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Immune Response to Erythrocyte-specific Antigens

By

Krystalyn E. Hudson

Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Immunology and Molecular Pathogenesis

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Immune Response to Erythrocyte-specific Antigens

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B.S., Georgia Institute of Technology, 2003

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Abstract

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Antibodies to red blood cell (RBC) antigens can occur spontaneously or as a consequence of antigen exposure through infection, transfusion, transplantation, or pregnancy. Some antibodies against RBC antigens are clinically insignificant, whereas others are clinically relevant and can result in hemolysis, pose a barrier to future transplants and complicate finding future compatible RBC units for transfusion. Defining the underlying mechanisms that contribute to the breakdown of tolerance to RBC antigens and factors that regulate the rates of alloimmunization are complex.

These data demonstrate that RBC-specific B cells escape both central and peripheral tolerance mechanisms, encounter and present self-antigen, and are receptive of CD4+ T cell help. Therefore, in the event that T cell tolerance is broken, autoimmunity can be initiated. We have further demonstrated that molecular mimicry of CD4+ T cell epitopes shared between a pathogen and an RBC antigen can enhance alloimmune responses. As such, it is plausible that pathogen-elicited CD4+ T cells, through molecular mimicry and/or linked recognition of cryptic (e.g. transmembrane or cytosolic) peptides from RBCs could break T cell tolerance and lead to autoimmune pathology.

Maintenance of RBC structure and function is essential for sustaining life. As such, RBC transfusions are life-saving therapies for persons with inherited or acquired anemia and also those who suffer blood loss as a consequence of trauma, surgery or childbirth. Thus, understanding the underlying mechanisms behind the breakdown of B and/or T cell tolerance to RBC-specific self- and allo-antigens is important.

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Chapter 1

Introduction

The Importance of Red Blood Cells

Structure and Function

Red blood cells (RBCs) or erythrocytes, differentiate from hematopoietic stem cells in the bone marrow in adults. Erythrocytes are essential for life; their primary function is to deliver oxygen from the lungs to tissues throughout the body. To achieve this, RBCs are rich with hemoglobin, an iron-containing protein that has a high affinity for oxygen (1). Additionally, RBCs express over 29 blood groups containing over 600 antigens (2). Many of these antigens are important to transfusion medicine due to their propensity to elicit alloantibody responses (that is, recipient antibodies generated against foreign antigens or polymorphisms within an antigen present on the donor RBCs), but many of them are also important to maintain the functional and structural integrity of the RBC (3).

RBC antigens are assigned to broad categories based on their function: membrane proteins, enzymes and structural proteins. With the exception of antigens in the Rhesus (Rh) blood group, most RBC antigens can be found on other tissues (e.g. Duffy expression in the kidney) and in secretions (e.g. saliva) (4-7). Examples of blood groups containing antigens that are important for maintaining the structure and function of RBCs are Kidd (Jk), Diego and Duffy (Fy). Jk facilitates the transport of urea across the membrane of not only RBCs but also endothelial cells of the descending vasa recta in the kidney. Similarly, Band 3 (part of the Diego blood group) is an ion transport channel that aids in efficient transport of respiratory gases. Band 3 also tethers the plasma

membrane to the cytoskeleton of the RBC and regulates glycolytic pathways (8). Finally, Fy is 7-pass membrane protein expressed on RBCs and endothelial cells. Fy functions as a chemokine sink, binding excess inflammatory chemokines and ultimately influencing immune responses (9). Taken together, blood group antigens perform diverse functions not only within RBCs, but also in the periphery.

In addition to structural and functional attributes of RBC antigens, the absence or presence of particular antigens can influence an individual's susceptibility to infection and/or the development of a disease. Blood group antigens can serve as receptors for parasites, bacteria and viruses (10). Endemic to Africa, Asia and South America, malaria is a mosquito-borne disease caused by infection with the parasite *Plasmodium*. There are 5 species of *Plasmodium*, each exploiting blood group antigens to invade erythrocytes. One such species, *P. vivax*, utilizes Fy to gain entry; erythrocytes deficient in Fy antigens are resistant to infection. Similarly, RBCs lacking glycophorin A or B are resistant to infection by *P. falciparum* (3, 11). Thus, due to selection pressures, individuals living in regions where malaria is endemic have unique RBC antigen expression (e.g. lacking Fy) that can confer protection against *Plasmodium* infection.

Another correlation between blood group antigen expression and infection is observed in individuals with blood type O. When compared to persons with other ABO blood types, those with blood type O have an increased occurrence of peptic ulcers. The infectious bacterium *Helicobacter pylori* is associated with gastritis and is thought to be a major cause of peptic ulcers. *H. pylori* bacteria bind residues unique to blood type O and colonize gut epithelia leading to inflammation and subsequent development of ulcers (10-

12). *H. pylori* infection of gut epithelia is one example of a negative consequence of promiscuous expression of blood group antigens.

RBCs in Transfusion Medicine

Why Transfusions are Necessary

The primary function of RBCs is to deliver oxygen to the rest of the tissues in the body. A defect in erythropoiesis (generation of new RBCs) or erythrocyte function can affect and hinder efficient delivery of oxygen to vital organs and tissues and can have detrimental effects on the quality of life and survival of the host (13, 14). Therefore, to maintain sufficient oxygenation of tissues, transfusion is one method of treatment for individuals suffering from decreased numbers or malformed RBCs (due to inherited or acquired anemia) (15-17). Additionally, transfusions of RBCs may be necessary to reconstitute blood volume due to loss after an injury resulting in trauma, childbirth, or surgery (18, 19).

Clinically Relevant Antigens

Some RBC antigens are more prone to inducing an alloimmune response than others. Indeed, this is how most RBC antigens were discovered. The assigning of antigens to blood groups is based on sequence similarity (3, 20). Most antigens within a blood group have varying degrees of polymorphisms (e.g. one amino acid difference that gives rise to Fy^a and Fy^b) (21). Polymorphisms that stimulate a detectable antibody response are considered immunogenic.

The phrase “clinically relevant” implies that a given polymorphism within a blood group is immunogenic and may elicit an immune response in a recipient. This represents a potential barrier to future transfusions and transplantations or, worse, may lead to a hemolytic transfusion reaction (22-24). The instance in which one antigen will be more immunogenic when compared to another depends on the frequency of alloantibodies generated against the antigen on the RBC and the characteristics of the resultant alloantibodies (e.g. immunoglobulin subtype) (25). Hence, clinically relevant antigens are (in order of immunogenicity): the carbohydrates that comprise the ABO group, Rhesus (Rh), Kell (K), Duffy (Fy) and Kidd (Jk) (3, 26).

The ABO components are ranked highly in immunogenicity due in part to homology shared with intestinal flora. Bacterial occupants of the gut express carbohydrate structures similar to ABO and elicit an antibody response specific for blood antigens that the host does not express (i.e. an individual who is blood type A will make anti-B antibodies) (27). Thus, an ABO incompatible transfusion may result in hemolysis due to the presence of anti-ABO antibodies. Futhermore, in retrospective studies, acute hemolysis due to ABO incompatible transfusions were responsible for over 50% of transfusion related fatalities (28, 29).

The next clinically relevant antigens belong to the Rhesus (Rh) blood group. The Rh blood group is comprised of over 49 antigens, of which many are immunogenic. The D antigen is the most immunogenic; in Rh D-negative recipients of Rh D-positive RBCs, 20-80% of patients make anti-D alloantibodies (30-32). Unlike some RBC antigens that are polymorphic at only one amino acid, most individuals are either Rh D-positive or

negative. Because Rh D-negative individuals lack the entire Rh D antigen, when exposed to an Rh D-positive RBC, it is more likely that a suitable peptide can be presented in the major histocompatibility complex (MHC) class II, thus prompting an immune response (33). This is one reason why it is hypothesized that Rh D is highly immunogenic.

The formation of anti-D alloantibodies is clinically significant for many reasons. Patients who readily make anti-D alloantibodies are more likely to become alloimmunized to antigens outside of the Rh blood group (3, 34); these alloantibodies can make finding future transfusion units difficult. Furthermore, anti-D antibodies can elicit a hemolytic transfusion reaction, causing hemolysis of not only the transfused unit, but also the recipients' RBCs (35). Lastly, anti-D antibodies may be especially clinically significant in immunized mothers carrying Rh-D positive fetuses, with anti-D antibodies leading to hemolytic disease of the fetus and newborn (See section titled "Hemolytic Disease of the Fetus and Newborn). In an attempt to avoid anti-D alloimmunization, Rh D-negative recipients are transfused with Rh D-negative RBCs.

The Rhesus blood group also contains antithetical antigens c/C and e/E, albeit less immunogenic than D. In patients who receive few transfusions, high frequencies of alloimmunization are directed towards both c and E. However, in multiply-transfused patients (e.g. those with sickle cell disease) the most common specificities are C and E (36). Antibodies against these antigens can also elicit hemolytic reactions. Thus, in combination with the D antigen, the Rhesus blood group is very immunogenic.

Outside the context of ABO and Rh D (which are donor and recipient matched prior to transfusion), Kell, Duffy and Kidd are the next most immunogenic. Alloantibodies directed against these antigens are involved in both acute and delayed hemolytic transfusion reactions (21). Furthermore, alloantibodies generated against these antigens pose a greater risk of not finding future compatible units of RBCs and may pose a barrier to transplantation (37).

Hemolytic Disease of the Fetus and Newborn

Hemolytic Disease of the Fetus and Newborn (HDFN) is a condition that shortens the lifespan of an infant's RBCs as a result of antibody-mediated hemolysis or suppression of erythropoiesis. The presence of antibodies is a consequence of prior maternal immunization (e.g. prior transfusion, transplantation or pregnancy) or as a result of the current pregnancy when the fetal RBCs are released into the maternal circulation. These antibodies, which are directed against antigens or polymorphisms of antigens on the infant's RBCs, are different from the mother, may cross the placenta and mediate hemolysis of the fetus' RBCs (thereby causing severe anemia or death) (38).

Many blood group antigens have been implicated in causing HDFN. The most severe cases involve an Rh D-negative woman who is pregnant with an Rh D-positive fetus. As a consequence of the immunogenicity of the Rh D antigen, anti-D antibodies are generated and the newborn will be at high risk of severe hemolytic anemia with possible mortality (39). Current prophylactic approaches to decrease the mortality associated HDFN caused by anti-Rh D antibodies is achieved through passive

administration of anti-Rh D immunoglobulins (RhoGAM, patented by Johnson & Johnson) (40). RhoGAM has been proven to be highly efficacious and prevents rejection of the fetus. The exact mechanisms of action are unknown. Other Rhesus antigens and antigens within Kell, Duffy and Kidd blood groups have also been implicated in causing HDFN (41). Current therapeutic approaches used to decrease mortality and anemia associated symptoms employ a combination of intravenous immunoglobulin and intra-uterine transfusion with antigen-negative RBCs.

Adverse Reactions to Transfusions

While transfusions provide life-saving treatments and therapies, there are adverse side effects. Alloimmunization to RBC antigens has already been discussed as a negative side effect that can influence the time it takes to find future compatible units, increase risk for hemolytic transfusion reactions and pose as a barrier to transplantation. Another consequence of receiving a transfusion is a febrile reaction that may be a result of contaminating leukocytes (e.g. lymphocytes). Most febrile reactions are non-hemolytic, but spur a cytokine storm that causes a sudden rise of body temperature that can be accompanied with nausea, headache and back pain (42).

In addition to contaminating leukocytes, transfused plasma may also contain donor antibodies that react with the recipient's tissues and cells. These antibodies are reactive primarily against human leukocyte antigens (HLA) class I or II and human neutrophil antigens. Donor-derived antibodies bind to recipient tissue and fix complement, initiating release of pyrogenic cytokines and recruitment of neutrophils into

tissue (primarily the lung) causing transfusion-related lung injury (TRALI) (43). Prior to the utilization of male only plasma products, TRALI was a leading cause of transfusion-related mortality, with symptoms of hypoxia, tachycardia, fever, chills and leukocyte infiltration of the lungs (visualized via x-ray) (44).

Transfusion recipients can suffer from both acute and delayed hemolytic reactions. Acute hemolytic reactions are associated with pre-existing alloantibodies specific for RBC antigens and/or complement-mediated intravascular hemolysis mediated by IgM. Acute hemolytic reactions occur within hours of transfusion. In contrast, delayed hemolytic reactions are associated with a secondary, anamnestic immune response, with alloantibodies appearing 3 to 10 days post transfusion. Symptoms of a hemolytic reaction include a change in urine color, fever, chills, a drop in hemoglobin concentration, jaundice and in some cases, death (45, 46). In addition to experiencing a hemolytic reaction, patients may also become further alloimmunized to antigens on the donor RBCs, further complicating the search for compatible transfusion units.

Finally, despite stringent attempts to screen blood and blood products for viruses, parasites and bacteria, trace amounts have been identified in the blood supply and have persisted to cause disease in new hosts. Indeed, viruses such as human immunodeficiency virus and hepatitis C virus were once prevalent in the blood supply (47, 48). However, the development of screening tools has significantly decreased the incidence of transfusion transmitted HIV and hepatitis (49). Despite rigorous attempts to eliminate all pathogens, cytomegalovirus, Babesia, Trypanosomes, Dengue and *Plasmodium* still pose a risk of infecting new hosts through transfusions, though

leukoreduction significantly reduces the risk of cytomegalovirus transmission (50). Additionally, emerging pathogens (e.g. West Nile Virus at the beginning of the 21st century) may be difficult to detect in blood units, especially soon after their emergence (51). Aside from viruses and parasites, bacterial contamination has proven to be very serious as bacterial components are recognized as foreign by the innate immune system and a cytokine storm results (52). Animal studies suggest that the state of inflammation of the recipient can affect the rates of alloimmunization (53-55). Thus, infectious agents in the blood supply can transmit disease and induce alloimmunization in transfusion recipients.

Tolerance to Self Erythrocyte Antigens

Development and Maturation of RBCs

Erythropoiesis is the process by which new RBCs are generated. Throughout life, erythropoiesis can occur in the spleen, liver and bone marrow from hematopoietic stem cells (56, 57). The development and maturation of erythrocytes is regulated by the hormone erythropoietin and guides the transition of early-stage erythroblasts to reticulocytes (immature RBCs) and finally to mature RBCs (58). Differentiation of erythrocytes is accompanied by a decrease in cell size, expulsion of the nucleus, increased capacity to transport hemoglobin and expression of cell surface antigens.

Receptors and antigens, some lineage specific, begin to be expressed in the early erythroblast stage. The earliest expression of antigens are the carbohydrates that comprise ABO, during the erythroblast stage (3). Also expressed on erythroblasts are

antigens within the blood groups such as Rh, Kell, Kidd and Duffy and adhesion molecules such as CD44 and intracellular adhesion molecule-4 (59). As the erythroblast matures, expression of surface adhesion molecules wanes and the number of blood group antigens increases. Accompanying blood group antigen expression is the up-regulation of proteins necessary for functional attributes of the RBCs, such as Band 3 (58).

Lymphocyte Tolerance

There are two main types of lymphocytes: B lymphocytes (B cells) and T lymphocytes (T cells). Within each type, there are subtypes of cells that participate in the innate and adaptive immune response. The following section will focus on B cells. B cells help bridge the innate and adaptive immune response because they can function as antigen presenting cells as well as actively participate in the clearance of pathogens through antibody secretion.

Stages of B Cell Development (Making the B Cell Receptor)

B cell lymphocytes differentiate from hematopoietic stem cells in the bone marrow. These cells were originally discovered in the bursa fabricius of chickens (hence "B" cells). To be a mature B cell, cells must express a fully functional B cell receptor (BCR) to be used for antigen recognition and signaling; the BCR confers antibody specificity (60).

A BCR is comprised of heavy and light polypeptide chains held together by disulfide bonds and expressed on the surface of the B cell. Each polypeptide chain is comprised of a variable (V) and joining (J) region; only the heavy chain has a diversity

(D) region which contributes additional variety. While only one gene segment from each of these regions is selected to be expressed, there are multiple functional genes that can be selected from. For example, the human heavy chain contains 40 V, 25 D and 6 J genes. It is due to this broad repertoire that an estimated 2×10^{11} possible combinations of heavy and light chain genes can come together and form a functional BCR (61). In theory, there is a BCR combination that can recognize any foreign antigen.

B cells go through several stages of development and checkpoints, each necessary for the generation of a complete BCR. Early pro-B cells differentiate from stem cells. Beginning at this stage, recombinase activating genes (RAG) are turned on and facilitate the joining genes from the diversity and joining segments of the heavy chain (61). After joining, the variable region is combined with the newly formed DJ segment. The newly-formed VDJ segment is paired with a constant region to constitute a complete heavy chain.

Productive rearrangement of the heavy chain (μ) is expressed on the surface of the developing large pre-B cell. The μ chain associates with a surrogate light chain to form a pre-BCR. At this point, the pre-BCR is tested for reactivity to self antigen. Progression of B cell development will be halted if the pre-BCR reacts strongly with self antigen; if there is little or no reactivity with self antigen, a positive signaling event is received, prompting division and rearrangement of the light chain (62). Upon successful light chain rearrangement (i.e. no or weak reactivity to self-antigen), the heavy and light chains are paired together, forming a complete IgM molecule, and expressed on the surface of the immature B cell (63, 64). Immature B cells emigrate from the bone marrow into the

periphery where they can undergo further differentiation into a mature (naïve) B cell, with expression of both IgM and IgD immunoglobulins on the cell surface.

Checkpoints During B Cell Development

There are 3 critical checkpoints during B cell development. The first checkpoint is the assembly and rearrangement of the μ heavy chain (65). The heavy chain is comprised of variable, diversity, joining and constant genes. There are multiple genes to be chosen from and not all VDJ combinations form a functional heavy chain. Evaluating the VDJ heavy chain combination is completed in the pre-B cell stage, when the μ heavy chain is paired with a surrogate light chain (SLC).

Association of the heavy chain with the SLC forms the pre-BCR and serves as the second checkpoint. Elimination of components of the pre-BCR (e.g. heavy chain through genetic manipulation) blocks development (66). Pairing with the SLC serves as a checkpoint for the correct folding of the heavy chain such that when pairing with the real light chain, it forms an antibody binding site (67). Thus, successful rearrangement of the heavy chain and pairing with the SLC prompts a proliferative burst. If the heavy chain does not pair well with the SLC, then this heavy chain will likely not form a good BCR. Pre-B cells with incompetent BCRs will not signal effectively, clonally expand or downregulate RAG expression (68).

The third checkpoint is the formation of a complete BCR through association of both the heavy and light chains displayed on the surface of the B cell. During this stage of development, the B cell with the newly expressed BCR encounters antigen (62).

Antigens that strongly cross-link the BCR have at least three outcomes: cells are eliminated through clonal deletion, cells become nonresponsive (anergic) or cells continue to undergo rearrangement of their BCR (receptor editing) (69, 70). Successful generation of the BCR leads to down-regulation of RAG expression and signals the completion of B cell development, ending with an immature B cell.

Types of B Cells

B lymphocytes (B cells) are responsible for the production of antibodies, the effectors of humoral immunity. There are 2 main populations of B cells, commonly referred to as B-1 and conventional (B-2). While both of these B cell types have distinct and overlapping phenotypes and functions, B-1 cells have been mostly associated with innate immunity and conventional B cells with the adaptive immune response. Both types of B cells are necessary to maintain a fully functional and complete immune system because deficiencies in one or more B cell subtypes leaves the host vulnerable (71, 72).

Conventional (B-2) B Cells

Conventional B cells differentiate from hematopoietic stem cells in the bone marrow where they mature and express surface BCR after antigen-induced positive and negative selection. Conventional B cells comprise the majority of B cells, consisting of both follicular and marginal zone B cells. These B cells are most often thought of as the mature, re-circulating B cells that populate the bone marrow and secondary lymphoid organs; they are intimately tied to the adaptive immune response, participating in

germinal center reactions and production of effector antibodies capable of facilitating the eradication of an invading pathogen.

Follicular B cells re-circulate between the spleen, bone marrow and lymph nodes. Follicular B cells are very efficient at presenting T-dependent antigens to T cells in secondary lymphoid organs. Likewise, follicular B cells can be induced to express the necessary costimulatory molecules (e.g. CD40) for complete B cell activation and differentiation into short and long term plasma cells. Traditional follicular B cell activation in response to a T-dependent antigen will result in memory B cell formation and long-lived plasma cells that reside in the bone marrow (73).

Follicular B cells are less efficient at responding to T-independent antigens, antigens that strongly crosslink the BCR thus circumventing the need for T cell interaction prior to differentiation into a plasma cell. Follicular B cells express toll like receptors (i.e. receptors that recognize pathogen associated molecular patterns) that can facilitate BCR crosslinking upon recognition of a pathogen (74). However, ligation of TLRs on follicular B cells cannot alone drive differentiation into plasma cells despite BCR crosslinking (75).

Marginal zone B cells are very similar to innate B cells because they primarily respond to T-independent antigens; they can differentiate into short lived plasma cells without BCR ligation. Marginal zone B cells reside primarily in the marginal sinus of the spleen and do not re-circulate in the lymph. Functionally, marginal zone B cells are highly efficient at transporting immune complexes (an antibody bound to a soluble

antigen) to follicles within the spleen (76). Activation of marginal zone B cells through TLR ligation results in migration to the red pulp and differentiation into short lived plasma cell. While marginal zone B cells predominately participate in T-independent responses, they do express costimulatory molecules and can present antigens to T cells (77).

Conventional B cells are phenotypically discriminated by expressing high levels of CD45R, IgD and low levels of IgM (78). Marginal zone and follicular B cells can be distinguished by expression of CD21/35 (complement receptor 2) and CD9 (79). Marginal zone B cells express high levels of CD21/35 and this is thought to aid in their efficiency of immune complex recognition. Functionally, marginal zone B cells will respond to T-dependent antigens quicker than follicular. However, follicular B cells are responsible for most of the generation of memory B cells after an immune response. Additionally, antibodies generated as a result of an immune response also differ between marginal zone and follicular B cells with marginal zone favoring IgM and IgG3 compared to a predominance of IgG1 as seen with follicular B cells (80).

Innate (B-1) B Cells

A subpopulation of Ly-1 (CD5) expressing B cells (later to be known as B-1a B cells) was first identified in 1983 (81). This B cell subset was of interest because there was an elevation in Ly-1^+ B cell percentages in autoimmune mice and an association with production of autoantibodies (82). After many years of research, this population of B cells (along with its B-1b counterpart; collectively known as B-1 B cells) has been

described to be distinct from the better-known conventional B cells in regards to origin, location, phenotype and antibody repertoire (78, 83, 84).

For over 30 years, it has been debated whether B-1 B cells arise from common lymphoid progenitors present found the bone marrow or, perhaps, in extramedullary compartments (85-87). Several *in vivo* experiments tested these hypotheses by performing either bone marrow or fetal liver transplants into irradiated recipients (88). These experiments demonstrated that B-1 B cells could not be derived from progenitors in the bone marrow; only fetal liver stem cells could recapitulate the B-1 B cell repertoire. However, in a series of recently published papers, it has become clear that there has been a B-1 cell progenitor in the bone marrow identified (89-91). The identification of the progenitor does not devalue the extramedullary contributions to the B-1 cell repertoire, however, as solely performing a bone marrow transplant does not completely recapitulate the numbers and percentages of B-1 B cells (and ratios of subtypes) obtained when using fetal liver progenitor cells (87, 92, 93). The question of origin of B-1 cells is pertinent to the understanding of B cell tolerance mechanisms outside of the bone marrow. If B-1 B cells arise from an extramedullary compartment, it is feasible that the stringent positive and negative selection mechanisms that conventional B cells are exposed to, and the repertoire of antigens to which conventional B cells are tolerized, are not available for B-1 B cells.

B-1 B cells populate mostly the pleural spaces, including the peritoneal cavity and chest cavity; there is a small subset of B-1 cells that are found in the spleen and lymph nodes (83, 94). B-1 B cells express high levels of surface IgM, intermediate levels of

B220 (CD45R) and low levels of IgD. Other surface antigen markers that allow discrimination of B-1 B cells from conventional B cells are the expression of CD43 and absence of CD23 (94, 95). B-1 B cells can be readily identified in the peritoneal cavity by expression of the myeloid marker, CD11b, whereas this surface marker is absent on the population in the spleen (96). Additionally, a subset of B-1 B cells in the peritoneal cavity expresses CD5 (B-1a) whereas CD5- B-1 B cells are referred to as B-1b (97, 98). These two subsets are different functionally as B-1a B cells have the capacity to secrete IgM antibodies in the absence of T-cell help whereas B-1b cells are more similar to conventional B cells and require T cell interaction to secrete antibodies (99).

Innate B cells are responsible for "natural antibodies" that populate the immune system prior to the maturation of the adaptive immune response. These natural antibodies are most important during the first couple of months of life, prior to the development of a fully functional immune system. They are especially important to protection against bacterial infections, but can also protect against viral pathogens (100, 101). Most antibodies produced are of the IgM subtype, but B-1 B cells can also make IgG3 and IgA, all of which are important in natural immunity (84). Unlike their conventional B-2 counterparts, B-1 B cells have a long half life and have the capacity for self-renewal (102, 103). The complete maintenance of the B-1 B cell population is dependent on the presence of the spleen. In splenectomized mice, the percentages of B-1a B cells are decreased (B-1b numbers are steady) within 2 weeks and these numbers do not rebound (72). These data are consistent with several hypotheses, including: 1) The splenic architecture is required for B-1a B cells to maintain their self-renewal capacity, 2)

A soluble factor from the spleen is necessary for the survival of B-1a B cells or 3) A B-1a progenitor is found in the spleen. Most notably, however, loss of B-1a cells leaves the host unable to mount a protective immune response against bacterial polysaccharides. This further demonstrates the importance of this B cell subset.

Despite the controversy surrounding the origin of B-1 B cells, it is known that these cells, like their conventional counterparts, express low levels RAG enzymes and rearrange their BCR (104-106). Unlike conventional B cells, B-1 B cells have a restricted repertoire of variable region of the heavy chain (V_H) usage. The most common V_H chains associated with B-1 B cells are V_H11 and V_H12, with minimal N nucleotide additions (98, 107, 108). Because of this limited usage of V_H chains, the BCR has broad specificity and is weakly self-reactive. Most of the antibodies secreted from B-1 B cells (specificity conferred by their BCR), are reactive against thymic independent antigens such as phosphorylcholine and phosphatidylcholine (80, 109).

Tolerance to Soluble and Membrane-bound Antigens

B cells are known to only be tolerized to antigens they encounter during development. For most B cells, antigen encounter occurs during development in the bone marrow, where active positive and negative selection is occurring. The optimal BCR expresses no self-reactivity and the matched heavy and light chains that form the receptor is capable of receiving a signal when tested with antigen.

B cells encounter many different types of self-antigen, not only in the bone marrow but also in the periphery. The type of self antigens and their locations can have

profound effects on the mature B cell repertoire. Self-antigens can be categorized as either soluble or membrane-bound, and depending on the form and location that self-antigen is encountered, different mechanisms of B cell tolerance are employed: deletion, receptor editing, anergy or ignorance.

One of the earliest models that demonstrated B cell tolerance was described by Goodnow and colleagues and utilized the hen egg lysozyme (HEL) BCR transgenic mouse (110). To generate the immunoglobulin (Ig) HEL transgenic mouse (IgHEL), the heavy and light chain from the Balb/c hybridoma HyHEL10 were cloned out and the constant regions were replaced with μ and δ, thus allowing for surface expression of IgM and IgD production. Additionally, the HEL Ig was inserted into a C57BL/6 mouse, allowing for allotypic discrimination between B cells specific for HEL (allotype 'a') and those from endogenous rearrangement (allotype 'b') (110). Using this approach, over 90% of splenic B cells in the IgHEL mouse are $IgM^{a+}IgD^{a+}$, with over 60% reactive with HEL. Thus, in the IgHEL mouse alone, some of the Balb/c-derived (HEL specific) heavy chains pair with endogenous light chains, perhaps through BCR editing.

To test whether the form and/or concentration of antigen influenced the establishment of B cell tolerance, Goodnow and colleagues introduced a HEL transgene as a self antigen in both membrane-bound and soluble forms. The HEL membranous form (mHEL) expression is driven under the $H\text{-}2k^{b}$ promoter (MHC class I) while soluble HEL (sHEL) antigen is induced under the metallothionein promoter and can be regulated by administration of zinc (111, 112). Interestingly, depending on the form of antigen encountered by the B cell, different mechanisms of tolerance are employed. In the F1 of

IgHEL and mHEL mice, mice carrying both transgenes exhibit a profound depletion of HEL-reactive (allotype ‘a’) B cells in the periphery (spleen and bone marrow) (112, 113). In contrast, autoreactive B cells persist in the periphery of mice from sHEL/IgHEL double transgenic mice. Phenotypically, the persistent autoreactive B cells that are detected in the periphery have a 20-fold decrease in surface IgM (111). Functionally, there is a decrease in HEL-reactive antibodies in the sera and the B cells are not responsive to antigenic stimulation (114, 115). Thus, these B cells are termed anergic (e.g. a state of unresponsiveness) (116-118).

Ferry and colleagues directly tested whether the location of antigen expression affects the autoreactive B cell repertoire by using the mHEL-KK transgenic mouse, expression of membrane-bound HEL restricted to the endoplasmic reticulum (119). Unlike data from the mHEL/IgHEL and sHEL/IgHEL double transgenic mice, neither deletion nor anergy is observed with the mHEL-KK/IgHEL mice. Compared with mHEL/IgHEL double transgenic mice, mHEL-KK/IgHEL mice have 25-fold more antibody secreting cells and more than 10,000-fold increase of anti-HEL IgM antibodies in their sera. Additionally, there is an enrichment of B-1 B cells in the peritoneum, which was not observed in mHEL/IgHEL or sHEL/IgHEL mice (119, 120). Thus, these data demonstrate that intracellular expression of HEL does not tolerize autoreactive B cells but instead promotes autoantibody production and expansion of B-1 B cells. Taken together, the combination of data generated by Ferry and Goodnow demonstrate that the B cell repertoire is greatly influenced on the type (soluble or membrane-bound) and location (extracellular or intracellular) of antigen.

A second model of B cell tolerance was established by Nemazee and colleagues and utilized an anti-MHC I (H-2k) transgene (3-83) to create an anti-MHC class I BCR transgenic mouse (121). The BCR has affinity for both H-2k^{k} and H-2k^{d} but not H-2^{d}. Thus, 3-83 mice were bred with Balb/k mice (thus expressing the autoantigen) or Balb/c (H-2^{d}, thus no autoantigen) to evaluate the B cell tolerance. Similar to what was observed in the IgHEL/mHEL double transgenic mice, clonal deletion of autoreactive B cells is observed, which correlates with an absence of autoantibodies.

Similar to Ferry and colleagues, Nemazee questioned whether antigen location influenced the degree of B cell tolerance established. To begin to answer this question, another BCR transgenic mouse whose BCR was specific for anti-MHC class I K^{b} was bred with the MT-K^{b} mouse, transgenic K^{b} expression restricted to the liver (122). In the F1 of these two transgenic mice, local depletion of autoreactive B cells in the liver is observed and there are no detectable anti-MHC class I K^{b} antibodies in the sera. Thus, despite incomplete central tolerance to the K^{b} antigen, some level of B cell tolerance is established. This data demonstrates that B cell tolerance mechanisms are not aborted once a B cell leaves the bone marrow, but it is a continual process and occurs in the periphery as well as during development.

Perhaps the most seminal finding reported by Nemazee and colleagues is that B cell tolerance can be achieved through receptor editing of the autoreactive BCR. Again using 3-83 mice and crossing them with mice expressing the autoantigen (either K^{k} or K^{b}), clonal deletion is observed. In addition, however, it was noticed that most of the deletion-resistant B cells express lambda light chains (whereas the 3-83 transgene

contains a kappa light chain). It was also observed that autoreactive B cells in the bone marrow express elevated levels of RAG expression; only B cells that express a lambda light chain were permitted to emigrate from the bone marrow (123). Thus, these data demonstrate that during development, autoreactive B cells will undergo BCR editing as an attempt to become less self-reactive and if BCR editing is not successful, autoreactive B cells undergo clonal deletion.

The compilation of work completed with the HEL and MHC antigens have demonstrated many mechanisms of establishing B cell tolerance. In addition to these model systems, other model antigens (e.g. rheumatoid factor) and transgenic mouse models (e.g. anti-Smith BCR) have been described with similar results (124-127). Taken together, antigen encounter during development and in the periphery continually shape the B cell repertoire. Interestingly, B cell tolerance to autoantigens in these systems is never complete; even in mHEL/IgHEL and 3-83/K^k double transgenic mice, small percentages of autoreactive B cells persist in the periphery (112, 123). While this could be an artifact of using transgenic murine models, it does beg the question of whether autoreactive B cells are present and under which circumstances are they receptive to the necessary signals to promote autoantibody secretion.

A Murine Model of B cell Tolerance to Erythrocyte Antigens

Tolerance to erythrocyte antigens was first investigated using an autoimmune hemolytic anemia (AIHA) murine model described by Okamoto and colleagues (128). AIHA is a clinical disorder in which autoantibodies bind to self-erythrocyte antigens and

promote destruction of RBCs (129). Symptoms of AIHA relate directly to the hemolysis caused by the RBC hemolysis and can include fever, abdominal or back pain, splenomegaly, and in some cases, death. Onset of AIHA can occur spontaneously, concurrently with another disorder or infection (130), or in association with a RBC transfusion.

Okamoto et al. generated transgenic mice (HL) using an anti-RBC (specifically Band4.1) monoclonal antibody (4C8) derived from New Zealand Black mice (131). HL mice experience a range of AIHA symptoms from tolerance to severe anemia, depending on the state of inflammation and housing conditions (132, 133). B cell tolerance in HL mice is abnormal. Conventional anti-RBC B cells are deleted in the spleen and other secondary lymphoid organs. Conversely, anti-RBC B-1 B cells are enriched in the peritoneal cavity and lamina propia lymphocytes (134). Additionally, there is a correlation between deletion-resistant B-1 B cells and anti-RBC antibodies. The presence of anti-RBC antibodies induces a range of autoimmune hemolytic anemia symptoms. Elimination of B-1 B cells by intraperitoneal injection of deionized water or antibodies against helper cytokines (e.g. IL10) induces apoptosis of B-1 B cells (135-137). After deletion of B-1 B cells, the symptoms of AIHA are resolved. Conversely, activation of B-1 B cells by oral administration of LPS increases anti-RBC autoantibodies and exacerbates AIHA symptoms (134, 138, 139).

Data generated using the HL model system demonstrates that in this transgenic system, B cell tolerance to erythrocyte antigens is incomplete. Additionally, it re-enforces the differences between tolerance mechanisms ascribed to both B-1 and B-2 subtypes of

B cells, highlighting that extramedular differentiation might not result in complete tolerance to self antigens. Furthermore, peripheral tolerance to anti-RBC antigen specific B-1 B cells might not be established due to sequestration of B-1 B cells to pleural spaces, peritoneal cavity and spleen. And, finally, anti-erythrocyte autoreactive B cells are receptive to the necessary signals to become plasma cells and secrete autoantibodies.

T Cell Tolerance

Similar to B cells, T cells participate in both the innate and adaptive immune response. Development of T cells is analogous to that of B cells as T cells differentiate from hematopoietic stem cells in the bone marrow, but maturation occurs in the thymus. T cells undergo positive and negative selection as the T cell receptor (TCR) rearranges (61). First, T cells are selected based on whether they can recognize MHC class I or II on antigen presenting cells. If T cells recognize the MHC, they proliferate and proceed to another portion of the thymus; this region of the thymus is responsible for negative selection. During negative selection, T cells that respond too strongly with self antigen undergo clonal deletion (140). While T cells can undergo receptor rearrangement, a large majority of autoreactive T cells undergo apoptosis and do not leave the thymus.

In addition to positive and negative selection in the thymus, it is also hypothesized that expression of the autoimmune regulator (AIRE) gene is critical for establishment of central tolerance. Expression of AIRE contributes to central tolerance by inducing the transcription of many otherwise tissue specific antigens in the thymus (141). In mice and

humans, absence or mutation of AIRE leads to the development of a multi-organ syndrome known as polyendocrinopathy-candidiasis-ectodermal-dystrophy syndrome (APECED) that is characterized by systemic autoimmune pathology resulting from decreased clonal deletion of autoreactive T cells (142).

The form of antigen (soluble versus membrane-bound) can have different effects on the establishment of T cell tolerance. Zhang and colleagues generated transgenic mice with expression of mHEL or sHEL under the lens αA-crystallin promoter (143). HEL transgenic mice were mated with HEL-specific TCR 3A9 mice to assess T cell tolerance. Data from these studies demonstrate that membrane-bound antigen induce clonal deletion of autoreactive T cells. In contrast, anergic autoreactive T cells are found in the periphery in sHEL expressing mice. Another study suggested that both forms of HEL (under a different promoter) were efficient at inducing deletion (144). Taken together, the form of antigen can have varying effects on the mature T cell repertoire.

It is unknown whether RBC antigens are expressed in the thymus. Additionally, due to differential effects on T cell tolerance in response to the form of antigen, tolerance to some RBC antigens could be more complete than others. For example, T cell tolerance to ABO antigens might be more complete because ABO can be found in both membrane-bound and secreted forms (5). Thus, whether and what form of RBC antigens are present in the thymus and how this shapes the T cell repertoire is unknown.

Breaking Tolerance to Erythrocyte Antigens

The Effect of Antibodies Specific for RBC Antigens

Antibodies against RBC antigens can occur naturally or be induced through exposure (e.g. transfusion or pregnancy) (145). Antibody binding to RBCs can have many outcomes including clinically insignificant (i.e. binding to antigen but not inducing hemolysis or inducing non-hemolytic antigen loss) or can be clinically significant by inducing hemolysis (28, 146). Naturally occurring antibodies can occur spontaneously, through oral exposure or infection (147). In most cases, naturally occurring antibodies play a role in maintaining homeostasis and protection against infection; few have adverse effects. In contrast, antibodies generated in response to a transfusion, transplantation or environmental factors have the propensity to induce RBC hemolysis or antigen loss. Unlike hemolysis, antigen loss does not promote destruction of the RBC. Antibodies involved in antigen loss cause the antigen itself to become weakened or undetectable after antibody binding while overall RBC function is unaltered (146). Hemolysis (as previously discussed) leads to anemia and has the potential to increase rates of alloimmunization to additional RBC antigens. Current understanding does not allow the prediction of which antibodies will be innocuous or induce hemolysis.

Mechanisms Hypothesized to Break Tolerance to RBC Antigens

Breaking tolerance to erythrocyte antigens can lead to autoimmunity. Autoantibody induction has been hypothesized to occur by several mechanisms; these can be categorized as spontaneous, bystander activation, epitope spreading, molecular mimicry, or drug metabolism (148-150). Autoantibodies that occur spontaneously can be harmless (e.g. naturally occurring) or initiate hemolysis, as in individuals who suffer from primary AIHA in the absence of underlying disease or infection (129).

The role of bystander activation and epitope spreading in the initiation of autoimmune pathology has been well documented (151, 152). Infections with pathogens result in antigen-specific and antigen-nonspecific immune responses. One type of an antigen-nonspecific immune response is the production of local and systemic cytokines and chemokines. An adverse effect of inflammation is bystander activation (that is, activation of autoreactive T cells or B cells in the absence of cognate antigen). Bystander activation has been studied in the context of viral infection. For example, in the murine model of type 1 diabetes, local inflammation due to Coxsackie virus V4 infection initiates autoimmunity in nonobese diabetic (NOD) mice (153). Another consequence of Coxsackie viral infection is epitope spreading; during infection, tissue damage exposes *de novo* self antigen from islets in the pancreas. Due to sequestration of self antigen, central and peripheral tolerance might be incomplete and lead to activation of autoreactive T and B cells. Thus, unveiling cryptic antigens perpetuates autoimmune pathology to additional autoantigens (154). The sequence of bystander activation and epitope spreading has also been implicated in exacerbation of pathology in experimental autoimmune encephalomyelitis (EAE), a murine model for multiple sclerosis (MS).

Molecular mimicry is an instance in which a pathogen expresses an antigen (e.g. a protein) which shares a high degree of sequence or structure to a host self antigen. During infection, the immune system mounts a response against the pathogen-specific antigen. As a result of this exposure, the responses generated (either activated T cells or antibodies) cross-react with the host antigen, thereby initiating an autoimmune response. Molecular mimicry can occur at both the T and B cell level. Molecular mimicry is the

mechanism that is most favored for explaining the initiation of MS, Myasthenia gravis and Rheumatoid arthritis (155-158).

Finally, although rare, autoimmunity can be initiated through metabolism of drugs. Drug-induced autoimmunity has been linked to many agents including halothane, penicillin and, more recently, cephalosporins. Breakdown of halothane generates reactive metabolites that are capable of covalently binding to self-protein. This complex can be recognized by the immune system as a foreign antigen and lead to development of autoimmune-mediated hepatitis (150). Both penicillin and cephalosporins have been implicated in promoting drug-induced hemolytic anemia (159). It is hypothesized that these drugs bind to and alter the structure of erythrocyte structure thereby promoting complement-mediated destruction.

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Chapter 2

Regulation of Primary Alloantibody Response through Antecedent Exposure to a Microbial T cell epitope

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Abstract

Humoral alloimmunization to RBC antigens is a clinically significant problem that can lead to transfusion reactions and difficulty in locating future compatible blood for transfusion. However, alloimmunization rates are variable, and factors regulating responder/non-responder status are only partially understood. Herein, we identify a series of microbes with 100% identity in 8-9 amino acid peptides containing the variant amino acids in Kell, Kidd, and Duffy blood group antigens. To test the hypothesis that infection with such a microbe could predispose to RBC alloimmunization, a mouse model was developed using murine polyomavirus expressing a defined CD4+ T cell epitope [$(OVA)_{323-339}$] and subsequent transfusion with RBCs expressing a B cell epitope [hen egg lysozyme (HEL)] fused to $(OVA)_{323-339}$. Whereas infection alone induced no detectable anti-HEL, subsequent RBC transfusion induced 100-1000 fold more anti-HEL in mice that had been previously infected compared to control mice. This effect did not occur with wild-type polyomavirus or RBCs expressing HEL alone. Together, these data indicate that prior exposure to a pathogen with small peptide homology to RBC antigens can lead to an enhanced primary alloantibody response. As such priming is not detectable by current clinical tests; it is unknown to what extent this occurs in human alloimmunization.

Introduction

Humoral immunization to red blood cell (RBC) alloantigens can occur as a result of transfusion or pregnancy. Antibodies against clinically significant blood group alloantigens, (i.e. RhD, Kell, Kidd etc.) can lead to both hemolysis of transfused RBCs and/or to hemolytic disease of the newborn (1, 2). However, unlike humoral immunization to microbial infection, which approaches 100% in immunocompetent individuals, exposure to RBC alloantigens induces a measurable antibody response in only a subset of recipients. Alloimmunization to the RhD antigen on RBCs ranges from 20-80%, with only 3-10% of recipients becoming immunized to the remaining RBC antigens (e.g. Kell, Duffy, Kidd, etc.), despite chronic transfusion (3-5). The reason why some transfused patients but not others become alloimmunized is unclear, and factors influencing alloimmunization have been only partially defined. Immunogenetics plays some role in variability of alloimmunization to blood products, as antibody responses to certain alloantigens are confined to distinct recipient HLA types (6-8). In addition, genetic variants outside of HLA may regulate RBC alloimmunization (9). Environmental differences between recipients also likely affects alloimmunization, as genetically identical animals still have variable alloantibody responses to transfused RBCs (10, 11). One such environmental variable may be the inflammatory status of the recipient, which has been shown to have a substantial effect upon alloimmunization to transfused RBCs in mice (10, 12, 13), and potentially in humans(14) . In the current report, we hypothesize that an additional potential factor is small peptide homology between microbial-derived peptides and blood group antigens.

It has long been appreciated that alloimmunity can be induced through exposure to microbial antigens that mimic the three-dimensional structure and epitopes of alloantigens (molecular mimicry); thus, antibodies generated against the microbial antigen can cross-react with alloantigens. This is a well documented event with anti-ABO antibodies, where humans make antibodies against "non-self" ABO antigens without any prior exposure through transfusion, due to immunization with gut microbes that express A and/or B antigens(15). In the context of IgG responses to protein alloantigens, limited crossreactivity of anti-RBC antibodies and microbes has been observed for K and Jk^b antigens (16, 17). However, the possibility of significant molecular mimicry inducing antibodies to non-ABO RBC antigens has been largely rejected, because alloantibodies against other alloantigens (e.g. RhD, RhCc, RhEe, Kell, Duffy, Kidd, etc.) are rarely if ever detected in the absence of prior exposure to alloantigen through transfusion or pregnancy (2, 18).

A second potential mechanism of molecular mimicry, which has not been thoroughly evaluated in the context of humoral alloimmunization, is similarity at the level of $CD4^+$ T cell epitopes in the absence of mimicry of the three-dimensional blood group antigen recognized by antibodies. In this case, mimicry would be restricted to homology of short peptide sequences presented by MHC II. Herein, we report a series of microbial peptides with substantial similarity or identity to peptides containing the polymorphisms responsible for three pairs of clinically significant antithetical human RBC alloantigens (K/k, Fy^a/Fy^b, and Jk^a/Jk^b). Based upon these findings, we hypothesize that $CD4^+$ T cell responses to some microbes cross-react with $CD4^+$ T cell epitopes of RBC alloantigens. Alloantibody binding to blood group alloantigens

typically requires precise three dimensional structure of the antigen. Because the identified $CD4^+$ T cell epitopes only represent small linear peptides surrounded by non-homologous amino acids, it is predicted that exposure to the microbes would result in isolated activation of $CD4^+$ T cells but not an induction of an alloantibody. The potential significance of this is that if the authentic RBC alloantigen were encountered during transfusion, the patient would have pre-formed $CD4^+$ helper T cell immunity, which could then provide the required help to allow B cells to make alloantibodies. As generation of $CD4^+$ helper T cells is often a key regulatory event in humoral immunization, which may not occur in response to the weak stimulus of RBCs alone, microbial mimicry at the $CD4^+$ T cell level represents an additional hypothesis to explain why some but not other transfusion recipients make an anti-RBC antibody

Current clinical methodologies in transfusion biology are limited to serological analysis, and do not evaluate $CD4^+$ T cell immunity. Therefore, evaluating this hypothesis in humans with existing clinical data is not technically feasible. To allow a rigorous testing of the hypothesis in an ethical fashion, we engineered a murine model by generating a novel recombinant virus with insertion of a known CD4+ T cell epitope ($OVA_{323-339}$) in frame with a viral protein. We also utilized a transgenic mouse expressing an RBC specific antigen containing a distinct B cell epitope fused in frame with $OVA_{323-339}$ (19). This model recapitulates the observed scenario in humans, by providing a microbe with homology limited to a small peptide, and not the entire blood group antigen. Utilizing this system, we report that an antecedent microbial infection substantially enhances the alloantibody response upon subsequent exposure to the whole

antigen on a transfused RBC, yet does not in of itself induce detectable antibodies in the absence of exposure to the relevant RBC antigen.

Materials and Methods

Mice

C57BL/6, B10.BR, and C57BL/6.PL-Thy1.1 mice were purchased from Jackson Laboratories (Bar Harbor, ME). HOD, FVB, OT-II, OT-II Thy1.1, mHEL, and C57BL/6 x B10.BR mice were bred by the Emory University Department of Animal Resources. Mice were maintained on standard rodent chow and water in a temperature- and light-controlled environment and used at 8-12 weeks of age. All experiments were performed according to approved Institutional Animal Care and Use Committee (IACUC) procedures.

Viruses

These studies utilized two viruses: wild-type polyomavirus (PyV.WT) and polyomavirus expressing $OVA_{323-339}$ (PyV.OVA-II), a model $CD4^+$ T cell ovalbumin epitope. PyV.OVA-II was generated by inserting the I-A^b-restricted $OVA_{323-339}$ (ISQAVHAAHAEINAGR) epitope from chicken ovalbumin at a unique Blp I site in the genome of mouse polyomavirus (PyV) strain A2 in frame with middle T-antigen. A fragment containing sequence for $OVA_{323-339}$ was generated by PCR using high-fidelity *Taq* polymerase (Invitrogen, Carlsbad,CA) with an overhanging forward primer encoding $OVA_{323-339}$ and the Blp I recognition sequence (F: 5'-GTGTTGCTGAGCATCTCACAAGCTGTTCATGCAGCACACGCGGAAACAACGA AGCGGGAAGA AGCCCGATGACACGATATCC-3', BlpI site underlined) and a reverse primer that included an EcoRI recognition site (R: 5'-TCAGAATTCGGGCCTGAACTTCC-3', EcoRI site underlined). Genomic DNA from

mouse polyomavirus (PyV) strain A2 was digested by BamHI and EcoRI to yield a small fragment and a large fragment, which were subcloned into pUC19. The PCR product encoding $OVA_{323-339}$ was digested with BlpI and EcoRI then cloned into the BlpI/EcoRI region of small fragment in place of the original sequence. Insertion of $OVA_{323-339}$ was confirmed by DNA sequencing. The large fragment and the recombinant small fragment were excised from pUC19 and ligated to form full-length recombinant PyV.OVA-II DNA. PyV.OVA-II DNA was transfected into primary baby mouse kidney (BMK) cells by Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Virus stocks were prepared on BMK cells and titered by plaque assay using BALB/3T3 clone A31 cells (ATCC, Manassas, VA), as previously described (20). Full length PyV.OVA-II DNA was sequenced to confirm retention of the $OVA_{323-339}$ insert.

Treatment of mice and transfusion of blood

C57BL/6 or C57BL/6 x B10.BR recipient mice was infected with $2x10^5$ pfu of PyV.WT or PyV.OVA-II via footpad injection. Two weeks post-infection, recipients were transfused with 70 µL packed mHEL, FVB, or HOD RBCs, as previously described (10). Given the ubiquitous expression of mHEL on all cells, mHEL RBCs were leukoreduced with Pall neonatal leukoreduction filters prior to transfusion as previously described (10). Serum was obtained pre-infection, pre-transfusion, and 7 and 14 days post-transfusion. Intracellular cytokine staining and quantitative real time PCR (qRT-PCR) were performed 7-10 weeks after infection.

Blast homology search with Kell, Kidd, and Duffy antigens.

On October 5th, 2009, a blast search was carried out using peptides containing the polymorphisms that constitute the antithetical RBC antigens K/k, Fy^a/Fy^b, and Jk^a/Jk^b. 20 amino acid inputs were entered into the BLAST tblastn database and the genome search was restricted to microbial sequences. To focus on short nearly exact matches, the following search parameters were used: word size=2, expect threshold=30,000, Matrix=PAM30, Gap Costs= Existance:9 Extension 1, low complexity filter was turned off, and compositional adjustments was set to "no adjustment".

ELISA for anti-HEL and anti-OVA humoral response

Enzyme-linked immunosorbent assays (ELISAs) for anti-HEL IgG and anti-OVA IgG were performed on sera run in triplicate at dilutions from 1:50 to 1:500,000 as previously described (10). Fold difference in antibody production was determined in the linear range of optical density.

Flow cytometric crossmatching

Sera from experimental mice were diluted 1:10 and incubated with HOD, mHEL or FVB RBCs in fluorescence activated cell sorter (FACS) buffer (PBS + 0.2mg/mL bovine serum albumin [Sigma, St. Louis, MO] + 0.9mg/mL EDTA [Sigma] + 2% fetal bovine serum [Hyclone, Logan, Utah]). Goat anti-mouse immunoglobulins conjugated to allophycocyanin (Becton-Dickinson) were used as a secondary antibody.

Intracellular cytokine staining and flow cytometry

Spleens were digested with collagenase (Sigma) and resuspended in complete media (RPMI 1640 + 1% L-glutamine + 1% penicillin/streptomycin + 1% 1M HEPES + 1%

sodium pyruvate + 1% non-essential amino acids all from Cellgro [Herndon, VA] + 2-beta mercaptoethanol [EM Science, Darmstadt, Germany] + 10% fetal bovine serum [Hyclone]). Splenocytes were restimulated *in vitro* using the BD Cytofix/Cytoperm plus Fixation/Permeabilization kit with BD Golgiplug (BD Pharmingen, San Diego, CA) as per manufacturer's instructions. Briefly, $2x10^6$ splenocytes were restimulated with 1uM $LT_{359-368}$, the dominant D^b-restricted $CD8^+$ T cell epitope for polyomavirus, in the presence of GolgiPlug. After 5 hours of stimulation, cells were stained with a directly conjugated antibody against surface CD8 (eBiosciences, Clone 53-6.7, San Diego, CA). Cells were then permeabilized and fixed. Cells were treated with Fc block (BD Pharmingen 2.4G2, San Diego, CA) and were stained intracellularly with a directly conjugated antibody against IFN□ (BD Pharmingen, Clone XMG1.2)). All staining was performed in FACS buffer. Samples were acquired using a six color BD FACS CANTO flow cytometer.

Quantitative real time PCR for viral genome copy level

DNA was extracted from sections of spleen by using QIAamp DNA mini kit [Qiagen, Valencia, CA]. Quantitative real time PCR (qRT-PCR) was set up as previously described (21). The TaqMan probe used was 5'-AGA CGA AAT CCT TGT GTT GCT GAG C-3'. The forward primer was 5'-CGC ACA TAC TGC TGG AAG AAG A-3', and the reverse primer was 5'-TCT TGG TCG CTT TCT GGA TAC AG-3'. A standard curve of known polyomavirus genome copy numbers was used to determine the threshold cycle of detection values for experimental samples, which were used to calculate genome

copies per milligram of splenic tissue. The detection limit of this assay is 10 copies of viral genomic DNA.

Adoptive transfer of cells

OTII spleens were digested in collagenase and resuspended in complete RPMI. Cells were washed twice, counted and resuspended at $20x10^6$ splenocytes/mL in PBS. C57BL/6.PL-Thy1.1 recipients were injected via lateral tail vein with $10x10^6$ total splenocytes. For studies characterizing viral responses, C57BL/6 recipient mice were injected via lateral tail vein with 1×10^5 OT-II splenocytes.

Statistical Analysis

Statistical significance was determined by using PRISM software and performing a Student's T-test for comparison of 2 samples or two-way repeated measure analysis of variance (ANOVA) with a Bonferroni post-test for 3 or more samples with multiple conditions. The measure of statistical significance was set at $p<0.05$.

Results

Homology of microbial peptides and polymorphisms that define human blood group antigens

To investigate potential homology at the peptide level between existing microbial sequence data and clinically relevant human blood group antigens, the BLAST database was searched using peptides from blood group antigens that contain allelic polymorphisms known to give rise to antithetical antigens. Peptides from Kell (K/k), Duffy (Fy^a/Fy^b), and Kidd (Jk^a/Jk^b) antigens were analyzed (Table 1). An eight amino acid peptide was identified from *Haemophilis Influenzae* with 100% identity to the Cellano (k) antigen. Likewise, a 9 amino acid peptide was found in *Bacteroides Fragilis* that had 100% identity to a rare form of Kell (K) and varied from Cellano only in the amino acid constituting the K/k polymorphism. Finally, an 8 amino acid peptide from *Vibrio species* with 100% identity to Cellano (k) was observed. For Duffy, a common 8 amino acid identity to Fy^b was found in *Vibrio Cholerae*, *Yersinia Pestis*, and *Salmonella enterica*. An additional 9 amino acid sequence was found that varied from Fy^a by 1 amino acid. Less significant homology was observed for Kidd antigens, but homologous sequences were observed from *Bordetella parapertussis*, and species of *Bacteroides* and *Clostridium*. For each of the observed sequences, identity/homology was restricted to 8-9 amino acids, and no significant homology was noted on flanking amino acids on either N or C ends.

Antecedent viral infection enhances initial humoral alloimmunization to subsequent RBC transfusion

The homology data presented above suggest that in at least some humans (depending upon HLA), certain microbial peptides may mimic $CD4^+$ T cell epitopes from blood group antigens, without themselves constituting the antibody binding epitope of humoral blood group antigens. The significance of such homology in small peptide epitopes depends upon the capacity of variant peptides on RBC antigens to display linked recognition characteristics with associated humoral alloantigens. We assessed this capacity by engineering a murine model system to test the hypothesis that a microbe expressing a $CD4^+$ T cell epitope from a blood group antigen can prime a recipient mouse for a strong humoral response to a subsequent RBC transfusion. Based upon this hypothesis, two outcomes can be predicted: 1) the microbial infection itself will not result in an immunization event that is detectable by serology (i.e. no anti-RBC antigen antibodies), and 2) the microbial infection will convert an animal that would have otherwise had no response or a very weak response to a given RBC antigen into a strong responder.

The experimental design to test this hypothesis consisted of infection of recipient mice with either polyomavirus encoding $OVA_{323-339}$ (PyV.OVA-II) or control strain wild-type polyomavirus (PyV.WT). Two weeks post-infection, recipient mice were then transfused with RBCs from transgenic HOD or control FVB donors (Figure 2.1A). The HOD mouse expresses a model antigen consisting of a well characterized B cell epitope, hen egg lysozyme (HEL), fused in frame with a portion of ovalbumin containing the $OVA_{323-339}$ T cell epitope, and anchored to the RBC membrane by a human blood group antigen (Duffy) (Figure 2.1B). Thus, the humoral HEL epitope is linked to $OVA_{323-339}$ on

the same molecule; HOD is expressed selectively on RBCs and is not detectable on leukocytes or platelets (19).

The model reagents used in the above experimental design were tested to determine if the $OVA_{323-339}$ peptide was processed and presented on MHC II. $CD4^+$ T cells were harvested from mice that express a TCR transgene specific for $OVA_{323-339}$ presented by $I\text{-}A^b$ (OT-II mice) (22, 23). Splenocytes from OT-II mice on a Thy1.1 background were adoptively transferred into C57BL/6 mice followed by PyV.WT or PyV.OVA-II infection. After 8 days, splenocytes were harvested and stained for CD4 and Thy1.1. OT-II cells expanded 10 fold in mice infected with PyV.OVA-II compared to mice infected with PyV.WT (Figure 2.1C). Similarly, to test if HOD protein on RBCs can be processed such that $OVA_{323-339}$ is presented on MHC II, OT-II cells were adoptively transferred into C57BL/6.PL-Thy1.1 mice followed by a HOD RBC or control FVB RBC transfusion 24 hours later. After 4 days, splenocytes were harvested and stained for CD4 and Thy1.2. OT-II cells expanded 10 fold in mice that received a HOD RBC transfusion compared to mice that received OT-II cells alone. OT-II expansion was specific to peptides presented from the HOD antigen as there was a lack of expansion in mice that received FVB RBC transfusion (Figure 2.1D). Thus, the OVA323-339 peptide from both the HOD antigen and the PyV.OVA-II virus can be efficiently processed and presented on MHC II of APCs.

The experimental design shown in figure 2.1A was carried out using the characterized reagents. In 3 of 3 experiments (5 mice/group/experiment), mice infected with PyV.OVA-II prior to HOD RBC transfusion had a dramatic increase in anti-HEL

IgG compared to control mice that received PyV.WT prior to HOD RBCs or HOD RBCs alone ($p<0.05$ at 1:50 dilution) (Figure 2.2A). Titration of sera from 1:50-1:50,000 and extrapolation on the linear part of the curve indicated a 1000 fold higher level of anti-HEL IgG in mice infected with PyV.OVA-II prior to HOD RBC transfusion compared to mice receiving HOD RBCs alone. No anti-OVA IgG was detected in any group, indicating that HEL is a highly immunodominant antibody epitope for the HOD antigen (Supplemental Figure 2.1). Consistent with our prior observation that inflammation in and of itself can enhance RBC alloimmunization (10), infection with PyV.WT resulted in a small and reproducible, but statistically insignificant, degree of enhancement (Figure 2.2A). Taking into account this inflammatory effect, PyV.OVA-II still had a 100 fold greater enhancement of anti-HEL IgG than infection with PyV.WT. To assess the ability of the antibody response to bind native HOD antigen on the RBC surface, flow cytometric crossmatch was performed using indirect immunofluorescence. Antibodies that bound HOD RBCs were detected in sera from mice infected with PyV.OVA-II prior to HOD RBC transfusion (Figure 2.2B). This binding was specific to the HOD antigen, as no signal was observed with RBCs from wild-type FVB donors. Likewise, no anti-HOD RBC antibodies were detected by flow cytometric crossmatch in mice that were uninfected or infected with PyV.WT prior to HOD RBC transfusion. Lack of detectable antibody in these latter two conditions is consistent with our previous report that the ELISA is substantially more sensitive than flow crossmatch (10).

To test the possibility that PyV.OVA-II infection induced anti-HEL IgG on its own (and transfusion with HOD RBCs is just boosting a secondary antibody response), specimens collected post-infection but prior to transfusion were analyzed; no anti-HEL

antibodies were detected in any group (Supplemental Figure 2.2). Mice infected with the virus but not transfused were tested as late as 18 weeks following infection and no anti-HEL IgG was detected (data not shown). Together, these data demonstrate that a combination of infection with PyV.OVA-II and subsequent transfusion with HOD RBCs is required for the observed enhancement of anti-HEL IgG.

Analysis of immune responses to PyV.WT and PyV.OVA-II

Based upon the above data, we hypothesized that PyV.OVA-II induced a $CD4^+$ T cell response, which was then able to provide T cell help to B cells that encountered the HOD antigen after RBC transfusion. However, it was also possible that the addition of $OVA_{323-339}$ to PyV.OVA-II created a more virulent strain that could serve as a stronger adjuvant to any antigen encountered. Alternatively, it was possible that failure of Py.WT to enhance anti-HEL IgG was due to lack of infectivity. To assess these possibilities, viral infection and cellular immune response was assessed.

Quantitative RT-PCR was performed to enumerate polyomavirus genome copy number. DNA was amplified using polyoma-specific primers and probes that recognized both PyV.WT and PyV.OVA-II. The threshold cycle (Ct) values were used to calculate the genome copy number per milligram of spleen. In 3 of 3 experiments (n=3-5 mice/group/experiment), there was no statistically significant difference between polyoma genome copy number in recipient mice of PyV.WT or PyV.OVA-II infection (Figure 2.3). RT-PCR was specific for viral infection, as mice that received HOD RBCs alone (uninfected) had no detectable polyoma DNA (data not shown). These data

indicate that the decreased enhancement of HOD alloimmunization by PyV.WT infection was not due to a lack of infectivity.

The similar viral genome copy number suggested a similar immunological clearance of either strain of virus. However, to quantify the extent of anti-viral immunity, intracellular cytokine staining was performed. Splenocytes were restimulated *in vitro* with the immunodominant D^b-restricted PyV $CD8^+$ T cell epitope ($LT_{359-368}$), and then surface stained for CD8 and intracellularly stained for IFN□□□ Infection with either strain resulted in an increase in $CD8^+$ T cells that expressed IFN□ compared to background levels in uninfected mice (Figure 2.4A). However, in 2 of 3 experiments, there was a significant increase in the percentage of viral specific $CD8^+$ T cells in PyV.OVA-II infected mice as compared to PyV.WT infected mice (Figure 2.4B; representative experiment). Together, these data indicate that both PyV strains were infectious and induced a detectable $CD8^+$ T cell anti-viral response, excluding the possibility that enhancement of anti-HEL IgG by PyV.OVA-II and not PyV.WT was due to a lack of viral infectivity by PyV.WT.

Enhancement of alloimmunization to HOD transfusion by PyV.OVA-II requires linkage of $OVA_{323-339}$ to HEL

We hypothesized that PyV.OVA-II induced $CD4^+$ T cells specific for $OVA_{323-339}$ presented by MHC II, resulting in enhanced anti-HEL IgG responses to HOD RBC transfusion due to antigen specific linked recognition. However, the increased $CD8^+$ T cell response to infection with PyV.OVA-II compared to PyV.WT (see Figure 2.4) raised the possibility that increased anti-HEL IgG in response to HOD following PyV.OVA-II

was simply an adjuvant effect. To directly test the requirements for linked recognition, a cohort of mice were transfused with mHEL RBCs instead of HOD RBCs. mHEL RBCs express the same HEL epitope on their surface fused to a transmembrane domain, but lack fusion to OVA, and thus are not linked to the $OVA_{323-339}$ peptide (see Figure 2.1B) (24). In contrast to what was observed with HOD RBCs, in 2 of 2 experiments (5 mice/group/experiment, utilizing both C57BL/6 as well as C57BL/6 x B10.BR recipients), no enhancement of anti-HEL IgG in response to mHEL RBC transfusion was observed in mice previously infected with PyV.OVA-II, compared to uninfected or PyV.WT infected controls at 7 days (Figure 2.5) or 14 days (data not shown) after transfusion. In aggregate, these findings demonstrate that anti-HEL IgG responses to HEL on transfused RBCs is not enhanced by antecedent infection with PyV.OVA-II when $OVA_{323-339}$ is not linked to the HEL.

Discussion

Herein, we demonstrate homology between infectious microbes such as *Haemophilis Influenzae, Yersinia Pestis* and *Bordetella parapertussis* and the RBC antigens Kell, Duffy, and Kidd, respectively. Although the homology was generally restricted to 8-9 amino acid stretches, the MHC II molecule has open ends to its presentation pocket (both in humans and mice), which allows substantial promiscuity in anchor residues. Peptides can be of variable lengths (often 13 amino acids) and obligate residues adjacent to the peptide recognized by the T cell receptor are less constrained than MHC I and more difficult to predict (25). Thus, whether adjacent amino acids allow the peptides to be presented on MHC II is difficult to surmise based upon known MHC II biology, and likely varies depending upon the particular HLA type of a given individual (26). However, it is likely that at least certain HLA types will be capable of presenting microbial peptides containing the observed homologous sequences.

We hypothesize that exposure to these infectious agents results in activation of CD4+ T cells, which would not be detectable by current serology based blood banking methodologies. Subsequent transfusion with RBCs containing a peptide sequence shared with the microbe then leads to RBC alloimmunization, with help from the preformed CD4+ T cells. Thus, molecular mimicry at the CD4+ T cell level may be a previously unappreciated factor influencing rates of RBC alloimmunization.

To formally test this hypothesis in a model system, we recapitulated in mice the scenario hypothesized to occur in humans. Using a virus containing a $CD4^+$ T cell epitope shared with a small peptide on the donor RBCs, we have shown that infection

with PyV.OVA-II enhances alloimmunization to HOD RBCs (containing linked HEL and OVA peptides). This enhancement requires that both the virus and the RBC antigen share a $CD4^+$ T cell epitope; the effect is lost if the epitope is absent from the virus (PyV.WT infection followed by HOD transfusion) or if the epitope is absent from the RBC antigen (PyV.OVA-II infection followed by mHEL transfusion).

It is worth noting that the baseline responses to mHEL RBCs are substantially lower than to HOD RBCs (see Figure 2.5). There are several possible reasons, including decreased levels of mHEL transgene expression compared to HOD (data not shown) and lack of additional $CD4^+$ T cell epitopes provided by the fusion partners in HOD. However, peptide(s) from HEL are known to be efficiently presented by MHCII encoded by the $H\text{-}2^k$ haplotype. Accordingly, these experiments were carried out in C57BL/6 x B10.BR recipients ($H\text{-}2^b$ x $H\text{-}2^k$) as well as C57BL/6 recipients ($H\text{-}2^b$), with similar results. We have previously reported that anti-HEL responses to mHEL transfusion can be significantly enhanced by inflammation (with poly (I:C)) (10) and are also markedly enhanced by increasing $CD4^+$ T cells specific for a peptide contained in HEL itself (12). Thus, lack of increased anti-HEL in response to mHEL was not due to an inability to be enhanced by either inflammation or increased $CD4^+$ helper T cells.

Our interpretation of these data is that following HOD transfusion, anti-HEL B cells selectively recognize and, through receptor-mediated endocytosis, internalize the HOD antigen (whole or partial RBC) via their HEL specific B cell receptor. Because the HEL antigen is linked to OVA, both antigens are endocytosed, broken down into peptides, and presented in the MHC II of B cells as well as professional antigen

presenting cells (i.e. dendritic cells). We hypothesize that $OVA_{323-339}$ specific $CD4^+$ T cells that were previously generated in response to the PyV.OVA-II infection persist, recognizing OVA peptide presented by MHC II on anti-HEL B cells that have processed HOD antigen. Through this interaction, anti-HEL B cells are activated and differentiate into antibody secreting plasma cells with the help of viral induced $OVA_{323-339}$ specific $CD4^+$ T cells (Figure 2.6).

Additional considerations include the possibility that PyV.OVA-II had a B cell epitope that mimicked HEL, such that PyV.OVA-II infection induced a primary anti-HEL antibody response and the enhancement of immunization with HOD RBC transfusion was actually due to boosting. However, we reject this hypothesis since no anti-HEL antibody was detected after PyV.OVA-II infection in the absence of transfusion (up to 18 weeks after inoculum). Moreover, there was no increase of anti-HEL in response to mHEL RBC transfusion, demonstrating that introducing the HEL B cell epitope(s) was not sufficient for augmentation by PyV.OVA-II. Finally, we rule out that the failure of the PyV.WT to enhance anti-HEL was an artifact due to the viral stock not being infectious, as a strong $CD8^+$ T cell antiviral response was observed and viral genome copy number was consistent with an active infection.

As the current genomic databases for microbes are far from complete, discovery of additional homology with ongoing microbial sequence analysis is possible. Moreover, the likelihood of peptide mimicry is increased by the recent appreciation of additional polymorphisms in alloantigens outside of those traditionally detected by patient serology (27). However, crossreactivity would not be alone sufficient, as the peptide in question would have to fit into the particular MHC encoded by a given recipient's HLA. Although

monitoring of $CD4^+$ T cell based immunity is not currently available in the routine clinical setting, it may be possible to predict a response to a future alloantigen based on prior pathogen exposure or gut flora. Further, identification of environmental antigens that cross-react with alloantigen $CD4^+$ T cell epitopes may lead to the development of specific diagnostic MHC II tetramers capable of detecting $CD4^+$ T cell based immunization to relevant peptides. Together, these advancements would help identify patients at high risk for humoral alloimmunization and provide peptide based therapeutic interventions.

In summary, the current findings provide additional insight into factors that may regulate RBC alloimmunization. A patient may be primed to respond to an RBC antigen through prior exposure to a microbial infection with a shared CD4+ T cell epitope, yet this initial priming would go undetected by current serology based blood banking methodologies. While the described studies utilize an RBC transfusion model, the principle demonstrated could extend to platelet and HLA antigens, with potential relevancy to both solid organ and bone marrow transplantation. Future comprehensive genomic and proteomic studies, in the context of our growing database of known sequences, will be useful to investigate potential homology between microbial peptides and RBC, WBC, and platelet antigens.

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Figure Legends

Table 2.1: Peptide homology of microbes and polymorphisms that define human blood group antigens. Peptide sequences from Kell, Duffy and Kidd antigens were entered into the BLAST database (see materials and methods). Peptide identity and/or homology were detected for the indicated microbes. Red letters denote the predominant amino acids while blue indicate polymorphisms. Green letters highlight amino acid variants between the blood group antigen and microbial amino acid sequences.

Figure 2.1: Experimental design and presentation of $OVA_{323-339}$ peptide after exposure to either PyV.OVA-II or HOD RBCs. Recipient mice were infected with wild type polyomavirus (PyV.WT) or polyomavirus expressing a known T-cell epitope (ovalbumin $(OVA)_{323-339}$), (PyV.OVA-II). Two weeks post-infection, recipient mice were transfused with RBCs from control FVB donors (expressing no HEL epitope), mHEL donors (expressing HEL on RBCs), or HOD donors (expressing RBC specific HEL fused to OVA). (**A**) Diagram of experimental design: Sera was collected prior to transfusion and at 7 and 14 days post-transfusion. (**B**) Diagram of B and T cell epitopes expressed in PyV.WT and PyV.OVA-II, as well as in FVB RBCs, mHEL RBCs, and HOD RBCs. Presence of the $OVA_{323-339}$ epitope is indicated by the green line. **(C)** OT-II Thy1.1 splenocytes were adoptively transferred into C56BL/6 mice followed by infection with either PyV.WT (C, left) or PyV.OVA-II (C, right). Eight days post infection, splenocytes were harvested and stained for CD4 and Thy1.1. **(D)** OT-II splenocytes were adoptively transferred into C56BL/6.PL-Thy1.1 mice followed 24 hours later by transfusion with either HOD RBC or FVB RBC. Four days post transfusion, splenocytes were harvested

and stained for CD4 and Thy1.2. Groups included mice receiving OT-II cells alone (D, left), OT-II cells and HOD transfusion (D, right), or OT-II cells and FVB transfusion (D, bottom).

Figure 2.2: Prior infection with PyV.OVA-II significantly enhances alloimmunization to a subsequent HOD RBC transfusion. C57BL/6 mice were infected with wild type polyomavirus (PyV.WT) or polyomavirus expressing $OVA_{323-339}$ (PyV.OVA-II). Additional control mice were uninfected. All groups received subsequent transfusion with HOD RBCs. Alloimmunization was assessed 2 weeks later by anti-HEL ELISA. (**A**) A representative experiment with 5 mice/group is shown, with mean ± S.E.M. shown at sera dilutions of 1:50 to 1:50,000. (**B**) Enhancement was also evident by flow cytometric crossmatching with FVB or HOD RBC targets. These experiments have been reproduced 3 times (5 mice/group/experiment), with similar results.

Supplemental Figure 2.1: Alloantibody production is not directed against OVA. C57BL/6 mice were infected with wild type polyomavirus (PyV.WT), polyomavirus expressing $OVA_{323-339}$ (PyV.OVAII) or uninfected. All groups were then transfused with HOD RBCs. Sera was analyzed for anti-OVA IgG at 7 days post transfusion. A representative experiment with 5 mice/group is shown. Sera from mice immunized with OVA emulsified with complete Freund ’s adjuvant was used as a positive control for anti-OVA detection (data not shown).

Supplemental Figure 2.2: PyV.WT or PyV.OVA-II infection alone does not induce anti-HEL IgG. C57BL/6 mice were either infected with wild type polyomavirus (PyV.WT) or polyomavirus expressing $OVA_{323-339}$ (PyV.OVA-II). Additional control mice were uninfected. Infection alone did not lead to detectable anti-HEL IgG by ELISA. Representative ELISAs with sera at 1:50 dilution are shown (5 mice/group); this experiment has been reproduced 3 times (5 mice/group/experiment), with similar results.

Figure 2.3: Quantitative Real Time PCR enumeration of viral genome copy number. DNA was extracted from splenic tissue taken 7-10 weeks post infection from recipient mice infected with wild type polyomavirus (PyV.WT) or polyomavirus expressing $OVA_{323-339}$ (PyV.OVA-II) and then transfused with HOD RBCs. Quantitative RT-PCR was used to determine the polyomavirus genome copy number. No statistically significant difference in genome copy number between the 2 groups was seen. A compilation of data from 3 individual experiments (3-5 mice/group/experiment) is shown; uninfected mice had an undetectable viral genome copy number (data not shown).

Figure 2.4: Enumeration of $CD8^+$ T cell anti-viral responses to both PyV.WT and PyV.OVA-II. Intracellular cytokine staining was performed on splenocytes 7-10 weeks post infection with either wild type PyV.WT or PyV.OVA-II; uninfected controls were also analyzed. Splenocytes were re-stimulated with and without a dominant MHC class I restricted PyV peptide ($LT_{359-368}$) and stained with anti-IFN□. Representative flow plots are shown (**A**), and compiled data is presented from a representative experiment (**B**). This experiment has been repeated 3 times (3-5 mice/group/experiment). In all cases, $CD8^+$ T cells from infected mice expressed IFN□ upon peptide stimulation. In 2 of 3

experiments, a greater percentage of IFN producing $CD8^+$T cells were seen in PyV.OVA-II infected mice compared to PyV.WT infected mice; the data shown are from a representative experiment displaying this difference.

Figure 2.5: **Enhanced alloimmunization to HOD transfusion by PyV.OVA-II requires linkage of $OVA_{323-339}$ to HEL on the antigen.** C57BL/6 mice were infected with PyV.WT or PyV.OVA-II and transfused with mHEL or HOD RBCs (uninfected and/or untransfused control groups were also included). One week post transfusion, sera was analyzed for anti-HEL IgG. Similar results were observed at 14 days (data not shown). A representative experiment with 5 mice/group is shown (mean and standard deviation depicted, with sera at 1:50 dilution); this experiment has been repeated twice (utilizing C57BL/6 as well as C57BL/6 x B10.BR recipients) with similar results.

Figure 2.6: Schematic of proposed enhanced alloimmunization. Response to infection. Peptides containing a polymorphism (designated by A) from a microbe will be processed and presented by host antigen presenting cells; peptides presented in MHCII will be recognized by $CD4^+$ T cells. However, in the absence of a B cell epitope, the B cells will not be able to receive $CD4^+$ T help to generate an antibody response. **Response to transfusion.** Upon a second antigenic exposure (transfusion) which contains the same polymorphism A, the polymorphism in the blood group constitutes not only a $CD4^+$ T cell epitope but also a *de novo* B cell epitope. Through receptor-mediated endocytosis, naïve B cells phagocytose the polymorphism-containing blood group molecule. The polymorphism is then presented on the MHCII of B cells to the preformed helper or memory $CD4^+$ T cells generated against the microbial infection. The B cells are then

stimulated to differentiate into plasma cells which secrete antibodies against the polymorphism.

Table 2.1

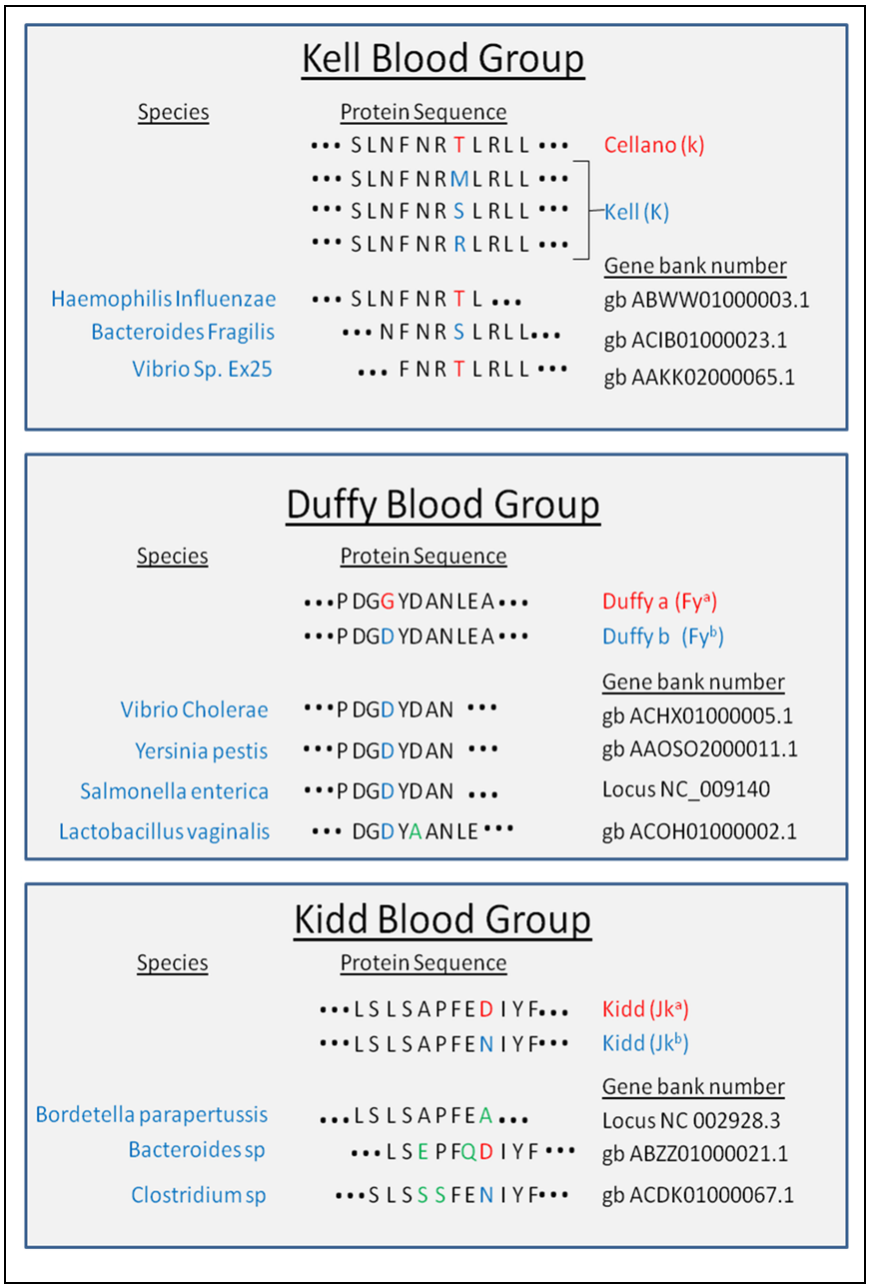
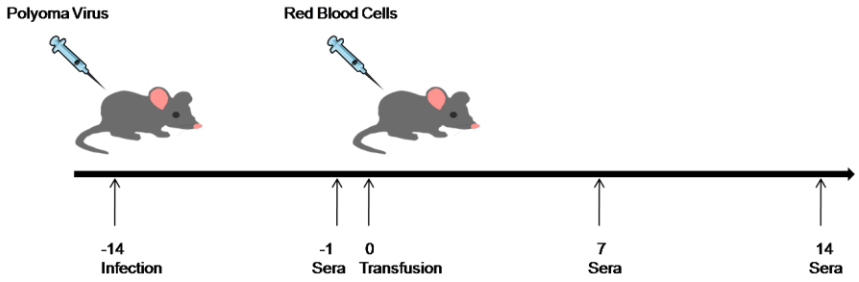
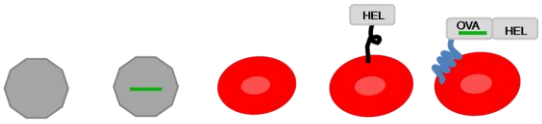

Kell Blood Group
Species
Protein Sequence
••• S L N F N R T L R L L •••
Cellano (k)
••• S L N F N R M L R L L •••
••• S L N F N R S L R L L •••
Kell (K)
••• S L N F N R R L R L L •••
Gene bank number
Haemophilis Influenzae
••• S L N F N R T L •••
gb ABWW01000003.1
Bacteroides Fragilis
••• N F N R S L R L L •••
gb ACIB01000023.1
Vibrio Sp. Ex25
••• F N R T L R L L •••
gb AAKK02000065.1
Duffy Blood Group
Species
Protein Sequence
•••P DGG YDANLEA •••
Duffy a (Fy[a])
•••P DGD YDANLEA •••
Duffy b (Fy[b])
Gene bank number
Vibrio Cholerae
•••P DGD YDAN •••
gb ACHX01000005.1
Yersinia pestis
•••P DGD YDAN •••
gb AAOSO2000011.1
Salmonella enterica
•••P DGD YDAN •••
Locus NC_009140
Lactobacillus vaginalis
••• DGD YA ANLE •••
gb ACOH01000002.1
Kidd Blood Group
Species
Protein Sequence
•••L S L S A P F E D I Y F•••
Kidd (Jk[a])
•••L S L S A P F E N I Y F•••
Kidd (Jk[b])
Gene bank number
Bordetella parapertussis
•••L S L S A P F E A •••
Locus NC 002928.3
Bacteroides sp
•••L S E P F Q D I Y F •••
gb ABZZ01000021.1
Clostridium sp
•••S L S S S F E N I Y F•••
gb ACDK01000067.1

Figure 2.1

A



B



Epitopes	PyV.WT	PyV.OVAII	FVB	mHEL	HOD
B cell epitope for HEL	-	-	-	+	+
T cell epitope for $OVA_{323-339}$	-	+	-	-	+
PyV T cell epitope	+	+	-	-	-

Figure 2.1

C

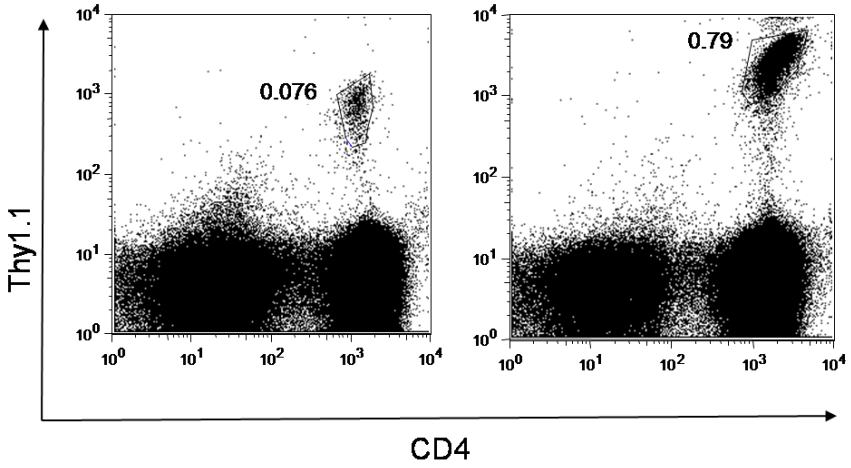


Figure 2.1

D

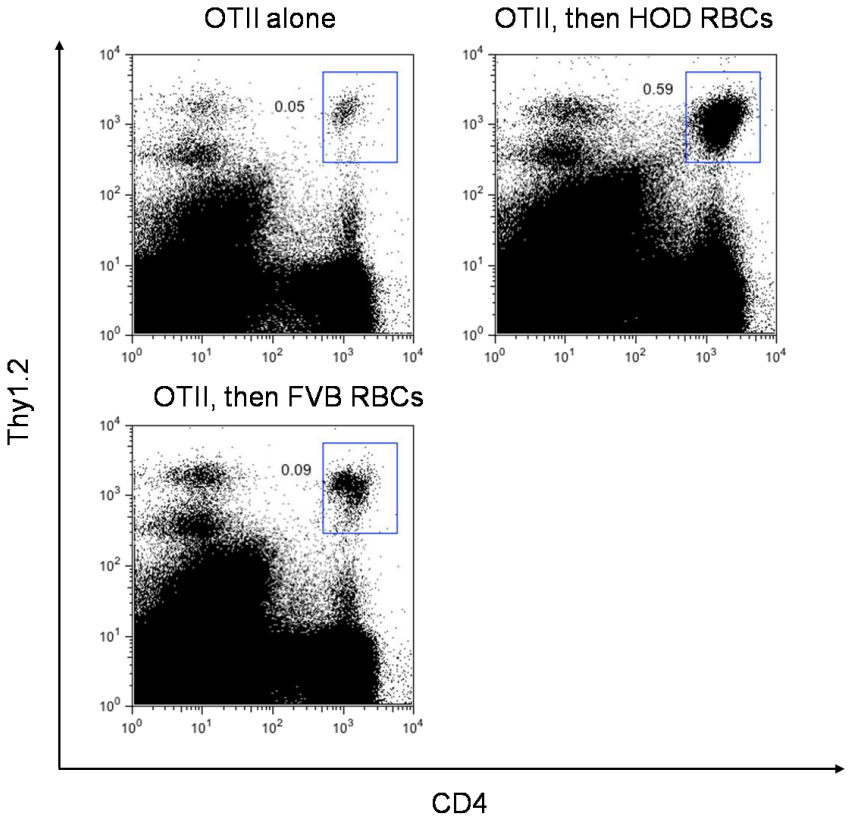
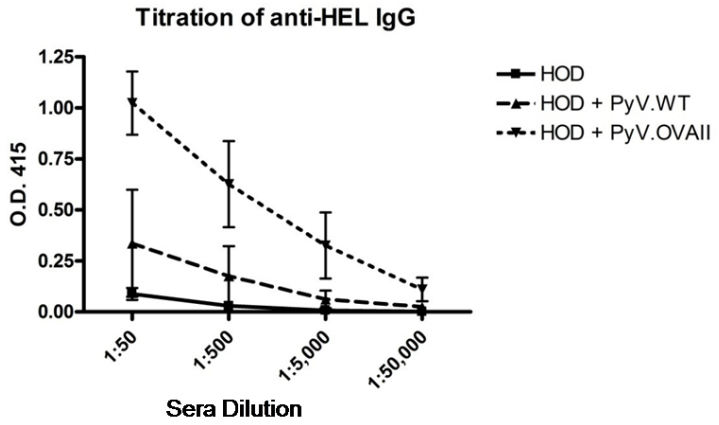
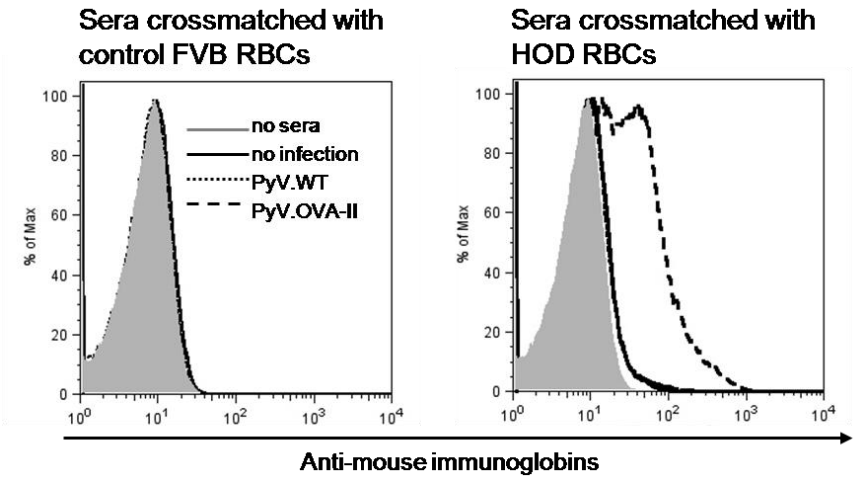

OTII alone
OTII, then HOD RBCs
OTII, then FVB RBCs
0.05
0.59
0.09
Thy1.2
CD4

Figure 2.2

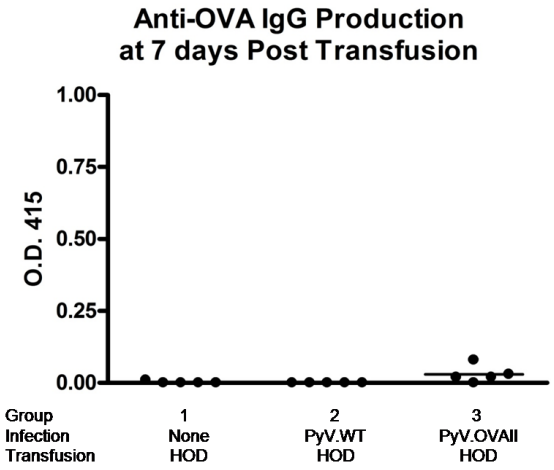
A


Titration of anti-HEL IgG
O.D. 415
1.25
1.00
0.75
0.50
0.25
0.00
1:50
1:500
1:5,000
1:50,000
Sera Dilution
HOD
HOD + PyV.WT
HOD + PyV.OVAII

B


Sera crossmatched with control FVB RBCs
Sera crossmatched with HOD RBCs
% of Max
no sera
no infection
PyV.WT
PyV.OVA-II
Anti-mouse immunoglobins

Supplemental Figure 2.1



Supplemental Figure 2.2

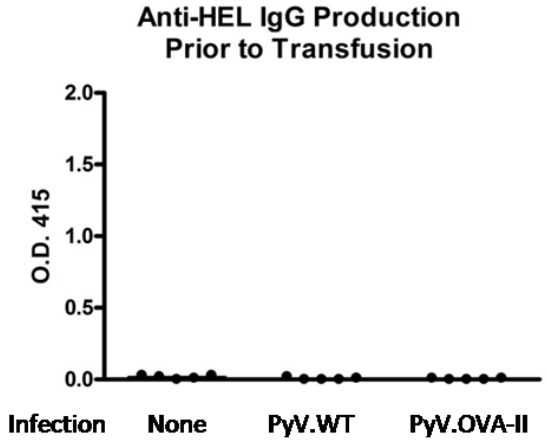


Figure 2.3

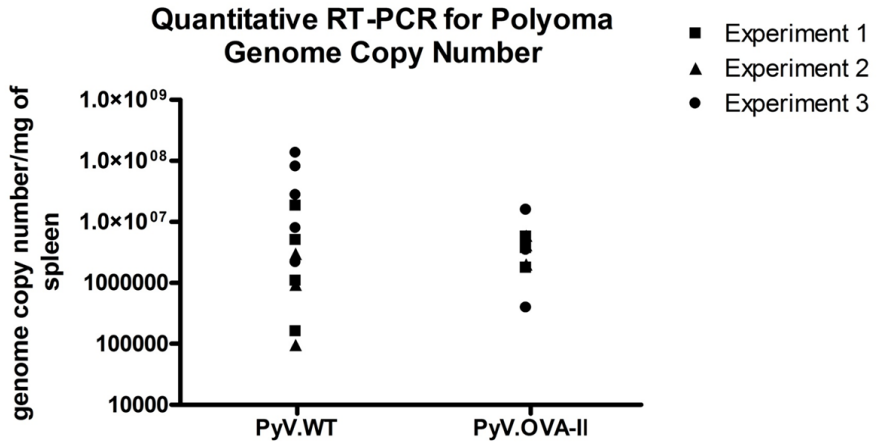
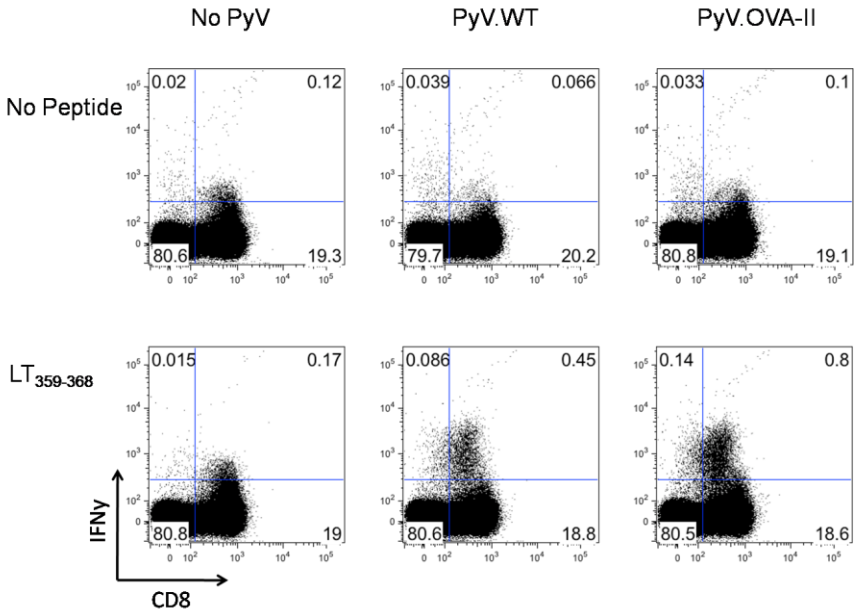

Quantitative RT-PCR for Polyoma Genome Copy Number
genome copy number/mg of spleen
1.0×10^09
1.0×10^08
1.0×10^07
1000000
100000
10000
PyV.WT
PyV.OVA-II
Experiment 1
Experiment 2
Experiment 3

Figure 2.4

A


No PyV
PyV.WT
PyV.OVA-II
No Peptide
0.02
0.12
80.6
19.3
0.039
0.066
79.7
20.2
0.033
0.1
80.8
19.1
LT359-368
0.015
0.17
80.8
19
0.086
0.45
80.6
18.8
0.14
0.8
80.5
18.6
IFNy
CD8

B

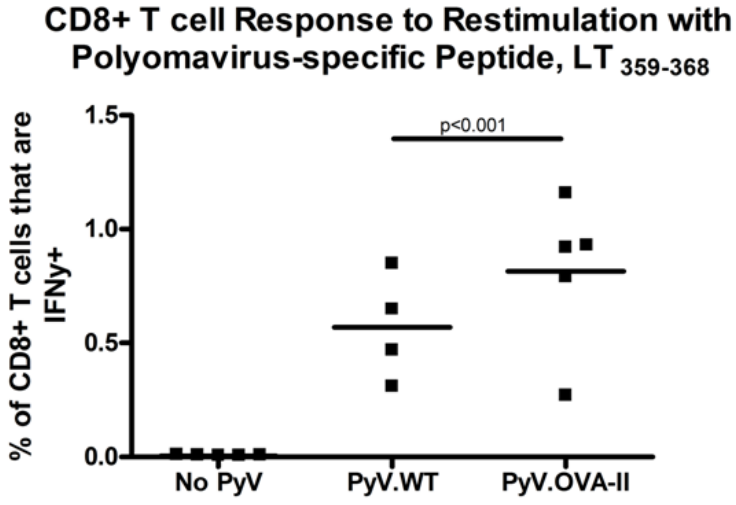

CD8+ T cell Response to Restimulation with Polyomavirus-specific Peptide, LT 359-368
% of CD8+ T cells that are IFNy+
1.5
1.0
0.5
0.0
p<0.001
No PyV
PyV.WT
PyV.OVA-II

Figure 2.5

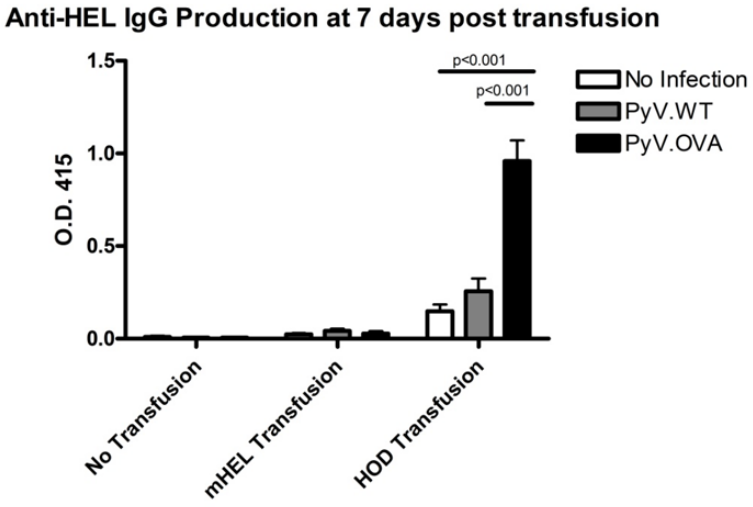
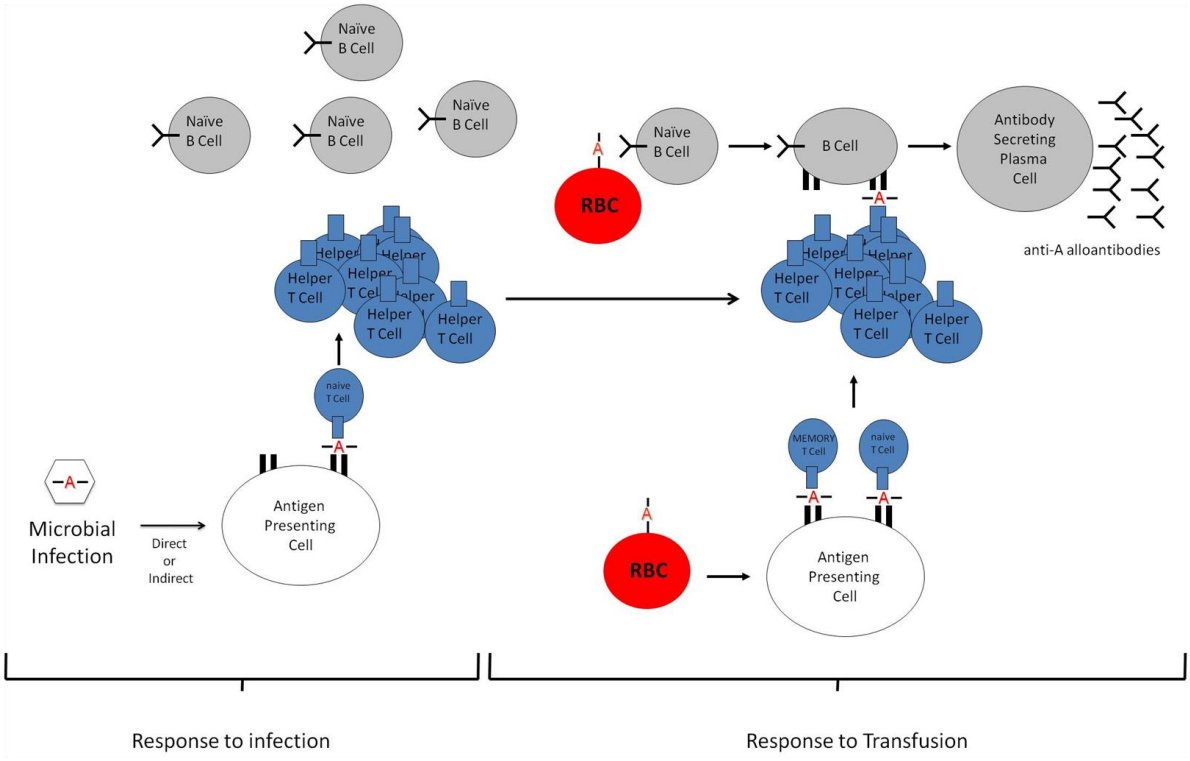

Anti-HEL IgG Production at 7 days post transfusion
O.D. 415
1.5
1.0
0.5
0.0
p<0.001
p<0.001
No Infection
PyV.WT
PyV.OVA
No Transfusion
mHEL Transfusion
HOD Transfusion

Figure 2.6


Naïve B Cell
Naïve B Cell
Naïve B Cell
Naïve B Cell
Helper T Cell
Helper T Cell
Helper T Cell
Helper T Cell
naive T Cell
A
Microbial Infection
Direct or Indirect
Antigen Presenting Cell
RBC
Naïve B Cell
B Cell
Antibody Secreting Plasma Cell
anti-A alloantibodies
MEMORY T Cell
naive T Cell
RBC
Antigen Presenting Cell
Response to infection
Response to Transfusion

Chapter 3

Patterns of Deletion of RBC Antigen Specific Autoreactive B cells in a Murine Model

Abstract

Autoimmune hemolytic anemia (AIHA) is a clinical disorder in which autoantibodies bind to self-RBC surface antigens and promote destruction. Why some individuals develop AIHA is unknown. To investigate baseline B cell tolerance, the loss of which leads to the production of erythrocyte-specific autoantibodies, we generated a novel non-hemolytic model of erythrocyte autoreactivity. We utilized the F1 of the HOD mouse (with RBC specific expression of a triple fusion antigen consisting of hen egg lysozyme (HEL), ovalbumin (OVA) and the human Duffy blood group antigen) and the IgHEL mouse (a BCR transgenic with specificity for HEL). Herein we demonstrate that erythrocyte specific autoreactive conventional B cells were decreased 2-10 fold in the bone marrow and secondary lymphoid organs in autoimmune $HOD^{+}IgHEL^{+}$ mice. In contrast, there was a relative enrichment of B-1 B cells in the peritoneal cavity, which correlated with an increased production of anti-HEL IgM antibodies. In aggregate, these data demonstrate that B cell tolerance to the erythrocyte-specific HOD antigen is not complete in this model, and suggest that in the natural state, RBC autoreactive B cells are tolerized differently based upon B cell subtypes and anatomical location.

Introduction

Establishment and maintenance of immunological tolerance is essential to prevent autoimmunity. Breakdown of tolerance to red blood cell (RBC) self-antigens can lead to autoantibody production; anti-RBC autoantibodies may occur in association with infection, lymphoproliferative disorders, and blood transfusions (1, 2). In some cases, antibody coated RBCs may have a normal circulatory half life (3). In other instances, however, autoantibodies may lead to autoimmune hemolytic anemia (AIHA), a severe and sometimes fatal autoimmune disease (4).

Tolerance to self-antigens is achieved through central and peripheral mechanisms that are responsible for deletion and/or preventing activation of autoreactive lymphocytes (5). B cell tolerance to self-antigen occurs concurrently with B cell development in the bone marrow. Autoreactive B cells that respond strongly with self-antigen undergo deletion, anergy or B cell receptor (BCR) editing to reduce autoreactivity (6-10). B cells that do not encounter their cognate antigen are permitted to enter the periphery. However, upon autoantigen exposure in the periphery, autoreactive B cells are susceptible to similar tolerance mechanisms as those employed in the bone marrow (11). Similarly, autoreactive T cells are susceptible to deletion or inactivation through tolerance mechanisms in the thymus and periphery (12, 13). As such, by requiring B cells to interact with T cells prior to becoming fully activated, another layer of tolerance to self-antigens is achieved (14).

Despite rigorous tolerance mechanisms, nearly 3-5% of individuals suffer from some form of autoimmunity (15). A subtype of B cells, called B-1, has been implicated

as a source for autoreactive B cells in many autoimmune diseases; B-1 B cells readily respond to T-independent antigens (16, 17). T-independent antigens extensively crosslink the BCR thereby obviating the need for T cells to help B cells activate (18). As a result, B cells that recognize and respond to T-independent antigens bypass a critical safeguard to protect against autoimmunity. Furthermore, most B-1 B cells express self-reactive BCRs (19, 20). And, unlike classical B cells, B-1 B cells are hypothesized to develop from extramedullary precursors (21-23). Thus, the same stringent central tolerance mechanisms to control autoreactive B cells observed in the bone marrow might not be available for B-1 B cells that develop extramedullary. Lastly, due to their anatomical sequestration, exposure to self-antigens not present in the peritoneal cavity and pleural spaces might be limited and peripheral tolerance mechanisms circumvented (24, 25).

The involvement of B-1 B cells and production of autoantibodies directed towards erythrocyte antigens has been studied in a murine model of AIHA. Okamoto and colleagues utilized a BCR transgenic mouse that expresses an immunoglobulin transgene specific for an antigen dependent upon erythrocyte protein, Band 4.1 (26). Transgenic mice experience a range of AIHA severity from tolerance to considerable anemia (27). B cell development is abnormal; conventional B cells are absent in the bone marrow and secondary lymphoid organs. In contrast, enrichment of B-1 B cells is observed in the peritoneal cavity and lamina propria lymphocytes. Upon activation of B-1 B cells through oral administration of the B cell mitogen lipopolysaccharide, the B-1 B cell

population expands; this proliferation correlates with secretion of autoantibodies (28). As a consequence of autoantibody production, the mice become anemic.

While great knowledge about tolerance to RBC antigens was attained through use of the AIHA model, there are several limitations. The baseline distribution of the BCR transgenic B cells in the absence of autoantigen could not assessed; Band 4.1 is essential to maintain the structure and function of the erythrocyte (29). Additionally, autoimmune mice experience severe hemolysis and chronic inflammation thereby complicating the analysis of tolerance mechanisms, as the overall pathophysiology has been altered. Lastly, there is a lack of reagents available that can be used to track autoreactive B cells and accompanying T cells.

Herein, we describe a novel model of erythrocyte autoreactivity, which allows for analysis of autoantibody production in the absence of hemolysis (Cadwell, CM *et al* in preparation). To generate this model, we crossed the recently- generated FVB.HOD mouse (a transgenic mouse with erythrocyte-restricted expression of a triple fusion protein consisting of hen egg lysozyme (HEL), ovalbumin (OVA) and human blood group molecule, Duffy) with the IgHEL mouse (a BCR transgenic with specificity for HEL) (7, 30). We observed a 2-10 fold range of deletion of conventional B cells in the bone marrow and secondary lymphoid organs in double transgenic autoimmune $HOD^{+}IgHEL^{+}$ mice. In contrast, there was an enrichment of B-1 B cells in the peritoneal cavity of transgenic autoimmune mice, which correlated with anti-HEL IgM antibody production. In aggregate, these data demonstrate that B cell tolerance to the erythrocyte-

specific HOD antigen is not complete in this model, nor is it similarly efficient among B cell subtypes or anatomical compartments.

Materials and Methods

Mice

FVB.HOD mice were created as previously described (30). IgHEL mice (of the MD4 variety) were obtained from Jackson labs (Bar Harbor, ME) (31). Breeding of the FVB.HOD, IgHEL mice, and the F1 cross between them was carried out by the Emory Division of Animal Resources Animal Husbandry service. Mice were maintained on standard rodent chow and water in a temperature- and light-controlled environment. All procedures were performed according to approved Institutional Animal Care and Use Committee (IACUC) procedures.

Isolation of leukocytes

Spleens and draining lymph nodes were harvested in complete RPMI (RPMI 1640 + 1% L-glutamine + 1% penicillin/streptomycin + 1% 1M HEPES + 1% sodium pyruvate + 1% non-essential amino acids all from Cellgro [Herndon, VA] + 2-beta mercaptoethanol [EM Science, Darmstadt, Germany] + 10% fetal bovine serum [Hyclone]). Spleens were cut into fragments and then were digested at 37C with 1mg/mL collagenase IV (Sigma, St. Louis, MO) in complete RMPI. Draining lymph nodes were digested with collagenase type IV at 37C. After incubation, tissue was pushed through a 70um filter and washed with complete RPMI. Red blood cells were lysed with RBC lysis buffer (Sigma). Splenocytes and leukocytes from lymph nodes were resuspended to a final concentration of $10x10^6$ cells/mL in complete media. Leukocytes from the intestines were isolated as previously described (32). Briefly, intestines were removed and cleaned of their

mesentery. Peyer's patches were excised from the small intestine. Both the small and large intestine were cut longitudinally and washed in Hanks' Balanced Salt Solution (Cellgro, Manassas, VA). Intestines were cut into 0.5 cm fragments and transferred to 50 mL conical tubes containing Hanks supplemented with 5% FBS (Hyclone) and 2 mM EDTA (Sigma). Tubes were shaken at 250 rpm for 20 min at 37 C. This process was repeated 2 times. Cells were passed through a strainer and intestinal tissue was washed, minced and transferred to 50 mL conical tubes containing Hanks supplemented with 5% FBS and type VIII collagenase (1.5 mg/mL, Sigma). Intestinal pieces were digested at 37 C for 20min. Leukocytes were collected, passed through a cell strainer and pelleted by centrifugation at 300 g. Peyer's patches and mesenteric lymph nodes were digested at 37C for 20min with type VIII collagenase (1.5 mg/mL) in Hanks containing 5% FBS. Tissue was passed over a cell strainer and leukocytes were collected. Leukocytes were resuspended to a final concentration of $10x10^6$ cells/mL in complete RPMI. Peritoneal leukocytes were harvested by peritoneal lavage with 10 mL cold PBS.

Staining leukocytes

A total of $1x10^6$ cells were stained for flow cytometry in FACS buffer (PBS + 0.2mg/mL bovine serum albumin [Sigma] + 0.9mg/mL EDTA [Sigma] + 2% fetal bovine serum [Hyclone]). Hen egg lysozyme (HEL) was conjugated to alexa fluor-647 (HEL-AF-647) using Alexa Fluor monoclonal antibody labeling kit (Invitrogen, Carlsbad, CA), per manufacturer's instructions. Briefly, 100 ug HEL was incubated for 1 hour at room temperature with AF-647. Mixture was then dialyzed three times against PBS at room temperature. Leukocytes were stained with directly-conjugated antibodies against IgM^a

(BD Pharmingen, San Diego, CA, clone DS-1), CD19 (BD Pharmingen, clone 1D3), B220 (eBiosciences, San Diego, CA, clone RA3-6B2), CD43 (Miltenyi Biotec, Auburn, CA, clone L11), IgD (eBioscience, clone 11-26c) and HEL-AF647. All staining was performed in FACS buffer. Samples were acquired using a six color BD FACS CANTO flow cytometer.

ELISA for anti-HEL humoral response

Enzyme-linked immunosorbent assays (ELISAs) for anti-HEL IgM were performed on sera run in triplicate at a 1:50 dilution as previously described (33).

ELISPOT

PVDF Enzyme-linked immunosorbent spot (ELISPOT) plates (Millipore, Billerica, MA) were coated with 10 ug/mL of HEL or PBS and incubated overnight at 4 C. Plates were washed with PBS-T (PBS + 0.001% Tween 20 [Sigma]) and blocked with RPMI-10 for 2-4 hr at room temperature. Splenocytes were resuspended to final concentration of $5x10^6$ cells/mL in RPMI-10 (RPMI + 10% FBS) and added in triplicate to the ELISPOT plate, making 4 five-fold dilutions with RPMI-10. Plates were incubated at 37 C for 16 hr. Plates were washed with PBS-T and anti-mouse IgM biotin (eBiosciences, clone ll/41) primary antibody was added and incubated at room temperature for 2hrs. Plates were washed with PBS-T and horseradish peroxidase avidin D (Vector Laboratories, Burlingame, CA) secondary antibody was added and incubated at room temperature for 1 hr. Plates were developed with 3-amino-9-ethylcarbazole (Sigma) and spots were visualized using ELISPOT reader (Cellular Technology, Ltd., Cleveland, OH).

Statistical Analysis

Statistical significance was determined by using PRISM software and performing a Student's T-test for comparison of 2 samples. The measure of statistical significance was set at $p<0.05$.

Results

Autoreactive B cells are decreased in primary and secondary lymphoid organs

IgHEL mice express an IgM^a transgene derived from Balb/c mice that confers anti-HEL specificity to the BCR (7). Due to allelic exclusion, it was predicted that all of the B cells in HOD^-IgHEL^+ mice express IgM^a. Splenocytes from IgHEL mice were stained with anti-IgM^a, anti-CD19 and HEL-AF-647. It was observed that most $CD19^+$ B cells expressed IgM^a; of $IgM^{a+}C19^+$ B cells, over 95% of were reactive with HEL (Figure 3.1a). Reactivity with HEL-AF-647 was not a result of non-specific binding to the fluorophore AF-647 as $IgM^{a+}C19^+$ B cells from 3-83 (transgenic BCR specific for anti-MHC I (H-2k)) mice did not stain positively with conjugated HEL (Figure 3.1b).

FVB.HOD mice are transgenic for erythrocyte-restricted expression of a triple fusion protein consisting of hen egg lysozyme (HEL), ovalbumin (OVA) and human blood group antigen, Duffy (30). All of the B cells in the FVB.HOD mice express IgM^a. Therefore, the double transgenic mice from the IgHEL and FVB.HOD mice (HOD^+IgHEL^+) are predicted to have over 95% of $IgM^{a+}C19^+$ B cells reactive with HEL. Staining splenocytes from double transgenic autoimmune mice revealed that over 87% of $IgM^{a+}C19^+$ B cells were reactive with HEL (Figure 3.1c).

To test the hypothesis that HEL-reactive B cells were deleted in the presence of autoantigen, B cells from HOD^-IgHEL^+ and HOD^+IgHEL^+ (autoimmune) mice were compared. Leukocytes were harvested from the bone marrow, spleen and lymph nodes and stained with anti-B220 and anti-IgM^a. Flow cytometric analysis revealed that

$B220^{+}IgM^{a+}$ B cells in the bone marrow of $HOD^{-}IgHEL^{+}$ mice constituted approximately 7% of the total population. In $HOD^{+}IgHEL^{+}$ mice, there was a significant 2-fold decrease in $B220^{+}IgM^{a+}$ B cells, when compared to mice without the HOD antigen (n = 6 mice/group, p = 0.01) (representative flow plots Figure 3.2a, compilation 3.2b). Similarly, it was observed that there was a 2-fold decrease of HEL-reactive B cells in the spleen of autoimmune mice ($p < 0.001$, representative flow plots Figure 3.2c, compilation 3.2d). Likewise, there was a profound decrease of HEL-reactive B cells observed in peripheral and mesenteric lymph nodes of autoimmune mice (Figure 3.2e and f, respectively).

Anti-HEL antibodies are increased in autoimmune mice

To test whether the decreased numbers of B cells correlated with decreased antibody production, ELISAs were performed on sera collected from $HOD^{-}IgHEL^{+}$ and $HOD^{+}IgHEL^{+}$ mice. Due to the transgenic nature of the IgHEL mice, they cannot class-switch to IgG. Therefore, anti-HEL IgM ELISAs were performed. When compared to $HOD^{-}IgHEL^{+}$ mice, $HOD^{+}IgHEL^{+}$ mice made statistically more anti-HEL IgM than controls ($p < 0.001$ at a 1:50 dilution, Figure 3.3a). To test whether the deletion-resistant HEL-reactive B cells in the autoimmune mice were making more anti-HEL IgM or whether there were an increased number of antibody secreting cells (ASC), ELISPOTs were performed on splenocytes. In 3 individual mice, $HOD^{-}IgHEL^{+}$ mice had an average of 40-50 anti-HEL IgM ASC per million splenocytes compared to 3500-4000 ASC in autoimmune $HOD^{+}IgHEL^{+}$ mice, which represented a 100-fold increase (Figure 3.3b).

Thus, the increased anti-HEL in $HOD^{+}IgHEL^{+}$ mice was likely a result of a quantitative increase in number of anti-HEL secreting plasma cells.

B-1 B cells are enriched in the peritoneal cavity of autoimmune mice

Due to the decreased numbers of B cells in the bone marrow and spleen but increased production of autoantibodies, we hypothesized that there was another compartment that contained autoreactive B cells. The peritoneal cavity is comprised mostly of innate B-1 B cells and macrophages; there is a small population of conventional B cells (34). B cell subtypes can be distinguished by expression of IgD. Therefore, peritoneal lavages were performed on control (HOD-IgHEL-), $HOD^{-}IgHEL^{+}$ and $HOD^{+}IgHEL^{+}$ mice and leukocytes were stained with antibodies against B220, IgM^{a} and IgD. Control mice (on an FVBxB6 background) have IgM^{a} and IgM^{b} allotypic B cells. Of IgMa+ B cells, approximately 30% display a conventional B cell phenotype ($IgD^{+}IgM^{alow}$), whereas 60% were innate B-1 B cells ($IgD^{+}IgM^{ahi}$) (Figure 3.4a). In contrast to what is observed in control mice, conventional B cells comprised the majority of B cells in the $HOD^{-}IgHEL^{+}$ mice, with a very low percentage of B-1 B cells (representative flow plot Figure 3.4b left, compilation c, left). In autoimmune mice, B-1 B cells were enriched in the peritoneum (representative flow plot Figure 3.4b, right, compilation c, right).

B cell enrichment is not a generalizeable gut phenomenon

We hypothesized that B cells in the gut were not deleted because of lack of antigen exposure. To test this hypothesis, we analyzed B cells from the large intestine,

Peyer's patches, and of $HOD^{-}IgHEL^{+}$ and $HOD^{+}IgHEL^{+}$ mice. Low percentages of HEL-reactive B cells were detected in the large intestine of $HOD^{-}IgHEL^{+}$ mice (Figure 3.5a). But, similar to the lymphoid organs, $B220^{+}IgM^{a+}$ HEL-reactive B cells were decreased 5-7 fold in the large intestines of $HOD^{+}IgHEL^{+}$ mice. Peyer's patches line the small intestine and serve as a niche for B cells in the gut. Again, similar to lymphoid organs, HEL-reactive B cells were decreased 10-fold in Peyer's patches of $HOD^{+}IgHEL^{+}$ mice, compared to $HOD^{-}IgHEL^{+}$ (Figure 3.5b). In correlation with decreased B cell numbers, there was a noticeable decrease in the size of Peyer's patches in $HOD^{+}IgHEL^{+}$ mice, when compared to $HOD^{-}IgHEL^{+}$ (Figure 3.5c). Very few B cells were detected in the small intestines of either type of mouse (Figure 3.5d).

Discussion

Herein, we have developed and characterized a model of erythrocyte autoreactivity, by crossing mice with the RBC specific HOD antigen with the IgHEL mouse (a BCR transgenic specific for HEL). In mice expressing both transgenes, conventional B cells were decreased 2-10 fold in the bone marrow as well as in secondary lymphoid organs. In contrast, there was an increase of anti-HEL antibodies in the autoimmune mice, which correlated with an enriched B-1 population in the peritoneal cavity. The enrichment of B cells was not generalizeable to the gut, as deletion of B cells was also seen in the large intestines and Peyer's patches.

There are at least two distinct hypotheses to explain the presence of HEL-reactive B cells in the bone marrow of autoimmune mice. The first hypothesis supposes that central tolerance mechanisms are overwhelmed due to the extremely high precursor frequency of autoreactive B cells in the marrow. In contrast to non-transgenic mice (in which 40-60% of developing B cells may display self-reactivity prior to central tolerance mechanisms), over 95% of B cells in the IgHEL mouse have a high affinity for HEL (31, 35). Thus, it is plausible that these numbers are simply too high for complete tolerance to be established. An alternative hypothesis, however, supposes that the RBC-specific HOD antigen is not being encountered in the bone marrow. However, this explanation is not favored, as autoimmune mice have decreased percentages of HEL specific B cells in the marrow, suggesting that at least some of HEL-specific B cells are encountering their cognate antigen. Thus, the data support that, HEL-specific B cells do encounter the HOD

antigen in the bone marrow, but these B cells cannot all be tolerized due to their high precursor frequency.

Other tolerance mechanisms that are available to be used are receptor editing, anergy and ignorance (36). There was a 3-fold increase in the percentage of $CD19^{+}IgM^{a+}$ B cells that were not reactive with HEL-AF-647 in autoimmune mice, compared to HOD-IgHEL+ (see Figure 3.1). These data suggest that the BCR has undergone receptor rearrangement and no longer recognize HEL. However, receptor editing does not describe the majority of deletion-resistant B cells in the autoimmune mouse. Furthermore, the deletion-resistant B cells are not all anergic, given the presence of anti-HEL antibody and HEL-specific ASCs. Thus, if deletion-resistant B cells were anergic, encountering their cognate antigen would not elicit antibody production or differentiation into plasma cells (31). Lastly, the deletion-resistant B cells are not ignorant. If the B cells were ignorant, they would be in a compartment devoid of HOD antigen; thus, no anti-HEL antibodies or ASC would be detected. Taken together, we hypothesize that the predominate mechanism used to tolerize anti-HEL B cells to the HOD antigen is deletion.

In contrast to the profound deletion of autoreactive B cells in the lymphoid organs, there was an enrichment of B-1 B cells in the peritoneal cavity. We hypothesize that the observed enrichment of B-1 cells was a consequence of extramedullary development of B-1 B cells. It has been well documented that B-1 B cell precursors are found predominately in the fetal liver (23, 37). Thus, it is plausible that B-1 B cells are not exposed to the same stringent positive and negative selection pressures that B-2 B cells undergo as they develop in the bone marrow. Thus, B-1 B cells would not be

tolerized to self-erythrocyte antigens. Additionally, peripheral tolerance mechanisms might not apply to B-1 B cells as erythrocyte antigens might not be encountered by B-1 B cells due to their restricted location (peritoneal cavity and pleural spaces) (38). An alternative hypothesis is that B-1 B cells arise from stem cells in the bone marrow and are exposed to the same selection pressures, but due to inherent signaling differences between B-2 and B-1 B cells, B-1 B cells are preferentially selected to develop and emigrate from the bone marrow to the periphery.

At baseline, $HOD^{-}IgHEL^{+}$ mice made low levels of anti-HEL IgM and had 40-50 million ASC per million splenocytes. However, in the presence of the HOD antigen in the autoimmune mice, significantly more anti-HEL IgM antibodies were detected, which correlated with a 100-fold increase of ASC compared to $HOD^{-}IgHEL^{+}$ mice. We hypothesize that B-1 B cells are the source of anti-HEL antibodies and ASC; B-1 B cells traffic to the spleen where they encounter the HOD antigen, which prompts differentiation into plasma cells. In support of this hypothesis, B-1 B cells were enriched in the peritoneum whereas there was profound deletion of B-2 cells throughout primary and secondary lymphoid organs and in the gut. While the ELISPOT data demonstrate anti-HEL ASC in the spleen, it is known that B-1 B cells can traffic between the peritoneal cavity and spleen. Additionally, when B-1 B cells become activated, they migrate to the mesenteric lymph node or spleen to differentiate into plasma cells. We cannot rule out the contribution of B-2 B cells in the spleen or other lymphoid organs, however. B-2 B cells typically require T-cell help to secrete antibodies whereas B-1 and marginal zone B cells do not. Given that B cells in the IgHEL mouse are unable to class-

switch, examination of IgG subtypes is not meaningful. Thus, it is impossible to know which subtype of B cell subtype(s) contribute to the anti-HEL antibody production.

In summary, the current findings demonstrate that there is incomplete B cell tolerance to the RBC specific HOD antigen in autoimmune $HOD^{+}IgHEL^{+}$ mice. A unique strength of this model lies in the ability to study the failure of tolerance mechanisms in the absence of hemolysis and chronic inflammation (Cadwell, CM *et al* in preparation), with additional strengths being the ability to directly access deletion patterns of autoreactive B cells in mice expressing or not expressing the HOD antigen. Ongoing studies utilizing HEL-specific BCR transgenic mice capable of class-switching will provide additional insight into the subtypes of B cells responsible for antibody production, as well as mechanisms of central tolerance failure. A better understanding of questions such as these may ultimately benefit patients with autoimmunity, in particular those suffering from AIHA.

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Figure Legends

Figure 3.1: $CD19^{+}IgM^{a+}$ B cells in IgHEL mice are specific for HEL. Splenocytes were stained with anti-IgM^{a}, anti-CD19 and HEL-AF-647. Left: Representative flow plots of $CD19^{+}IgM^{a+}$ B cells from splenocytes and (right) percentage of $CD19^{+}IgM^{a+}$ B cells reactive with HEL from (A) $HOD^{-}IgHEL^{+}$, (B) 3-83 and (C) $HOD^{+}IgHEL^{+}$ mice.

Figure 3.2: HEL-reactive B cells are decreased in the bone marrow and secondary lymphoid organs in autoimmune $HOD^{+}IgHEL^{+}$ mice. Leukocytes were isolated from lymphoid organs of $HOD^{-}IgHEL^{+}$ and autoimmune $HOD^{+}IgHEL^{+}$ mice and stained with anti-B220 and anti-IgMa. Graphical representation of the percentage of $B220^{+}IgM^{a+}$ B cells in the (A) representative flow plots B) of bone marrow of $HOD^{-}IgHEL^{+}$ and $HOD^{+}IgHEL^{+}$ mice (n = 6 mice/group); (C) representative flow plots (D) of spleen (n = 11 mice/group); (E) peripheral lymph nodes (n = 12 mice/group) and (F) mesenteric lymph nodes (n = 8 mice/group).

Figure 3.3: Autoimmune mice make more anti-HEL IgM and have more antibody secreting cells compared to $HOD^{-}IgHEL^{+}$ mice. (A) Sera from $HOD^{-}IgHEL^{+}$ and autoimmune $HOD^{+}IgHEL^{+}$ mice was analyzed by ELISA for anti-HEL IgM antibodies (n = 8 mice/group). (B) ELISPOTs were performed on splenocytes from $HOD^{-}IgHEL^{+}$ and autoimmune mice and antibody secreting cells were enumerated (n = 3 mice/group).

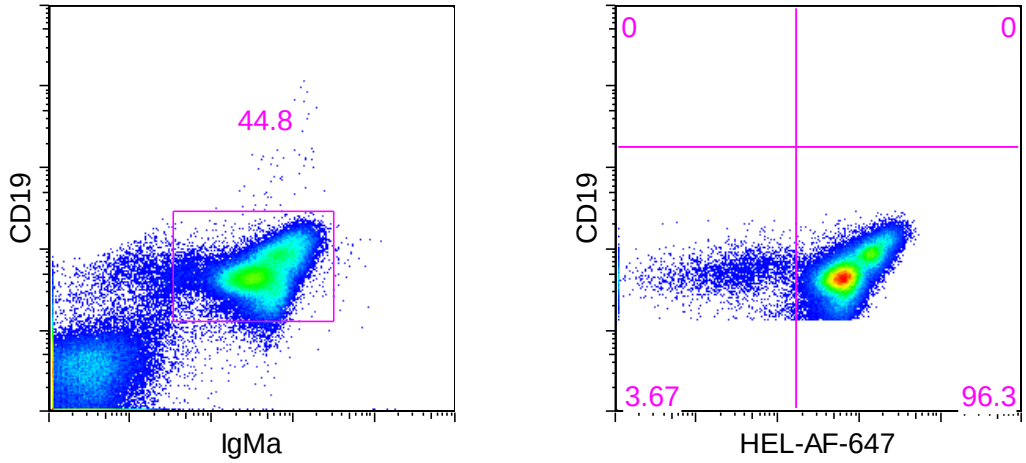
Figure 3.4: B-1 B cells are enriched in the peritoneal cavity of autoimmune mice. Peritoneal lavages were performed on control, $HOD^{-}IgHEL^{+}$ and autoimmune mice. Leukocytes were stained with anti-B220, anti-IgMa and anti-IgD. (A) Representative

flow plot of B-1 and conventional B-2 B cells of peritoneal leukocytes of control mice and (B) B cell subsets and (C) compilation of B cell percentages of B-1 and conventional B cells in $HOD^{-}IgHEL^{+}$ (left) and $HOD^{+}IgHEL^{+}$ (right) mice.

Figure 3.5: B cell enrichment in autoimmune mice was not generalizeable to the gut. Leukocytes were harvested from gut associated lymphoid tissue and stained with anti-B220 and anti-IgMa. Graphical representation of the percentage of $B220^{+}IgM^{a+}$ B cells in the (A) large intestine (B) Peyer's patches with (C) photograph of Peyer's patches and (D) small intestine from $HOD^{-}IgHEL^{+}$ and $HOD^{+}IgHEL^{+}$ mice.

Figure 3.1

A


44.8
CD19
IgMa
0
0
CD19
3.67
96.3
HEL-AF-647

B

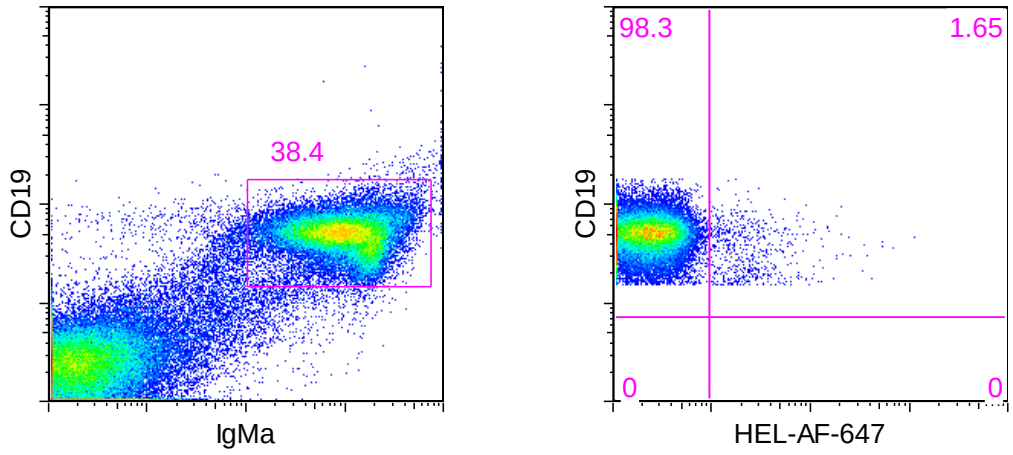

38.4
CD19
IgMa
98.3
1.65
CD19
0
0
HEL-AF-647

Figure 3.1

C

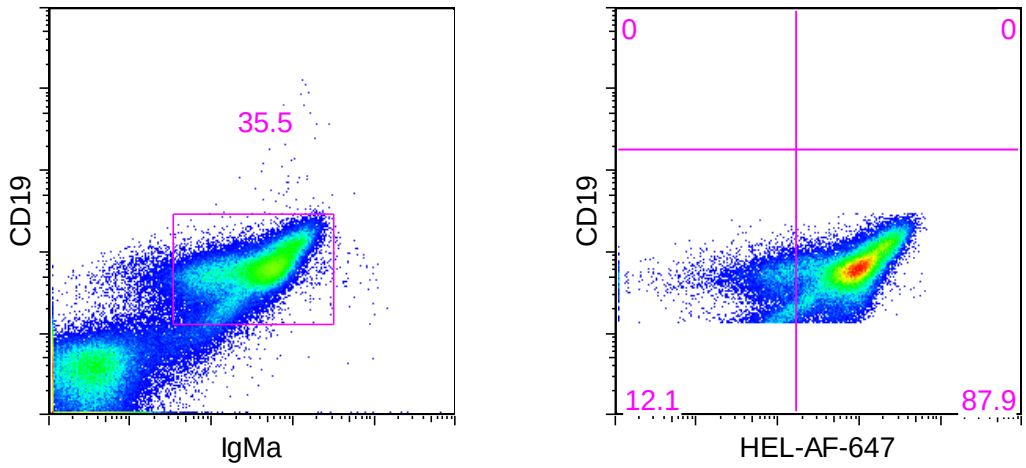
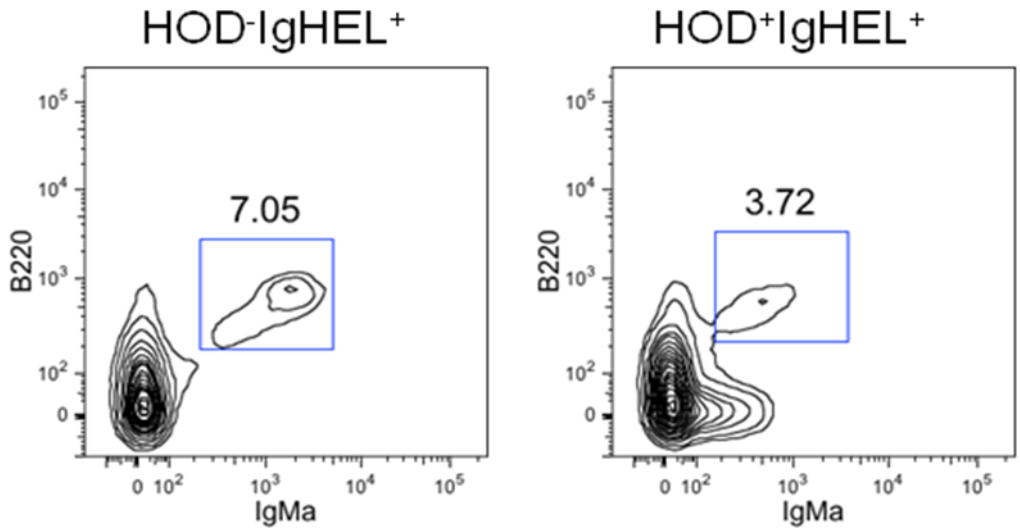


Figure 3.2

A


HOD-IgHEL+
HOD+IgHEL+
7.05
3.72
B220
IgMa
0
10^2
10^3
10^4
10^5

B

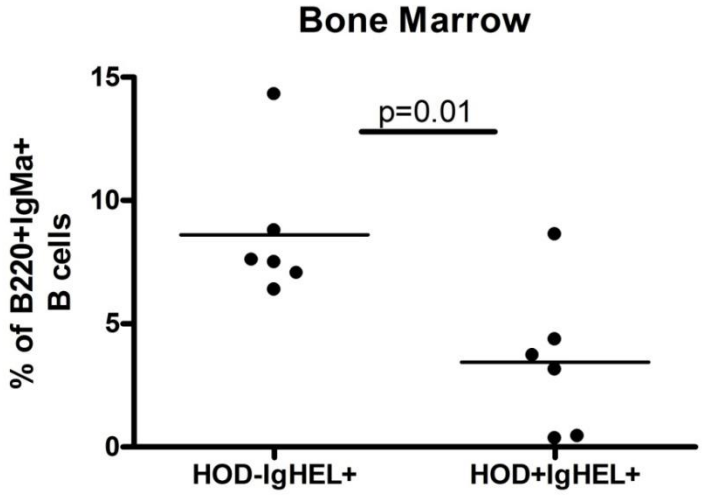
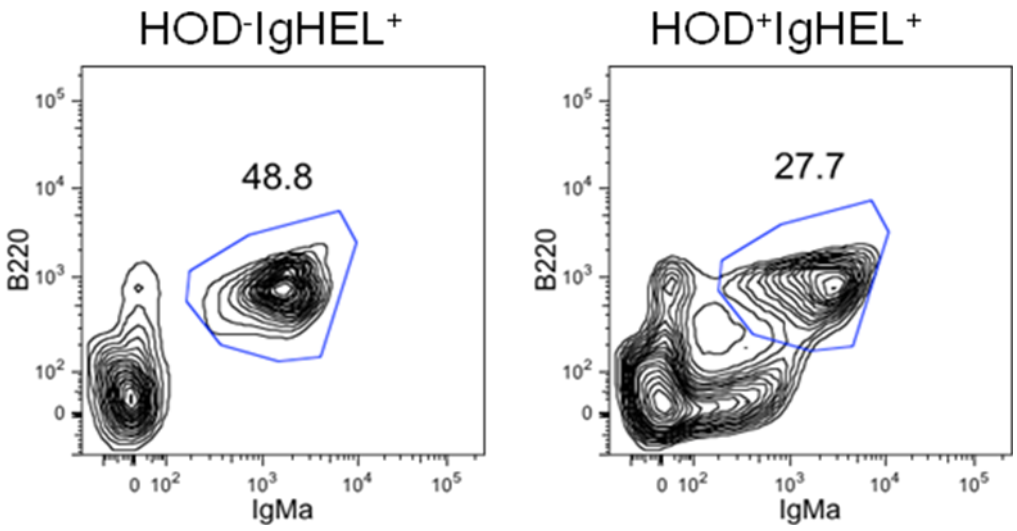

Bone Marrow
% of B220+IgMa+ B cells
p=0.01
15
10
5
0
HOD-IgHEL+
HOD+IgHEL+

Figure 3.2

C


HOD-IgHEL+
HOD+IgHEL+
48.8
27.7
B220
IgMa
0
10^2
10^3
10^4
10^5

D

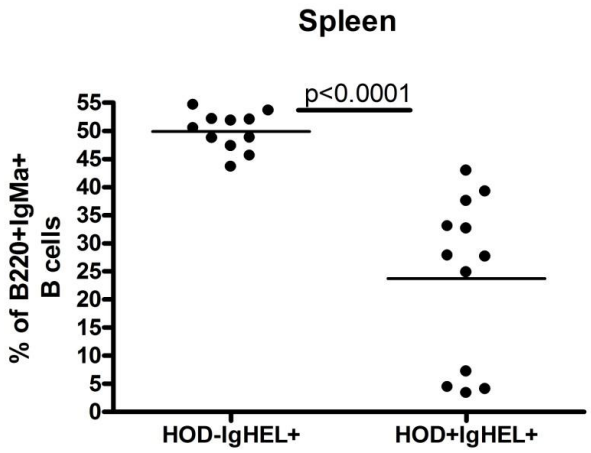
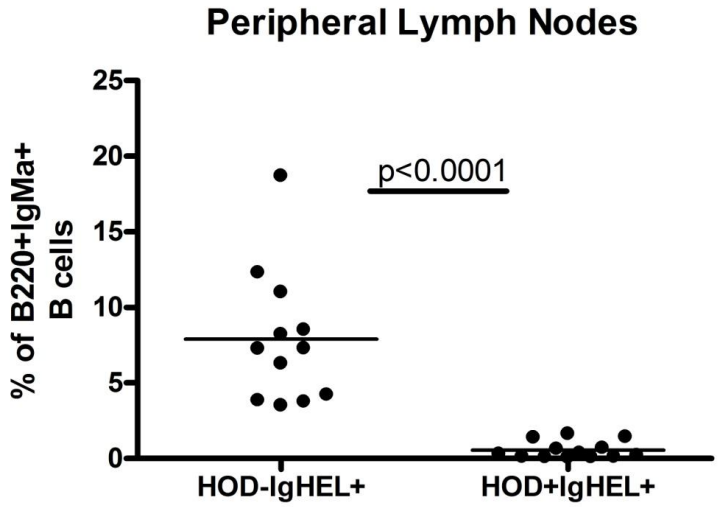

Spleen
p<0.0001
% of B220+IgMa+ B cells
55
50
45
40
35
30
25
20
15
10
5
0
HOD-IgHEL+
HOD+IgHEL+

Figure 3.2

E


Peripheral Lymph Nodes
% of B220+IgMa+ B cells
25
20
15
10
5
0
p<0.0001
HOD-IgHEL+
HOD+IgHEL+

F

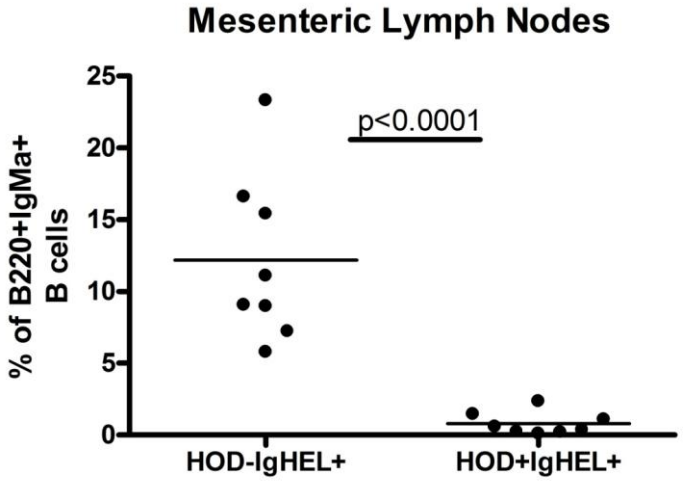
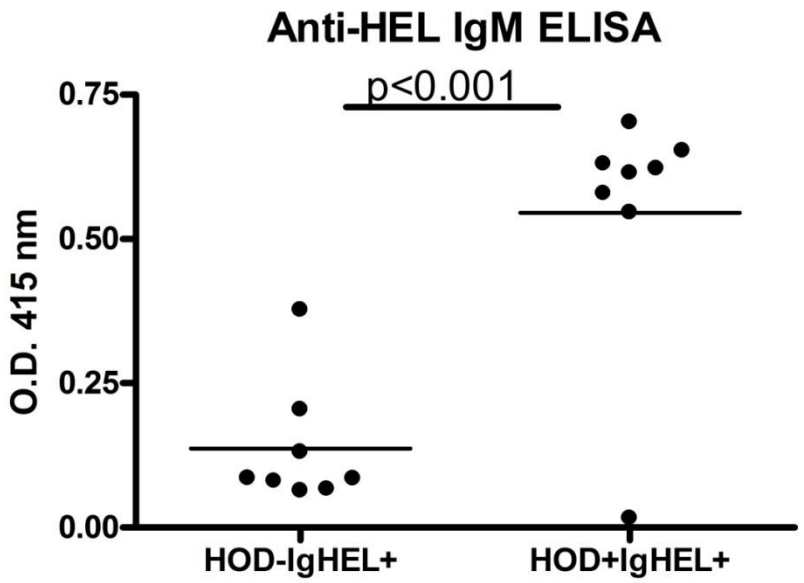
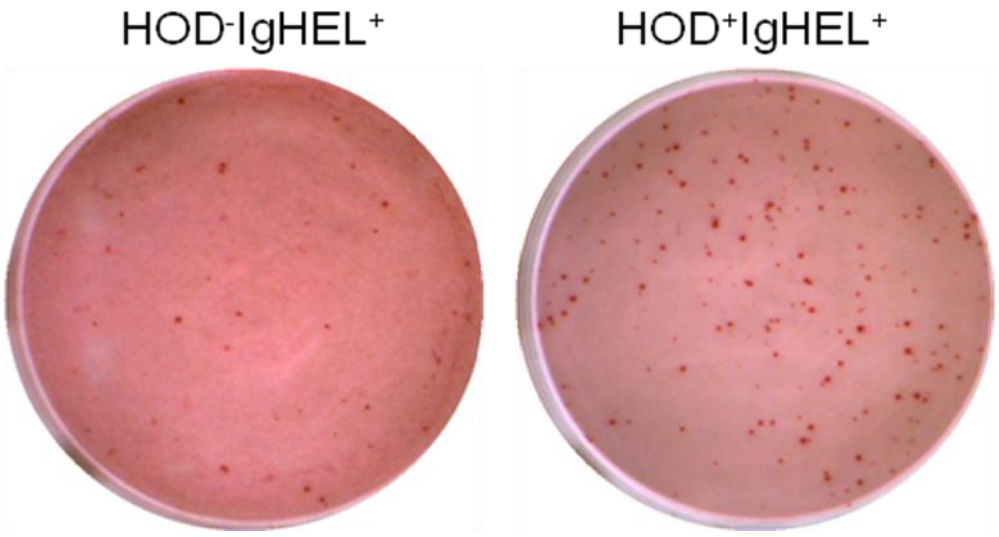

Mesenteric Lymph Nodes
% of B220+IgMa+ B cells
25
20
15
10
5
0
p<0.0001
HOD-IgHEL+
HOD+IgHEL+

Figure 3.3

A



B

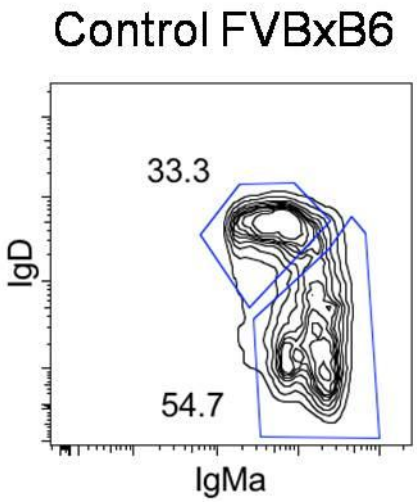


40-50 ASC/million splenocytes

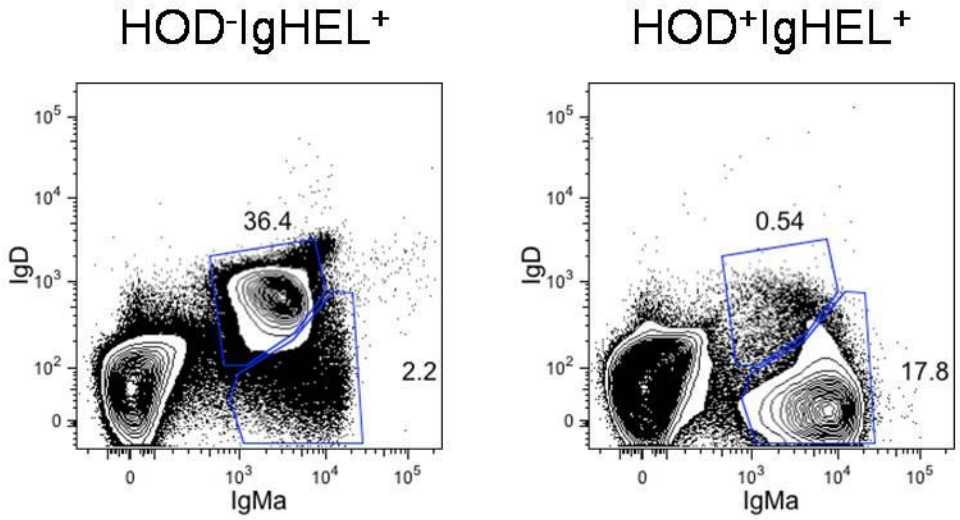
3500-4000 ASC/million splenocytes

Figure 3.4

A


Control FVBxB6
33.3
54.7
IgD
IgMa

B


HOD-IgHEL+
HOD+IgHEL+
36.4
2.2
0.54
17.8
IgD
IgMa
0
10^2
10^3
10^4
10^5

C

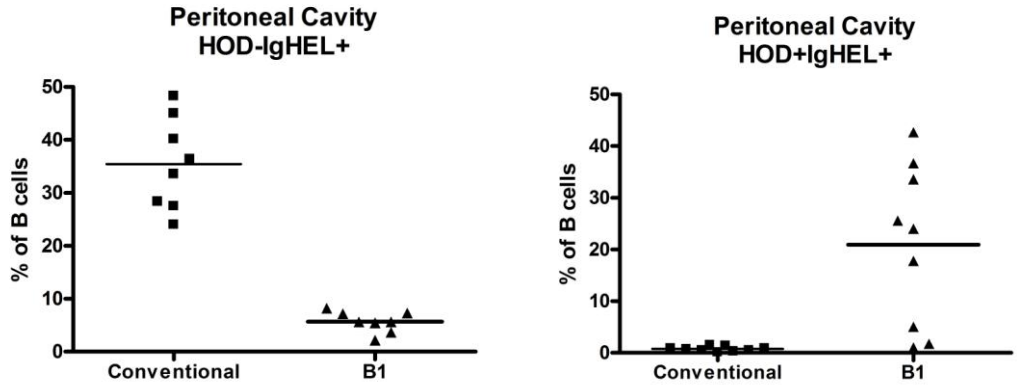
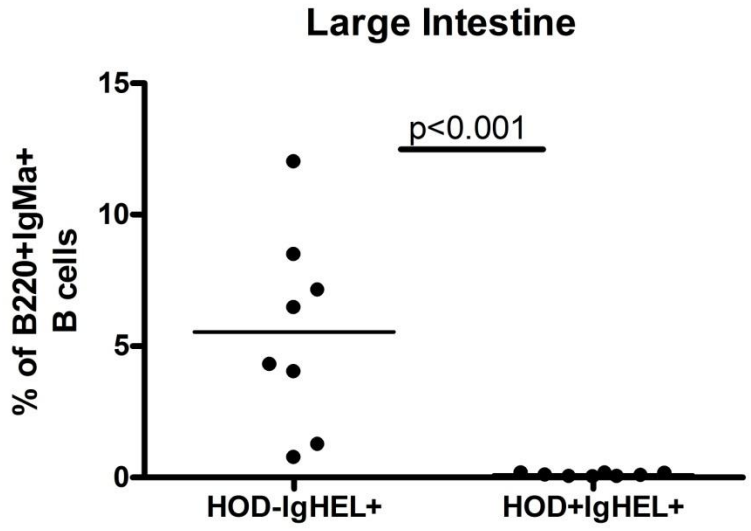
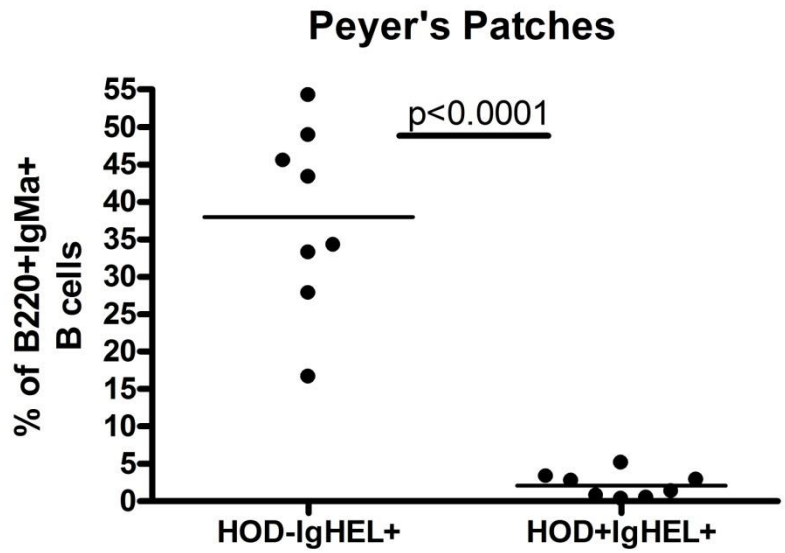

Peritoneal Cavity
HOD-IgHEL+
% of B cells
50
40
30
20
10
0
Conventional
B1
Peritoneal Cavity
HOD+IgHEL+
% of B cells
50
40
30
20
10
0
Conventional
B1

Figure 3.5

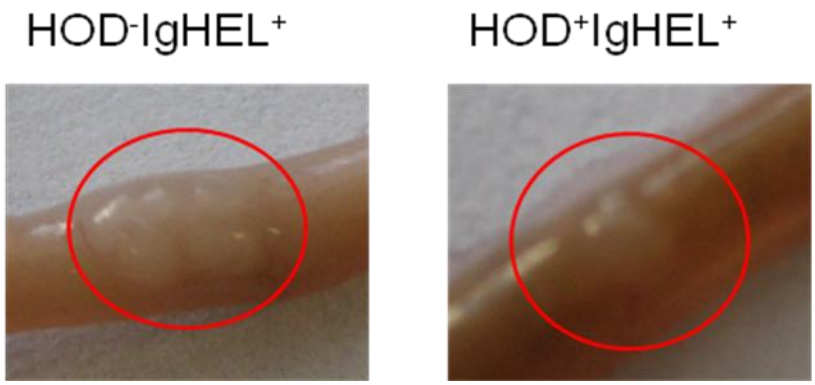
A


Large Intestine
% of B220+IgMa+ B cells
15
10
5
0
p<0.001
HOD-IgHEL+
HOD+IgHEL+

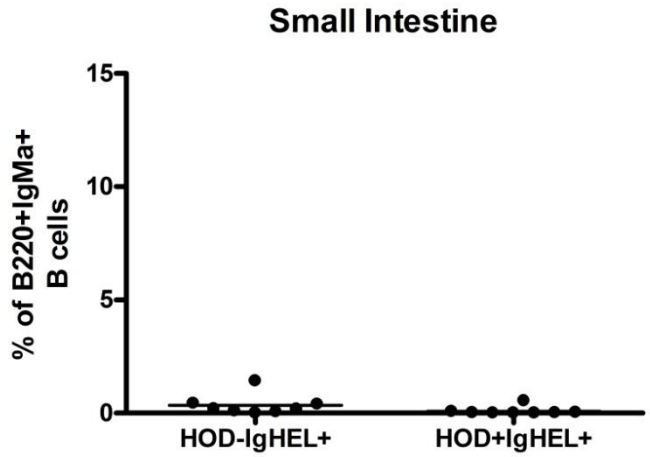
B


Peyer's Patches
% of B220+IgMa+ B cells
55
50
45
40
35
30
25
20
15
10
5
0
p<0.0001
HOD-IgHEL+
HOD+IgHEL+

C


HOD-IgHEL+
HOD+IgHEL+

D


Small Intestine
% of B220+IgMa+ B cells
15
10
5
0
HOD-IgHEL+
HOD+IgHEL+

Chapter 4

Central B cell Tolerance to Erythrocyte-Specific Antigens is Incomplete

Abstract

Breakdown of humoral tolerance to red blood cell (RBC) antigens can lead to autoimmune hemolytic anemia (AIHA), a severe and sometimes fatal disease. The underlying mechanisms that lead to the breakdown of B and/or T cell tolerance to RBC antigens are poorly understood. B cell receptor (BCR) transgenic mice have been used extensively to characterize central and peripheral tolerance mechanisms ascribed to B cells. Additionally, BCR transgenics have served as a good model for studying AIHA. While great knowledge regarding B cell tolerance has been gained through the use of BCR transgenics, there are several caveats inherent in these model systems including a high autoreactive B cell precursor frequency, an affinity-matured B cell receptor, and (specifically to the murine model of AIHA) chronic inflammation and hemolysis of RBCs. These limitations make studying the mechanisms of the failure of B and/or T cell tolerance to RBC antigens difficult. Thus, we describe a novel model used to define the B and T cell tolerance to antigens expressed only on RBCs. We utilized the recently-generated HOD mouse (which expresses an RBC-specific triple fusion protein consisting of hen egg lysozyme, ovalbumin and human blood group molecule, Duffy). By circumventing any potential T cell tolerance through adoptive transfer of OT-II T cells, we demonstrate that B cell tolerance to the HOD antigen is incomplete. Additionally, these data suggest that T cell tolerance to RBC-specific antigens is functionally complete, and in the event that T cell tolerance breaks down, AIHA can be initiated. To our

knowledge, this is the first report to analyze B cell tolerance to an RBC-specific antigen without the use of BCR transgenic mice.

Introduction

Autoimmune hemolytic anemia (AIHA) consists of loss of tolerance to self antigens on red blood cells (RBCs) in the humoral compartment (1). When this occurs, profound hemolysis can ensue, which leads to substantial morbidity and mortality. Response to pharmacologic immunesuppression and/or surgical splenectomy is variable, and in extreme cases, patients fail to respond to any of the established interventions (2). Because the antibodies formed during AIHA are often against common antigens expressed by essentially all humans, even support with transfusion may not be possible, as all units of RBCs will be incompatible (3). It is for these reasons that severe cases of AIHA can lead to death due to profound auto-hemolysis.

Some forms of AIHA are known to be secondary to infectious disease or immune dysregulation as a result of neoplasia (4, 5). However, in the primary form of AIHA, no inciting cause is identified (6). The basic pathogenesis of primary AIHA is poorly understood, but is presumably the result of failure of tolerance mechanisms at the level of T cell and/or B cells. Approximately 9000 cases of AIHA are observed annually in the United States (1). However, it is essential to note that the majority of antibodies against RBC antigens, both for alloantibodies from transfusion and autoantibodies, do not result in hemolysis (7, 8). Indeed, antibodies against many blood group antigens are considered "clinically insignificant" by transfusion medicine, as incompatible blood can be transfused with impunity for such antigen/antibody pairs. Such also appears to be the case for autoantibodies. Based upon large scale analysis of blood donors, the frequency of autoantibodies to RBCs in asymptomatic patients is as frequent as 0.1%. Thus,

baseline humoral tolerance to RBC antigens appears to fail in up to 1/1000 humans, indicating that tolerance mechanisms to RBC antigens are lost with considerable frequency (8).

The relative inefficiency of humoral tolerance to RBC antigens is not predicted, given the known characteristics of central B cell tolerance. Central tolerance in the B cell compartment occurs as a result of exposure to auto-antigen at several checkpoints during B cell development (9). Establishment of tolerance can lead to deletion, anergy, or receptor editing such that the immunoglobulin is no longer autoreactive (10, 11). Like B cells, erythrocyte precursors differentiate into mature RBCs in the bone marrow; blood group antigens are expressed on RBC precursors during their development (12-14). As such, B cells undergo central tolerance induction in close proximity to a rich source of RBC antigens; thus, it is a reasonable hypothesis that central B cell tolerance to RBC antigens would normally be an efficient and robust process.

In contrast to classic B cells that develop in the bone marrow (typically B-2 B cells), B-1 B cells are known to develop in visceral cavities outside of the normal hematopoietic compartments (15, 16). As such B-1 B cells may not be exposed to RBC antigens during development. It has thus been hypothesized that central tolerance to RBC antigens does not extend to extramedullary B cells, and that B-1 B cells represent a reservoir containing specificities that are autoreactive to RBC antigens (17, 18). Studies of B-1 B cells in this context have made extensive use of immunoglobulin transgenic mice expressing a B cell receptor (BCR) that is autoreactive to a murine RBC antigen (19). While deletion of the autoreactive B cells does occur in these mice, there is

substantial breakthrough of autoreactive B cells, with a strong preference for B-1 B cells in the peritoneal cavity (20). The resulting mice develop clinical AIHA, with a range of severity, regulated in part by baseline innate immune activation and interaction with gut flora (21, 22).

The use of mice transgenic for an RBC autoreactive immunoglobulin has been a highly innovative and fruitful approach to analyzing tolerance/autoimmunity to RBC antigens. However, there are also several limitations to this approach. First, the extremely high precursor frequency (essentially all B cells) in the transgenic mice is an unnatural challenge to tolerance mechanisms; thus, it is unclear if surviving auto-reactive B cells reflect the natural state or are the outcome of an extremely high precursor frequency. Second, the transgene is an affinity matured immunoglobulin, and thus the B cells have an unnaturally strong BCR signaling upon first encounter with antigen. Finally, the clinical development of AIHA in these mice is an excellent model of autoimmune disease, but it also introduces complicating biology of Fc receptor ligation, complement activation, anaphylotoxin release, saturation of the reticuloendothelial system, and extreme iron metabolism. Each of these factors, while relevant to modeling overall AIHA pathology, may influence the development of the immune system, and thus interfere with analysis of baseline tolerance immunology.

So as to build on the current mechanistic understanding, we report herein the engineering and use of a new model of tolerance/autoimmunity to RBC antigens that does not depend upon immunoglobulin transgenic mice and does not involve the pathophysiology co-incident with clinically significant AIHA. We have recently

described the HOD mouse, which expresses an erythrocyte-specific transgene consisting of hen egg lysozyme (HEL) fused to ovalbumin (OVA) fused to the human Duffy blood group antigen (HEL-OVA-Duffy (HOD)) (23). CD4+ T cell tolerance was experimentally circumvented through the adoptive transfer of CD4+ T cells from OT-II mice into HOD mice on a C57BL/6 background (B6.HOD). Because the HOD antigen contains the peptide recognized by OT-II cells when presented in I-A^b (OVA $_{323-339}$), then if HOD reactive B cells exist, and they have processed and presented HOD antigen on MHC II, then the OT-II T cells can provide help. We report that transfer of enriched CD4+ OT-II T cells into B6.HOD mice does indeed induce high titers of autoantibodies against HOD RBCs. Autoantibody production is dependent on antigen-specific T cell help and cannot be recapitulated with increased numbers of CD4+ T cells or bystander activation. Together, the data demonstrate that in the context of an otherwise unaltered immune system, B cell tolerance to RBC-specific antigens is incomplete. To the best of our knowledge, this represents the first report of B cell tolerance biology to RBC antigens in the absence of BCR transgenic mice, and suggests that loss of CD4+ T cell tolerance to RBC antigens is all that is required for the induction of AIHA.

Materials and Methods

Mice

C57BL/6 and Balb/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). B6-[TG]TCR-OTII-RAG1 tm1Mom mice (OTII/RAG1ko) were obtained from Taconic Farms, Inc. through the NIAID Exchange program, NIH mouse line #4234. SMARTA mice were a generous gift from Dr. Evavold. B6.HOD, mHEL, mOVA, OT-IIxThy1.1, TCR75 mice were bred by the Emory University Department of Animal Resources. Mice were maintained on standard rodent chow and water in a temperature- and light-controlled environment and used at 8-12 weeks of age. All experiments were performed according to approved Institutional Animal Care and Use Committee (IACUC) procedures.

Manipulation of mice: surgical splenectomy, transfusion and immunization

Splenectomies were carried out using standard murine surgical techniques; control mice were unmanipulated. Mice that received an adoptive transfer of enriched CD4+ T cells from TCR75 donors also received a transfusion of 100uL of packed Balb/c whole blood in 500uL PBS 24 hr post transfer. B6 and B6.HOD mice were immunized subcutaneously with 100 ug of OVA (Sigma, St. Louis, MO) emulsified in complete freunds adjuvant (OVA, OVA/CFA). Two weeks later, mice were boosted with 100 ug OVA (Sigma) in incomplete freunds adjuvant. Sera was collected 7 and 14 days post boost. Allotypic antibodies against HEL were generated in Balb/c mice. Two weeks post

immunization, mice were exsanguinated and sera was collected. The dominant immunoglobulin subtype was IgG1 as determined by ELISA.

Immunofluorescence

Tissue was snap frozen in OCT and stored at -80C. 8-10 um sections were cut from frozen tissues with a Leica Cryostat (Leica Microsystems, Bannockburn, IL). Sections were fixed with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA) and endogenous biotin reactivity was quenched by incubation at 37 C for 20 minutes with 3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ). Sections were blocked with PBS + BSA (Sigma) for 1 hour at room temperature in a humidified chamber. Sections were stained sequentially with each antibody addition incubation 1 hour at room temperature: Balb/c anti-HEL, anti-mouse $IgG1^a$ biotin (BD Pharmingen, San Diego, CA, clone 10.9) streptavidin-APC (BD Pharmingen). Secondary lymphoid organ tissues were additionally stained with anti-Thy1.2 (eBioscience, San Diego, CA, clone 53-2.1) and anti-B220 (BD Pharmingen, clone RA3-6B2). Non-lymphoid organs were stained with anti-Desmin (Abcam, Cambridge, MA) or anti-E-cadherin (eBioscience, clone DECMA-1). Sections were mounted with Prolong gold with DAPI and allowed to dry at room temperature. Slides were stored at 4C until imaging. Tissues were imaged with Zeiss LSM 510 Meta (Carl Zeiss Microimaging, Thornwood, NY).

ELISA for anti-HEL and anti-OVA humoral response

Enzyme-linked immunosorbent assays (ELISAs) for anti-HEL IgG and anti-OVA IgG were performed on sera run in triplicate at a 1:50 dilution as previously described.

Monoclonal anti-HEL IgG antibody (4B7) was used as an internal standard to allow for comparison between experiments.

Staining Leukocytes

A total of $2x10^6$ leukocytes from bone marrow were stained for flow cytometry in FACS buffer (PBS + 0.2mg/mL bovine serum albumin [Sigma] + 0.9mg/mL EDTA [Sigma] + 2% fetal bovine serum [Hyclone, Logan, Utah]). Duffy-reactive MIMA-29 and HEL-reactive 4B7 monoclonal antibodies were conjugated to alexa fluor-647 (HEL-AF-647) using Alexa Fluor monoclonal antibody labeling kit (Invitrogen, Carlsbad, CA), per manufacturer's instructions. Briefly, 100 ug of monoclonal antibody was incubated for 1 hour at room temperature with AF-647. Mixture was then passed over a size exclusion column to separate unconjugated fluorophore. Bone marrow leukocytes were stained with anti-CD71 (BD Pharmingen, clone C2), anti-TER119 (BD Pharmingen, clone TER119), and MIMA-29 AF-647 or 4B7-AF-647.

Peripheral blood was collected from mice in ACD. RBCs were stained in FACS buffer with anti-HEL, anti-OVA and MIMA-29 primary antibodies. Streptavidin-APC or anti-ms immunoglobulins were used as secondary antibodies. Peripheral white blood cells were stained with antibodies against CD19 (BD Pharmingen, clone 1D3), CD41 (BD Phmingen, clone MWReg30) or CD45 (BD Pharmingen, clone 30-F11) and primary antibodies against HEL. Streptavidin APC was used as a secondary. Samples were analyzed with a 4 color Accuri Cytometer (Ann Arbor, MI).

ELISPOT

PVDF Enzyme-linked immunosorbent spot (ELISPOT) plates (Millipore, Billerica, MA) were coated with 10 ug/mL of HEL or PBS and incubated overnight at 4 C. Plates were washed with PBS-T (PBS + 0.001% Tween 20 [Sigma]) and blocked with RPMI-10 for 2-4 hr at room temperature. Splenocytes were resuspended to final concentration of $5x10^6$ cells/mL in RPMI-10 (RPMI + 10% FBS) and added in triplicate to the ELISPOT plate, making 4 five-fold dilutions with RPMI-10. Plates were incubated at 37 C for 16 hr. Plates were washed with PBS-T and anti-mouse IgM biotin (eBiosciences, clone ll/41) or anti-mouse IgG biotin (eBiosciences, clone) primary antibody was added and incubated at room temperature for 2hrs. Plates were washed with PBS-T and horseradish peroxidase avidin D (Vector Laboratories, Burlingame, CA) secondary antibody was added and incubated at room temperature for 1 hr. Plates were developed with 3-amino-9-ethylcarbazole (Sigma) and spots were visualized using ELISPOT reader (Cellular Technology, Ltd., Cleveland, OH).

Flow cytometric crossmatching

Sera from experimental mice were diluted 1:10 and incubated with HOD, mHEL or B6 RBCs in FACS buffer. Goat anti-mouse immunoglobulins conjugated to allophycocyanin (Becton-Dickinson) were used as a secondary antibody.

CD4+ T cell purification and adoptive transfer of cells

Leukocytes from the spleens of OTIIxThy1.1, SMARTA, TCR75 and OTII/RAG1ko mice were liberated by physical disruption with microscope slides. Leukocytes were resuspended in complete RPMI. Mice were adoptively transferred with $10x10^6$ total

splenocytes from OTIIxThy1.1. In instances where purified CD4+ T cells were used, $CD4^+$ T cell enrichment was performed using the CD4+ T cell isolation kit (Miltenyi Biotec, Auburn, CA), per manufacturer's instructions. Post purity of CD4+ T cell enrichment was performed by staining the bound and unbound fractions with anti-CD19 and anti-CD4 with average purity of CD4+ T cells to be 70% (OTIIxThy1.1), 85% (SMARTA), 88% (TCR75). In each instance, the percentage of leukocytes $CD19^+CD4^-$ was less than 1.5% of the total, ungated population. B6 and B6.HOD recipients were injected via lateral tail vein with 1-5x10^6 enriched $CD4^+$ T cells. .

Statistical Analysis

Statistical significance was determined by using PRISM software and performing a Student's T-test for comparison of 2 samples or two-way repeated measure analysis of variance (ANOVA) with a Bonferroni post-test for 3 or more samples with multiple conditions. The measure of statistical significance was set at $p<0.05$.

Results

Expression of the HOD antigen is restricted to erythroid cells in the B6.HOD mouse

Most RBC antigens are not expressed on platelets or leukocytes, but can have varied expression on non-hematopoieic tissues (e.g. Duffy is expressed in kidney and Kell is expressed in skeletal muscle) (24, 25). In contrast, the RhD antigen has erythrocyte-restricted expression, and has not been detected on non-RBC elements in either hematopoietic or non-hematopoietic tissues (26, 27). Of autoantibodies to RBC antigens, antibodies to RhD dependant antigens are by far the most common (28). Thus, so as to model the most common RhD RBC autoantigen, and to ask an isolated question of what are the effects of antigen expression on RBCs on establishment of tolerance, we utilized a system designed to have RBC-specific expression of a model antigen.

The HOD mouse expresses a triple fusion transgene containing the well-characterized antigens, hen egg lysozyme (HEL) fused to ovalbumin (OVA) fused to Duffy expressed under an RBC specific regulatory element (beta-globin promoter an enhancer) (23). Previous characterization of peripheral blood in FVB.HOD mice revealed HOD expression on erythrocytes but not on platelets or leukocytes. For the current studies, the HOD transgene was backcrossed 10 generations onto a C57BL/6 background (B6.HOD), and tissue expression of HOD was characterized. Similar to what was observed on the FVB background, the HOD antigen is only detected on RBCs in peripheral blood, with no detectable HOD on WBCs or platelets (Figure 4.1a and b).

Spleen, kidney, draining lymph nodes, mesenteric lymph nodes, Peyer's patches, small and large intestine, heart, pleural viscera and peritoneal vicera were each harvested and subjected to immunofluorescence staining. C57BL/6 mice were used as negative controls, while mHEL mice that express HEL under the ubiquitous MHCI promoter were used as a positive control (29).

Secondary lymphoid organs were stained with anti-B220 and anti-Thy1.2 to delineate B cell and T cell zones, respectively. HOD expression was detected by indirect immunofluorescence with anti-HEL as the primary antibody. In 3 of 3 experimental B6.HOD mice, anti-HEL staining was equivalent to the wild-type B6 control, indicating no HOD expression was detected in any lymphatic tissue, including spleen, draining lymph nodes, mesenteric lymph nodes and Peyer's patches. This was not due to the inability to detect HEL expression by immunofluorescence, as staining of mHEL mice gave a strong signal in these tissues. (Figure 4.2, representative spleen). Anti-HEL staining of non-lymphatic HOD tissues did not detect expression above control B6 mice in any of the other organs analyzed. For the non-lympatic tissues of the mHEL mouse, only trace levels were detected in lung and liver and no staining was detected on other organs; however, the same conditions were used as gave strong signal on the mHEL lymphatic tissue.

To assess HOD antigen expression in bone marrow, femoral marrow was harvested and stained with reticulocyte marker anti-CD71 and erythrocyte-specific anti-TER119 to delineate the stages of erythrocyte maturation (Figure 4.3a). To analyze expression of the HOD antigen, cells were stained with two monoclonal antibodies

specific for different portions of HOD (MIMA-29 recognizes Duffy anti-HEL (clone 4B7) binds HEL; all samples were assessed by flow cytometry. In 3 of 3 experiments (5 mice/group/experiment), a subset of $CD71^{hi}TER119^{lo}$ (RI) proerythroblasts had low levels of detectable staining above control C57Bl/6 mice when incubated with MIMA-29. Most $CD71^{hi}TER119^{+}$ (RII) basophilic erythroblasts in B6.HOD mice had high levels of MIMA-29 expression, which persisted on $CD71^{-}TER119^{+}$ (RIV) mature erythrocytes (Figure 4.3b, middle and right). Expression of HEL, as determined by reactivity with 4B7, coincided with MIMA-29 expression in proerythroblasts (Figure 4.3c, left). HEL was expressed during each stage of erythrocyte development, but the intensity decreased at later stages of erythrocyte differentiation (Figure 4.3c, middle and right). Final expression in mature RBCs was higher than the latter stages of erythrocyte differentiation. No detectable signal with MIMA29 or anti-HEL was observed in erythroid cells from C57BL/6 mice at any stage of development. Taken together, these data demonstrate that the HOD antigen is expressed throughout multiple stages of erythropoiesis in B6.HOD bone marrow and on mature RBCs, but is not detectable on any other tissue analyzed.

B cell Tolerance to the HOD antigen is incomplete

We hypothesized that if B cell tolerance were incomplete, then providing HOD specific CD4+ T cell help would result in differentiation into plasma cells and anti-HEL antibody production. We have previously reported that OT-IIs enhance anti-HEL

antibody production in C57BL/6 mice transfused with HOD RBCs, presumably by recognizing $OVA_{323-339}$ presented by self $I\text{-}A^b$ (30). Therefore, any potential T cell tolerance was circumvented through adoptive transfer of 10 x 10^6 OT-II splenocytes into C57BL/6 and B6.HOD recipient mice. Sera was collected at 7 and 14 days post adoptive transfer and analyzed for anti-HEL antibody production by ELISA. In 3 of 3 experiments, B6.HOD mice that received OT-IIs made high levels of anti-HEL IgG compared to control B6 mice that had low or undetectable signal ($p<0.001$ at 1:50 dilution, Figure 4.4a).

To enumerate anti-HEL secreting plasma cells, HEL-specific ELISPOTs were performed on cells from the spleen, bone marrow and peritoneal cavity. B6.HOD mice that received OT-II splenocytes had 15-20 fold more HEL-specific antibody secreting cells (ASCs) in the spleen, compared to control B6 mice that received the same number of OT-II splenocytes (Figure 4.4b). Similarity, B6.HOD mice also had 5-10 fold more HEL-specific ASCs in the bone marrow, compared to B6 mice (Figure 4.4c). No HEL-specific ASCs were detected in the peritoneal cavity from either the B6 or B6.HOD mice (data not shown). In no case did either B6 or B6.HOD mice have significant levels of anti-HEL signal or significant numbers of HEL specific ASCs in the absence of adoptive transfer of OT-II cells.

To test the possibility that antibody production was due to adoptive transfer of naïve HEL-reactive B cells from donor OT-II mice, OT-II splenocytes from donors on a RAG1ko background were used, so as to prevent recombination of immunoglobulin genes in the OT-II donors. Because CD4+ T cells from OT-II/RAG1ko express non-

traditional T cell markers and develop in the absence of B cells, enrichment of CD4+ T cells from OT-II mice were also carried out by negative selection. Similar trends with slight differences in magnitude were observed using either OT-II/RAG1ko or enriched OT-II cells (Figure 4.5a and b, respectively). In aggregate, these data demonstrate that the anti-HEL IgG made in B6.HOD mice after OT-II adoptive transfer was not due to contaminating anti-HEL B cells from donor animals.

To assess the antigen-specificity of anti-HEL induction by OT-II transfer, LCMV-specific CD4+ T cells from SMARTA mice were adoptively transferred into C57BL/6 and B6.HOD mice. SMARTA mice are similar to OT-II mice, except that the recognized antigen is from a murine pathogen and is not contained in the HOD transgene. In 3 of 3 experiments no anti-HEL was induced by transfer of SMARTA CD4+ T cells as measured by ELISA ($p<0.001$ at 1:50 dilution, Figure 4.6a). Similarly, no HEL-specific ASCs above background were detected in the spleen or bone marrow, as determined by ELISPOT (Figure 4.6b). These data suggest that the anti-HEL IgG observed with OT-II adoptive transfer was not an artifact of an abundance of non-specific CD4 T cell help.

Although the SMARTA cells control for CD4+ T cell number, unlike the OT-II, their antigen is not present in the system and thus they do not control for CD4+ T cell activation. To test whether anti-HEL antibodies are generated non-specifically in response to bystander activation of CD4+ T cells, TCR75 mice were utilized. TCR75 mice are T cell transgenic mice with specificity for a peptide derived from Balb/c H-$2k^d$ MHC I presented in C57BL/6 I-A^b MHC II. B6 and B6.HOD mice were adoptively transferred with enriched CD4+ T cells from TCR75 splenocytes. To introduce the

antigen recognized by TCR75, mice were transfused with 100uL of packed Balb/c whole blood 24 hours after adoptive transfer. Sera was collected 7 and 14 days later. Adoptive transfer of TCR75 CD4+ T cells induced high levels of BALB/c reactive antibodies that were not detected in recipients of BALB/c whole blood only. Both B6 and B6.HOD mice that received TCR75 and BALB/c whole blood made similar amounts anti-Balb/c antibodies, as determined by flow crossmatch with Balb/c splenocytes targets (Figure 4.7a, representative mice). In contrast, neither group made anti-HEL antibodies (by ELISA) nor HOD reactive antibodies (by flow crossmatch with B6.HOD RBC targets) (Figure 4.7b and c, respectively). The absence of detectable anti-HOD antibodies by flow crossmatch was not due to the assay not working, as B6.HOD mice that received an adoptive transfer of CD4+ OT-II T cells make anti-HOD antibodies (Figure 4.7c, left). These antibodies were specific for the HOD antigen as they did not react with B6 RBCs (Figure 4.7c, right). Taken together, these data demonstrate that induction of anti-HEL antibodies by adoptive transfer of OT-II T cells is due to recognition of cognate antigen presentation, and not an artifact of either increased CD4+ T cell number or bystander activation.

T cell tolerance to the HOD antigen is functionally complete

Thus far, the data demonstrate that B cell tolerance to the HOD antigen is incomplete. In consideration of the lack of anti-HOD antibodies observed at baseline in B6.HOD mice, we hypothesized that T cell tolerance to epitopes within the HOD antigen is complete. To test this hypothesis, B6 and B6.HOD mice were immunized with OVA/CFA. Two weeks post immunization, mice were boosted with OVA/IFA and

serum was analyzed for anti-HEL and anti-OVA IgG production by ELISA and flow crossmatch. B6 and B6.HOD immunized mice made similar amounts of anti-OVA IgG, but no detectable anti-HEL IgG (Figure 4.8a). Likewise, B6 and B6.HOD mice made detectable antibodies to mOVA splenocytes, as determined by flow crossmatch (Figure 4.8b). In contrast, B6 but not B6.HOD mice made anti-HOD antibodies; antibodies detectable against the HOD antigen were directed to the OVA portion as no signal was observed with mHEL RBC targets (Figure 4.8b).

The spleen is not required for Induction of RBC specific autoantibodies by loss of T cell tolerance.

Surgical splenectomy is a treatment for AIHA that is refractory to pharmacological intervention. It is generally assumed that the efficacy is predominantly a function of diminishing the phagocytic capacity of the reticuloendothelial system. However, as patients remit, antibody titers often decrease. Thus, it is also important to understand the extent to which the spleen plays a role in initiating and/or maintaining autoantibody responses to RBC antigens. To test if a spleen is required for anti-RBC autoantibody induction upon loss of T cell tolerance, B6.HOD mice were surgically splenectomized, allowed to recover for two weeks, and then adoptively transferred with CD4+ enriched OT-II T cells. Although titers of anti-HEL IgG were lower in splenectomized mice compared to naïve animals, significant anti-HEL was nevertheless detected in 7/10 splenectomized animals (Figure 4.9, both experiments shown, n = 5 mice/group). This was a specific effect of introducing OT-II T cells, as no antibody was detected in control animals (splenectomized or naïve) that did not receive adoptive

transfer. The spleen contains several specialized compartments known to participate in clearance of and humoral response to circulating cells and particles. While these data suggest that the spleen may participate to some extent in the process of autoimmunization to a RBC antigen, they reject the hypothesis that a spleen is required.

Discussion

Autoreactive B cells that encounter their cognate antigen in the bone marrow undergo deletion, anergy or receptor editing as a consequence of establishment of central tolerance to self-antigen. Concurrent with B cell tolerization, erythrocytes undergo erythropoiesis in the bone marrow; RBC antigens are expressed on early erythrocyte precursors. Thus, through the establishment of central tolerance to self-antigens, we hypothesized that B cells developing in the bone marrow are tolerized to erythrocyte self-antigens.

To test this hypothesis, we utilized the HOD mouse that expresses an erythrocyte-specific model antigen consisting of HEL, OVA and blood group antigen, Duffy. Any potential T cell tolerance to erythrocyte antigens was circumvented by adoptive transfer of OTII/Thy1.1 T cells; OTII/Thy1.1 T cells recognize a portion of OVA contained within the HOD antigen. As such, adoptive transfer induced anti-HOD autoantibody production. Autoantibody generation was not an artifact of excess CD4+ T cell numbers or bystander activation. Further, B cell activation did not require the presence of a spleen. Taken together, contrary to what would be predicted as a consequence of central tolerance, autoreactive B cells specific for erythrocyte self-antigens is not complete. Additionally, these data demonstrate that autoreactive B cells encounter and present self-antigen; and upon CD4+ T cell help are driven to differentiate and secrete autoantibodies, suggesting that in this model autoreactive B cells are not anergic or ignorant.

There are at least two distinct non-exclusive hypotheses that can explain these data. One hypothesis supposes that central tolerance mechanisms fail in establishing tolerance to erythrocyte-specific antigens. The data demonstrate that in the B6.HOD mouse, HOD-specific B cells are present and can secrete antibodies that can bind native HOD antigen. Thus, the known forms of central tolerance (deletion, anergy and receptor editing) did not affect erythrocyte-specific autoreactive B cells. The other hypothesis is that the autoreactive B cells develop extramedullary (outside of the bone marrow) and are exempt from central tolerance mechanisms. It has been well documented that B-1 B cells have extramedullary origin, express low-affinity self-reactive BCRs and do not require T cell help to secrete antibodies. However, we have no data to support or refute that B-1 B cells are the source of autoantibodies in the B6.HOD mouse.

An alternative interpretation of these data is that developing B cells do not encounter the erythrocyte-specific HOD antigen. The HOD antigen is expressed on early erythrocyte precursors, thus, it is likely that developing B cells encounter the HOD antigen. However, it is also plausible that precursors for B cells and erythrocytes are sequestered from each other in the bone marrow. However, data from Okamoto et al. demonstrate that deletion of B cells in the bone marrow is a consequence of cognate antigen recognition on RBCs. As such, it is likely that B cells encounter RBC antigens.

In our attempt to model blood group antigen Rh D, the HOD antigen was expressed under an RBC-specific promoter. Staining peripheral white blood cells demonstrate that the HEL protein of the HOD antigen cannot be detected on platelets or leukocytes. However, we cannot rule out that trace levels of the HOD antigen are

expressed on non-lymphatic tissues. Tissues from mHEL mice were used as a positive control for HEL-staining and immunofluorescence imaging. In contrast to the bright staining observed with lymphatic tissue, there was no detectable expression of HEL above background in non-lymphatic tissues other than lung and liver. The lack of detectable HEL expression on non-lymphatic tissue from mHEL mice might be due to low levels of MHC I expression on epithelial and skeletal muscle cells (the predominate cell types in the tissues analyzed). As such, neither lymphatic nor non-lymphatic tissue stained above background for expression of HEL in the B6.HOD mouse. However, regardless of whether trace amounts of HEL are present on other tissues (which would be more consistent with expression patterns of other RBC antigens), our data clearly demonstrate that antigen expression on RBCs is not alone sufficient to tolerize B cells to erythrocyte self-antigens.

While the data presented herein demonstrate that anti-HEL B cells are clearly present, this doesn't directly address CD4+ T cell tolerance. However, the fact that B cells make anti-HEL IgG upon CD4+ T cell help (e.g. OT-II), that OVA/CFA induces OVA specific CD4+ T cells in wild-type mice, and that OVA/CFA does not induce anti-HEL in HOD mice, gives a strong suggestion that CD4+ T cell tolerance is complete in HOD mice. Whether CD4+ T cells are deleted, anergized, or present but overcome by regulatory T cells is not clear; however, the data do suggest that functional CD4+ T cell tolerance is present.

Individuals who suffer from AIHA that cannot be managed with immunesuppression can have a splenectomy as an effort to decrease the occurrence of

new antibodies forming. However, our data demonstrate that a splenectomy is not required for initiation of autoantibody production. The levels of autoantibody formation is significantly decreased in splenectomized mice, but not completely inhibited. The implications of this finding are that autoreactive B cells might not be localized to the spleen and/or do not require the spleen for survival. Interestingly, this form of therapy might not outweigh the risk of being susceptible to capsulated bacteria, as there is no prevention of autoantibody formation.

In aggregate, these data demonstrate that B cell tolerance to RBC-specific antigens is incomplete. The autoreactive B cells that escape tolerance mechanisms are not ignorant to self-antigen as providing T cell help initiates autoantibody production. These data beg to question,\ whether B cell tolerance to hematopoietic-specific antigens is similarly incomplete. As such, these findings could apply to platelet antigens as well and could serve as an autoreactive B cell pool for the initiation of ITP. The breakdown of T cell tolerance might occur through molecular mimicry and/or linked recognition of non-exofacial polymorphisms (mostly expressed in the transmembrane region or intracellularly of RBCs). As such, exposure to pathogens or alloantigens through transfusions and transplantations could induce T cells that crossreact with a linear peptide contained within the NEP. Upon infection, T cells expand, and B cells that have processed and presented self-RBC antigens receive T cell help because of presentation of the NEP peptide and secrete autoantibodies. Through linked recognition of antigens, autoantibodies can form against many RBC antigens. Taken together, there are many opportunities for T cell tolerance to be compromised thus leading to autoimmunity.

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Figure Legends

Figure 4.1: HOD antigen is restricted to expression on erythrocytes in peripheral blood. Peripheral blood was collected from C57Bl/6, mHEL and B6.HOD. (A) HOD antigen expression on RBCs was detected by indirect immunofluorescence with anti-MIMA-29, anti-HEL or anti-OVA as the primary antibody. Peripheral white blood cells were stained with anti-HEL and (B, left) anti-CD41 or (B, right) anti-CD45 for detection of HOD expression on platelets or leukocytes, respectively.

Figure 4.2: HEL is not expressed on lymphatic tissue in B6.HOD mice. Spleens from C57Bl/6, mHEL and B6.HOD were snap-frozen and sectioned for immunofluorescence. Tissue was stained with antibodies against Thy1.2, B220 and HEL to detect expression of the HOD antigen.

Figure 4.3: HOD antigen is expressed on early precursors of erythrocytes. Bone marrow was harvested and stained with (A) anti-CD71, anti-TER119 to delineate stages of RBC development and either (B) anti-HEL monoclonal antibody 4B7 or (C) Duffy-reactive MIMA-29.

Figure 4.4: B6.HOD mice make anti-HEL when T cell tolerance is circumvented (given OTIIs). B6 and B6.HOD mice were adoptively transferred with 10x106 OTII splenocytes. Two weeks post adoptive transfer, (A) sera was analyzed for anti-HEL IgG by ELISA and (B) anti-HEL IgG antibody secreting cells were quantified in the spleen and (C) bone marrow by ELISPOT.

Figure 4.5: Anti-HEL Autoantibodies are not due to contaminating donor HEL-specific B cells. B6 and B6.HOD mice were adoptively transferred with (A) splenocytes from OTII/RAGko mice or (B) enriched CD4+ T cells from OT-II splenocytes. Sera was collected at 7 and 14 days post adoptive transfer and analyzed for anti-HEL IgG by ELISA.

Figure 4.6: Anti-HEL IgG autoantibody production is not an artifact of excess CD4+ T cell numbers. Enriched CD4+ T cells from SMARTA mice with specificity for an irrelevant antigen or OT-II mice were adoptively transferred into B6 and B6.HOD mice. (A) Sera was collected 7 and 14 days post adoptive transfer and analyzed for anti-HEL IgG by ELISA. (B) Anti-HEL IgG antibody secreting cells from splenocytes were quantified by ELISPOT

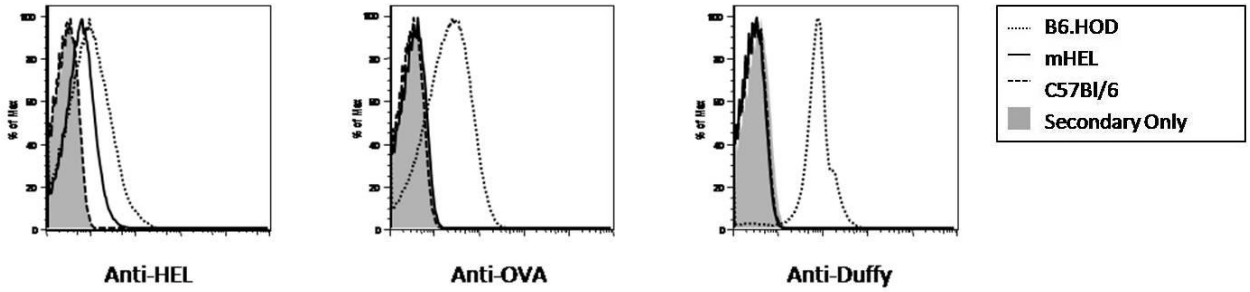
Figure 4.7: Activated CD4+ T cells cannot elicit anti-HEL IgG autoantibodies in B6.HOD mice. Enriched CD4+ T cells from splenocytes of TCR75 or OT-II mice were adoptively transferred into B6 and B6.HOD mice. 24 hours post adoptive transfer, recipient mice that received TCR75 CD4+ T cells were transfused with Balb/c whole blood. One group of B6 mice received Balb/c whole blood in the absence of TCR75 T cells. Sera was taken at 14 days post adoptive transfer and flow crossmatched with targets (A) Balb/c splenocytes, (B, left) B6.HOD RBCs and (B, right) B6 RBCs. Antibodies that reacted with antigens on target cells were visualized by anti-mouse immunoglobulins conjugated to APC.

Figure 4.8: T cell tolerance in B6.HOD mice is functionally complete. B6 and B6.HOD mice were immunized with OVA/CFA followed by an OVA/IFA boost. Sera was collected 7 days post immunization and analyzed for (A) anti-OVA IgG by ELISA and (B) anti-HEL, anti-HOD and anti-OVA antibodies by flow crossmatch with mHEL RBCs, B6.HOD RBCs and mOVA splenocytes, respectively.

Figure 4.9: The spleen is not required for induction of RBC specific autoantibody production. B6 and B6.HOD mice were splenectomized or unmanipulated. All groups received an adoptive transfer of enriched CD4+ OT-II T cells. Sera was collected at 7 and 14 days post adoptive transfer and analyzed for anti-HEL IgG by ELISA.

Figure 4.1

A


B6.HOD
mHEL
C57Bl/6
Secondary Only
Anti-HEL
Anti-OVA
Anti-Duffy

B

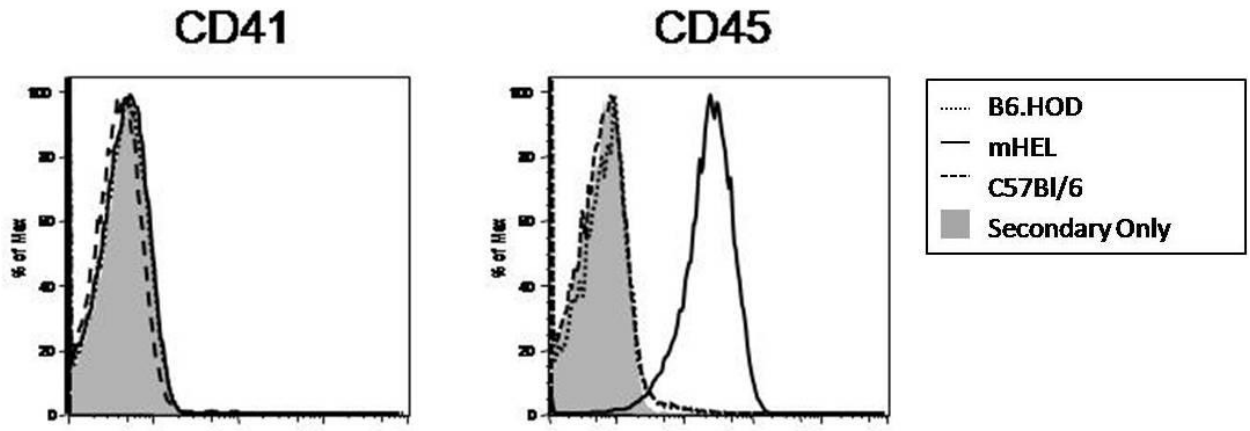

CD41
CD45
B6.HOD
mHEL
C57Bl/6
Secondary Only

Figure 4.2

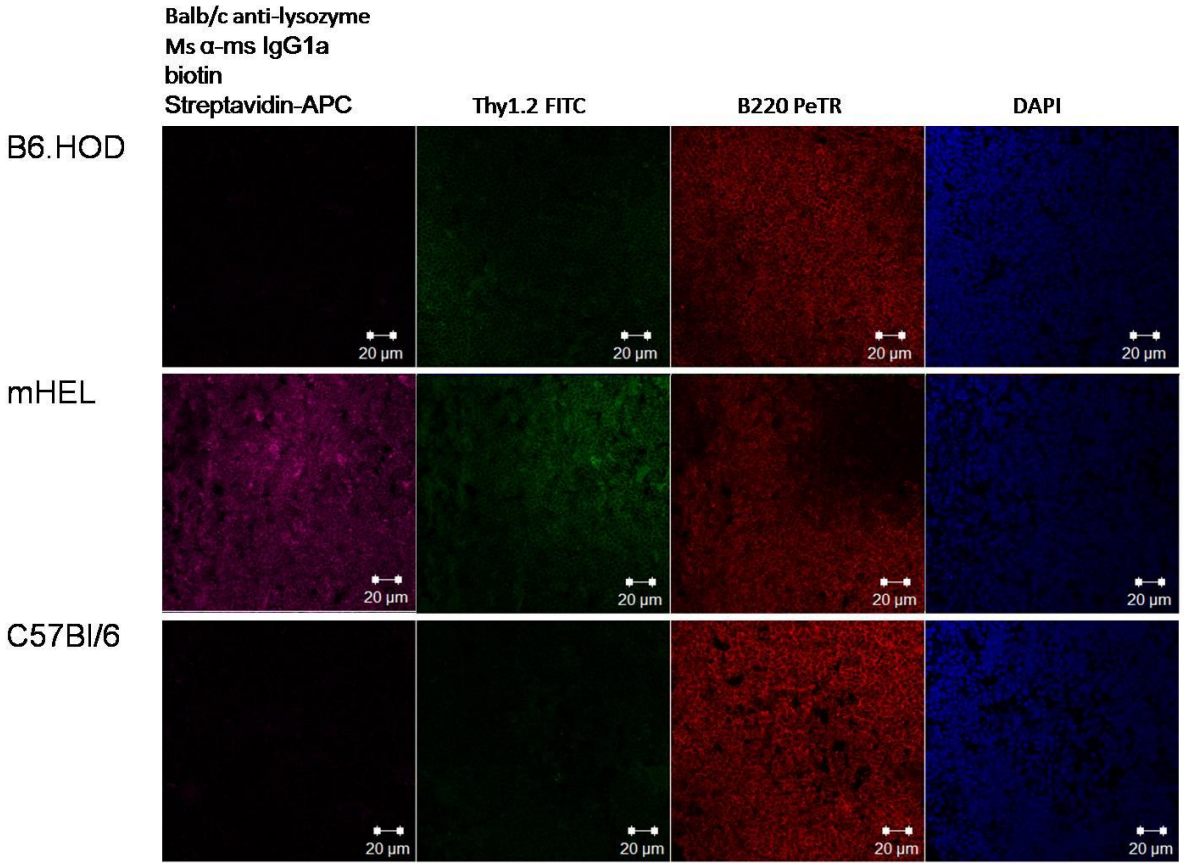
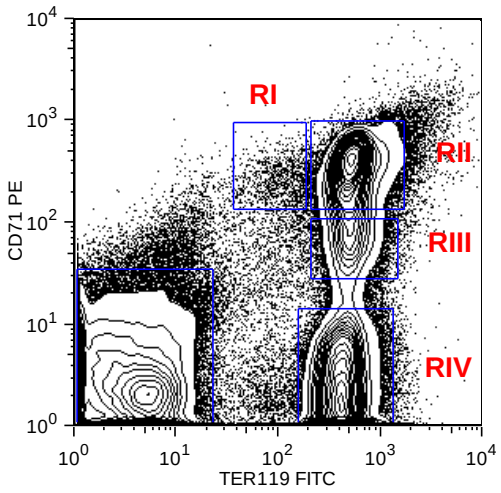

Balb/c anti-lysozyme
Ms α-ms IgG1a
biotin
Streptavidin-APC
Thy1.2 FITC
B220 PeTR
DAPI
B6.HOD
mHEL
C57Bl/6
20 µm

Figure 4.3

A


RI
RII
RIII
RIV
CD71 PE
TER119 FITC
10^0
10^1
10^2
10^3
10^4

B

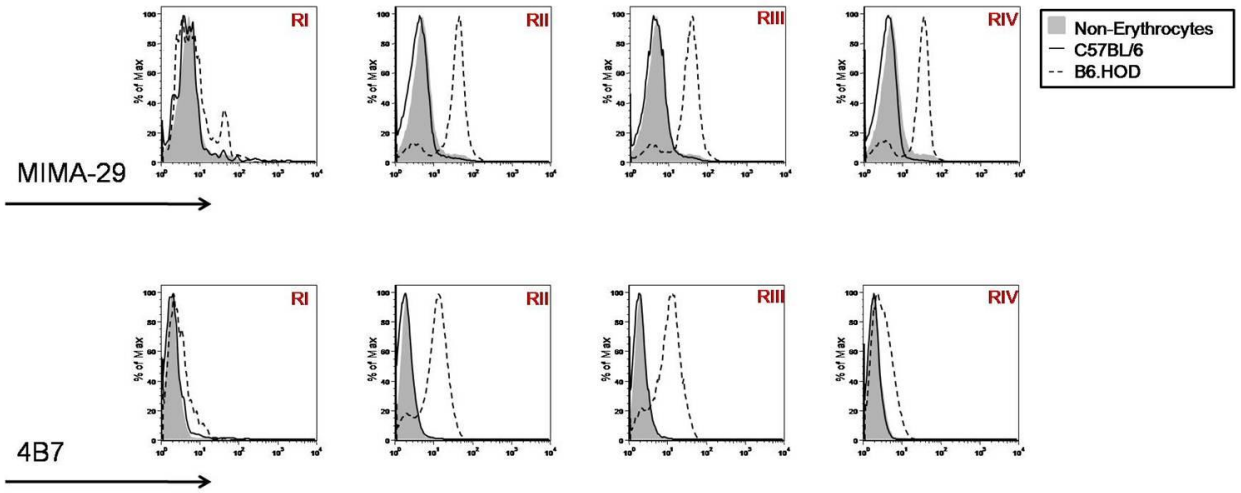
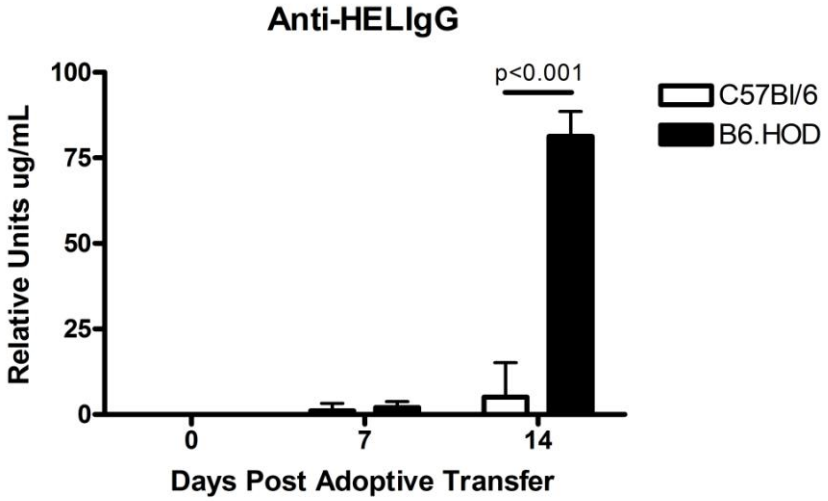

RI
RII
RIII
RIV
% of Max
MIMA-29
4B7
Non-Erythrocytes
C57BL/6
B6.HOD

Figure 4.4

A


Anti-HELIgG
p<0.001
C57Bl/6
B6.HOD
Relative Units ug/mL
100
75
50
25
0
0
7
14
Days Post Adoptive Transfer

B

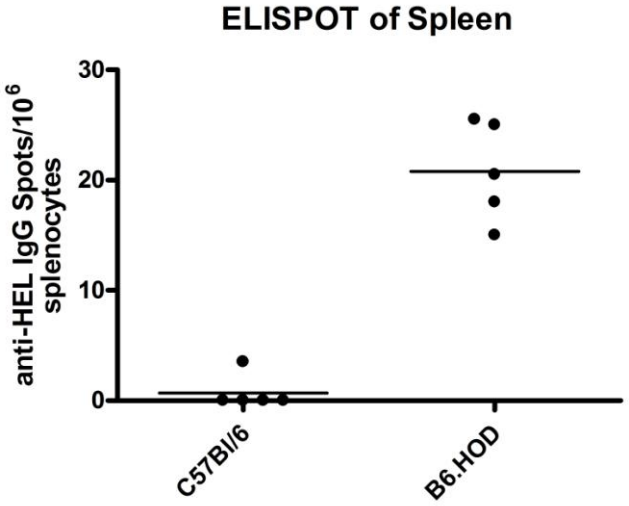

ELISPOT of Spleen
anti-HEL IgG Spots/10^6 splenocytes
30
20
10
0
C57Bl/6
B6.HOD

Figure 4.4

C

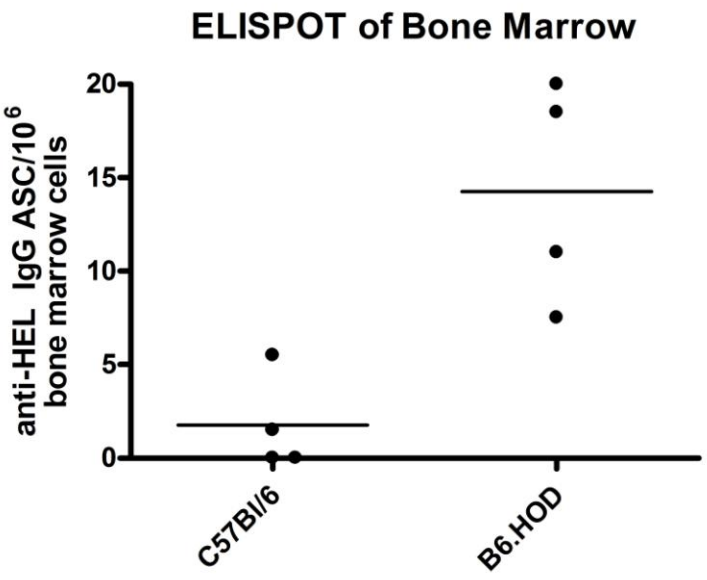
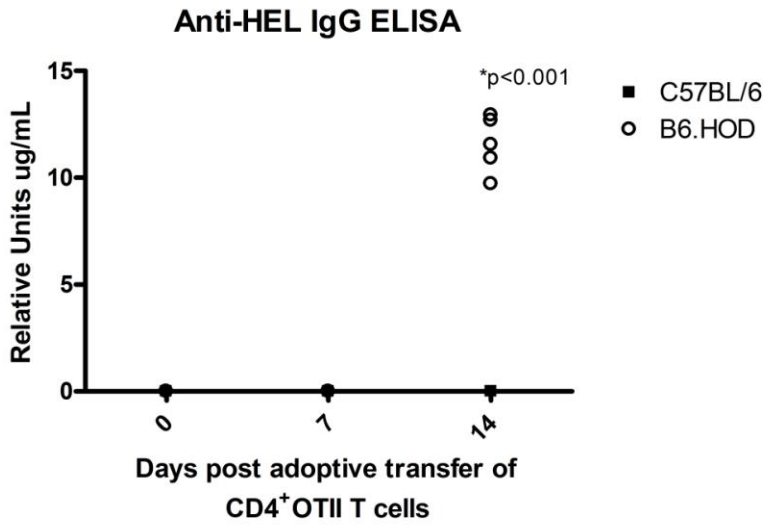

ELISPOT of Bone Marrow
anti-HEL IgG ASC/10^6 bone marrow cells
20
15
10
5
0
C57Bl/6
B6.HOD

Figure 4.5

A


Anti-HEL IgG ELISA
*p<0.001
C57BL/6
B6.HOD
Relative Units ug/mL
15
10
5
0
0
7
14
Days post adoptive transfer of
CD4+OTII T cells

B

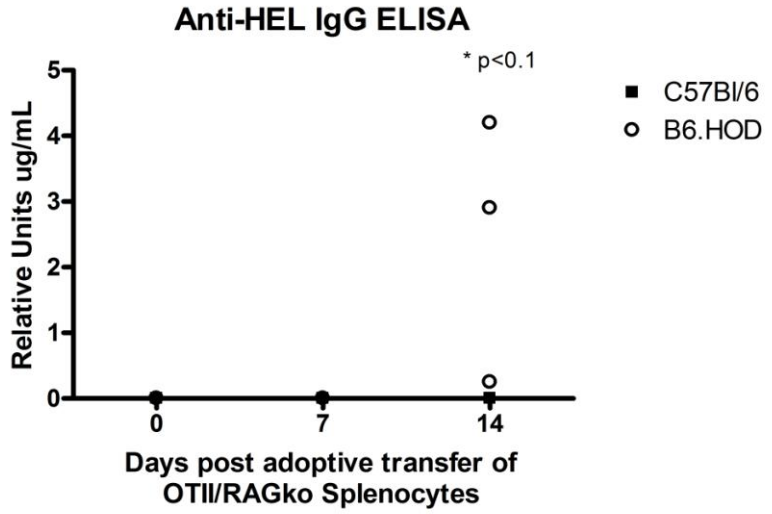
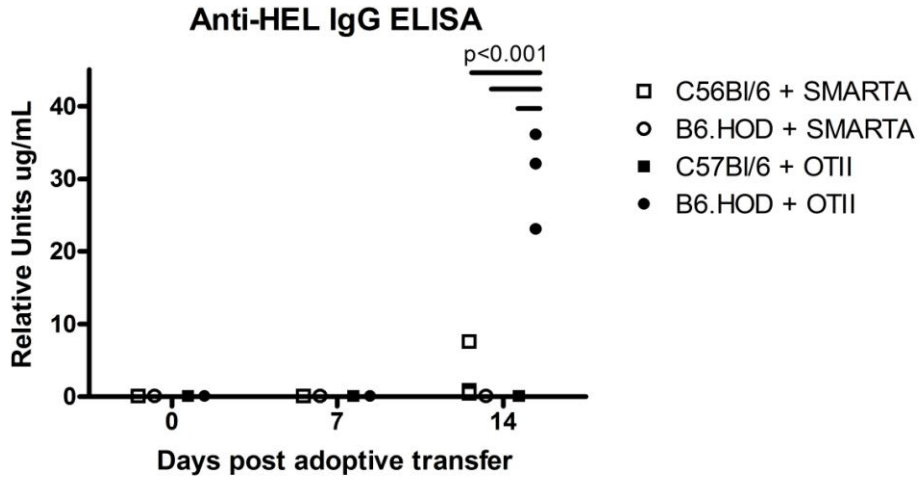

Anti-HEL IgG ELISA
* p<0.1
C57Bl/6
B6.HOD
Relative Units ug/mL
5
4
3
2
1
0
0
7
14
Days post adoptive transfer of
OTII/RAGko Splenocytes

Figure 4.6

A


Anti-HEL IgG ELISA
p<0.001
Relative Units ug/mL
40
30
20
10
0
0
7
14
Days post adoptive transfer
C56Bl/6 + SMARTA
B6.HOD + SMARTA
C57Bl/6 + OTII
B6.HOD + OTII

B

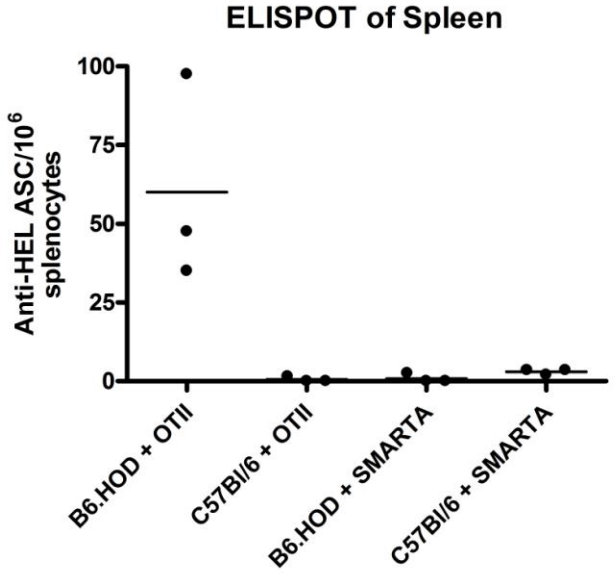
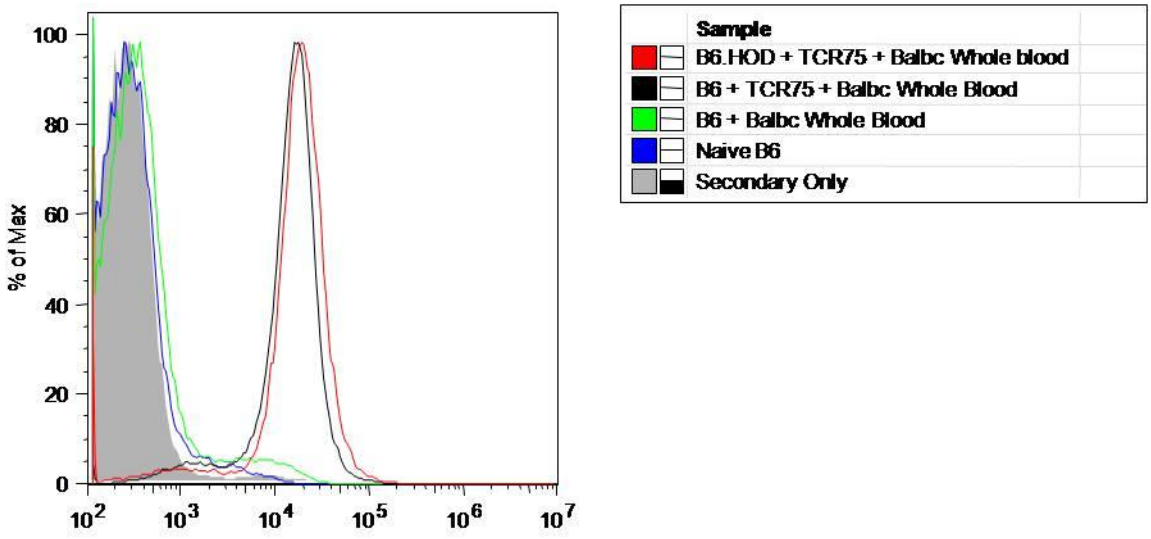
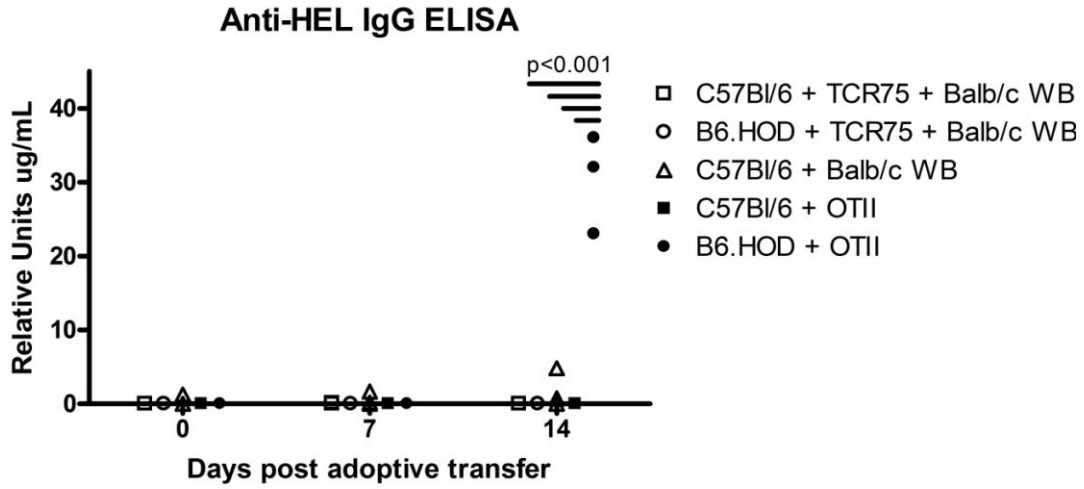

ELISPOT of Spleen
Anti-HEL ASC/10^6 splenocytes
100
75
50
25
0
B6.HOD + OTII
C57Bl/6 + OTII
B6.HOD + SMARTA
C57Bl/6 + SMARTA

Figure 4.7

A


Sample
B6.HOD + TCR75 + Balbc Whole blood
B6 + TCR75 + Balbc Whole Blood
B6 + Balbc Whole Blood
Naive B6
Secondary Only
% of Max
100
80
60
40
20
0
10^2
10^3
10^4
10^5
10^6
10^7

B


Anti-HEL IgG ELISA
p<0.001
C57Bl/6 + TCR75 + Balb/c WB
B6.HOD + TCR75 + Balb/c WB
C57Bl/6 + Balb/c WB
C57Bl/6 + OTII
B6.HOD + OTII
Relative Units ug/mL
40
30
20
10
0
0
7
14
Days post adoptive transfer

C

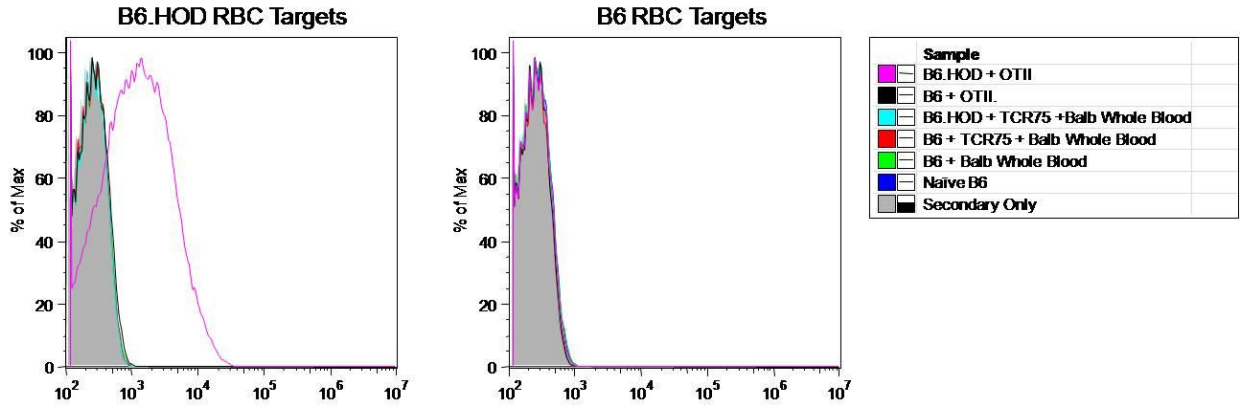
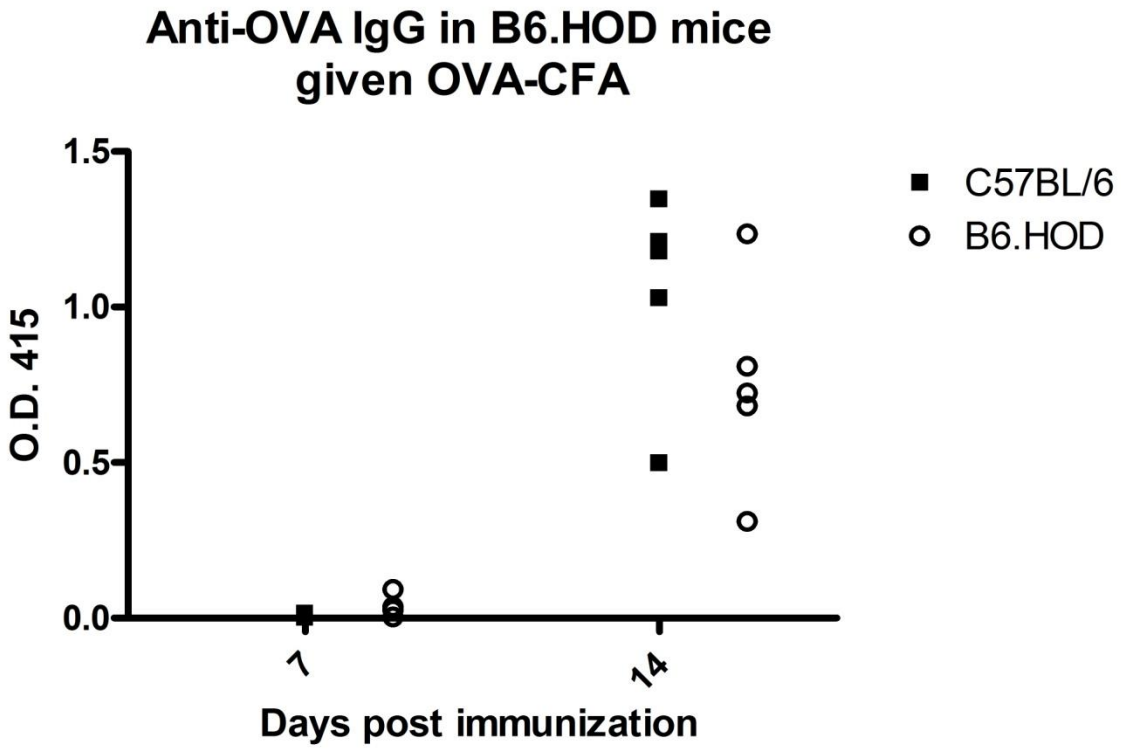

B6.HOD RBC Targets
B6 RBC Targets
% of Max
100
80
60
40
20
0
10^2
10^3
10^4
10^5
10^6
10^7
Sample
B6.HOD + OTII
B6 + OTII.
B6.HOD + TCR75 +Balb Whole Blood
B6 + TCR75 + Balb Whole Blood
B6 + Balb Whole Blood
Naive B6
Secondary Only

Figure 4.8

A


Anti-OVA IgG in B6.HOD mice given OVA-CFA
O.D. 415
1.5
1.0
0.5
0.0
7
14
Days post immunization
C57BL/6
B6.HOD

B

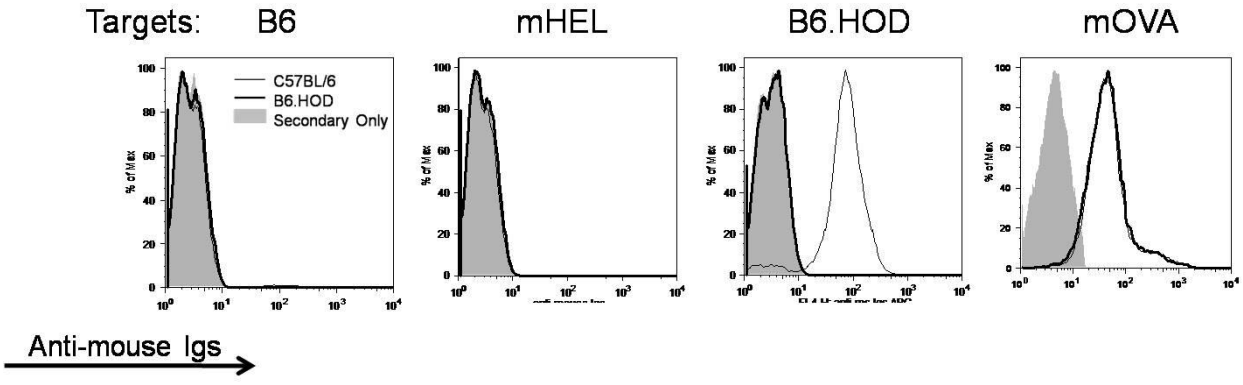
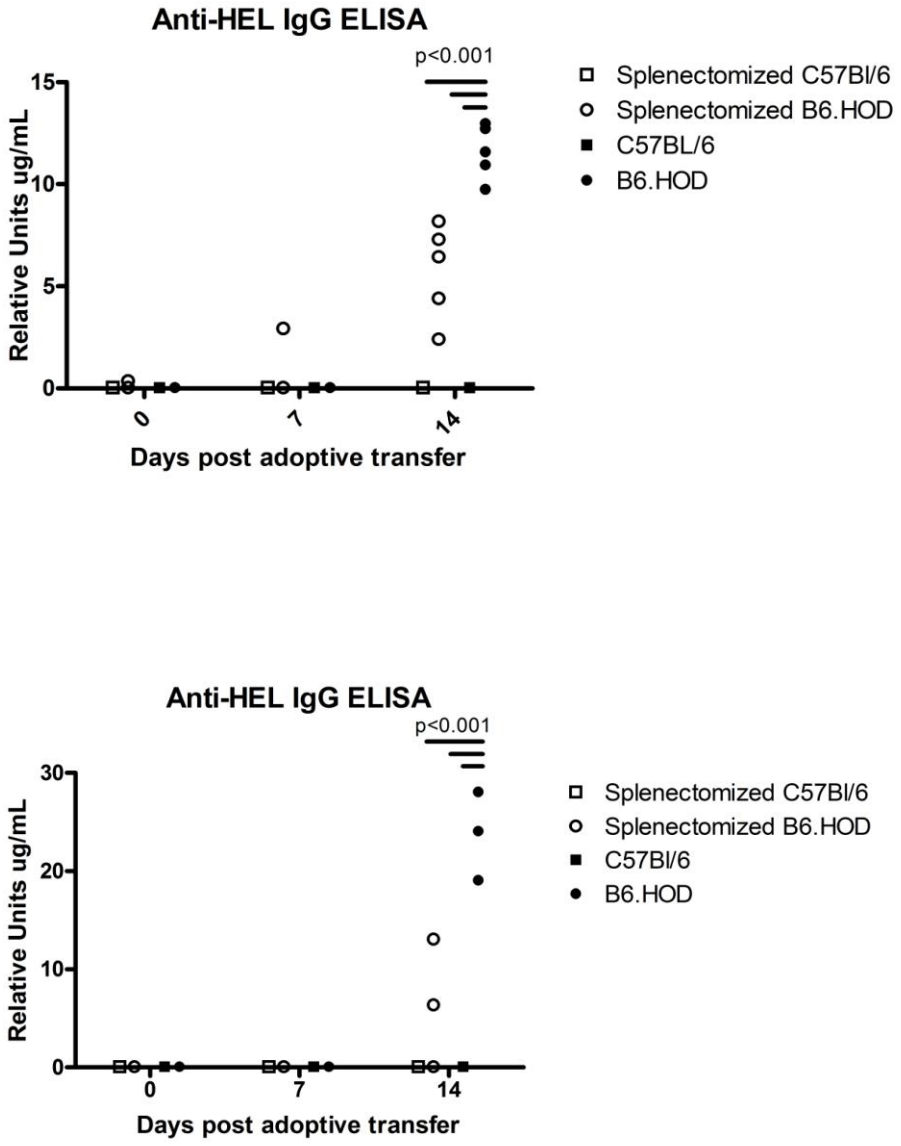

Targets:
B6
mHEL
B6.HOD
mOVA
C57BL/6
B6.HOD
Secondary Only
% of Max
Anti-mouse Igs

Figure 4.9


Anti-HEL IgG ELISA
p<0.001
Relative Units ug/mL
15
10
5
0
0
7
14
Days post adoptive transfer
Splenectomized C57Bl/6
Splenectomized B6.HOD
C57BL/6
B6.HOD
Anti-HEL IgG ELISA
p<0.001
Relative Units ug/mL
30
20
10
0
0
7
14
Days post adoptive transfer
Splenectomized C57Bl/6
Splenectomized B6.HOD
C57Bl/6
B6.HOD

Chapter 5

Discussion

Summary

Red blood cells (RBCs) differentiate from hematopoietic stem cells in the bone marrow; they are essential for life. The primary function of RBCs is to deliver oxygen to tissues and vital organs (1). To achieve this, RBCs contain hemoglobin, an iron bound protein with a high affinity for oxygen. Additionally, RBCs express over 29 blood groups containing over 600 antigens (2). Blood group molecules are necessary to maintain the structure and function of RBCs (3, 4). However, individuals express polymorphisms or have null and/or partial phenotypes of blood group antigens that can serve as a target for alloantibodies in response to exposure (e.g. transfusion) (5).

Humoral alloimmunization to erythrocyte antigens is a clinically significant problem. The presence of alloantibodies can lead to hemolytic transfusion reactions, pose a barrier to transplantation and can make finding future compatible RBC units for transfusion difficult (6-8). Alloantibodies can form as a consequence of transfusion, pregnancy or transplantation (9, 10). Rates of alloimmunization vary depending on the RBC antigen and frequency of exposure (i.e. number of transfusions). In addition, animal data suggest that the inflammatory status of the individual may play a role (11). However, in cohorts of multiply transfused patients, most individuals do not make alloantibodies (12-14). Understanding the mechanisms underlying why some individuals but not others make alloantibodies is complex. Genetics (e.g. HLA type) and environmental factors such as inflammation can influence whether an individual becomes alloimmunized (15-17).

The contribution of molecular mimicry to alloimmunization has been posited for many years. However, the realized contribution has never been fully evaluated. In an effort to avoid incompatible transfusion, prior to transfusion or transplantation, recipient serum is crossmatched against RBC antigens to determine the presence of allo- and/or auto-antibodies. One of the limitations to this approach is the inability to detect microbial-elicited $CD4^+$ T cells that could crossreact with RBC antigens presented on MHCII, and thus prime for humoral alloimmunization upon exposure. Therefore, molecular mimicry of B cells would be detected with current blood bank technology due to crossreactive antibodies. In contrast, T cells that recognize both microbial peptides and RBC antigens, but not generate antibodies until exposure, are undetectable with current methodology.

Thus, we developed a murine model to formally test whether molecular mimicry of $CD4^+$ T cell epitopes could prime for humoral alloimmunization. Data from these studies demonstrate that prior pathogen exposure enhances humoral alloimmunization upon transfusion through $CD4^+$ T cell molecular mimicry. The observed alloimmunization was dependent on the presence of the $CD4^+$ T cell epitope within the pathogen and RBC antigen, as removal ablates the enhanced alloantibody response. Both bystander activation and inflammation have been implicated in regulating alloimmune responses. However, in this model, neither was alone sufficient to modulate humoral alloimmunization.

In aggregate, these data demonstrate that an antecedent pathogen exposure can enhance alloimmunization. The current findings provide additional insight to factors that

may regulate alloimmunization. Thus, a patient may be primed to respond to an RBC antigen through prior exposure to a microbial infection with a shared $CD4^+$ T cell epitope, yet this initial priming would go undetected by current serology-based blood banking methodologies. These findings may extend beyond RBC transfusions and have relevancy to both platelet and HLA antigens.

Central and peripheral tolerance mechanisms are in place to prevent autoimmune pathology. However, despite attempts to prevent and/or control autoreactive lymphocytes, nearly 3-5% of individuals suffer from autoimmunity (18). Through the use of B cell receptor (BCR) transgenic mouse models, mechanisms of B cell tolerance have been described; autoreactive B cells developing in the bone marrow undergo deletion, anergy or BCR editing (to decrease the affinity for self-antigen) (19-21). Additionally, through the use of tissue-specific expression of autoantigen in combination with BCR transgenic mice, peripheral tolerance in the mature B cell repertoire has been described (22).

Patterns of autoantigen expression continually shape the mature B cell repertoire. Through a process called erythropoiesis, RBCs differentiate from precursors in the bone marrow, concurrent with B cell development. Expression of RBC antigens can be detected on early precursors (23, 24). Thus, it is predicted that B cells would be tolerized to RBC antigens. Furthermore, with the exception of the Rhesus blood group antigen D (Rh D), most RBC antigens are expressed on non-hematopoietic tissues (10, 25, 26). As such, peripheral expression of RBC antigens is predicted to add another layer of protection through peripheral tolerance mechanisms.

Contrary to what is predicted, loss of tolerance to RBC antigens occurs at a frequency of 1/1000 individuals (5). Whereas this failure in tolerance does not always lead to autoimmune pathology, the presence of autoantibodies demonstrates that tolerance to RBC antigens is not complete (27, 28). Interestingly, most autoantibodies are against Rh D, the only antigen with RBC-specific expression. Additionally, anti-D antibodies are clinically relevant and initiate RBC hemolysis (29, 30).

To further understand how RBC antigens influence the B cell repertoire, we generated a murine model that utilized an RBC-specific antigen, HOD (comprised of a triple fusion protein consisting of hen egg lysozyme, ovalbumin and the human blood group antigen, Duffy) (31). The F1 of HOD and IgHEL mice (a BCR transgenic with specificity for HEL) was used to assess patterns of deletion and/or enrichment of B cell subsets (20). Our data demonstrate that conventional B-2 B cells are decreased in the bone marrow and secondary lymphoid organs. In contrast, there was a relative enrichment of B-1 B cells in the peritoneal cavity. The increase of B-1 B cells correlated with an increase of anti-HEL IgM autoantibodies and anti-HEL antibody secreting cells.

Implications of the data generated with the HODxIgHEL model system must be carefully considered due to the many caveats with BCR transgenic mice. Thus, while the use of BCR transgenics have provided great insight to the mechanisms of tolerance through the ease of tracking autoreactive B cells, these models suffer a high precursor frequency of autoreactive B cells expressing an affinity matured BCR. As such, the efficiency of central tolerance mechanisms might be compromised by not only increasing the number of B cells that need to be tolerized to self-antigens, but also possibly

influencing the make-up of the mature B cell repertoire by altering the signaling thresholds.

To evaluate whether the observed patterns of deletion in the HODxIgHEL mice were recapitulated in the absence of BCR transgenics, central tolerance was assessed in B6.HOD mice. The HOD antigen contains epitopes that can be recognized by T cells. As such, any potential T cell tolerance was circumvented by adoptive transfer of $CD4^+$ T cells into B6.HOD mice. Data from this model demonstrate B cell tolerance to RBC-specific antigens is incomplete whereas T cell tolerance is functionally absent.

Taken together, our data define the nature of erythrocyte antigens from the standpoint of baseline tolerance mechanisms. Using two separate models (HODxIgHEL and B6.HOD), we demonstrate that B cell tolerance to RBC-restricted antigens is incomplete. Further, we document that these autoreactive B cells, upon antigen encounter, are capable of being functional thus differentiating into antibody secreting cells. These findings suggest that autoreactive B cells persist despite central and peripheral tolerance mechanisms; in the event of molecular mimicry or thymic dysregulation, tolerance can be broken thereby resulting in autoimmunity.

Discussion

Microbial Infection Can Enhance Alloimmunization to RBC Antigens

In Chapter 2, we demonstrated that molecular mimicry of $CD4^+$ T cell epitopes shared between a microbe and a red blood cell (RBC) antigen can enhance alloimmunization. Studies were performed with polyomavirus engineered to express a known $CD4^+$ T cell epitope from ovalbumin (OVA), $OVA_{323-339}$ (PyV.OVA-II), and RBCs expressing the HOD antigen (an RBC-specific triple fusion protein comprised of hen egg lysoszyme (HEL), OVA and human blood group antigen, Duffy), which also contains $OVA_{323-339}$ (31). Mice infected with PyV.OVA-II followed by transfusion with HOD RBCs made significantly more anti-HEL IgG than mice that received an infection or transfusion alone. The enhancement of alloimmunization was contingent on the microbe and the RBC antigen sharing $CD4^+$ T cell epitopes, as infection with wild-type polymomavirus (PyV.WT, without the $OVA_{323-339}$ epitope) did not significantly enhance alloimmunization to HOD transfusion. Likewise, infection with PyV.OVA-II did not enhance anti-HEL antibodies in mice transfused with mHEL RBCs (thus lacking the $OVA_{323-339}$ epitope) (20).

Aside from molecular mimicry, bystander activation has been implicated in propagating antibody formation (32, 33). We previously demonstrated that inflammation enhances alloantibodies against transfused RBCs (11). Thus, in this model, we evaluated the contribution of bystander activation through use of PyV.WT. If bystander activation was alone responsible for enhanced alloimmunization, then we would predict that

infection with PyV.WT followed by mHEL or HOD RBC transfusion would result in significant production of anti-HEL IgG. In contrast to our predictions, infection with PyV.WT followed by mHEL or HOD RBC transfusion did not result in significant anti-HEL IgG production, compared to transfusion alone. This observation was not due to non-infectious viral stock as viral titers were comparable between PyV.WT and PyV.OVA-II. Additionally, PyV.WT infection resulted in a robust $CD8^+$ T cell response with production of interferon gamma, which indicated that the immune system responded to the virus. Taken together, the enhanced alloimmunization to the HOD antigen was not a consequence of general inflammation. These data further support the hypothesis that enhanced alloimmunization to RBC antigens is dependent on sharing the CD4+ T cell epitope with the microbe.

We cannot rule out that the enhanced alloimmunization relies on the conservation of sequence between the PyV.OVA-II and HOD epitope; the virus and the RBC antigen contain an identical sequence of peptides from OVA. It is plausible that altering the sequence such that it is not identical would alter the magnitude of alloimmunization. Changing or mutating peptides recognized by T cells can affect the affinity and efficiency with which the epitope can be presented in MHC or the response by the T cell (e.g. induction of anergy or increasing affinity) (34, 35). However, in our BLAST homology searches (see Table 2.1), there were microbes identified that shared a high degree of sequence homology with RBC antigens; thus, the modeled situation is not completely theoretical.

Throughout these experiments we focused on the production of anti-HEL IgG. The HOD antigen also contains OVA epitopes, yet no anti-OVA IgG was detected in mice that received a HOD RBC transfusion in the absence or presence of polyomaviurs infection. The working hypothesis predicts that that upon PyV.OVA-II infection, CD4+ T cells specific for $OVA_{323-339}$ are generated. Following HOD RBC transfusion, HEL-specific B cells present HOD antigens (both HEL and OVA peptides) on MHC II; preformed $OVA_{323-339}$-specific CD4+ T cells give help to HEL-specific B cells and elicit antibody production. As such, it is likely that OVA-specific B cells would receive the same $OVA_{323-339}$-specific $CD4^+$ T cell help. However, due to the lack of detectable anti-OVA antibodies, we hypothesize that the OVA-specific B cells are absent, anergic or in a compartment sequestered away from CD4+ T cells. Alternatively, the binding of the anti-OVA BCR to the HOD antigen may alter the pattern of peptide generation such that OVA 323-339 is not presented.

We can rule out the hypothesis that OVA-specific B cells are absent because immunization of C57BL/6 mice with OVA-CFA results in anti-OVA IgG, which can recognize HOD RBCs. We also reject the hypothesis that OVA-specific B ells are anergic. There is evidence to suggest that anergic B cells can be activated with sufficient stimulation (36). Akin to CFA, polyomavirus infection induces a robust cytokine response (37). Additionally, polyomavirus is a chronic infection thereby constantly eliciting an immune response (38). Therefore, we predict that polyomavirus infection would reverse any potentially anergic B cells. However, we cannot rule out the hypothesis that OVA-specific B cells were anatomically sequestered from OVA-specific

T cells. OVA-CFA immunization was administered subcutaneously whereas polyomavirus infection was given in the footpad. Thus, the target compartment for the resultant primary immune response is different; one route might lead to activation of OVA-specific B cells while the other may not. However, both CFA and polyomavirus infection become systemic; thus it is likely that both stimuli activate B cells in similar compartments. Taken together, the lack of detectable anti-OVA IgG was not due to absence or anergy of OVA-specific B cells. It is equally unlikely that compartmentalization of OVA-specific B cells was responsible for the lack of detectable anti-OVA IgG.

The Use of BCR Transgenics to Understand B cell Tolerance to Erythrocyte Antigens

In Chapter 3, we demonstrated that B cell tolerance to antigens restricted to expression on erythrocytes is neither equal between B cell subtypes nor anatomical compartments. Through the use of a novel model of erythrocyte autoreactivity (the F1 of FVB.HOD and IgHEL mice), we observed a 2-10 fold decrease of autoreactive B cells (specific for HEL) in the bone marrow and secondary lymphoid organs of double transgenic autoimmune mice. In contrast, there was an enrichment of B-1 B cells in the peritoneal cavity, which correlated to an increase in anti-IgM antibodies and antibody secreting cells (ASCs). B cell enrichment was only observed in the peritoneal cavity and only with B-1 B cells.

B cells are tolerized only to antigens which they encounter during development or in the periphery. Conventional B cells differentiate from hematopoietic stem cells in the

bone marrow. During development in the bone marrow, B cells interact with self antigen. If a B cell receptor (BCR) reacts strongly with self-antigen, the B cell is susceptible to central tolerance mechanisms including deletion, anergy and receptor editing of the BCR to make it less autoreactive.

Similar to conventional B cells, erythrocytes differentiate from precursors in the bone marrow; 2-3 million new RBCs are generated every second (39, 40). Blood group antigen expression on RBCs is observed on early erythrocyte precursors. The final step in transition from immature to mature RBC involves the expulsion of the nucleus; mature RBCs cannot synthesize new antigens. Thus, we hypothesized that B cells that develop in the bone marrow encounter erythrocyte antigens. Data supporting this notion is demonstrated in Figure 3.2a. Bone marrow $B220^{+}IgM^{a+}$ B cells are decreased 2-fold in $HOD^{+}IgHEL^{+}$ mice, compared to control IgHEL mice; this decrease suggests that at least half of the autoreactive B cells in autoimmune mice encounter their cognate antigen (i.e. are exposed to the HOD antigen on erythrocytes) during development in the bone marrow. Furthermore, because there was enrichment of B-1 B cells in the peritoneum (see Figure 3.4), these data suggest that this population of B cells do not encounter erythrocyte antigens during development, do not encounter erythrocyte antigens in the periphery or are positively selected as a result of exposure to the HOD antigen.

It is hypothesized that B-1 B cells are derived from a progenitor in the fetal liver (41-43). Little is known about the establishment of central tolerance outside the bone marrow. Thus, if B-1 B cells develop outside the bone marrow, then it is plausible that B-1 B cells never encounter RBCs or their antigens during development. Additionally,

B-1 B cells are mostly restricted to the peritoneal cavity and pleural spaces; although a small population of B-1 B cells can be identified in the spleen (44, 45). Typically, RBCs are not found in the peritoneal cavity or within pleural spaces. Therefore, B-1 B cells are likely not exposed to erythrocyte antigens in the periphery. In aggregate, these data are most consistent with the hypothesis that B-1 B cells, due to their extramedullary origin and sequestration in the peritoneal cavity and pleural spaces, are not tolerized to erythrocyte antigens.

Conventional and B-1 B cells have differential signaling thresholds. Whereas conventional B cells that react strongly with self antigen are subject to central tolerance mechanisms, B-1 B cells receive positive signaling through their BCR and undergo a proliferative burst (46, 47). Thus, it is equally likely that B-1 B cells differentiate from hematopoietic stem cells in the bone marrow, but due to their inherent signaling differences, are positively selected for upon encounter with self-antigen.

An alternative interpretation of these data is that B cell tolerance mechanisms in the autoimmune $HOD^{+}IgHEL^{+}$ mice were overwhelmed due to the use of a BCR transgenic mouse with an extremely high precursor frequency of autoreactive B cells. An estimated 40-60% of newly-formed B cells express low affinity for self-antigens prior to establishment of central tolerance (48, 49). However, in most BCR transgenic mice, over 95% of B cells express the affinity-matured BCR transgene for the same antigen. Thus, attempting to tolerize most of the B cells in the autoimmune mouse might overwhelm tolerance mechanisms. Therefore, it is possible that the enrichment observed in the peritoneal cavity was a consequence of deletion of most autoreactive cells followed by

homeostatic proliferation of the deletion-resistant cells to fill the niche. Additionally, because B-1 B cells are the predominate lymphocyte in the peritoneum, they are most fit to proliferate in the peritoneal cavity. However, this line of reasoning does not apply to other compartments or tissues; homeostatic proliferation of other subtypes of B cells in the bone marrow or secondary lymphoid organs was not observed. Taken together, however, the extremely high precursor frequency of autoreactive B cells might contribute to the incomplete B cell tolerance observed. We cannot rule out that a titration of the frequency of autoreactive B cells down to a physiological percentage of total B cells would result in complete B cell tolerance to erythrocyte antigens.

Data generated from the bone marrow suggest that deletion is the predominate mechanism utilized for tolerizing erythrocyte-specific autoreactive B cells in autoimmune mice. The other mechanisms available to establish central and peripheral tolerance are receptor editing, anergy and ignorance (21, 50). To evaluate whether other tolerance mechanisms were employed, $HOD^{+}IgHEL^{+}$ splenocytes were stained with HEL-AF-647, anti-CD19 and anti-IgM^{a}. Flow cytometric analysis revealed that 90% of $CD19^{+}IgM^{a+}$ cells maintained their reactivity with HEL. Thus, in the autoimmune mouse, there was a small fraction of cells that underwent receptor editing (thereby maintaining expression of IgM^{a} but losing reactivity with HEL). Anergic B cells down-regulate expression of surface IgM and do not secrete antibody in response to cognate antigen (20). Splenocytes were stained with anti-B220 and anti-IgMa; flow cytometric analysis revealed that surface expression of IgM^{a} on B cells was similar between IgHEL and autoimmune mice. Further, ELISA and ELISPOT data demonstrate that deletion-resistant B cells in

autoimmune mice are activated (see Figure 3.3). Therefore, we reject the hypothesis that all autoreactive B cells are anergic. Taken together, these data demonstrate that deletion is preferentially used to deal with anti-RBC autoreactive B cells.

Limitations to Current BCR Transgenic Mouse Models

Much of the knowledge about the establishment of B cell tolerance has been defined through the use of BCR transgenic mice,. While the B cell tolerance and autoimmunity fields have progressed in the understanding of these mechanisms, there are many caveats to using BCR transgenic model systems. Even though some of the caveats associated with particular models can be addressed, many of the general limitations of using BCR transgenics cannot be circumvented.

The first caveat to using BCR transgenic mice to study the mechanisms of B cell tolerance is that the precursor frequency of autoreactive B cells is extremely high. Although, there is always some degree of leakiness associated with BCR transgenics (i.e. not all B cells in the IgHEL mice are IgM^{a+}), attempting to tolerize over 90% of newly-formed B cells might overwhelm tolerance mechanisms. However, through the generation of bone marrow chimeras, this limitation can be overcome.

Another limitation is that BCR transgenics are constructed with affinity-matured heavy and light chains; the B cell that responds best to the desired antigen is selected and subsequently used for cloning. These particular B cells are antigen experienced, have entered the germinal center and have undergone somatic hypermutation to have an

extremely high affinity for the antigen. It is unknown how this high affinity BCR passes through the multiple tolerance checkpoints in the bone marrow and if this has any additional effects on the immune system. However, due to the inherent signaling differences between B cell subtypes, it hypothesized that the high affinity BCR influences the composition of the mature B cell repertoire.

Another consideration when using BCR transgenics is that the BCR is a "fixed" part of the developing B cell. When B cells are developing, the pre-B cell first displays a heavy chain. The heavy chain associates with a surrogate light chain for stability purposes but also to begin the portion of development where antigen testing begins. It is through this association with the light chain that sends a positive or a negative signal to the B cell telling it whether the heavy chain is suitable for progression to a mature B cell. Transgenic BCRs might not be able to associate with the endogenous surrogate light chain. Thus, it is unknown what effects this has on the B cell; most of what we have learned by using BCR transgenics could just be a byproduct of not associating with a surrogate light chain.

Understanding B Cell Tolerance to Erythrocyte Antigens in the Absence of BCR Transgenics

In Chapter 4, we demonstrate that there is incomplete B cell tolerance to erythrocyte antigens in naïve B6.HOD mice. Through adoptive transfer of OTII/RAG1ko splenocytes, we observed anti-HEL IgG antibodies from B6.HOD mice.

We hypothesized, however, that T cell tolerance is functionally complete. Immunizing B6.HOD mice with OVA-CFA followed by OVA-IFA boost elicits anti-OVA antibodies, but only to the portion of OVA not contained within the HOD antigen. No anti-HEL antibodies are observed.

Activation of anti-HEL B cells is due to presence of specific help of the B cells. As such, excess CD4+ T cell help did not elicit antibodies, as adoptive transfer of SMARTA CD4+ T cells had no measurable anti-HEL response. Also in support of specific T cell help, excess activated CD4+ T cell help did not activate anti-HEL B cells as adoptive transfer of TCR75 CD4+ T cells followed by a Balb/c whole blood transfusion resulted in anti-Balb/c antibodies but not anti-HOD. We cannot rule out, however, the presence of miniscule numbers of 'passenger' anti-HEL B cells in the adoptive transfer. Despite using CD4 enrichment (which eliminated over 98% of CD19+ cells) and parallel experiments utilizing OTII/RAG1 knockout splenocytes, we nevertheless observed anti-HEL antibody production in B6.HOD mice but not B6. Thus, we reject the hypothesis that passenger B cells are required for induction of anti-HEL in B6.HOD mice that receive OT-II adoptive transfer.

One must be mindful of the possibility that there is incomplete B cell tolerance to the HOD antigen because it is not part of the normal, wild-type mouse genome; the HOD antigen was introduced to embryos thus is transgenic in nature. However, because the HOD antigen undergoes the same transcriptional and translational and post translational modifications as any other murine protein, to the mouse, it is a murine protein. However,

we cannot unequivocally rule out that this is a phenomenon of the introduction of a transgene into the murine system.

Future Directions

To expand our understanding of the failure of B and/or T cell tolerance to RBC antigens, there are many questions that remain to be tested. The following sections include short term experiments to may be performed to address some of the caveats of using BCR transgenic mice in evaluating B cell tolerance and T cell tolerance as well as long term future directions to assess the contributions of affinity between the BCR and autoantigen in relation to the resultant mature B cell repertoire.

Short-Term Future Directions

Molecular Mimicry-induced Alloimmunization with Acute and Chronic Viral Infection

Alloimmunization to transfused RBCs can be regulated by an array of factors including genetics and environmental differences. An additional factor that can influence alloimmunization is molecular mimicry through prior pathogen exposure. Many chronic viral infections have been associated with alloimmunization to RBC antigens. Chronic viral infections have 3 distinct phases: infection, latency and reactivation. During infection, viral antigen is constantly being encountered and the responding T and B cells can become exhausted. A consequence of infection is the generation of antigen-specific memory T and B cells. Due to immune system pressures, most chronic viruses enter latency and viral antigen is undetectable. Once reactivated, new viral-specific T cells are generated from naïve T cells and memory cells are reactivated. Distinct from chronic infections, acute infections are cleared from the immune system. Viral antigen is eliminated and antigen-specific memory T and B cell populations are formed. It is

unknown whether at the time of transfusion, recipients with chronic viral infections have an active infection.

To test whether there is a difference between a chronic and acute viral infection primed alloimmunization response, B6 mice will be infected with a chronic viral infection engineered to express $OVA_{323-339}$ (e.g. LCMV Clone 13) or an acute viral infection (e.g. LCMV Armstrong expressing $OVA_{323-339}$). Mice infected with Clone 13-OVA will continually mount an immune response to not only LCMV antigens, but also $OVA_{323-339}$. Similarly, Armstrong-OVA infected mice will mount an immune response to LCMV antigens and $OVA_{323-339}$, but will eventually eliminate the viral infection. Each group of mice will then be transfused with HOD RBCs. The magnitude and kinetics of anti-HEL alloantibodies will be analyzed. Through use of an acute viral infection, the enhanced alloantibodies previously observed in mice infected with Clone 13-OVA and transfused with HOD RBCs might be ablated due to the contracted $CD4^+$ memory T cell population and absence of newly-generated antigen-specific T cells. Alternatively, alloantibodies might be generated but with delayed kinetics.

Dissecting B cell Tolerance to Erythrocyte Antigens with BCR transgenics

Understanding the establishment of tolerance to RBC antigens is critical to our understanding of how the mechanisms of tolerance are broken in autoimmunity. In our initial studies with the F1 of FVB.HOD and IgHEL mice, we circumvented many of the caveats that plagued the HL model system of AIHA (most notably the chronic

inflammation and hemolysis). However, through the use of IgHEL BCR transgenic mice, our model still suffers from the general caveats of BCR transgenic mice. Specifically, the limitations in our model include a high precursor frequency of autoreactive B cells, the inability of HEL-specific B cells to class-switch and an affinity-matured BCR. As such, we cannot rule out that the patterns of deletion and enrichment are an artifact of the inability of autoreactive B cells to progress through development and activation naturally.

One approach to decrease the high precursor frequency of autoreactive B cells in $HOD^{+}IgHEL^{+}$ mice is to generate bone marrow chimeras. Irradiation and busulfan (an alkylating compound that preferentially depletes early hematopoietic stem cells) are both available and practical options (51). Pilot experiments have been performed using busulfan. Various ratios of B6.Thy1.2:B6.Thy1.1 bone marrow cells were transplanted into busulfan-treated B6.Thy1.2 recipients. Engraftment was assessed by staining peripheral white blood cells of recipient mice with anti-Thy1.1 and anti-Thy1.2 and calculating the percentage of $Thy1.1^{+}$ T cells in the total T cell population. By using this approach, we were able to reproducibly titrate down the number of $Thy1.1^{+}$ T cells to a more physiological frequency in recipient mice (Figure 5.1). For our studies, tracking B cell engraftment can be accomplished by using CD45 congenic markers. Through use of the F1 of IgHEL and B6.CD45.1, we can track the engraftment of autoreactive B cells after transplant into B6.HOD (CD45.2) mice. B cell engraftment can be assessed by the calculating percentage of $CD45.1^{+}CD45.2^{+}$ B cells (representing IgHEL B cells) in the total B cell population. A limitation of this approach lies within the ability to differentiate between unsuccessful engraftment and deletion of all autoreactive B cells.

To address this issue, B6 mice will also receive busulfan and transplantation with IgHEL.CD45.1 bone marrow. By comparing the frequency, distribution and phenotype of $CD45.1^{+}CD45.2^{+}$ HEL-reactive B cells in B6 and B6.HOD mice, the effects of HOD antigen expression on the B cell repertoire can be determined. Alternatively, through use of a double congenic mouse (for CD45 and Thy), T cell engraftment could be a surrogate to assess the success of bone marrow chimerism.

Another approach to decrease the number of autoreactive B cells is through the use of SW_{HEL} BCR transgenic mice with specificity for HEL (52). The utility of SW_{HEL} transgenic mice is 3-fold: B cells retain the ability to undergo receptor editing, the precursor frequency of HEL-specific B cells is estimated to be 40-60% (much lower than >95% in IgHEL mice), and B cells maintain isotype class-switching capability. Absence or mutation (as a consequence of receptor editing) of the transgene decreases the reactivity with HEL; antigen binding can be assessed by flow cytometric analysis by staining with HEL-AF-647. In addition to having a lower frequency of HEL-reactive B cells, SW_{HEL} B cells can switch to all immunoglobulin isotypes. As such, antibody subtypes can be analyzed in the double transgenic autoimmune $HOD^{+}SW_{HEL}+$ mice, providing insight to the B cell subset responsible for autoantibody secretion. Through a combination of utilizing SW_{HEL} mice and performing bone marrow chimeras in B6.HOD mice, recapitulating the natural frequency of autoreactive B cells while maintaining the ability to track and phenotype them can be achieved.

One concern with performing bone marrow chimeras is the ability to reconstitute all of the B cell subsets. It has been demonstrated that B-1 B cells derive predominately

from precursors in the fetal liver (42). Thus, in consideration of this and to ensure recapitulation of the entire B cell repertoire, parallel experiments will be performed using fetal liver from 14 day old embryos for transplantation.

It is known that most RBC antigens are also expressed on non-hematopoietic tissues thereby possibly adding another layer of tolerance (e.g. peripheral) to those particular antigens. Therefore, we question, how is tolerance to antigens expressed only on RBCs different from those expressed in multiple compartments? To answer this question, we need the same antigen expressed in two scenarios: 1) RBCs and a non-hematopoietic tissue and 2) RBC-specific. We currently have mHEL mice (expression of HEL under the MHCI promoter) and HOD mice (HEL in an RBC-specific manner). While both of these mice satisfy the initial criteria, HEL is a portion of the HOD antigen. As such, it cannot be used as an exact juxtaposition. Therefore, to directly compare RBC-specific HEL and HEL expressed on RBCs and other tissues, we generated another transgenic mouse, meHEL, in which HEL is expressed under an RBC promoter. Initial characterizations of meHEL mice demonstrate that HEL is expressed on RBCs and a subset of $CD11c^+$ leukocytes. By comparing patterns of deletion and phenotype of HEL-reactive SW_{HEL}^+ B cells from $mHEL^+SW_{HEL}^+$ and $meHEL^+SW_{HEL}^+$, we can dissect how RBC antigens influence the B cell repertoire.

Assessment of T cell Tolerance to Erythrocyte-specific Antigens

Breakdown of B and T cell tolerance to erythrocyte antigens can lead to the development of AIHA. To study the establishment of tolerance to RBC-specific antigens, we utilized B6.HOD mice. In Chapter 4, we demonstrate B cell tolerance to the HOD antigen is incomplete in B6.HOD mice. In contrast, the data suggest that HOD-specific T cells are functionally absent. Current immediate future directions are to investigate the mechanism of T cell tolerance (e.g. anergy or deletion).

To assess T cell tolerance to the HOD antigen in B6.HOD mice, there are several experimental approaches that can be used. B6 but not B6.HOD mice made anti-HOD antibodies after immunization with OVA/CFA. The HOD antigen contains only the C terminal half of OVA. As such, it is likely that $OVA_{323-339}$ is not the dominant $CD4^+$ T cell epitope within the OVA portion of the HOD antigen. To direct the T cell response to an epitope contained within the HOD antigen, B6 and B6.HOD mice will be immunized with $OVA_{323-339}$ peptide emulsified in CFA. Mice will be concurrently immunized with an irrelevant peptide, LCMV GP_{61-80} as an internal control for the immunization protocol and to assess whether B6.HOD mice are capable of mounting an immune response. At the peak of T cell responses, $OVA_{329-337}$ and LCMV GP_{66-77} tetramer positive T cells will be quantified by flow cytometry. By comparing B6 responses to B6.HOD responses, we can deduce whether $OVA_{323-339}$-specific $CD4^+$ T cells are capable of recognizing and expanding to OVA peptide. The immunization protocol will only detect T cells capable of responding to OVA and will not recognize anergic T cells. Therefore, to quantify endogenous OVA-specific T cells, we will utilize the tetramer pulldown assay (53). Data

from the combination of these two approaches will allow for discrimination between anergy and/or deletion T cell tolerance mechanisms.

Long-Term Future Directions

Effect of BCR Affinity, Antigen Density and Location on B Cell Tolerance to RBC Antigens

Strength of BCR binding to cognate antigen and the amount and location of antigen encountered can have profound effects on B cell tolerance. One of the weaknesses of BCR transgenics is the use of affinity matured BCRs. As such, cognate antigen encounter is always high affinity. B cell subsets are differentially affected by high affinity antigen encounters; B-2 B cells are deleted when they encounter self-antigen that strongly crosslinks their BCR whereas B-1 B cells are positively selected (46). Therefore, BCR transgenic mice with varying affinities for HEL can be generated to test how the strength of signaling to erythrocyte self-antigens influences the B cell repertoire. In a reciprocal approach, transgenic mice with mutations within the HEL protein can be generated. Amino acids predicted to affect the binding affinity of the IgHEL and/or SWHEL B cells would be altered.

Density of antigen expression on RBCs might have effects on the threshold of tolerance induction (54). Goodnow and colleagues describe a series of mice with varying amounts of secreted HEL crossed with IgHEL mice (55). These studies demonstrate that low levels of secreted HEL induce ignorance in B cells with HEL-specificity, whereas higher levels of soluble HEL prompt deletion and anergy. Due to the inherent differences between soluble and membrane-bound antigens, generating B6.HOD mice with varying

copies of HOD antigen would test the amount of self-reactive antigen needed to trigger central tolerance mechanisms or avoid them.

Data presented with multiple autoimmune models utilizing BCR transgenic mice report an enrichment of autoreactive B-1 B cells in the peritoneal cavity (56, 57). The leading hypothesis is that B-1 B cells are not tolerized because they do not encounter autoantigen. To test this hypothesis and to model RBC antigens with non-hematopoietic tissue expression, the HOD antigen can be engineered to be expressed under a ubiquitous promoter with a stop sequence flanked by LoxP sites. Crossing the LoxP-STOP-LoxP-HOD (LoxP-HOD) mice with Cre recombinase mice specific for expression in the peritoneal epithelium (Cre-PeC) will induce expression of the HOD antigen. In this scenario, peritoneal HEL-specific B cells encounter their antigen peripherally. Direct comparison between HEL-reactive B cells in HOD+SW_{HEL}+ and the F1 of LoxP-HOD/Cre-PeC and SW_{HEL} will allow for assessment RBC antigen expression on peripheral tissue and the establishment of B cell tolerance.

Identification of the B Cell Subset(s) Responsible for Autoantibody Production

Through use of two models: a BCR transgenic (FVB.HOD x IgHEL) and B6.HOD transgenic mice, we have demonstrated that B cell tolerance to the RBC-specific HOD antigen is incomplete. In the BCR transgenic model, we hypothesized that incomplete B cell tolerance to RBC antigens was due to either the high precursor frequency of autoreactive B cells (due to the use of the IgHEL mouse) or deletion-

resistant B cells did not encounter RBC antigens. Due to the lack of BCR transgenic in B6.HOD mice, there is not an unusually high frequency of autoreactive B cells. While the B6.HOD mice recapitulate a more physiological precursor frequency of HEL-specific B cells, it is more difficult to identify and phenotype them. As such, a combination of approaches will be useful in determining the B cell subset(s) responsible for autoantibody production.

To determine the B cell subset(s) responsible for anti-HEL IgG in B6.HOD mice, many approaches can be used. First, identifying HEL-reactive B cells by flow cytometry would provide great insight to location and phenotype. Pilot studies have utilized tetramerized HEL to identify an expanded HEL-reactive B cell population after adoptive transfer of enriched $CD4^+$ OT-II T cells. Initial studies indicate that 14 days post OT-II adoptive transfer, HEL-specific activated B cells have already down-regulated subset-specific B cell markers. Therefore, future studies should include early time points to allow for B cell subset identification. There are a variety of indirect methods that can be used to narrow down which B cell subset is responsible for erythrocyte-specific autoantibodies. These approaches include using established protocols reported to delete or remove one B cell subset. The first strategy that has already been explored is splenectomy. Upon splenectomy, B-1a B cells are decreased (58). Additionally, the niche for the marginal zone B cells and a majority of follicular B cells has been lost. Therefore, the contribution of these subtypes of B cells can be evaluated. Another method is intraperitoneal injection of water (59). Several papers have documented that injection of water induces apoptosis of peritoneal cells. As such, the majority of B-1 B

cells can be eliminated and the contribution of B cell subsets that are found predominately in the spleen can be evaluated. And, finally, crossing the B6.HOD mice onto an IL7 knockout background might be useful. Mature IL7 knockout mice do not have follicular B cells, but have normal development of marginal zone and B-1 B cells (60). Taken together, using a combination of techniques, the B cell subset(s) capable of secreting anti-HEL autoantibodies in the B6.HOD mice can be identified.

Regardless of the offending B cell subset(s), it will be necessary to assess whether bone marrow-derived B cells encounter erythrocyte antigens. The data presented thus far are equally consistent with sequestration of RBC and B cell precursors. Therefore, to determine whether B cells and erythrocytes develop in close proximity to each other in the bone marrow, morphology of decalcified bone marrow sections from $HOD^{+}IgHEL^{+}$ and B6.HOD mice will be imaged with immunoflourescence or immunohistochemistry staining.

General Conclusions

Antibodies to red blood cell (RBC) antigens can occur spontaneously or as a consequence of antigen exposure through infection, transfusion, transplantation or pregnancy. Some antibodies against RBC antigens are clinically insignificant, whereas others are clinically relevant and can result in hemolysis, pose a barrier to future transplants and complicate finding future compatible RBC units for transfusion. Defining the underlying mechanisms that contribute to the breakdown of tolerance to RBC antigens and factors that regulate the rates of alloimmunization are complex.

The data in this thesis demonstrate that RBC-specific B cells escape both central and peripheral tolerance mechanisms, encounter and present self-antigen, and are receptive of CD4+ T cell help. Therefore, in the event that T cell tolerance is broken, autoimmunity can be initiated. We have further demonstrated that molecular mimicry of CD4+ T cell epitopes shared between a pathogen and an RBC antigen can enhance alloimmune responses. As such, it is plausible that pathogen-elicited CD4+ T cells, through molecular mimicry and/or linked recognition of cryptic (e.g. transmembrane or cytosolic) peptides from RBCs could break T cell tolerance and lead to autoimmune pathology.

Maintenance of RBC structure and function is essential for sustaining life. As such, RBC transfusions are life-saving therapies for persons with inherited or acquired anemias and also those who suffer blood loss as a consequence of trauma, surgery or

childbirth. Thus, understanding the underlying mechanisms behind the breakdown of B and/or T cell tolerance to RBC-specific self- and allo-antigens is important.

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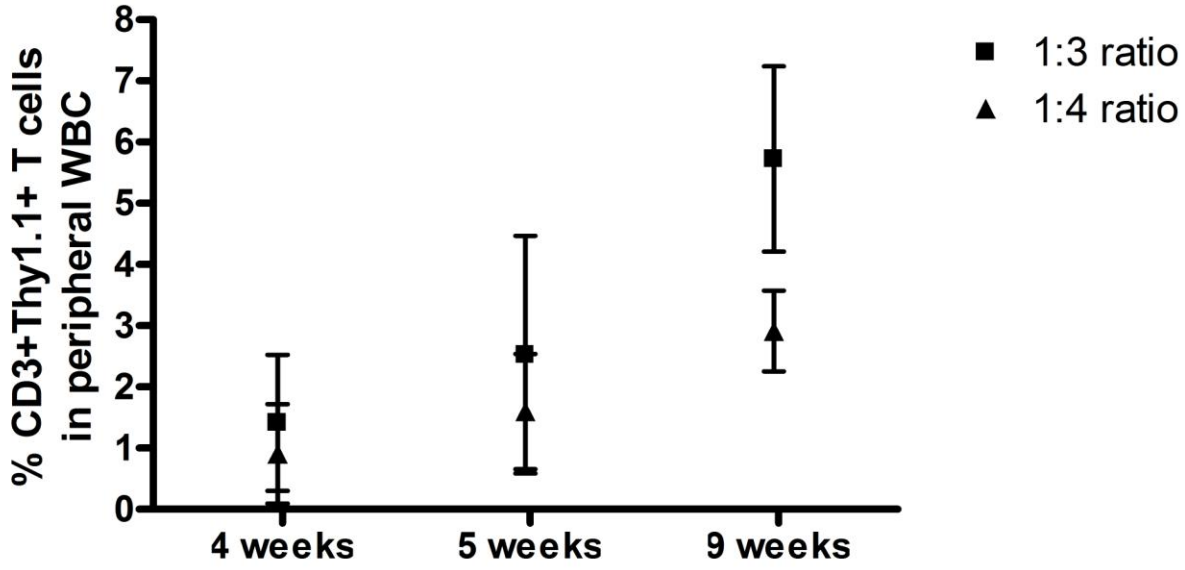
Figure Legends

Figure 5.1: Low precursor frequencies of CD3+Thy1.1+ T cells can be achieved through bone marrow chimeras and busulfan. C57Bl/6.Thy1.2 recipient mice were treated with busulfan one day prior to bone marrow transplant. Bone marrow from C57Bl/6.Thy1.1 and C57Bl/6.Thy1.2 donor mice were mixed at a ratio of 1:3 or 1:4 and transplanted into recipients. Peripheral white blood cells were stained with antibodies against CD, Thy1.1 and Thy1.2. Engraftment by calculating the percentage of $CD3^{+}Thy1.1^{+}$ T cells in the total T cell population. Data shown represents 5 mice/group, mean ± S.D.

Figure 5.1


% CD3+Thy1.1+ T cells in peripheral WBC
8
7
6
5
4
3
2
1
0
4 weeks
5 weeks
9 weeks
1:3 ratio
1:4 ratio