following secondary incubation, cells were washed once with FACS buffer and fluorescence was

detected using Attune NxT flow cytometer (Invitrogen). Data were analyzed using FlowJo software to obtain median fluorescence intensity (MFI). MFIs are presented as a ratio of HOD MFIs and FVB MFIs to control for technical variability between experiments, variability in flow cytometers and different secondary antibodies.

CD4+ T cell depletion: To deplete CD4+ T cells, mice were injected with either a CD4+ T cell depleting antibody (250 µg, BioXCell Cat: BE0003) or an isotype control antibody (250 µg, BioXCell Cat: BE0090) at days -4 and -2 via i.p injection. CD4+ T cell depletion was confirmed at days 0, 7 and 14 by flow cytometric staining of PMBCs using anti-CD4 AlexaFlour488 (1:200) (Biolegend Cat:100425), anti-CD8 Brilliant Violet 510 (1:200) (Biolegend Cat: 100751) and anti-B220 PE/Cy7 (1:200) (Biolegend Cat: 103221) using a protocol as explained above. At day 0, both groups of mice were injected with 5 x 10^4 PFU MCMV and transfused with HOD RBCs as explained previously.

IFNAR1 blocking experiments: To block IFNAR1 signaling, mice were injected with either an IFNAR1 blocking antibody (100 µg, BioXCell Cat: BE0241) or an isotype control antibody (100 µg, BioXCell Cat: BE0083) at days -1 and 1. At day 0, mice were injected with 5 x 10^4 PFU MCMV and transfused with HOD RBCs as explained previously.

Statistical analysis: Graphing of data and statistical analysis were performed using GraphPad Prism software. Mann-Whitney test was done to compare 2 groups. $p < 0.05$ was considered to be statistically significant and assigned *, whereas $p < 0.01$, $p < 0.001$, and $p < 0.0001$ were assigned **, ***, and ****, respectively.

Results:

Murine CMV infection leads to enhancement of anti-RBC antibody response:

To study the impact of ongoing CMV infections on anti-RBC antibody generation in mice, we transfused mice with HOD RBCs with or without prior infection with murine CMV virus (MCMV) (Figure 2.1A). We first measured the anti-HOD IgM response 1 week post transfusion. Interestingly, we observed that pre-infection with MCMV led to enhancement of the anti-HOD IgM response (Figure 2.1B). To measure the impact of MCMV infection on class switching to IgG, we measured various anti-HOD IgG isotypes at 2 weeks post transfusion. We first observed that MCMV infection enhanced total anti-HOD IgG (Figure 2.1C), consistent with previous data using poly I:C and influenza virus.[14,15,45] Regulation of IgG class switching into various subtypes has been shown to be influenced by the cytokine milieu generated downstream of the immunogenic stimuli. We found that, relative to transfusion with HOD RBCs alone, mice that were pre-infected with MCMV showed significantly elevated levels of anti-HOD IgG1, IgG2b, IgG2c and IgG3 (Figure 2.1D-G), suggesting that molecular pathways activated downstream of MCMV infection can support class switching to multiple IgG subtypes. Our data demonstrate that ongoing murine CMV infection at the time of transfusion with HOD RBCs leads to elevated anti-HOD antibody formation, suggesting a broad range of impact of CMV infection on all anti-RBC antibody subtypes. Given the enhancement of anti-RBC antibodies in the context of MCMV infection, we set out to investigate the cell types and molecular pathways that might be playing a role in this process.

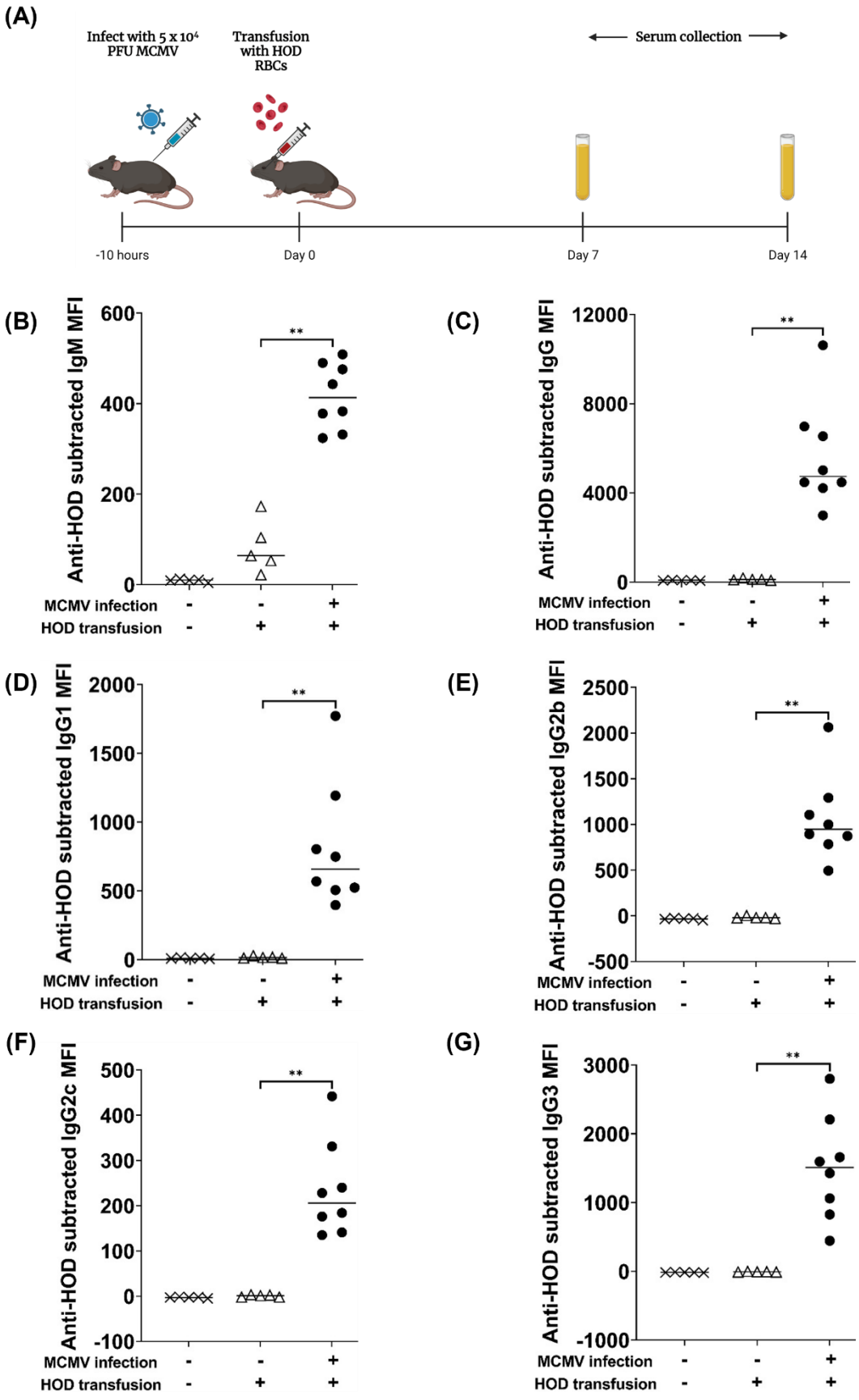

(A)
Infect with 5 x 10⁴ PFU MCMV
Transfusion with HOD RBCs
Serum collection
-10 hours
Day 0
Day 7
Day 14
(B)
Anti-HOD subtracted IgM MFI
600
400
200
0
**
MCMV infection - - +
HOD transfusion - + +
(C)
Anti-HOD subtracted IgG MFI
12000
8000
4000
0
**
MCMV infection - - +
HOD transfusion - + +
(D)
Anti-HOD subtracted IgG1 MFI
2000
1500
1000
500
0
**
MCMV infection - - +
HOD transfusion - + +
(E)
Anti-HOD subtracted IgG2b MFI
2500
2000
1500
1000
500
0
-500
**
MCMV infection - - +
HOD transfusion - + +
(F)
Anti-HOD subtracted IgG2c MFI
500
400
300
200
100
0
-100
**
MCMV infection - - +
HOD transfusion - + +
(G)
Anti-HOD subtracted IgG3 MFI
3000
2000
1000
0
-1000
**
MCMV infection - - +
HOD transfusion - + +

Figure 2.1: Murine CMV infection leads to enhancement of anti-RBC antibody response.

WT mice were transfused with HOD RBCs with or without pre-infection with MCMV. Anti-HOD antibody response was measured at days 7 and 14 post transfusion. (A) Schematic representation of experimental design. (B) Measurement of anti-HOD IgM. (C) Measurement of anti-HOD IgG. (D) Measurement of anti-HOD IgG1. (E) Measurement of anti-HOD IgG2b. (F) Measurement of anti-HOD IgG2c (G) Measurement of anti-HOD IgG3. Bars on plots show median values. Figure is representative of three independent experiments. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$; n.s. $p > .05$.

Type 1 interferon signaling is required for poly I:C-driven RBC alloimmunization, but mostly dispensable for CMV-driven RBC alloimmunization:

The cytokine milieu plays an important role in regulating antibody production to various immunogenic stimuli. Therefore, we set out to identify the cytokines that regulate anti-RBC antibody production. CMV infection in mice leads to production of a whole host of cytokines including IL-12, IFN-γ, TNF-α, type 1 IFNs and IL-6.[65–69] In addition, previous data in poly I:C and influenza-induced RBC alloimmunization have shown the requirement of type 1 IFN signaling pathways for the generation of anti-RBC antibody responses.[14,15] We therefore set out to test whether type 1 IFN signaling also played a role in CMV-induced RBC alloimmunization.

We first validated the role of type 1 IFN signaling in poly I:C driven RBC alloimmunization. IFNAR1 is the common receptor subunit required for signaling by type 1 IFNs (IFN-α and IFN-β). WT mice were either administered an isotype control antibody or IFNAR1 blocking antibody as described previously.[15] Both groups of mice were injected with poly I:C prior to transfusion with HOD RBCs (Figure 2.2A). We found that at 1 week post transfusion, IFNAR1 blocked mice had similar anti-HOD IgM levels compared to isotype controls (Figure 2.2B). We did observe a dramatic decrease in anti-HOD IgG levels in IFNAR1 blocked mice (Figure 2.2C), consistent with previous results.[15] We further measured anti-HOD IgG subclasses. We found that IFNAR1 blocking had a significant impact on IgG1, IgG2b, IgG2c and IgG3, suggesting that IFNAR1 signaling played an important

role and was required for the generation of poly I:C-driven anti-HOD IgG responses (Figure 2.2D-G).

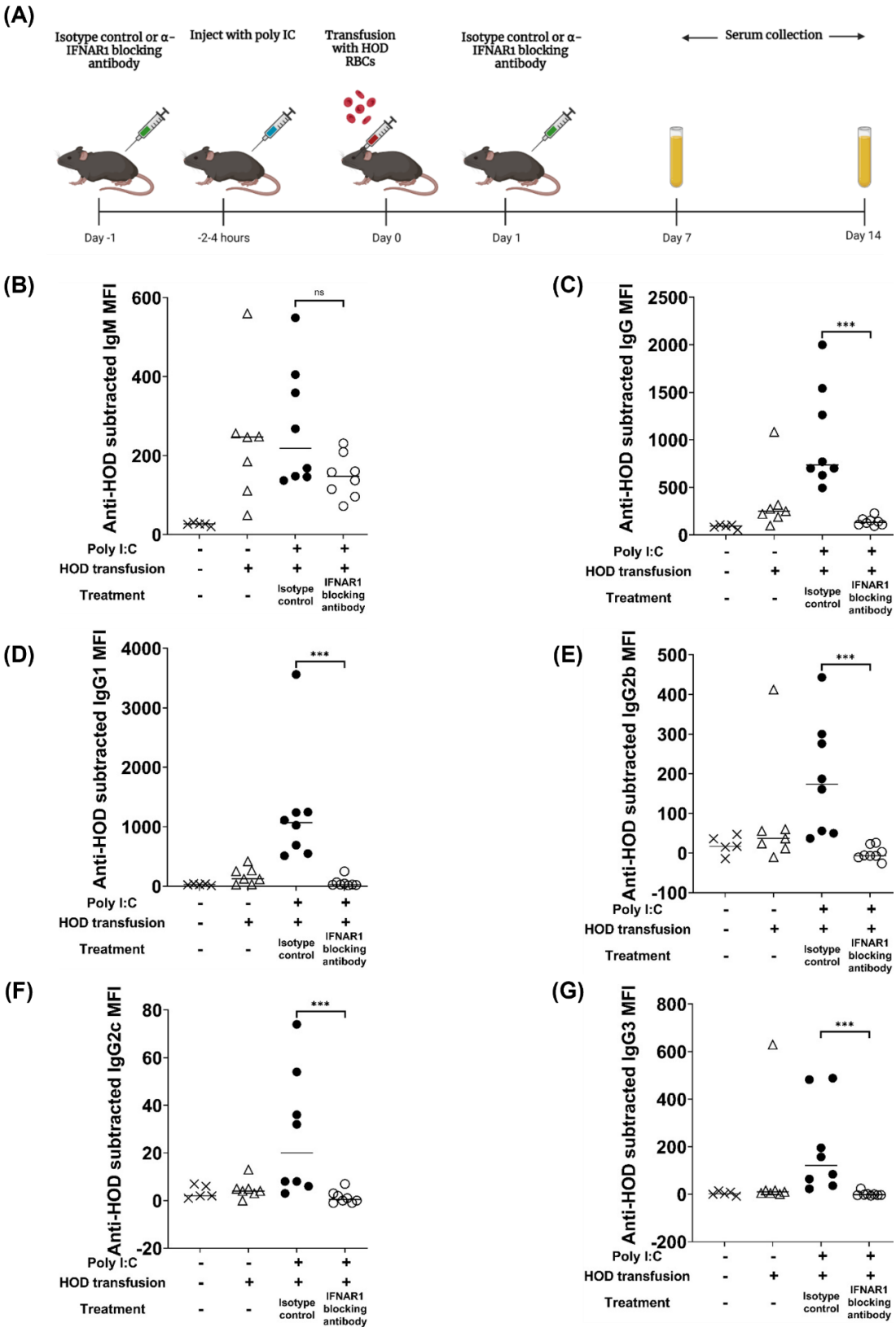

(A)
Isotype control or α-IFNAR1 blocking antibody
Inject with poly IC
Transfusion with HOD RBCs
Isotype control or α-IFNAR1 blocking antibody
Serum collection
Day -1
-2-4 hours
Day 0
Day 1
Day 7
Day 14
(B)
Anti-HOD subtracted IgM MFI
ns
(C)
Anti-HOD subtracted IgG MFI
***
(D)
Anti-HOD subtracted IgG1 MFI
***
(E)
Anti-HOD subtracted IgG2b MFI
***
(F)
Anti-HOD subtracted IgG2c MFI
***
(G)
Anti-HOD subtracted IgG3 MFI
***
Poly I:C
HOD transfusion
Treatment
Isotype control
IFNAR1 blocking antibody

Figure 2.2: Poly I:C-driven RBC alloimmunization requires IFNAR1 signaling.

WT mice were injected with an isotype control antibody or an IFNAR1 blocking antibody. These mice were then transfused with HOD RBCs following administration with poly I:C. Anti-HOD antibody response was measured at days 7 and 14 post transfusion. (A) Schematic representation of experimental design. (B) Measurement of anti-HOD IgM. (C) Measurement of anti-HOD IgG. (D) Measurement of anti-HOD IgG1. (E) Measurement of anti-HOD IgG2b. (F) Measurement of anti-HOD IgG2c (G) Measurement of anti-HOD IgG3. Bars on plots show median values. Figure is representative of three independent experiments. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$; n.s. $p > .05$.

In order to study if IFNAR1 signaling was required for MCMV-driven anti-HOD antibody responses, we disrupted type 1 interferon signaling by using blocking antibodies directed against IFNAR1. Mice were administered either anti-IFNAR1 blocking antibodies or an isotype control antibody prior to the experiment (Figure 2.3A). Both groups of mice were infected with MCMV and transfused with HOD RBCs as detailed above. We found that disruption of IFNAR1 signaling did not have an impact on the anti-RBC IgM response (Figure 2.3B). We further observed no differences in the total anti-RBC IgG response between the two groups of mice (Figure 2.3C). Interestingly, we did observe a small but significant decrease in anti-RBC IgG2b levels in mice that were deficient in IFNAR1 signaling (Figure 2.3E). However, we did not observe any differences in anti-RBC IgG1, IgG2c and IgG3 between the two groups (Figure 2.3D, F-G).

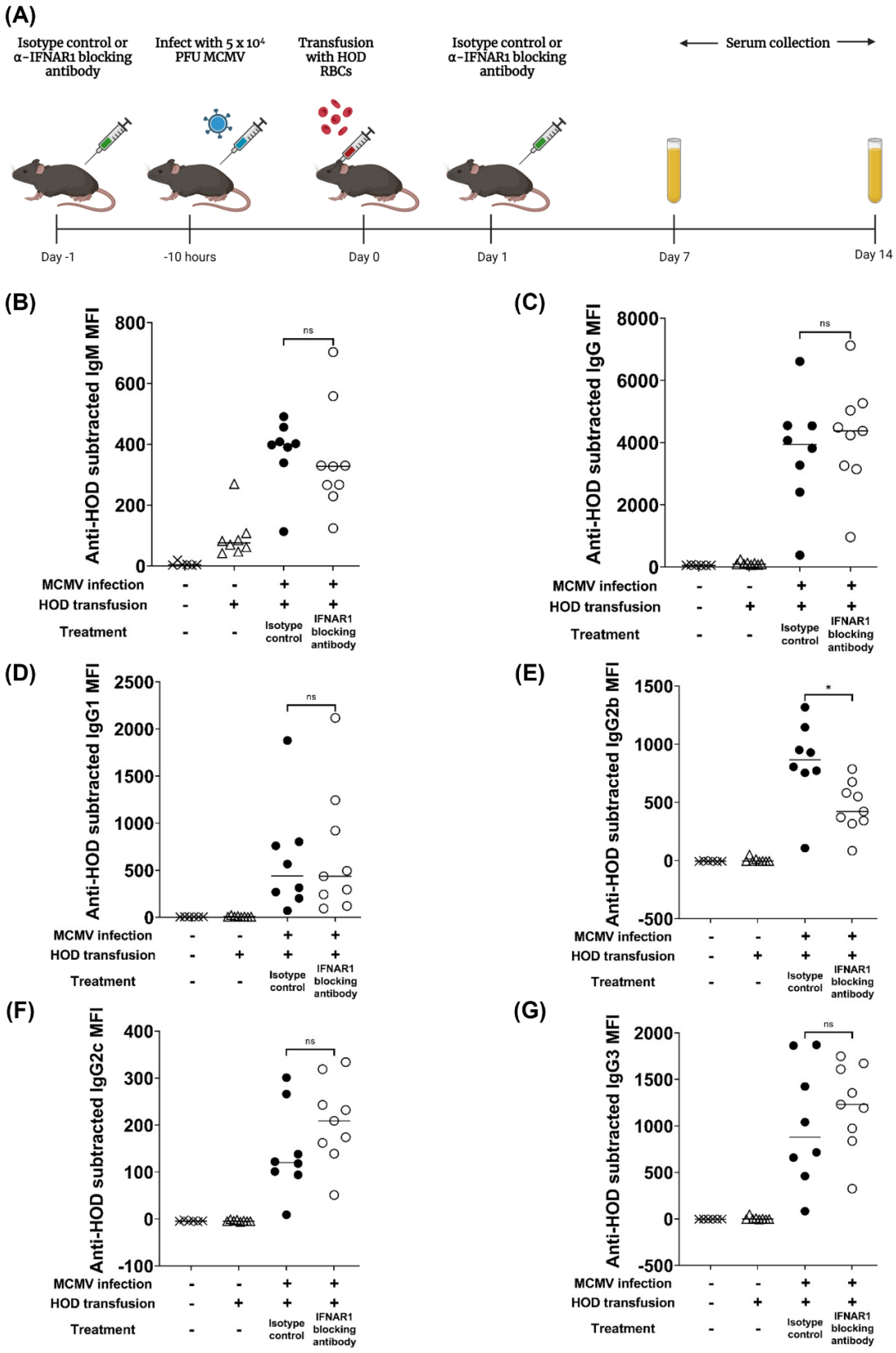

(A)
Isotype control or α-IFNAR1 blocking antibody
Infect with 5 x 10⁴ PFU MCMV
Transfusion with HOD RBCs
Isotype control or α-IFNAR1 blocking antibody
Serum collection
Day -1
-10 hours
Day 0
Day 1
Day 7
Day 14
(B)
Anti-HOD subtracted IgM MFI
ns
MCMV infection
HOD transfusion
Treatment
Isotype control
IFNAR1 blocking antibody
(C)
Anti-HOD subtracted IgG MFI
ns
(D)
Anti-HOD subtracted IgG1 MFI
ns
(E)
Anti-HOD subtracted IgG2b MFI
*
(F)
Anti-HOD subtracted IgG2c MFI
ns
(G)
Anti-HOD subtracted IgG3 MFI
ns

Figure 2.3: IFNAR1 signaling is mostly dispensable for MCMV-driven RBC alloimmunization.

WT mice were injected with an isotype control antibody or an IFNAR1 blocking antibody. These mice were then transfused with HOD RBCs following infection with MCMV. Anti-HOD antibody response was measured at days 7 and 14 post transfusion. (A) Schematic representation of experimental design. (B) Measurement of anti-HOD IgM. (C) Measurement of anti-HOD IgG. (D) Measurement of anti-HOD IgG1. (E) Measurement of anti-HOD IgG2b. (F) Measurement of anti-HOD IgG2c (G) Measurement of anti-HOD IgG3. Bars on plots show median values. Figure is representative of three independent experiments. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$; n.s. $p > .05$.

We further validated these results by using WT and IFNAR1 KO mice. Lack of IFNAR1 signaling in IFNAR1 KO mice was confirmed by stimulating splenocytes with IFN-β and measuring pSTAT1. We observed that while WT splenocytes showed robust pSTAT1 signal, splenocytes from IFNAR1 KO mice failed to show a pSTAT1 signal, showing that IFNAR1 KO mice were deficient in type 1 IFN mediated signaling (Figure 2.4).

WT and IFNAR1 KO mice were infected with MCMV and transfused with HOD RBCs as explained above (Figure 2.5A). Consistent with our experiments using IFNAR1 blocking antibodies, we observed no difference in the anti-RBC IgM response between WT and IFNAR1 KO mice (Figure 2.5B). We further observed that WT and IFNAR1 KO had similar levels of total anti-RBC IgG, IgG1, IgG2c and IgG3 (Figure 2.5C-G). However, we observed a significant decrease in anti-RBC IgG2b in IFNAR1 KO mice compared to WT mice, consistent with our data using IFNAR1 blocking antibodies (Figure 2.5E).

Taken together, our data show that while IFNAR1 signaling is partially required for anti-RBC IgG2b production, it is mostly dispensable for the CMV-induced anti-RBC antibody response. This is in stark contrast to our data using poly I:C and previously published data using poly I:C and influenza, [14,15] and highlights the differences between the anti-RBC antibody response enhanced by various viral infections. Given that IFNAR1 signaling was largely dispensable for anti-RBC antibody generation, we set out to explore the cytokines might be required for anti-RBC antibody responses. To get a better understanding of

cytokines regulating anti-RBC antibodies, we first identified the cell types that play a key role in the antibody response.

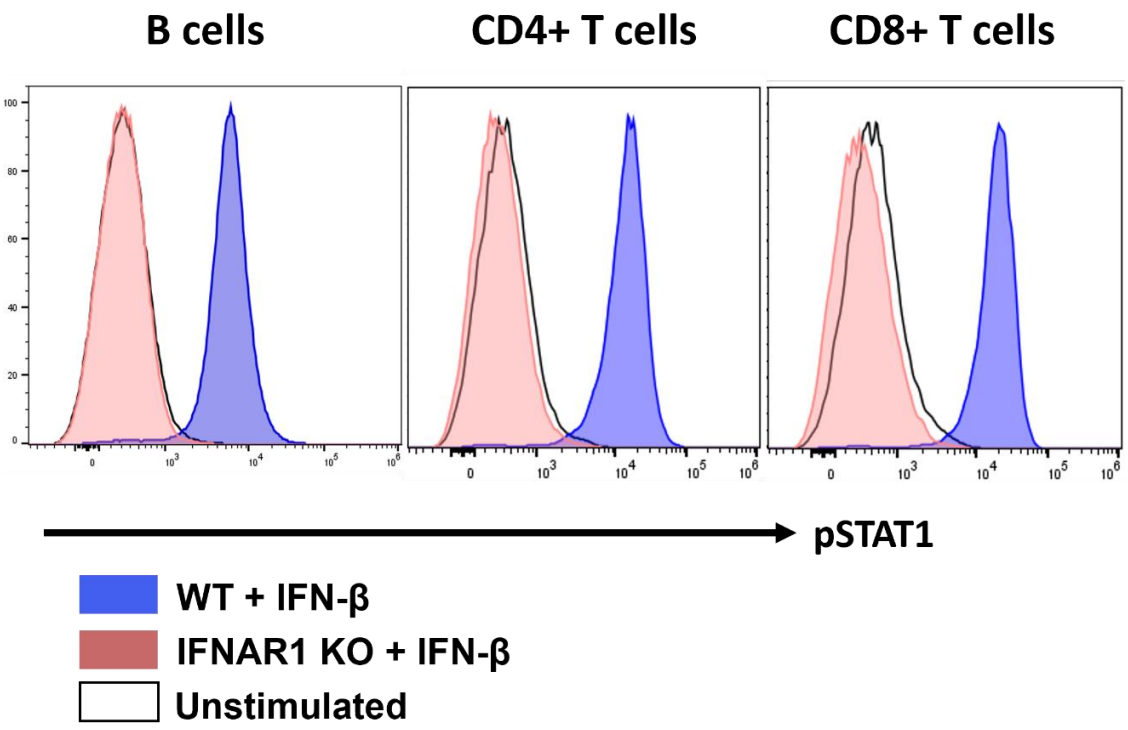


Figure 2.4: IFNAR1 KO mice are deficient in IFN-β driven pSTAT1 signaling.

Splenocytes from WT and IFNAR1 KO mice were stimulated in vitro with IFN-β. pSTAT1 was measured in various cell populations by flow cytometry

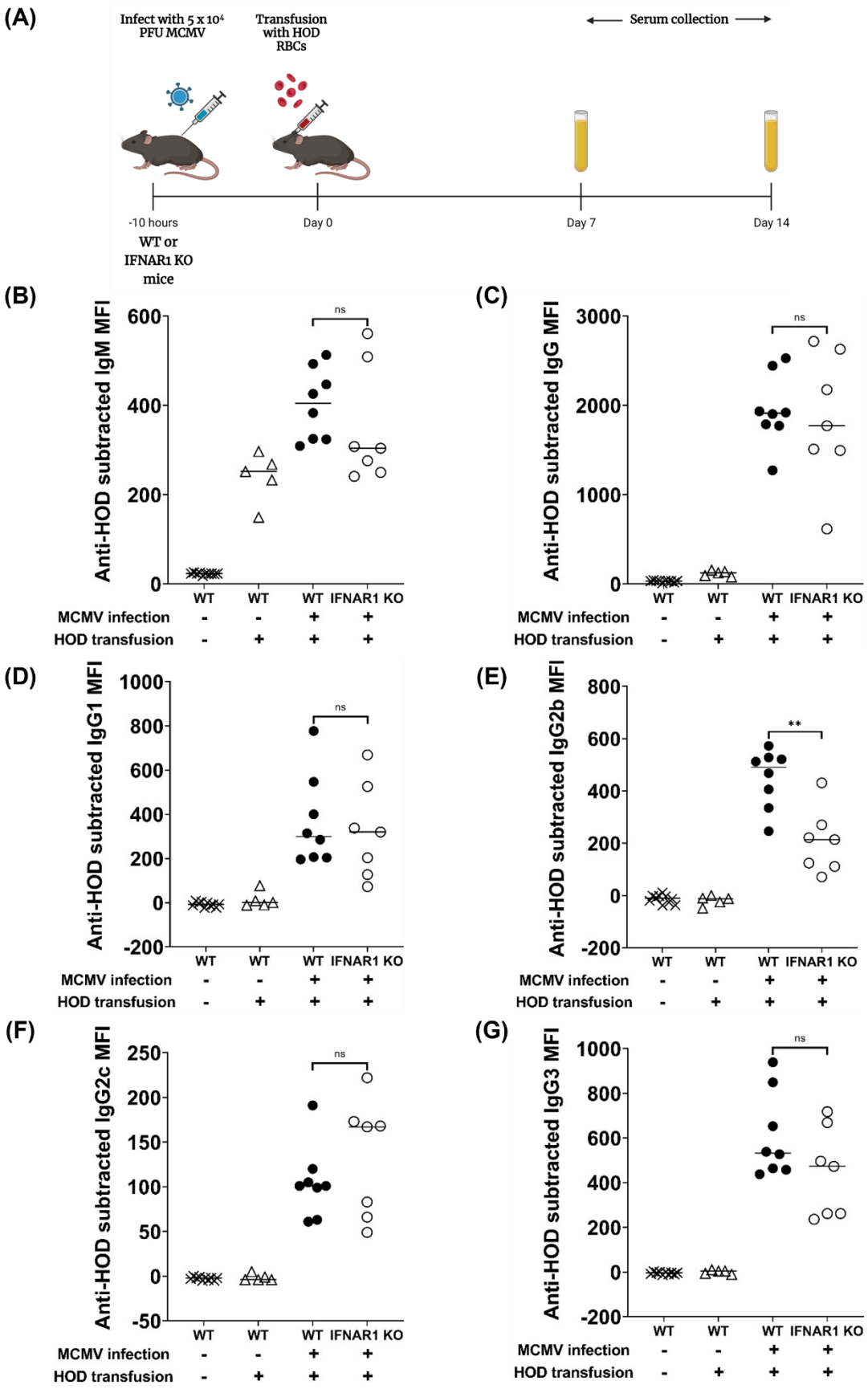

(A)
Infect with 5 x 10⁴ PFU MCMV
Transfusion with HOD RBCs
Serum collection
-10 hours
Day 0
Day 7
Day 14
WT or IFNAR1 KO mice
(B)
Anti-HOD subtracted IgM MFI
(C)
Anti-HOD subtracted IgG MFI
(D)
Anti-HOD subtracted IgG1 MFI
(E)
Anti-HOD subtracted IgG2b MFI
(F)
Anti-HOD subtracted IgG2c MFI
(G)
Anti-HOD subtracted IgG3 MFI
ns
**
WT
IFNAR1 KO
MCMV infection
HOD transfusion

Figure 2.5: IFNAR1 signaling is mostly dispensable for MCMV-driven RBC alloimmunization.

WT and IFNAR1 KO mice were transfused with HOD RBCs following infection with MCMV. Anti-HOD antibody response was measured at days 7 and 14 post transfusion. (A) Schematic representation of experimental design. (B) Measurement of anti-HOD IgM. (C) Measurement of anti-HOD IgG. (D) Measurement of anti-HOD IgG1. (E) Measurement of anti-HOD IgG2b. (F) Measurement of anti-HOD IgG2c. (G) Measurement of anti-HOD IgG3. Bars on plots show median values. Figure is representative of three independent experiments. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$; n.s. $p > .05$.

CD4+ T cells are largely required for enhanced anti-RBC antibody formation following MCMV infection:

Our previous data showed that ongoing MCMV infection can lead to enhancement of anti-RBC antibody production and is largely independent of IFNAR1 signaling. To better understand the cytokine mechanism, we first investigated the cell types that might be required for the CMV-driven RBC alloimmunization. CD4+ T cells play an important role in antibody secretion by producing cytokines and providing help to cognate B cells in various antigenic systems. In fact, previous work has shown that antibody responses to stored HOD RBCs is dependent on CD4+ T cells.[12,33]

To study the role of CD4+ T cells in the MCMV-driven enhancement of anti-RBC antibody responses, mice were treated with a CD4+ T cell depleting antibody or an isotype control antibody prior to the experiment (Figure 2.6A). CD4+ T cell depletion was confirmed by flow cytometric analysis of PMBCs (Figure 2.6B). Following confirmation of CD4+ T cell depletion, mice were infected with MCMV and then transfused with HOD RBCs as explained previously. We first measured the anti-HOD IgM response 1 week post transfusion. Interestingly, we observed that CD+ T cell depletion had no impact on the anti-HOD IgM response, suggesting that CD4+ T cells might not be required for MCMV-enhanced anti-HOD IgM formation (Figure 2.6C). We next measured anti-HOD IgG subtypes following CD4+ T cell depletion. In contrast to our IgM data, we observed that anti-HOD IgG was significantly diminished following CD4+ depletion (Figure 2.6D). We further found that anti-HOD IgG1, IgG2b, IgG2c and IgG3 were dramatically impacted by

CD4+ T cell depletion (Figure 2.6E-H). Our results show that MCMV-driven enhancement of anti-RBC IgM is CD4+ T cell independent. We also show that the anti-RBC IgG response is largely T cell dependent, with IgG1, IgG2b, IgG2c and IgG3 being impacted by loss of CD4+ T cells.

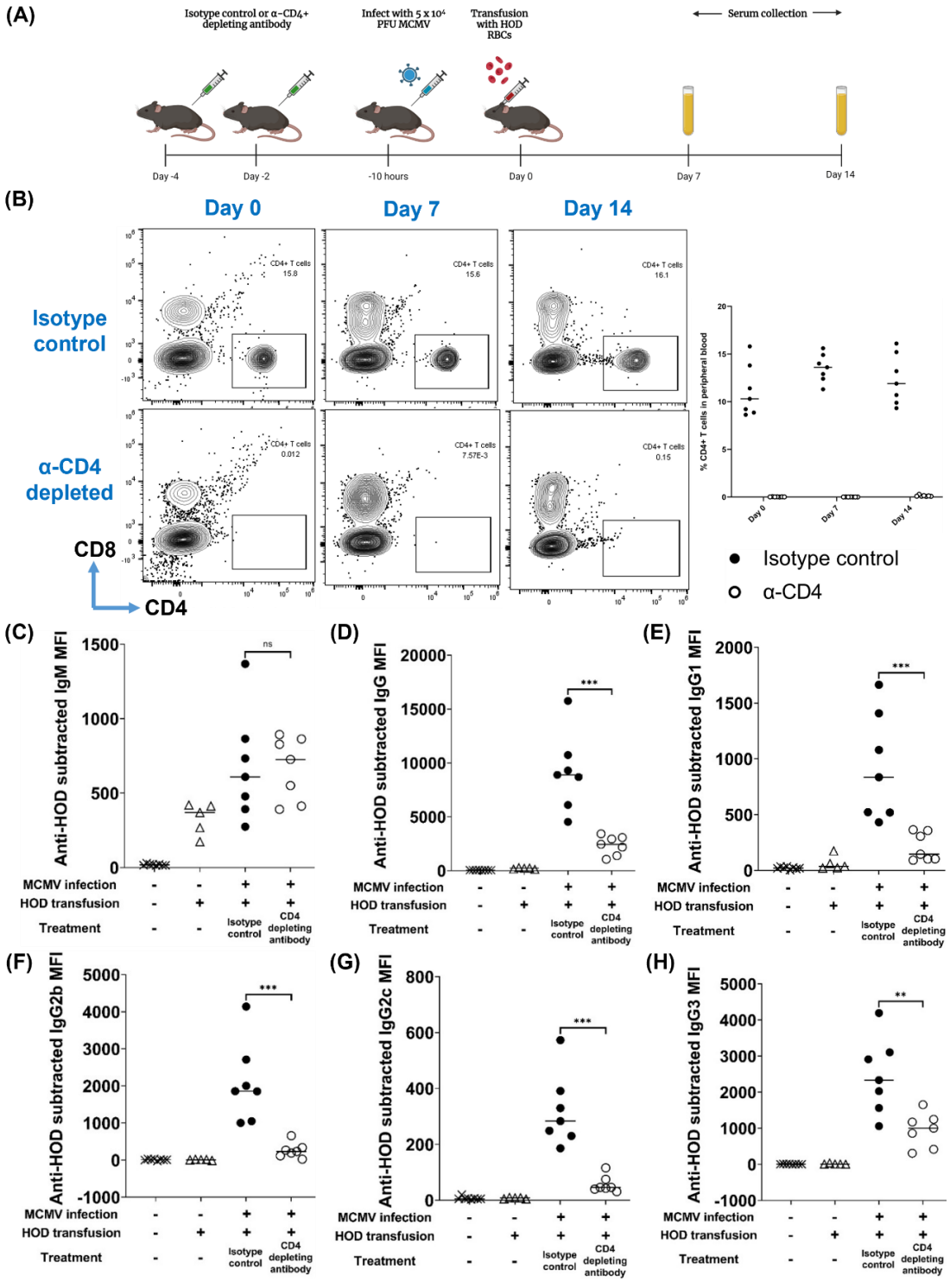

(A)
Isotype control or α-CD4+ depleting antibody
Infect with 5 x 10⁴ PFU MCMV
Transfusion with HOD RBCs
Serum collection
Day -4
Day -2
-10 hours
Day 0
Day 7
Day 14
(B)
Day 0
Day 7
Day 14
Isotype control
α-CD4 depleted
CD8
CD4
CD4+ T cells 15.8
CD4+ T cells 15.6
CD4+ T cells 16.1
CD4+ T cells 0.012
CD4+ T cells 7.57E-3
CD4+ T cells 0.15
% CD4+ T cells in peripheral blood
Isotype control
α-CD4
(C)
Anti-HOD subtracted IgM MFI
ns
(D)
Anti-HOD subtracted IgG MFI
***
(E)
Anti-HOD subtracted IgG1 MFI
***
(F)
Anti-HOD subtracted IgG2b MFI
***
(G)
Anti-HOD subtracted IgG2c MFI
***
(H)
Anti-HOD subtracted IgG3 MFI
**
MCMV infection
HOD transfusion
Treatment
Isotype control
CD4 depleting antibody

Figure 2.6: CD4+ T cells are mostly required for MCMV-driven RBC alloimmunization.

WT mice were injected with an isotype control antibody or a CD4+ T cell depleting antibody. These mice were then transfused with HOD RBCs following infection with MCMV. Anti-HOD antibody response was measured at days 7 and 14 post transfusion. (A) Schematic representation of experimental design. (B) Confirmation of CD4+ depletion. (C) Measurement of anti-HOD IgM. (D) Measurement of anti-HOD IgG. (E) Measurement of anti-HOD IgG1. (F) Measurement of anti-HOD IgG2b. (G) Measurement of anti-HOD IgG2c (H) Measurement of anti-HOD IgG3. Bars on plots show median values. Figure is representative of three independent experiments. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$; n.s. $p > .05$.

Anti-RBC CD4+ T cells show increased expansion and differentiation following MCMV infection:

Given that CD4+ T cells were largely required for the MCMV driven RBC alloimmunization, we set out to investigate the impact of MCMV infections on anti-RBC CD4+ T cells. To study anti-HOD RBC specific CD4+ T cells, we made use of the TCR transgenic OT-II mouse model, where most of the CD4+ T cell compartment is specific for $OVA_{323-339}$ peptide, which is an important component of the HOD transgenic protein. Adoptive transfer of naïve OT-II cells into HOD RBC recipients allows us to track the anti-HOD CD4+ T cell response. Therefore, in order to measure the impact of MCMV infection on anti-HOD CD4+ T cells, congenically marked naïve OT-II cells were adoptively transferred into C57BL/6 mice. These mice were infected with MCMV and transfused with HOD RBCs as explained previously (Figure 2.7A). Splenic OT-II cell numbers were measured at day 5 post transfusion to measure the anti-HOD CD4+ T cell response. We found that while transfusion with HOD RBCs alone only led to a modest increase in OT-II cell numbers, infection with MCMV prior to transfusion led to a robust expansion of OT-II cells (Figure 2.8B). Mice that were infected with MCMV without transfusion with HOD RBCs failed to show any expansion of OT-II cells. Activation of naïve CD4+ T cells leads to increased expression of the chemokine receptor CXCR5 that supports migration towards the B cell follicle. In multiple immunization models, activated CD4+ T cells can provide help to cognate B cells, either at the B-T zone interface (extrafollicular) or inside the follicle (germinal center). CD4+ T cell help can support class switching, affinity maturation and differentiation into long lived

plasma cells.[39] Given the increased numbers of OT-II cells seen in HOD transfused mice following MCMV infection, we wanted to investigate CD4+ T cell activation in this setting. We used expression of CXCR5, PD-1 and Bcl-6 to identify activated T cells. It is important to note that classically, expression of these proteins have been used to identify germinal center localized follicular helper T cells (Tfh). However, while these proteins are important in germinal center formation, there is evidence suggesting that they are also expressed by activated T cells outside the germinal center (extrafollicular T cells).[75–77] We therefore used these proteins as markers of RBC-specific CD4+ T cell activation. We identified these cells by measuring co-expression of CXCR5 and PD-1 or CXCR5 and BCL6. Interestingly, we found that while HOD RBCs alone showed no T cell activation, pre-infection with MCMV led to robust expression of these markers, suggesting enhanced RBC-specific T cell activation following CMV infection (Figure 2.8C-D). Our data demonstrate that infection with MCMV prior to RBC transfusion leads to enhanced expansion of anti-RBC CD4+ T cells and increased activation, suggesting better CD4+ T cell help in the generation of anti-RBC antibodies.

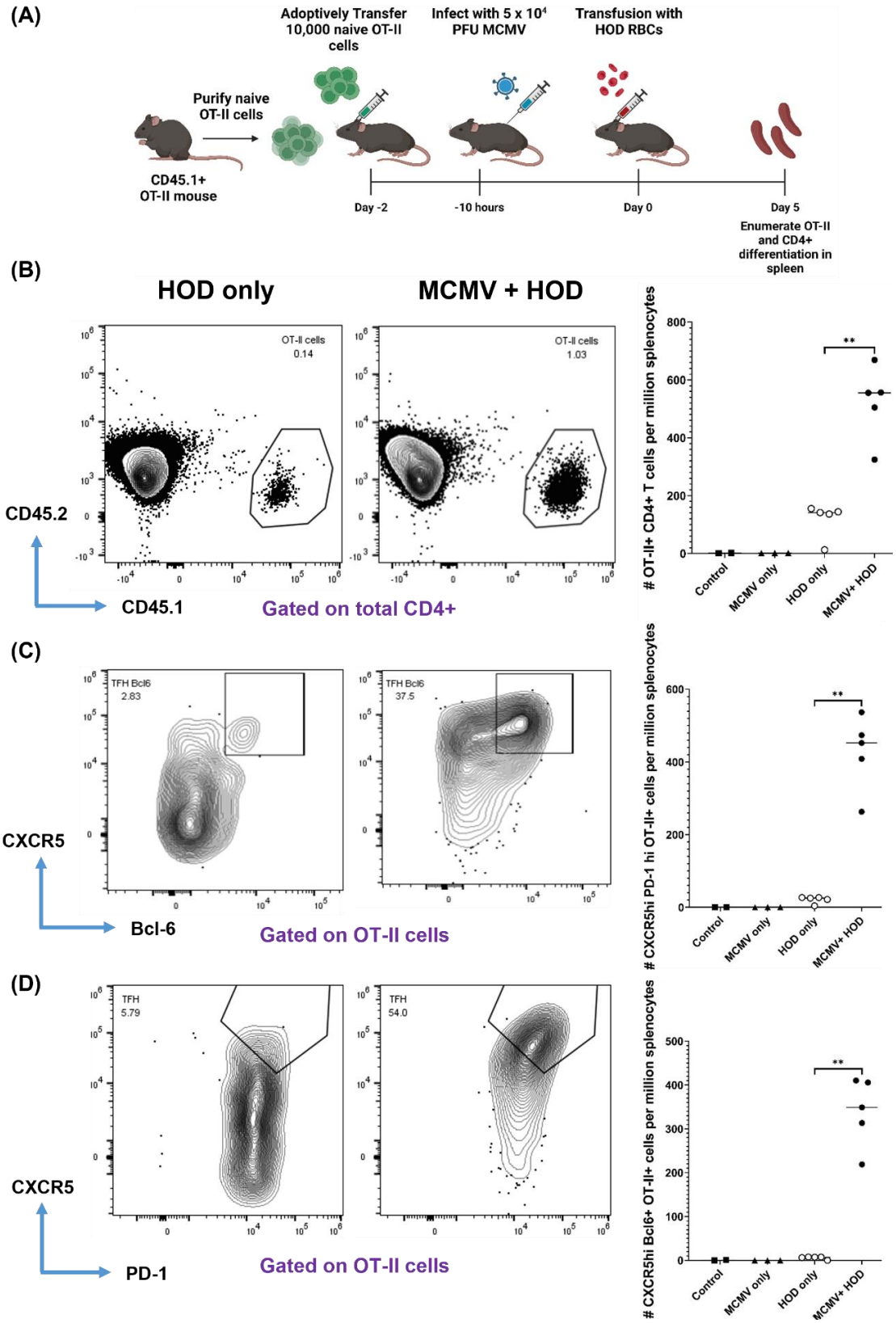

(A)
Adoptively Transfer 10,000 naive OT-II cells
Infect with 5 x 10⁴ PFU MCMV
Transfusion with HOD RBCs
Purify naive OT-II cells
CD45.1+ OT-II mouse
Day -2
-10 hours
Day 0
Day 5
Enumerate OT-II and CD4+ differentiation in spleen
(B)
HOD only
MCMV + HOD
OT-II cells 0.14
OT-II cells 1.03
CD45.2
CD45.1
Gated on total CD4+
OT-II+ CD4+ T cells per million splenocytes
Control
MCMV only
HOD only
MCMV+ HOD
(C)
TFH Bcl6 2.83
TFH Bcl6 37.5
CXCR5
Bcl-6
Gated on OT-II cells
CXCR5hi PD-1 hi OT-II+ cells per million splenocytes
(D)
TFH 5.79
TFH 54.0
CXCR5
PD-1
Gated on OT-II cells
CXCR5hi Bcl6+ OT-II+ cells per million splenocytes

Figure 2.8: MCMV infection leads to increased expansion and differentiation of anti-RBC CD4+ T cells.

Naïve OT-II cells were adoptively transferred into WT mice. These mice were then transfused with HOD RBCs following infection with MCMV. OT-II cells were quantified at day 5 post-transfusion. (A) Schematic representation of experimental design. (B) Measurement of OT-II cell numbers in the spleen. (C) Measurement of $CXCR5^{hi}$ $PD\text{-}1^{hi}$ OT-II cells. (D) Measurement of $CXCR5^{hi}$ $Bcl6^{hi}$ cells. Bars on plots show median values. Figure is representative of three independent experiments. *p < .05; **p < .01; ***p < .001; ****p < .0001; n.s. p > .05.

IL-21 signaling is required for IgM and IgG3 in CMV-induced RBC alloimmunization:

Our previous results showed that type 1 IFN signaling was only partially required for anti-RBC antibody responses. We further observed that CD4+ T cells play an essential role in the generation of anti-RBC antibody responses. We therefore set out to explore what CD4+ T cell-derived cytokines might be involved in the generation of anti-RBC antibodies. IL-21 is an important cytokine that is secreted by CD4+ T cells and has previously been shown to regulate antibody production in multiple immunization models.[78–81] Furthermore, IL-21 is an important cytokine for TFH generation and germinal center B cell differentiation.[80,82–84]

Given our data that MCMV infection leads to robust CD4+ T cell responses, we hypothesized that IL-21 might play a role in the CMV-driven anti-RBC antibody production. To study the role of IL-21 in anti-RBC antibody generation, WT and IL21R KO mice were infected with MCMV and transfused with HOD RBCs (Figure 2.9A). We first measured the anti-RBC IgM response 1 week post-transfusion. We found that mice deficient in IL21R showed significantly lower anti-RBC IgM levels compared to WT mice (Figure 2.9B). We next measured anti-RBC IgG in both groups of mice. We found that IL21R KO mice had significantly lower total anti-RBC IgG compared to WT (Figure 2.9C). While we did observe lower levels of anti-RBC IgG1, IgG2b and IgG2c in IL21R KO mice, it did not reach statistical significance (Figure 2.9D-F). Interestingly, we observed that IL21R KO mice had significantly lower anti-RBC IgG3 levels compared to WT mice (Figure 2.9G). Our

data suggests that IL-21 signaling plays a significant role in the generation of anti-RBC IgM and IgG3.

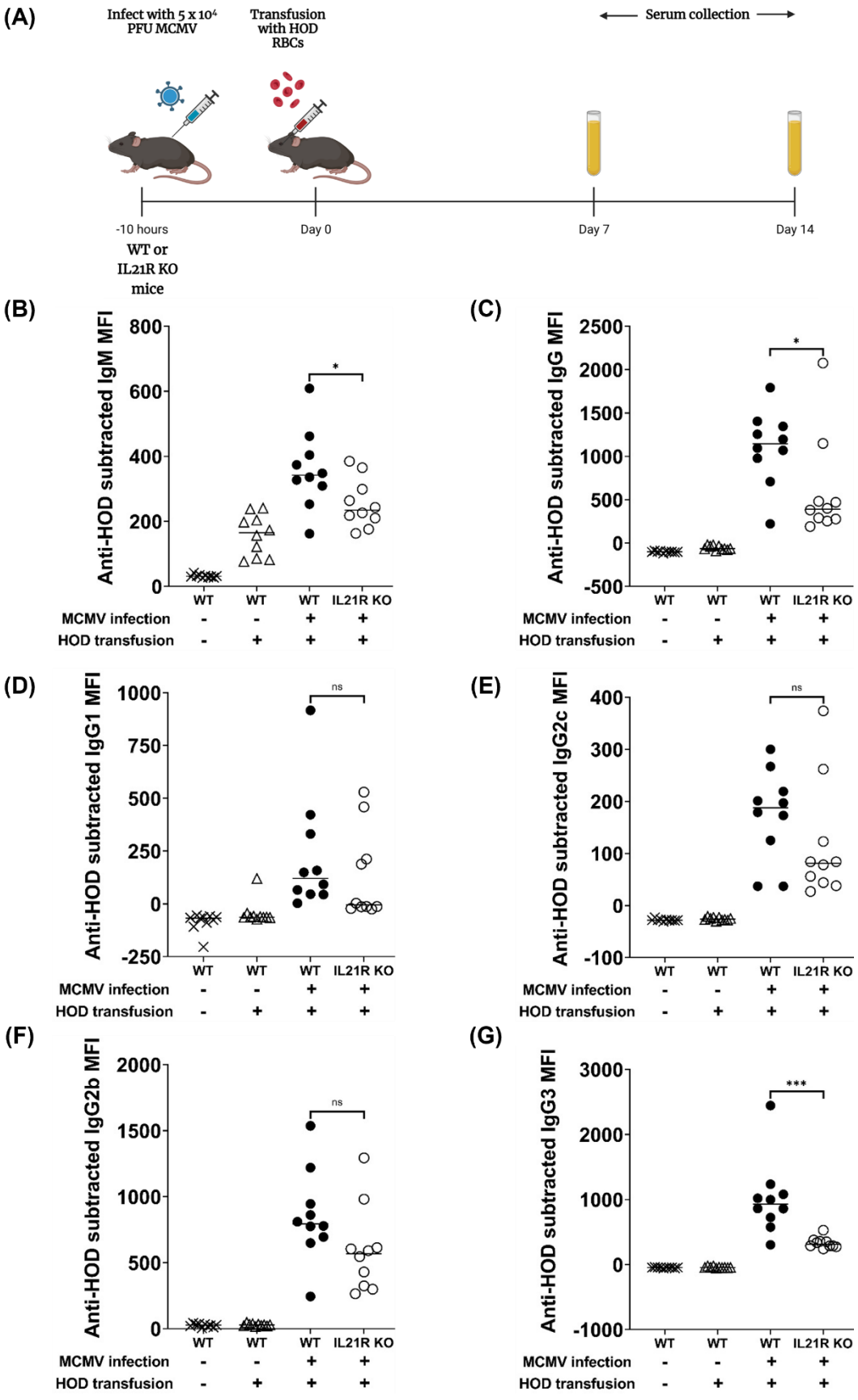

(A)
Infect with 5 x 10⁴ PFU MCMV
Transfusion with HOD RBCs
Serum collection
-10 hours
WT or IL21R KO mice
Day 0
Day 7
Day 14
(B)
Anti-HOD subtracted IgM MFI
(C)
Anti-HOD subtracted IgG MFI
(D)
Anti-HOD subtracted IgG1 MFI
(E)
Anti-HOD subtracted IgG2c MFI
(F)
Anti-HOD subtracted IgG2b MFI
(G)
Anti-HOD subtracted IgG3 MFI
WT WT WT IL21R KO
MCMV infection - - + +
HOD transfusion - + + +
ns
*
***

Figure 2.9: IL-21 is required for anti-HOD IgM and IgG3 in MCMV-driven RBC alloimmunization.

WT and IL-21R KO mice were transfused with HOD RBCs following infection with MCMV. Anti-HOD antibody response was measured at days 7 and 14 post transfusion. (A) Schematic representation of experimental design. (B) Measurement of anti-HOD IgM. (C) Measurement of anti-HOD IgG. (D) Measurement of anti-HOD IgG1. (E) Measurement of anti-HOD IgG2b. (F) Measurement of anti-HOD IgG2c (G) Measurement of anti-HOD IgG3. Bars on plots show median values. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$; n.s. $p > .05$.

CMV positivity is associated with RBC alloimmunization in patients:

Given our data showing that murine CMV infections can enhance anti-RBC antibody responses, we investigated if underlying CMV infections at the time of RBC transfusions can impact human RBC alloimmunization. Our collaborators at Leiden University in the Netherlands were able to look in their patient dataset consisting of HSCT patients who had received RBC transfusions. Investigation of this patient dataset revealed that patients who had CMV viremia showed an adjusted RR of 1.91, suggesting a higher risk of RBC alloimmunization (Figure 2.10). This result supports our murine data suggesting that CMV infections can enhance the generation of anti-RBC antibodies.

RBC Alloimmunization

	Cases (N (%))	Controls (N (%))	RR (95% CI) *	Adjusted RR (95% CI) †
CMV infection				
Absent	502 (99.4)	1,001 (99.1)	ref.	ref.
Present	3 (0.6)	9 (0.9)	0.68 (0.39-1.18)	1.91 (0.84-4.33)

Figure 2.10: CMV is associated with human RBC alloimmunization.

Association between CMV positivity and RBC alloimmunization was measured in a patient population primarily comprising of allogeneic HSCT patients.

Discussion:

Given that only a small percentage of transfusion recipients generate anti-RBC antibodies, we set out to understand the underlying factors that might lead to enhanced alloantibody production. Due to the clinical importance of cytomegalovirus (CMV) infections and their coincident occurrence with multiple transfusions, we investigated the impact of acute CMV infections on RBC alloimmunization. Using mouse models, we observed that underlying CMV infections at the time of RBC transfusions led to enhanced anti-RBC IgM, suggesting that CMV infections led to activation of pathways that can support the early wave of anti-RBC IgM. We further observed that CMV infections enhanced total anti-RBC IgG and the various IgG subclasses- IgG1, IgG2b, IgG2c and IgG3. These results are very interesting given that IgG class-switching to one subtype over others is regulated by the cytokine milieu. The effect of CMV infections on anti-RBC IgG suggests that CMV infection leads to the production of a broad range of cytokines that can support class switching to multiple different IgG subclasses.

Given that type 1 IFN signaling is required for poly I:C and influenza-driven anti-RBC antibody production, we tested the role of type 1 IFNs in the CMV model of alloimmunization. We did not observe an impact of IFNAR1 blockade on anti-RBC antibody production. Although anti-RBC IgG2b showed a small, but significant decrease, anti-RBC IgM and IgG responses were largely IFANR1 independent. These results suggest that the cytokine mechanisms regulating anti-RBC antibodies in the context of CMV infections is different from that observed in the poly I:C and influenza models of RBC alloimmunization. We therefore set out

to identify the cytokines that are required for CMV-driven alloimmunization. However, we first identified the cellular players to gain a better understanding of the mechanisms regulating anti-RBC antibody production.

Given the importance of CD4+ T cells in driving class switching in various immunization systems, we studied the role of CD4+ T cells in regulating the CMV-driven anti-RBC response. We observed that CD4+ T cells were not required for anti-RBC IgM production. This is consistent with several reports using various immunization systems and suggests that the B cell production of anti-RBC IgM does not require CD4+ T cell help.[33,85–88] We did observe that CD4+ T cell depletion led to a near complete abrogation of the total anti-RBC IgG response and anti-RBC IgG subclasses. This is consistent with previous experiments in various immunization models showing that antigen specific IgG responses are largely CD4+ T cell dependent. Although anti-RBC IgG3 was significantly impacted by the loss of CD4+ T cells, we did detect IgG3 above background, suggesting that there might be a CD4+ T cell independent component to the IgG3 response. This is consistent with several reports, where IgG3 is the major IgG subclass that is observed in T-independent antigenic systems.

Given that CD4+ T cells were largely required for anti-RBC antibody IgG production, we tested the impact of CMV infections on RBC-specific CD4+ T cell expansion and differentiation. We observed that CMV infection led to increased expansion of RBC-specific CD4+ T cells. We further observed that CMV infection also enhanced CD4+ T cell differentiation into “helper cells” capable of providing B cell help and drive IgG class switching. These “helper cells” were measured by

expression of CXCR5, PD-1 and Bcl-6. While expression of these proteins have been classically associated with germinal center localized follicular T cells (Tfh), there is evidence suggesting that they are also expressed by activated T cells outside the germinal center (extrafollicular T cells).[75–77] Therefore, we show that CMV infection drives the generation of RBC-specific CD4+ T cells that differentiate into helper cells that can potentially drive B cell class switching and antibody production. Future studies using microscopy will help in distinguishing between these cell types based on their localization.

Given the key role of CD4+ T cells in anti-RBC IgG production following CMV infections, we hypothesized that CD4+ T cell-derived cytokines might be playing an important role in regulating CMV-driven RBC alloimmunization. We first identified some of the key CD4+ T cell-derived cytokines that can drive antibody production. IL-21 is an important cytokine that is secreted by CD4+ T cells and has previously been shown to regulate antibody production in multiple immunization models.[78–81] Furthermore, IL-21 is an important cytokine for TFH generation and germinal center B cell differentiation.[80,82–84] We, therefore set out to investigate the role of IL-21 in regulating anti-RBC antibody responses. Interestingly, we observed that mice deficient in IL-21 signaling showed significantly decreased IgM production. We also observed that IL-21 signaling was partially required for anti-RBC IgG class switching. While IgG3 was dramatically impacted by IL-21R deficiency, the effect on IgG1, IgG2b and IgG2c was less dramatic. This is an interesting finding because CD4+ T cell depletion leads to a near complete abrogation of IgG1, IgG2b and IgG2c. These experiments suggest that CD4+ T

cell-derived cytokines other than IL-21 might also be playing a role in IgG1, IgG2b and IgG2c class switching. In fact, class switching to IgG1 has been shown to be regulated by IL-4 and it's downstream signaling molecule STAT6.[89–104] Furthermore, previous reports have shown that cytokines such as IL-12 and IFN-γ can induce class switching to IgG2 subtypes.[105–109] Therefore, it is possible that these cytokines might also regulate class switching to IgG1, IgG2b and IgG2c in RBC alloimmunization. Further investigation using blocking antibodies and cytokine deficient mice would help in the interrogation of these pathways.

Given our data showing that murine CMV infections can drive the enhancement of anti-RBC antibody responses, we were very interested in understanding the relevance of human CMV infections in RBC alloimmunization. Our collaborators in the Netherlands looked in patient datasets to ask if CMV viremia during an alloimmunization risk period had an impact on alloimmunization risk. Their data suggested that CMV viremia in their patient dataset was associated with a higher incidence of RBC alloimmunization. These findings are supportive of our data using mouse models and suggest that CMV is associated with increased RBC alloimmunization in mice and humans.

Ours is the first study to investigate the impact of cytomegalovirus (CMV) infections on the development of anti-RBC antibodies in mice and humans. While previous reports have studied RBC alloimmunization in the context of poly I:C and influenza, ours is the first to use a clinically important viral infection model. One of the caveats of our experiments is that we use an acute infection model to study RBC alloimmunization. However, it is important to note that most clinical CMV

cases in HSCT patients receiving RBC transfusions are due to reactivation of latent CMV. Therefore, studying murine CMV reactivation and RBC alloimmunization in the context of immunosuppression would provide a more physiologically accurate model. Interestingly, several studies of CMV reactivation show induction of a similar cytokine profile to acute infection (IL-6, IFN-g, IFN-a, TNF-a).[110–112] While it is plausible that RBC alloimmunization in acute CMV infection vs CMV reactivation might occur via different mechanisms, we believe that they might be regulated by similar pathways, given similar cytokine profiles. Future studies using models of CMV reactivation could be used to study the impact of CMV reactivation on RBC alloimmunization.

Chapter 3: Class switching is differentially regulated between RBC alloimmunization and vaccination

This chapter was adapted from: Prakash A, Medved J, Arneja A, Niebuhr C, Li AN, Tarrah S, Boscia AR, Burnett ED, Singh A, Salazar JE, Xu W, Santhanakrishnan M, Hendrickson JE, Luckey CJ. Class switching is differentially regulated in RBC alloimmunization and vaccination. Transfusion. 2023 Mar 12. doi: 10.1111/trf.17301. Epub ahead of print. PMID: 36907655.

Introduction

Red Blood Cell alloimmunization is a major problem for those patients who require chronic transfusions.[113] Allogenic anti-RBC IgG antibodies can make it difficult to find compatible blood for many patients. For others, production of anti-RBC IgG antibodies can induce delayed hemolytic transfusion reactions (DHTRs) that remain an all too common cause of patient morbidity and mortality.[3,4] Despite the clinical importance of anti-RBC alloantibodies, we do not completely understand how transfusion of foreign RBCs leads to the production of class-switched IgG antibodies.

IgG antibodies are not homogenous, as individual antibodies are expressed as a particular IgG isotype that has unique effector properties. Expression of different IgG isotypes occurs when B cells are induced to undergo class-switching, a process by which a fixed antigen binding region of the heavy chain locus is rearranged so that it is expressed along with one of four different unique IgG constant regions.[44] Since each of these constant regions interacts with different effector molecules, class-switching serves as a mechanism to diversify the potential effector functions of a given antibody specificity. Indeed, each IgG isotype has its own unique half-life, serum abundance, affinity for specific Fc receptors, and ability to activate complement. Thus, knowing which isotypes of antibodies predominate in a given immune response can inform you of the potential functional outcome.

Given their functional differences, it is not surprising that the production of specific IgG subclasses is carefully regulated. Though most immune responses

generate antibody class switching to all IgG subclasses, the relative amount of each subclass has been shown to differ as a result of specific immunogenic stimuli.[114,115] In mouse models, viral infections and mRNA vaccinations predominantly drive class switching to IgG2 subtypes in response to Th1 cytokines such as IFN-γ.[116–122] Parasitic infections and alum vaccination preferentially drive class-switching to IgG1 in response to Th2 cytokines such as IL-4 and IL-13.[89,121–125] Encapsulated bacteria and pneumococcal vaccines preferentially favor the IgG3 isotype via a largely T cell-independent mechanism.[126–130] Thus, IgG isotype distributions can provide important information on the specific class of immune stimuli and resultant cytokine signaling pathways that drive a given antibody response.

There are a remarkably limited number of studies that have looked at the relative amounts of IgG subclasses that are generated in patients in response to RBC transfusion, most dating back to the 1960s and 1970s. The majority of these studies focused on either pregnancy related anti-RhD antibodies or RhD negative volunteers who were intentionally immunized and boosted with RhD positive blood.[131] There were however a few studies that looked at patient responses to transfusions.[41–43] Collectively, the predominant isotypes of anti-RBC alloantibodies generated in response to transfusion are IgG3 and IgG1, while IgG2 and IgG4 were either not detected or present at very low titers in the majority of cases.[41–43] Interestingly, the observed profile of IgG isotypes identified in patients does not fit well with any clear Th1, Th2 or T-independent profile since in humans, where Th1 favors IgG3 and suppresses IgG1, Th2 favors IgG1 and IgG4 while suppressing

IgG3, and T independent responses favor IgG2.[132] Thus, it is unclear how selective IgG isotypes are driven in response to transfusion in patients.

In order to better understand the molecular drivers of RBC alloimmunization and the induction of class-switching to IgG, we turned to the experimentally tractable HOD mouse model of RBC alloimmunization. The HOD model is a widely used system to understand mechanisms of storage-dependent RBC alloimmunization. HOD transgenic mice express a fusion protein consisting of Hen Egg Lysozyme (HEL), Ovalbumin (OVA) and human Duffy on the surface of RBCs.[74] Multiple studies have shown that transfusion of HOD RBCs into recipient mice after storage leads to a class switched anti-HEL IgG response.[13,133–135] Furthermore, we have previously shown that like most infectious stimuli,[114] HOD transfusion into C57BL/6 mice induces an anti-HEL IgG response consisting of all four of the C57BL/6 mouse isotypes: IgG1, IgG2b, IgG2c and IgG3.[13] However, our understanding of the molecular regulators of transfusion-induced class-switching remains limited.

To measure the relative ability of transfused RBCs to induce a given IgG subtype, we directly compared HOD RBC transfusion with HEL-OVA protein emulsified in Alum. Protein in Alum vaccination is a well-studied immunization model that has served as the gold standard for the study of IgG class switching in mice. This allowed us to compare and contrast the class-switching observed in response to transfusion with that observed in response to Alum vaccination.

To better understand the molecular mechanisms regulating IgG isotype specific class switching in response to RBC transfusion, we further tested the

impact of STAT6 deficiency on class switching to both transfusion and Alum vaccination. STAT6 is a well-known regulator of class switching where, in mice, it supports IgG1 and suppresses IgG2 subtypes.[89,92–104] Thus, transfusion of STAT6 KO mice allow us to directly test whether the molecular mechanisms that regulate class-switching in response to RBC transfusion are similar or different from those that regulate class-switching in response to protein in Alum vaccination.

Materials and Methods:

Mice: 8-10 weeks old C57BL/6J mice (Jackson Laboratories Strain # 000664) and STAT6 KO mice (described below) were housed at the University of Virginia Animal Care Facility. HOD transgenic mice were maintained on an FVB background as previously described.[74] HOD transgenic RBCs contain the triple fusion protein of Hen Egg Lysozyme, Ovalbumin and Duffy. All mouse protocols were approved by Institutional Animal Care and Use Committees of University of Virginia, Charlottesville.

Murine blood collection and transfusion: Blood from HOD mice was aseptically collected by cardiac puncture into the anticoagulant citrate phosphate dextrose adenine (CPDA-1, Boston Bioproducts IBB-420). The final volume was adjusted to 20% CPDA-1 (v/v). Collected HOD blood was leukoreduced using whole blood cell leukoreduction filter (Pall, AP-4851). Leukoreduced blood was centrifuged at 1200 x g for 10 minutes, adjusted to a final hematocrit of 75% and stored 4°C for 12 days. Recipient mice received 100µl of 75% Hct HOD RBCs intravenously via retroorbital injection.

Alum/HEL-OVA vaccination: For immunizations with Alum/HEL-OVA, mice were administered HEL-OVA (100µg) emulsified in aluminum hydroxide (Alhydrogel, InvivoGen Cat# vac-alu-250) via intraperitoneal (i.p.) injection.

Measurement of anti-HEL antibodies via ELISA: Antibody responses to the Hen egg lysozyme (HEL) portion of the HOD antigen or HEL-OVA were measured by HEL-specific enzyme-linked immunosorbent assay (ELISA), as previously

described.[13,134] High-binding polystyrene plates (Corning # 9018) were coated for 1 hour at 37°C with 10 µg/ml HEL (Sigma-Aldrich Cat: L6876) in PBS. Plates were then washed (0.05% Tween-20 in PBS) and incubated with blocking buffer (2% BSA and 0.05% Tween-20 in PBS) overnight at 4°C. Sera samples were serially diluted (4-fold dilutions starting at 1:50, diluted 12 times) in blocking buffer and incubated in coated plates for 1 hour at room temperature. Wells were then incubated for 1 hour at room temperature with one of the following horseradish peroxidase conjugated secondary antibodies: goat anti-mouse IgM, goat anti-mouse IgG, goat anti-mouse IgG1, goat anti-mouse IgG2b, goat anti-mouse IgG2c, goat anti-mouse IgG3 (Jackson ImmunoResearch Codes: 115-035-075, Jackson ImmunoResearch Codes: 115-035-008, Jackson ImmunoResearch Codes: 115-035-205, 115-035-207, 115-035-208, 115-035-209 respectively). Wells were developed using 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate (SeraCare Cat# 52-00-03) and quenched with 2 N H_2SO_4 after 10 min. Optical densities were measured at 450 nm. End-point titers were calculated using GraphPad Prism through interpolation of the cutoff value from the fit of the optical density versus (1/serum dilution) curve for each sample using the “plateau followed by one-phase decay” model. The cutoff value was defined as the average plus 3 standard deviations (SDs) of signals from background wells (i.e., signal values from wells incubated with blocking buffer alone). O.D values that started off below background and unable to be interpolated were assigned a titer value of 100.

Measurement of total serum IgG and IgM: In order to measure total serum IgM and IgG levels in WT and STAT6 KO mice, serum was collected from naïve 8-week-old

mice. Total IgM was measured using IgM mouse uncoated ELISA kit (ThermoFisher Cat: 88-50470-22) and total IgG was measured using IgG (Total) mouse uncoated ELISA kit (ThermoFisher Cat: 88-50400-22). ELISA was performed based on manufacturer's instructions.

Generation of STAT6 KO mice: CRISPR/cas9 genome editing technology was used to generate STAT6 KO mice. Three gRNAs targeting SH2 coding exons were selected using Desktop Genetics (DESKGEN) guide picker.[136] crRNA, tracrRNA and Cas9 were purchased from IDT (Coralville, Iowa). The crRNA sequences are listed here: crRNA1- 3'-TCCGGAGACAGCGTTTGGTG-5', crRNA2- 3'-AGGTCCCATCTGGCTCATTG-5', crRNA3- 3'-GTGACTCACCATCCTGACCC-5'. crRNA and tracrRNA were diluted to 100 μM in RNase-free microinjection buffer (10mM of Tris-HCl, pH 7.4, 0.25mM of EDTA). 3 μl crRNA and 3 μl tracrRNA were mixed and annealed in a thermal cycler by heating the mixture to 95°C for 5 minutes and ramped down to 25°C at 5°C/min. Ribonucleic protein (RNP) complexes were prepared by mixing and incubating Cas9 at 0.2 μg/μl with three crRNA/tracrRNA (1μM) in RNase-free microinjection buffer at 37°C for 10 minutes. Embryos were collected from super-ovulated C57BL/6J females mated with C57BL/6J males. Three RNPs were co-delivered into the fertilized eggs by electroporation using NEPA21 super electroporator (Nepa Gene Co., Ltd. Chiba, Japan) under the following conditions: 2 pulses at 40 V for 3 msec with 50 msec interval for poring phase; 2 pulses at 7 V for 50 msec with 50 msec interval for transferring phase. The electroporated embryos were cultured overnight in KSOM medium (EMD Millipore, Billerica, MA) at 37°C and 5% CO2. The next morning,

embryos that had reached the two-cell stage were implanted into the oviducts of pseudopregnant ICR CD-1 mothers (Envigo Order code: 030). Pups born were bred with wildtype C57BL/6J mice and were further bred to generate homozygous knockout mice. Homozygous knockout mice were confirmed by stimulating splenocytes with IL-4 and pSTAT6 was measured by flow cytometry.

Intracellular pSTAT6 staining: To confirm STAT6 KO, mice were euthanized, and spleens were harvested and disrupted mechanically to obtain a splenocyte suspension. Splenocytes were resuspended in RPMI-1640 supplemented with 10% FBS (Sigma-Aldrich Cat# F2442), 1% sodium pyruvate, 1% non-essential amino acids, 1% Penn Strep, 1% L-Glutamine, 2.5% HEPES and stimulated with 50ng/ml IL-4 (R&D Systems Cat: 404-ML) for 15 minutes at 37°C. Splenocytes were then fixed using 1.6% paraformaldehyde for 10 minutes at room temperature. Cells were washed twice with 1x PBS, resuspended in Perm Buffer III (BD Biosciences Cat: 558050) and incubated on ice for 1 hour. Following this, cells were washed three times with FACS buffer (PBS+0.5% BSA+2% FBS+0.1% sodium azide) and stained with the following antibodies for 30 minutes: anti-CD4 PerCP/Cy5.5 (BioLegend Cat: 100540), anti-CD8a Brilliant Violet 510 (BioLegend Cat: 100751), anti-B220 FITC (BioLegend Cat: 103206), anti-STAT6 pY641 Alexa Fluor 647 (BD Biosciences Cat: 558242). Cells were then washed once with FACS buffer and analyzed on Attune NxT flow cytometer. Data were analyzed using FlowJo software.

Measurement of T and B cell numbers: To measure T and B cell numbers, WT and STAT6 KO mice were euthanized, and spleens were harvested. Spleens were

mechanically disrupted to obtain a splenocyte suspension. Splenocytes were stained with eBioscience Fixable Viability Dye eFluor 780 (Thermo Fisher Cat# 65-0865-14) in 1x PBS to exclude dead cells. Splenocytes were washed once and stained with the following antibodies for 30 minutes: anti-CD4 Alexa Fluor 488 (BioLegend Cat: 100529), anti-CD8a Brilliant Violet 510 (BioLegend Cat: 100751), anti-CD19 Brilliant Violet 421 (BioLegend Cat: 115538), anti-CD21 PE/Cy7 (BioLegend Cat: 123419), anti-CD23 PE (BioLegend Cat: 101607). Cells were then washed once with FACS buffer and analyzed on Attune NxT flow cytometer. Data were analyzed using FlowJo software.

Statistical Analysis: Graphing of data and statistical analysis were performed using GraphPad Prism software. Mann-Whitney test was done to compare 2 groups. $p < 0.05$ was considered to be statistically significant and assigned *, whereas $p < 0.01$, $p < 0.001$, and $p < 0.0001$ were assigned **, ***, and ****, respectively.

Results:

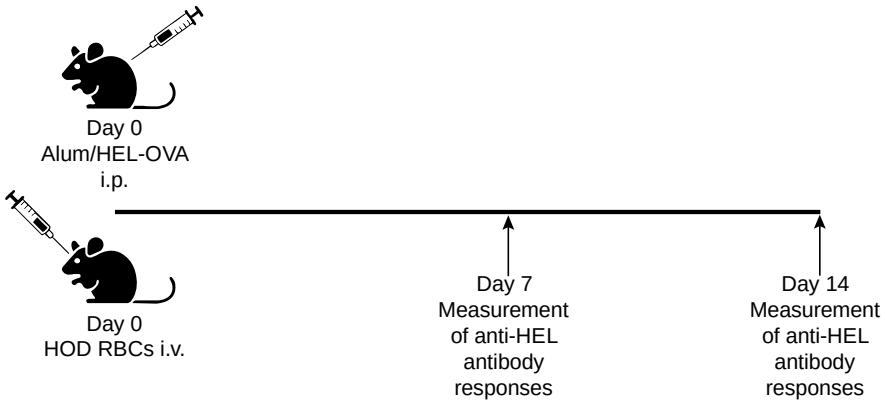
RBC transfusion induces a different IgG class switching profile than vaccination:

While previous reports have shown that HOD RBC transfusion leads to production of multiple IgG subclasses in mice,[13] it is unknown whether the class-switching that is induced by transfused RBCs is similar or different to other class-switch inducing stimuli. We therefore compared antibody production to HOD RBCs with protein emulsified in alum, the gold standard to study IgG isotype class switched antibody responses. Since HOD RBCs express a chimeric protein containing both HEL and OVA, we used HEL-OVA protein emulsified in Alum to minimize potential antigen-specific differences. We measured anti-HEL IgM at 7 days post transfusion/immunization and anti-HEL IgG and subtypes 14 days post transfusion/immunization using endpoint titer ELISAs in order to accurately quantify antibody levels (Figure 3.1A). Interestingly, transfused RBCs consistently led to anti-HEL IgM levels that were at least as high than those observed in response to alum vaccination (Figure 3.1B). This stood in stark contrast to anti-HEL total IgG levels, which were consistently several logs lower in response to transfusion relative to vaccination (Figure 3.1C). Both HOD transfusion and Alum/HEL-OVA vaccination led to class switching to each of the four mouse IgG isotypes found in C57BL/6 mice: IgG1, IgG2b, IgG2c and IgG3. However, the relative amounts of antibodies of each subtype differed dramatically between transfusion and vaccination. Relative to vaccination, transfusion with HOD RBCs consistently led to several logs lower anti-HEL IgG1, IgG2b and IgG2c titers (Figure 3.1D-F). This stood in stark contrast with IgG3 titers, which failed to

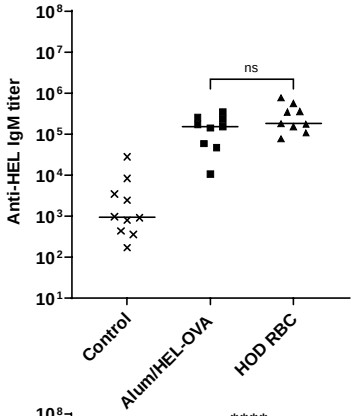
demonstrate consistently reproducible different titers (Figure 3.1G). Our data demonstrate that transfusion with HOD RBCs leads to a unique IgG subtype profile in mice that, relative to Alum vaccination, favors IgG3 at the expense of IgG1, IgG2b, and IgG2c.

Given the unique isotype profile observed in response to transfusion, we set out to investigate the mechanisms controlling class switching in this system. STAT6 is a signaling molecule that has been shown to differentially regulate IgG isotype class-switching in mice and humans.[89–104] We therefore set out to test whether STAT6 signaling similarly regulated class-switching in response to both transfusion and vaccination.

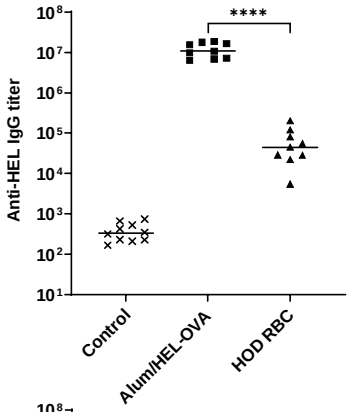
(A)


Day 0
Alum/HEL-OVA
i.p.
Day 0
HOD RBCs i.v.
Day 7
Measurement
of anti-HEL
antibody
responses
Day 14
Measurement
of anti-HEL
antibody
responses

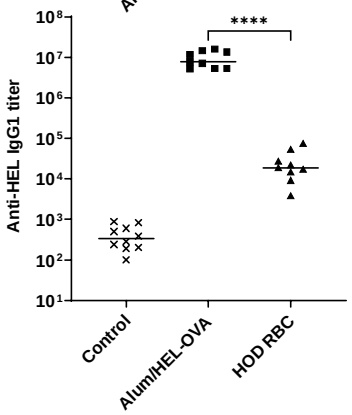
(B)


Anti-HEL IgM titer
ns
Control
Alum/HEL-OVA
HOD RBC

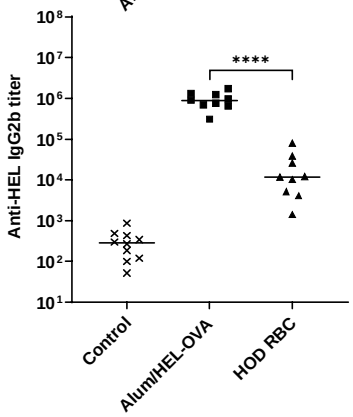
(C)


Anti-HEL IgG titer
****
Control
Alum/HEL-OVA
HOD RBC

(D)


Anti-HEL IgG1 titer
****
Control
Alum/HEL-OVA
HOD RBC

(E)


Anti-HEL IgG2b titer
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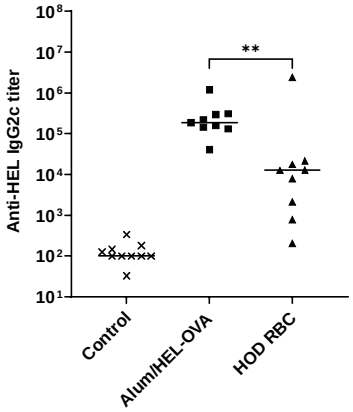
Control
Alum/HEL-OVA
HOD RBC

(F)


Anti-HEL IgG2c titer
**
Control
Alum/HEL-OVA
HOD RBC

(G)

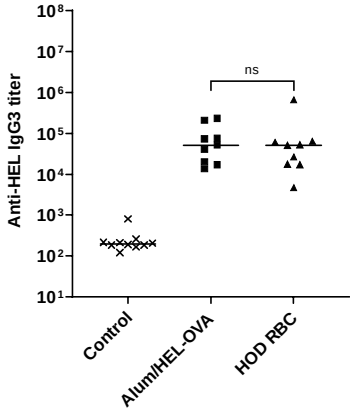

Anti-HEL IgG3 titer
ns
Control
Alum/HEL-OVA
HOD RBC

Figure 3.1: RBC transfusion induces a different IgG class switching profile than vaccination.

WT (C57BL/6) mice were either transfused with HOD RBCs or immunized with Alum/HEL-OVA. Anti-HEL titers were measured by ELISA at 14 days post transfusion/immunization. (A) Schematic representation of experimental design (B) Anti-HEL IgM (C) Anti-HEL IgG (D) Anti-HEL IgG1 (E) Anti-HEL IgG2b (F) Anti-HEL IgG2c (G) Anti-HEL IgG3. Each data point represents one mouse. Bars on plots show median values. Figure is representative of 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s. $p > 0.05$.

Generation of STAT6 KO mice:

In order to measure the impact of STAT6's function on class-switching in response to transfusion, we first generated a novel STAT6 KO mouse model using CRISPR/cas9 on a pure C57BL/6 background. Previously generated STAT6 KO mice[93,137] have used targeting constructs with neomycin resistance cassettes to disrupt STAT6 coding exons.[93,137] These mice were generated by electroporation into D3 ES cells derived from 129S2/SvPas mouse strain,[93,137] followed by injection into BALB/c blastocytes and multiple rounds of breeding with C57BL/6 mice to generate STAT6 KO mice on a C57BL/6 background.[137] This results in a STAT6 deficient mouse that contains not only a neomycin cassette, but also a large swath of tightly linked 129S2 genome surrounding the STAT6 targeted locus. Given the strain specific differences in mice with respect to immune responses[138] and potential off-target effects of neomycin resistance cassettes,[139,140] we believe that our STAT6 KO mouse model on a pure C57BL/6 background better reflects the wild-type C57BL/6 controls used in our experiments. The CRISPR/cas9 system consists of a crRNA/tracrRNA duplex (gRNA) and cas9 protein, an RNA-guided endonuclease. The crRNA part of the complex binds to targeted exons while the tracrRNA guides the cas9 to the targeted locus. Cas9 endonuclease introduces double-stranded breaks in the targeted region, triggering DNA repair pathways via non-homologous end joining (NHEJ). NHEJ introduces insertions and deletions (indels) and can cause frameshift mutations, leading to disruption of the targeted gene. In order to disrupt STAT6 function, we designed gRNAs to target SH2 coding domains of the STAT6 gene (Figure 3.2A, Figure 3.2B). STAT6 SH2 domains are

required for STAT6 dimerization and downstream effector function.[141–143] crRNA/tracrRNA duplexes were complexed with cas9 protein and electroporated into fertilized eggs from C57BL/6 mice, following previously published approaches.[144–146] Zygotes were transferred into foster mothers and pups were screened for STAT6 mutations and bred to homozygosity (Figure 3.2A). STAT6 KO homozygous mice were confirmed by stimulating splenocytes with IL-4 and measuring pSTAT6 by flow cytometry within multiple cell populations (Figure 3.2C). We further characterized the numbers of splenic lymphocyte subsets and observed no significant differences in follicular B cells, marginal zone B cells, CD4+ T cells and CD8+ T cells in STAT6 KO mice compared to WT mice (Figure 3.2D). Interestingly, while WT and STAT6 KO mice had similar levels of total IgM, STAT6 KO mice showed elevated levels of total IgG (Figure 3.2E). Our data demonstrate that we have effectively disrupted STAT6 function in our novel STAT6 KO mice.

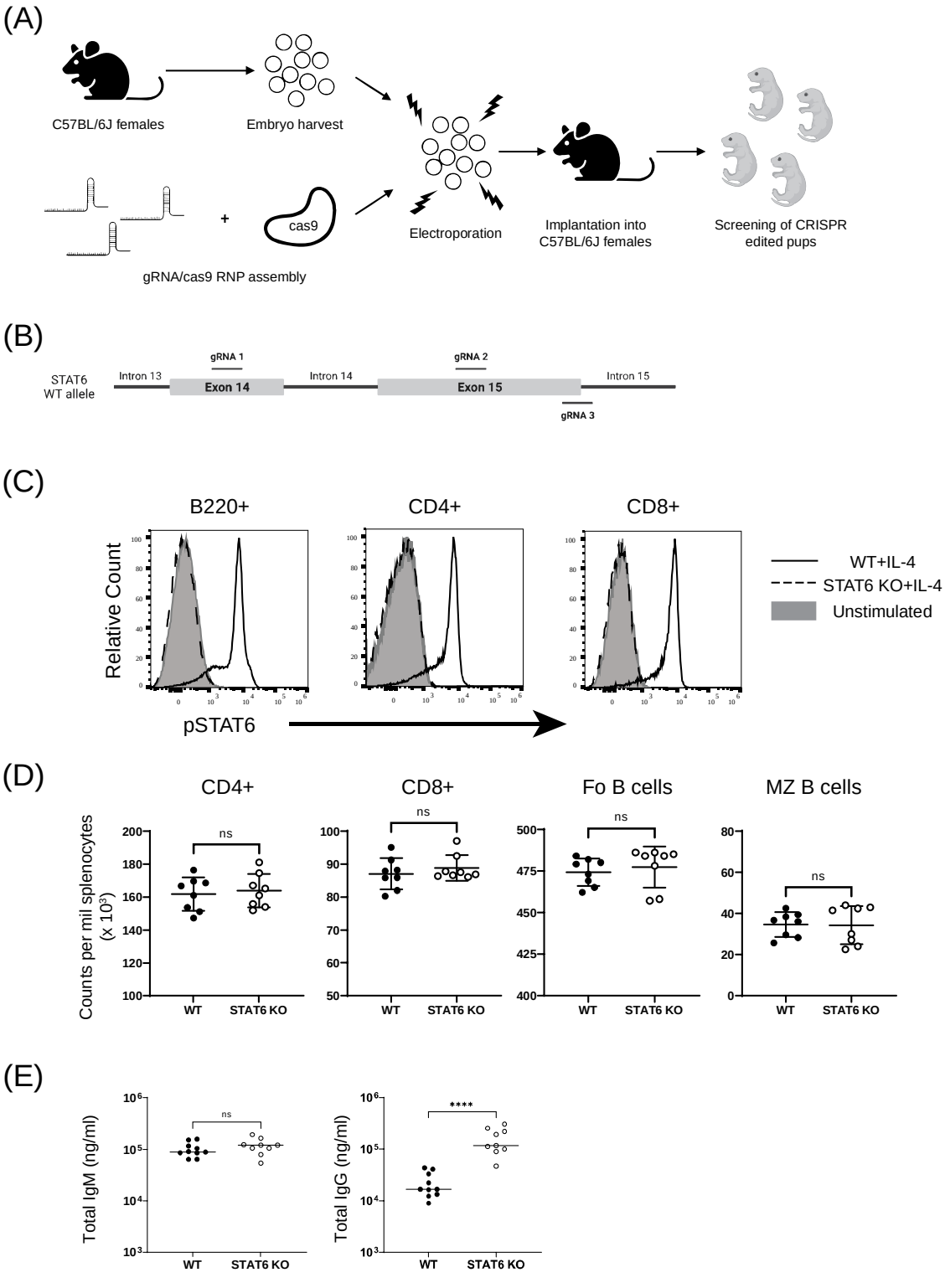

(A)
C57BL/6J females
Embryo harvest
cas9
gRNA/cas9 RNP assembly
Electroporation
Implantation into C57BL/6J females
Screening of CRISPR edited pups
(B)
STAT6 WT allele
Intron 13
Exon 14
Intron 14
Exon 15
Intron 15
gRNA 1
gRNA 2
gRNA 3
(C)
B220+
CD4+
CD8+
Relative Count
pSTAT6
WT+IL-4
STAT6 KO+IL-4
Unstimulated
(D)
CD4+
CD8+
Fo B cells
MZ B cells
Counts per mil splenocytes (x 10³)
ns
WT
STAT6 KO
(E)
Total IgM (ng/ml)
Total IgG (ng/ml)
ns
****
WT
STAT6 KO

Figure 3.2: Generation of STAT6 KO mice using CRISPR/cas9 gene editing.

(A) Schematic showing generation of STAT6 KO mice (B) A representative view of the STAT6 targeting strategy showing positions of selected gRNAs (C) pSTAT6 staining measured by flow cytometry following IL-4 stimulation. pSTAT6 in STAT6 KO (dotted line) was compared to WT (solid line). Unstimulated controls are shown using the gray background. (D) Quantification of CD4+ T cells, CD8+ T cells, Follicular B cells and Marginal Zone (MZ) B cells in WT and STAT6 KO spleens. (E) Concentration of total serum IgM and IgG in WT and STAT6 KO mice. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s. $p > 0.05$.

STAT6 regulates class switching to all IgG isotypes in Alum/HEL-OVA vaccination:

STAT6 is known to be the key transcription factor responsible for Th2 signaling downstream of IL-4 and IL-13. Since protein in alum vaccinations are well known to be Th2 dominant,[89] we first hypothesized that IgG isotype switching would be altered significantly in STAT6 deficient mice in response Alum/HEL-OVA vaccination. WT and STAT6 KO mice were immunized with Alum/HEL-OVA and anti-HEL IgM and IgG subclass titers were measured (Figure 3.3A). While total anti-HEL IgG and IgM titers remained similar (Figure 3.3B and Figure 3.3C), STAT6 KO mice had significantly lower anti-HEL IgG1 titers and elevated anti-HEL IgG2b and IgG2c titers compared to WT mice (Figure 3.3D-F), consistent with previous reports.[89] Interestingly, we found that STAT6 KO mice also had increased anti-HEL IgG3, a novel finding demonstrating that STAT6 also inhibits class switching to IgG3 following Alum/HEL-OVA immunization (Figure 3.3G).

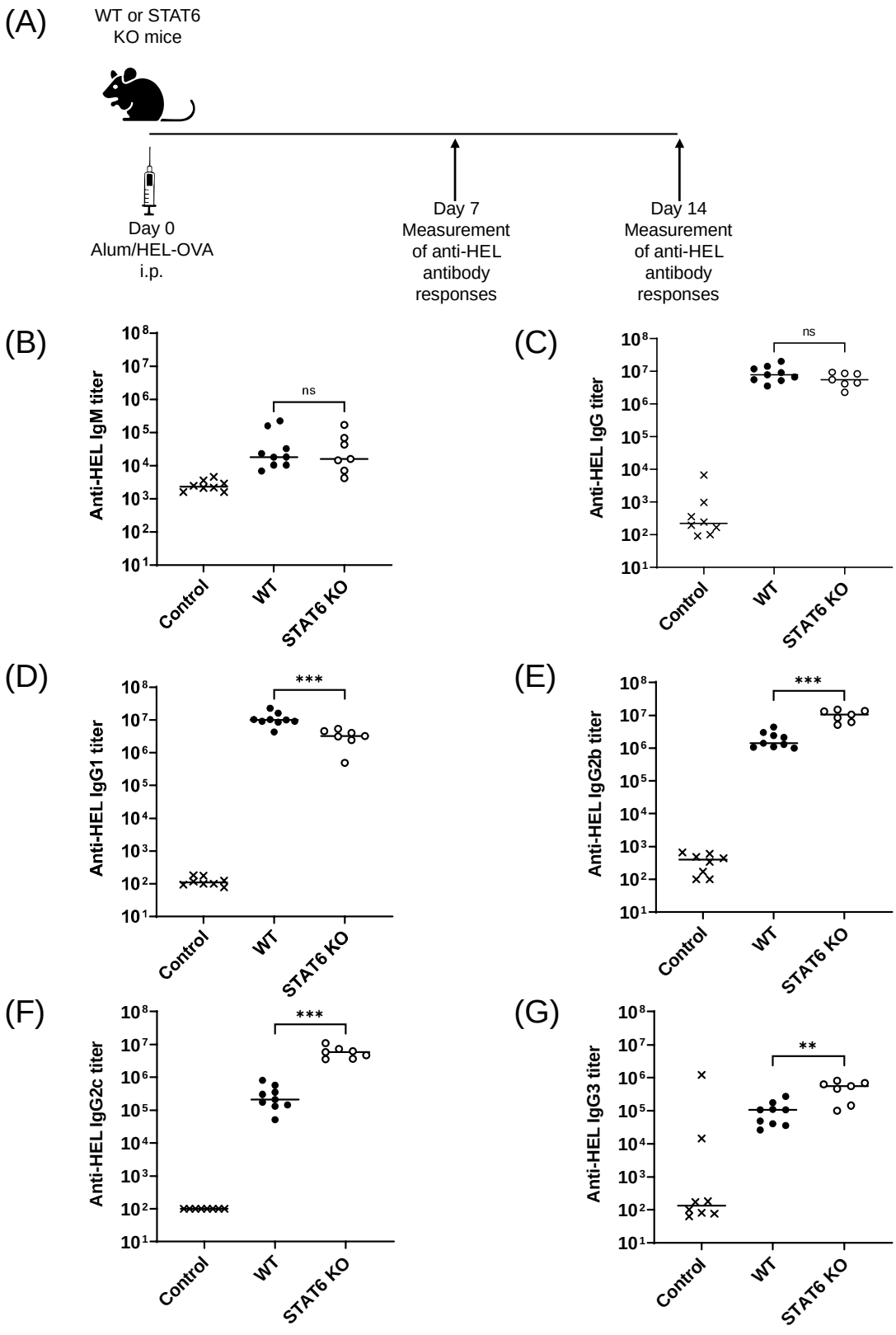

(A)
WT or STAT6 KO mice
Day 0 Alum/HEL-OVA i.p.
Day 7 Measurement of anti-HEL antibody responses
Day 14 Measurement of anti-HEL antibody responses
(B)
Anti-HEL IgM titer
ns
Control
WT
STAT6 KO
(C)
Anti-HEL IgG titer
ns
(D)
Anti-HEL IgG1 titer
***
(E)
Anti-HEL IgG2b titer
***
(F)
Anti-HEL IgG2c titer
***
(G)
Anti-HEL IgG3 titer
**

Figure 3.3: STAT6 regulates class switching in response to Alum/HEL-OVA vaccination.

WT and STAT6 KO mice were immunized with Alum/HEL-OVA. Anti-HEL IgG and subtype titers were measured by end point titer ELISAs at 14 days post immunization. (A) Schematic representation of experimental design showing mice immunized with Alum/HEL-OVA (B) Measurement of anti-HEL IgM (C) Measurement of anti-HEL IgG (D) Measurement of anti-HEL IgG1 (E) Measurement of anti-HEL IgG2b (F) Measurement of anti-HEL IgG2c (G) Measurement of anti-HEL IgG3. Each data point represents one mouse. Bars on plots show median values. Figure is representative of 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s. p > 0.05.

STAT6 has a minimal impact on class switching to most IgG isotypes in response to HOD transfusion:

In order to determine the impact of STAT6 on transfusion-induced class switching, WT and STAT6 KO mice were transfused with HOD RBCs and anti-HEL antigen specific titers were measured. We found that WT and STAT6 KO mice had similar anti-HEL IgM and IgG titers (Figure 3.4B and Figure 3.4C). We then measured anti-HEL IgG subclass titers 14 days post transfusion. To our surprise, STAT6 deficient mice had similar levels of IgG1, IgG2c and IgG3 in response to HOD transfusion (Figure 3.4D, Figure 3.4F and Figure 3.4G). We also observed that STAT6 KO mice showed increased IgG2b (Figure 3.4E). Collectively, our data demonstrates that transfusion-induced IgG class switching occurs in a largely STAT6-independent manner, suggesting that alternative pathways that are largely STAT6-independent regulate IgG class switching in response to RBC transfusions.

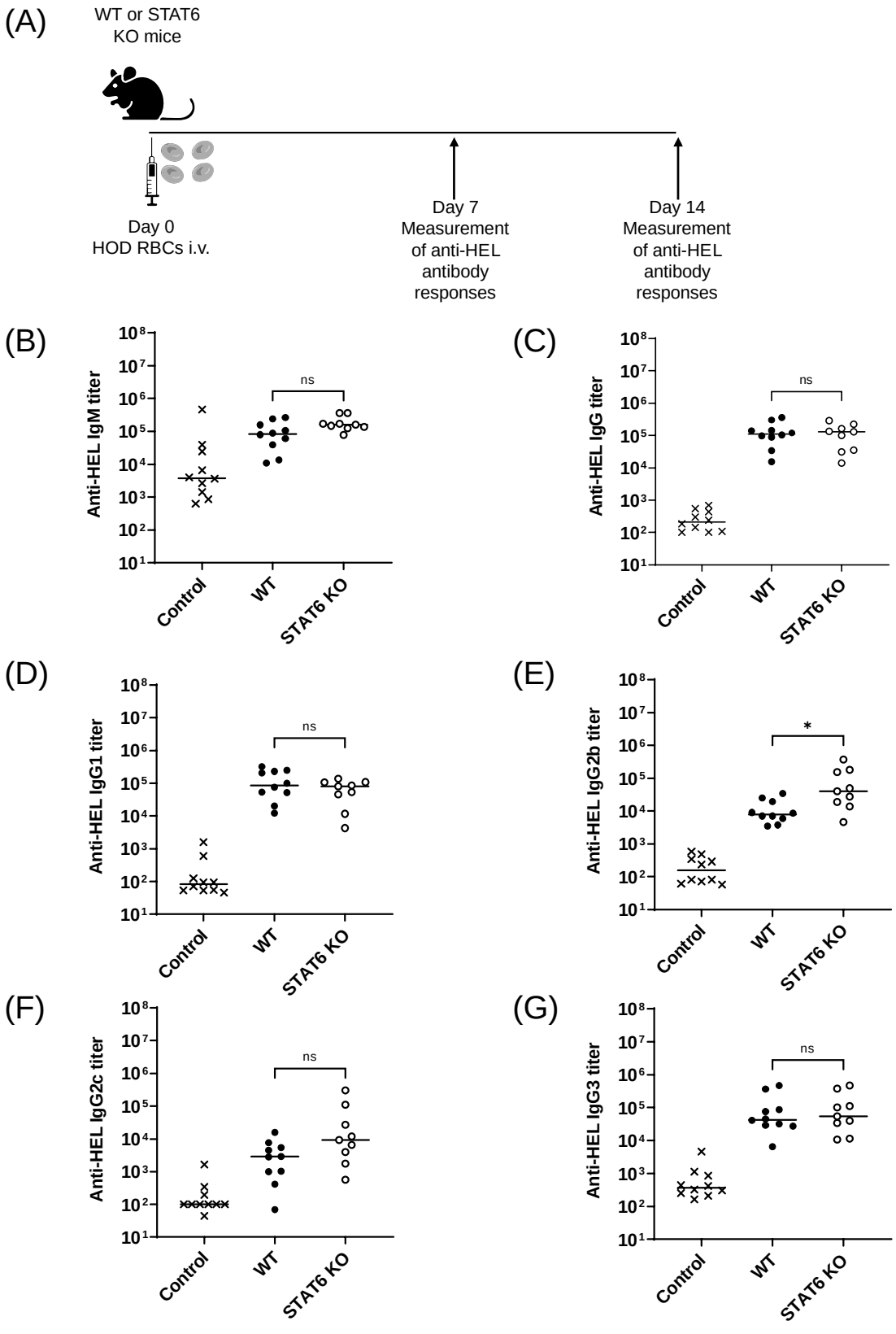

(A)
WT or STAT6 KO mice
Day 0 HOD RBCs i.v.
Day 7 Measurement of anti-HEL antibody responses
Day 14 Measurement of anti-HEL antibody responses
(B)
Anti-HEL IgM titer
(C)
Anti-HEL IgG titer
(D)
Anti-HEL IgG1 titer
(E)
Anti-HEL IgG2b titer
(F)
Anti-HEL IgG2c titer
(G)
Anti-HEL IgG3 titer
ns
*
Control
WT
STAT6 KO

Figure 3.4: STAT6 is mostly not required for class switching in response to HOD RBC transfusion.

WT and STAT6 KO mice were transfused with HOD RBCs. Anti-HEL IgG and subtype titers were measured by end point titer ELISAs at 14 days post transfusion. (A) Schematic of experimental design showing mice transfused with HOD RBCs (B) Measurement of anti-HEL IgM (C) Measurement of anti-HEL IgG (D) Measurement of anti-HEL IgG1 (E) Measurement of anti-HEL IgG2b (F) Measurement of anti-HEL IgG2c (G) Measurement of anti-HEL IgG3. Each data point represents one mouse. Bars on plots show median values. Figure is representative of 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, n.s. $p > 0.05$.

Discussion:

Our data in the HOD mouse model demonstrate that transfusion of HOD RBCs leads to consistent generation of IgG. However, we show that, compared to Alum/HEL-OVA vaccination, HOD RBC transfusion induces similar IgG3 titers but dramatically lower IgG1, IgG2b and IgG2c titers. This demonstrates that even though all IgG isotypes are produced in response to transfusion, the levels of a given isotype generated in response to transfused RBCs are different than those generated in response to protein in Alum vaccination.

The comparable level of IgG3 between Alum/HEL-OVA vaccination and HOD RBC transfusion suggests a preference towards IgG3 class switching in transfusion. This is an interesting finding for several reasons. IgG3 has largely been shown to be T-cell independent in mice. However, work in our lab has shown that depletion of CD4+ T cells leads to attenuation of IgG3 in response to HOD RBCs (unpublished), suggesting that there might be T-dependent mechanisms of IgG3 regulation in the HOD transfusion system. Furthermore, IgG3 is a strong activator of complement in mice.[147] Interestingly, previous reports have shown that there is deposition of complement C3 on the surface of HOD RBCs following transfusion.[148] Given the importance of IgG3 in activation of complement pathways, the deposition of C3 on the surface of RBCs might be dependent on IgG3 production. In addition, human IgG3 and IgG1 are also robust activators of complement.[149,150] In contrast, however, mouse IgG3 shows limited FcγR binding,[151,152] while human IgG3 and IgG1 can bind to all hFcγRs.[152,153] The predominance of complement activating/robust FcγR activating IgG isotypes in

human (IgG1 and IgG3) and complement activating/weak FcγR activating isotype in mice (IgG3) suggests both similar as well as divergent mechanisms of RBC alloimmunization between mice and humans.

Given the observed differences in isotype class switching induced by transfusion vs. vaccination, we were interested in determining whether the molecular regulators of class switching might also be different between these immune stimuli. Since STAT6 is a well-known regulator of IgG1 and IgG2 subtype class switching in multiple different vaccination and infectious settings[89–104], we created a novel STAT6 deficient mouse model to directly test whether STAT6 played a similar role in regulating isotype class-switching in response to transfusion. We used CRISPR/cas9 to target STAT6 in C57BL/6J embryos and confirmed successful disruption of STAT6 function. These mice represent, to our knowledge, the first STAT6 deficient strain generated on a pure C57BL/6 background. STAT6 deficient mice had similar levels of baseline polyclonal IgM, but elevated levels of baseline polyclonal IgG. This suggests that in response to the normal microflora found in our specific pathogen free (SPF) colony at University of Virginia, STAT6 deficient mice tend to have higher circulating total IgG levels relative to their wild type counterparts.

Having established the baseline polyclonal antibody production in STAT6 KO mice, we next asked what the impact of STAT6 deficiency in the production of antigen-specific antibody production in response Alum/HEL-OVA vaccination. Consistent with previous publications[89], STAT6 KO mice that were vaccinated with Alum/HEL-OVA expressed similar levels of antigen-specific IgM and antigen-

specific total IgG. Furthermore, STAT6 KO mice expressed lower levels of antigen-specific IgG1 while expressing significantly elevated levels of antigen-specific IgG2b, IgG2c and IgG3 subclasses in response to Alum vaccination. These data demonstrate that STAT6 plays a significant role in the relative class switching to all IgG isotypes in response to Alum vaccination, supporting IgG1 antigen-specific antibody production while suppressing IgG2b, IgG2c and IgG3 antigen-specific antibody production.

Importantly, STAT6 deficiency had a much different impact on antigen-specific IgG subclass production in response to transfused RBCs. Unlike what was observed in response to Alum vaccination; anti-RBC IgG1, IgG2c and IgG3 levels were not consistently or robustly different between wild type mice and STAT6 KO mice. While anti-RBC IgG2c levels did trend somewhat lower, they failed to consistently show a significant difference. We did however observe a small but reproducible enhancement in IgG2b levels in absence of STAT6. Thus, our data demonstrate that STAT6 clearly suppressed IgG2b (and to some degree IgG2c) class-switching in response to transfusion. However, there was no significant impact on anti-RBC IgG1 and IgG3 production in STAT6 deficient mice. This demonstrates that the molecular regulation of the antigen-specific IgG1 and IgG3 class-switching in response to transfusion is quite different from that induced by Alum vaccination.

There are several potential mechanisms that might account for the differences that we observed between HEL/OVA in Alum vaccination and HOD RBC transfusion. One potential explanation for the differences we observe may be

associated with differences in antigen doses between HEL/OVA in Alum vaccination and HOD transfusion. Although certainly a possibility, we do not favor antigen dose differences as a major explanation for our results for several reasons. First, experiments in our lab using a range of varying doses of HOD RBCs showed no significant drop-off in antibody titers for the doses tested (data not shown). Second, significant variation in the dose of Alum/HEL-OVA had only a small effect on overall titers and did not impact isotype distribution (data not shown). Thus, we do not think that intrinsic differences in antigen dosing account for the differences in class switching observed.

Another potential difference that might account for our observed differences in class-switching is the different route of antigen exposure: with the vaccination given intra-peritoneally (i.p.) while the transfusion is given intravenously (i.v.). Importantly, direct comparisons of Alum and transfusion are not possible since Alum cannot be given i.v. in mice due to toxicity, and i.p. transfusion of HOD RBCs fails to generate any measurable anti-RBC alloantibodies (data not shown). However, we do not think that the route of exposure accounts for the differences we observed since Alum used via other routes (intra-muscularly) gives similar results to i.p., and i.v. protein vaccination with i.v.-compatible adjuvants such as the Sigma Adjuvant System show similar robust induction to IgG1 and IgG2 subclasses relative to IgG3 to what we have observed in the i.p. Alum setting (data not shown). Furthermore, STAT6 controls IgG1 antigen-specific class switching to multiple different infectious agents that infect multiple different sites, including intestinal helminth infection,[91,154] intranasal pox viral vaccination,[104] and upper

respiratory flu viral infection.[155] Thus we believe that the i.p. vaccination gives similar results to vaccination and infection at many other sites. However, it is certainly possible that i.v. exposure to antigens that occurs via transfusion may account for some of the observed differences in class switching. Importantly, RBC transfusions, by definition entail i.v. antigen exposure via RBCs, and thus our model accurately depicts the unique properties of transfusion-associated induction of class-switching.

Though route of immunization and antigen dosing are potential explanations for the differences in IgG class-switching we have observed, we favor the hypothesis that antigens presented on transfused RBCs represent a unique costimulatory signal to the immune system. This is particularly true when sterile RBCs are transfused in the absence of exogenous adjuvants as is the case for our studies herein and for most clinical transfusions. We have used HEL/OVA in Alum vaccination as a comparator in our studies because protein in Alum vaccination has long served as the gold standard for the scientific study of class-switching, and protein in Alum vaccination continues to be the vaccine approach used in most childhood vaccines. Importantly, HEL/OVA in Alum vaccination faithfully represents the vast majority of the published data looking at infectious and vaccine induced class-switching in so far as STAT6 clearly supporting IgG1 class-switching and suppressing IgG2 isotype class switching. The key point here that we would like to emphasize is not that the IgG class-switching induced by transfusion is different from the IgG class-switching induced by HEL/OVA in Alum per se, but rather that the class-switching induced by transfusion is different from virtually

every other infectious and adjuvant-based system that we have looked at and that has been studied (Alum included). Ultimately, we hypothesize that foreign antigens that are expressed on transfused RBCs stimulate the immune system in a unique manner relative to most other vaccination or infectious stimuli, driving a relatively robust IgG3 response but dramatically weaker IgG1, IgG2b and IgG2c responses. Our data herein demonstrate that STAT6 fails to play a significant role in the production of most of the class-switched IgG antibodies generated in response to HOD transfusion, and certainly not IgG3 and IgG1. Given the unique nature of the IgG class switching observed in response to transfusion, we are particularly interested in future experiments aimed at understanding both the cellular and molecular regulators of IgG production in response to RBC-expressed foreign antigens.

Chapter 4: Discussion and Future Directions

This study was done to address some of the key questions that were identified in Chapter 1.

Do clinically relevant infections such as cytomegalovirus have an impact on RBC alloimmunization?

Given that the vast majority of transfusion recipients do not generate anti-RBC antibodies, we aimed to understand the underlying risk factors that drive RBC alloimmunization. We first set out to study the impact of clinically relevant infections like cytomegalovirus (CMV) on RBC alloimmunization. We found that murine CMV infections led to enhancement of anti-RBC antibody responses. We further showed that this enhancement did not require type 1 IFN signaling, a surprising finding, given that type 1 IFNs were required for poly I:C and influenza-driven RBC alloimmunization. We further showed that IL-21 was only partially required for anti-RBC antibody production following MCMV infection. In addition, we found that the antibody response was mostly dependent on CD4+ T cells and observed that MCMV infection led to increased expansion of anti-RBC CD4+ T cells and enhanced differentiation into “helper cells” that could potentially provide help to cognate B cells and drive antibody production. In addition to our work in mice, patient data from our collaborators in the Netherlands suggested that CMV infections can increase RBC alloimmunization risk in patients, supporting our mouse data. Taken together, our data shows that clinically relevant infections such as CMV can lead to enhancement of RBC alloimmunization. Ours is the first study to show that CMV infections can lead to enhancement of anti-RBC antibody

responses in mice and that is correlated with increased RBC alloimmunization in human patients.

What is the relative IgG subclass abundance and distribution in RBC alloimmunization?

Another question that we addressed in this study was the characterization of the anti-RBC antibody response. We, therefore, set out to study the relative abundance of IgG subclasses and their distribution in RBC alloimmunization. By comparing the anti-RBC antibody response to antibody responses generated to vaccines, we were able to characterize the anti-RBC IgG response. We observed that transfusion of RBCs led to the generation of anti-RBC IgG1, IgG2b, IgG2c and IgG3. We further found that IgG1, IgG2b and IgG2c were several folds lower in RBC transfusion compared to Alum vaccination. Interestingly, however, anti-RBC IgG3 levels were similar compared to alum vaccination.

What are the molecular regulators of anti-RBC IgG subclasses and how do they differ from antibody responses to vaccination?

Our previous findings prompted us to further investigate the molecular mechanisms regulating the anti-RBC IgG response. STAT6 is a transcription factor that regulates IgG production by controlling IgG1 vs IgG2 class switching in various immunization systems, including Alum vaccination models. We therefore tested if STAT6 played a role in the regulation of IgG class switching to transfused RBCs. We first studied the impact of STAT6 on IgG class switching in an alum vaccination setting. Consistent with previous reports, we observed that STAT6 regulated class

switching to IgG1 and IgG2 subtypes, with STAT6 KO mice having lower IgG1 titers and elevated IgG2b and IgG2c titers compared to WT mice. Interestingly, we also observed that STAT6 KO mice had elevated IgG3 titers, a novel finding in the alum vaccination system. We then tested the role of STAT6 in response to transfused RBCs. In contrast to the alum vaccination model, STAT6 KO mice had similar IgG1, IgG2c and IgG3, while demonstrating a small increase in IgG2b in response to RBC transfusion. Our data herein demonstrate that STAT6 fails to play a significant role in the production of most of the class-switched IgG antibodies generated in response to RBC transfusion, and certainly not IgG3 and IgG1. Given the unique nature of the IgG class switching observed in response to transfusion, we are particularly interested in future experiments aimed at understanding both the cellular and molecular regulators of IgG production in response to RBC-expressed foreign antigens.

Taken together, our study fills some major gaps in our knowledge and understanding of RBC alloimmunization. We now have a better understanding of underlying infections that can have an impact on RBC alloimmunization. Furthermore, this is the first study to carefully characterize the anti-RBC IgG response and compare it to a well-studied immunization model like alum vaccination. Additionally, we also provide insight into how canonical class-switching regulators like STAT6 might not play a significant role in RBC alloimmunization. However, there are still several unanswered questions that remain. We still do not understand how anti-RBC CD4+ T cells and B cells respond to RBC transfusions, in terms of their localization and differentiation state. In

addition to the cellular mechanisms, we still do not understand all the molecular regulators of the anti-RBC antibody response. Future experiments will address these questions, in an effort to improve our understanding of RBC alloimmunization.

Future Directions:

CMV-driven RBC alloimmunization

While we have shown that MCMV infections can increase the immunogenicity of RBC transfusion, future studies are needed to investigate cytokine and cellular mechanisms that might regulate the anti-RBC antibody response. Here are some potential questions that might explored in future experiments:

How does CMV infection enhance the innate immune response leading to increased anti-RBC antibody production?

While we now understand that CMV infections can lead to increased anti-RBC antibody production, we still do not understand how CMV infections might lead to enhancement of innate immune cells. Innate cells such as bridging channel DCs (cDC2s) in the spleen have been implicated in RBC uptake and CD4+ T cell activation in the storage induced HOD model of RBC alloimmunization. Future experiments can investigate the impact of CMV infections and HOD transfusion on DC maturation by measuring the expression of activation markers such as CD80, CD86, among others. Furthermore, purification of cDC2s post-transfusion, and co-culture with naïve OT-II cells can inform us about the potential of these DCs to

stimulate anti-RBC CD4+ T cells. This can further be confirmed by performing adoptive transfers of naïve OT-II cells in mice that lack cDC2s. These experiments can be done to further our understanding of innate cellular players that might be regulating anti-RBC antibody production.

What is the requirement of follicular B cells vs marginal zone B cells in CMV mediated RBC alloimmunization?

Our data in Chapter 2 shows that CMV infections leads to increased production of anti-RBC antibodies. However, we still have not identified the source of these antibodies. Previous data in the stored HOD system have shown that anti-RBC antibodies require MZ B cells and that follicular B cells are dispensable. Experiments using depleting antibodies and knockout mice can be done to determine the source of anti-RBC antibodies in the CMV-driven RBC alloimmunization model. This will further inform us about the function and relevance of follicular B cells and marginal zone B cells in RBC alloimmunization.

Where do anti-RBC CD4+ T cells and B cells localize in CMV-driven RBC alloimmunization?

Our OT-II adoptive transfer experiments show that CMV infection leads to expansion of anti-RBC CD4+ T cells in the spleen and that these cells can differentiate into cells that can provide help to cognate B cells. However, these helper cells represent a heterogeneous subset of T cells that can help B cells in the follicle (germinal center Tfh cells) or in the extrafollicular spaces (extrafollicular T cells). Microscopic analysis of the spleen following adoptive transfers of anti-

RBC CD4+ T cells can be done to study the localization of these cells, further informing us about the differentiation state of anti-RBC CD4+ T cells.

What cytokines regulate anti-RBC antibody production following CMV infection?

Our data shows that type 1 IFNs are mostly not required, and that IL-21 is only partially required for anti-RBC antibody responses following CMV infection. These results suggest that other cytokines are playing a role in regulating anti-RBC antibody production. Cytokines such as IL-12 and IFN-γ have been previously shown to regulate class switching to IgG2 subtypes in various viral infection models. In addition, IL-4 has been shown to control IgG1 class switching. The role of these cytokines can be investigated in the CMV setting using blocking antibodies and knockout mice.

Do physiological models of CMV reactivation lead to enhancement of anti-RBC antibodies?

Our experiments make use of an acute CMV infection model. However, most cases of CMV in HSCT patients occurs as a result of re-activation of the latent virus. Therefore, in order to study RBC alloimmunization in a clinically relevant CMV model, future experiments will use CMV reactivation models to study molecular and cellular mechanisms.

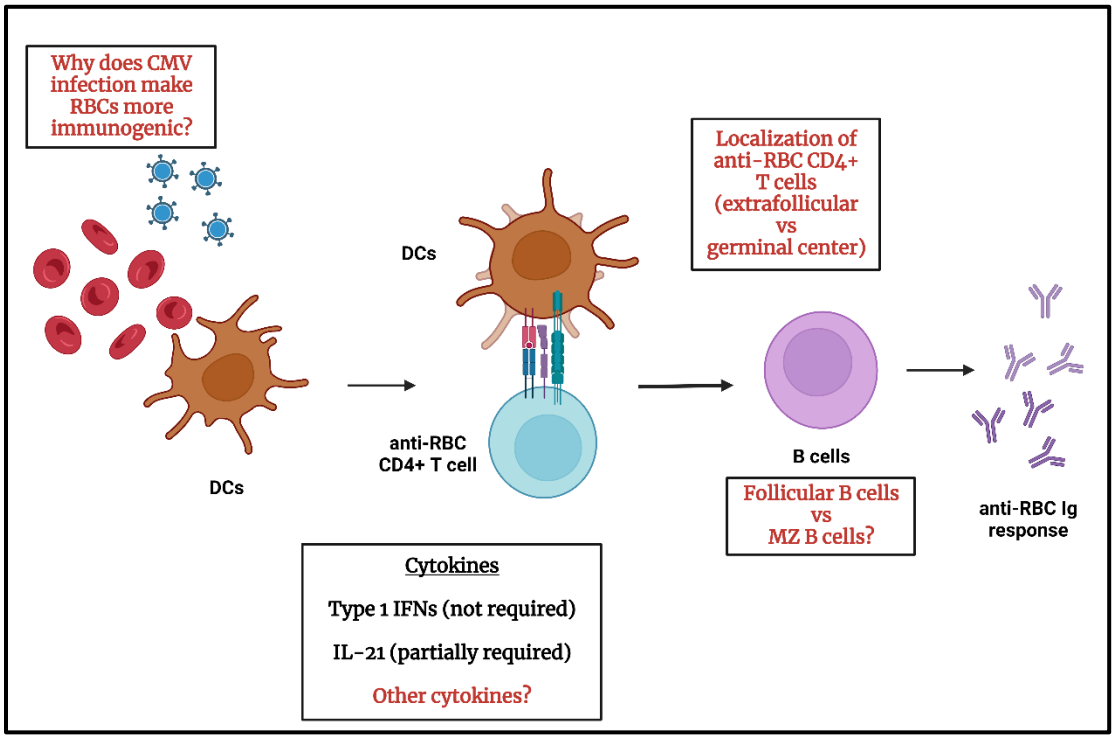


Figure 4.1: Schematic showing outstanding questions (in red) that will be answered in future experiments

Storage dependent RBC alloimmunization

What cytokines regulate anti-RBC class switching in the stored HOD model?

Our data in Chapter 3 shows that transfusions of stored HOD RBCs leads to production of all IgG subtypes: IgG1, IgG2b, IgG2c and IgG3. However, we further show that STAT6 (a known regulator of IgG1) is not required for IgG1 class switching in the HOD transfusion system. While these results are surprising, they open up further questions about what cytokines might be regulating the IgG response. Given the requirement of CD4+ T cells in regulating class switching in the HOD system, we hypothesize that T cell-derived cytokines might be regulating class-switching. We can identify these cytokines by adoptively transferring naïve OT-II cells and transfusing the recipients with HOD RBCs. These OT-II cells can then be purified and RNAseq or RT-PCR can be done to study the cytokine genes that are expressed. This can give us cytokine targets that can be further studied using knockout mice or blocking antibodies. These experiments can help us understand the cytokines that might be regulating the IgG response to HOD RBCs.

How do CD4+ T cells help MZ B cells and induce class switching?

While previous reports have shown that CD4+ T cells and MZ B cells are required for anti-HOD IgG production, it remains unknown how CD4+ T cells provide help to MZ B cells in the HOD system. Understanding the cytokines that regulate the alloantibody response to HOD RBCs can help us study the role of cytokine help in antibody production by marginal zone B cells. MZ B cell-specific knockouts for cytokine receptors can be used to study the role of cytokine help in

marginal zone B cell activation and antibody production. We can further study the differentiation of MZ B cells into plasmablasts or long-lived plasma cells.

Where do HOD-specific CD4+ T cells localize following RBC transfusion?

Previous experiments have shown that in an adoptive transfer setting, OT-II cells can express CXCR5 and Bcl-6 following transfusion of stored HOD RBCs. However, the exact location of these cells has not been previously studied. Using microscopy studies, we can investigate the location of these transferred CD4+ T cells. This will allow us to study if anti-HOD CD4+ T cells form germinal center localized Tfh cells or extrafollicular T cells.

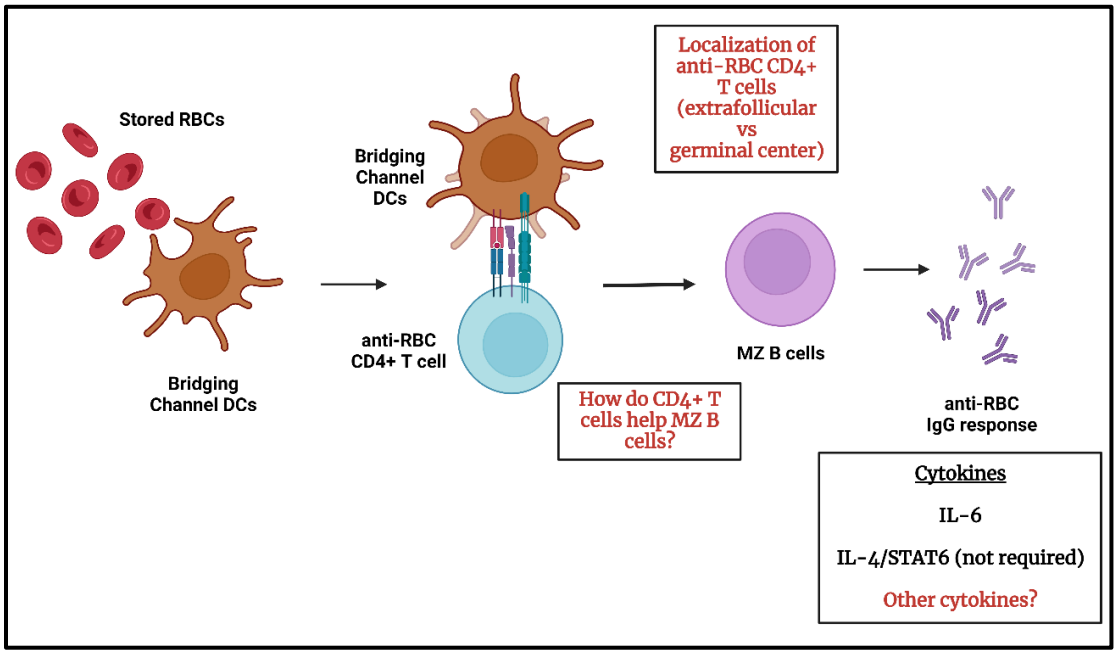


Figure 4.2: Schematic showing outstanding questions (in red) that will be answered in future experiments

Chapter 5: References

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