



Murine models of autoimmune hemolytic anemia

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Purpose of review

Pathogenic autoantibodies directed against red blood cells (RBCs) may lead to autoimmune hemolytic anemia (AIHA), a severe and sometimes fatal disease. Much of what is known about the etiology and pathogenesis of AIHA has been learned from observations made in human patients and murine models, but many questions remain; importantly, it is still unclear why some people generate RBC-specific autoantibodies. The combination of technological advancements applied to existing models and the development of new AIHA murine models will continue to provide considerable insight into the initiation of AIHA and provide a platform for the design of more effective therapies.

Recent findings

Advancements in well described murine models of AIHA show that reticulocytes are preferentially targeted by anti-RBC autoantibodies and an increase in oxidative stress may trigger autoantibody production. Additionally, a new murine model of erythrocyte autoreactivity demonstrates that T cell tolerance is the stopgap for autoimmunity. Moreover, unlike many self-antigens, data suggest that RBC self-antigens are not presented in the thymus thereby escaping the scrutiny of T cell central tolerance mechanisms and placing emphasis on peripheral tolerance instead. Information gained from this new model provide novel insight into how the immune system responds to RBC autoantigens and provides a tractable platform to discover new therapies for AIHA.

Summary

Murine models of AIHA have provided significant understanding into the risk factors for AIHA. The application of new technologies and models of erythrocyte autoreactivity is a pathway with the potential to elucidate how tolerance to RBC autoantigens is established, maintained, and broken down.

Keywords

autoimmune hemolytic anemia, autoimmunity, murine models of autoimmune hemolytic anemia, red blood cell autoantibodies, red blood cells, tolerance

INTRODUCTION

Autoimmune hemolytic anemia (AIHA) is a pathology characterized by shortened red blood cell (RBC) lifespan and detectable erythrocyte autoantibodies. There are two main types of AIHA: warm autoimmune hemolytic anemia (wAIHA) and cold agglutinin disease (CAD), differentiated by the optimal temperature for autoantibody binding to RBCs, autoantibody isotype, and complement activation (Table 1) [1–4]. Patients with AIHA may present with fatigue, pallor, hemoglobinuria, decreased hematocrit, and splenomegaly. Development of AIHA can be idiopathic or secondary, in which AIHA can coexist with a lymphoproliferative disorder or primary immunodeficiency. First-line treatment options include RBC transfusion (depending on hemoglobin levels) and/or corticosteroids, whereas second-line therapies include intravenous immunoglobulin (IVIg), splenectomy, and anti-CD20 monoclonal antibody (Rituximab). Unfortunately, treatment options for AIHA patients can be limited

and/or ineffective. For instance, transfusion support is challenging as autoantibodies are usually specific for ubiquitous RBC antigens (e.g., the Rh complex, Band 3) thereby making compatible units difficult, if not impossible, to locate. Moreover, relapse rates can be as high as 50% in patients that are refractory to steroids and receive a second-line treatment of

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KEY POINTS

- AIHA is a severe disease.
- Treatment options for AIHA patients are limited and sometimes ineffective; thus, there is a need to understand how AIHA is initiated such that better therapies can be designed.
- There are several risk factors that predispose individuals to break tolerance to their own RBCs, including a newly described involvement of oxidative stress.
- Autoreactive B cells escape tolerance mechanisms; T cell tolerance is robust but can be compromised.
- New understanding from murine models may provide additional insight into the initiation and perpetuation of AIHA.

splenectomy or Rituximab [5–7]. Thus, AIHA is a complex disease, which is difficult to manage and successfully treat.

The etiology and pathogenesis of AIHA has been an area of intense study – both from an observational standpoint in human AIHA patients and through the use of mouse models. With these

studies, several mechanisms have been purported to contribute to AIHA, including dysregulation of central and peripheral tolerance mechanisms, disruption of cytokine axes, molecular mimicry between autoantigens and pathogens, underlying disease pathologies (including lymphoproliferative disorders, primary immunodeficiency), and genetics. With each hypothesis, there are data from human studies or mouse models that provide significant support. This review provides an update on the current findings of existing animal models of AIHA (Table 2). It also discusses a new model of erythrocyte autoreactivity that provides insight into the tolerization of lymphocytes and potential mechanisms of initiation of autoimmunity.

RECENT PROGRESS IN WELL DESCRIBED MODELS OF AUTOIMMUNE HEMOLYTIC ANEMIA

New Zealand black mice

Summary of previous findings

One of the initial models of AIHA, New Zealand black (NZB) mice spontaneously develop RBC autoantibodies that lead to hemolytic anemia by

Table 1. Characteristics of AIHA

Name	Underlying pathology	Incidence	Autoantibody specificity	DAT and autoantibody characteristics	Presentation
Warm autoimmune hemolytic anemia	45–50% are idiopathic; 50–55% are secondary to lymphoproliferative disorders, benign tumors, immune dysregulation	Accounts for 65–70% of all AIHA cases 1 : 75 000–80 000	Majority: Rh complex, Band 3 Also reported: Wr ^b , En ^a , LW, U, Ge, Sc1, K	DAT positive for IgG or IgG + complement, autoantibody binds optimally at 37°C	Extravascular hemolysis
Cold autoimmune hemolytic anemia	Cold agglutinin syndrome Idiopathic/primary: chronic hemolytic disorder; secondary: underlying infections (EBV, mycoplasma pneumoniae), lymphoproliferative disorders	10–15% of AIHA cases; 1 : 100 000	majority: I/i also reported: Pr, M, P	DAT positive for complement, IgM autoantibody binds optimally <37°C	Intra or extra-vascular hemolysis
	Paroxysmal cold hemoglobinuria Idiopathic/primary: chronic hemolytic disorder; secondary: underlying infections (EBV, VZV, syphilis)	1–2% of AIHA cases, <1 : 150 000	P	Donath–Landsteiner test positive IgG autoantibody with highest avidity at <37°C but induces hemolysis at 37°C	Intravascular hemolysis
Mixed AIHA	Idiopathic; secondary to lymphoproliferative or autoimmune diseases	<10% of AIHA cases	IgM reactive against I/i IgG is panreactive	DAT positive for IgG + complement IgM autoantibody binds optimally <37°C and IgG autoantibody with optimal binding at 37°C	Intra and extra-vascular hemolysis

AIHA, autoimmune hemolytic anemia; DAT, direct anti-globulin test; EBV, Epstein-barr virus; VZV, Varicella-Zoster virus.

Table 2. Key Findings in Murine Models of AIHA

AIHA model system	Key findings
NZB mice	Genes can modulate AIHA predisposition Identification of RBC autoantibody targets: Band 3, Band 4.1, Rh complex Autoantibody subtype can dictate RBC hemolysis <i>New findings:</i> RBC oxidative stress may be a risk factor for AIHA
Playfair and Marshall–Clarke	RBCs from different species have cross-reactive T cell epitopes Dysregulation of regulatory T cells can lead to RBC autoimmunity Linked-recognition of foreign and self-derived peptides may break tolerance to RBC autoantigens <i>New findings:</i> Pathogenic anti-RBC autoantibodies target reticulocytes
HL transgenic mice	Tg mice that have B cells specific for Band 4.1 (a protein integral for RBC structure) induces severe RBC hemolysis in a subset of mice The degree of RBC hemolysis is influenced by exposure to inflammatory stimuli and environment Expansion and activation of innate B-1 B cells in the peritoneum directly correlate with AIHA onset and severity
HOD transgenic mice	Endogenous RBC-specific autoreactive B cells escape central and peripheral tolerance mechanisms and can secrete autoantibodies upon activation T cell tolerance is the stopgap for autoimmunity <i>Cross with BCR Tg mice</i> The technology utilized to create BCR Tg mice plays an important role in subsequent immune responses B-1 B cells may not responsible for antierythrocyte autoantibody production <i>Cross with TCR Tg mice</i> RBC autoantigens may not be presented in the thymus T cell peripheral tolerance mechanisms are key for AIHA prevention When tolerance breaks down: splenomegaly, age-related onset, female predominance, two subsets of autoantibody producers: relapse-remitting and sustained

AIHA, autoimmune hemolytic anemia; NZB, New Zealand black; RBC, red blood cell.

6 months of age [8,9]. The NZB model has been instrumental in demonstrating that genetic factors contribute to the development of AIHA [10,11]. Multiple susceptibility and suppression genes (e.g., *Aia1*, *Aia2*, *Aem1*) have been identified and correlated with antierythrocyte autoantibody production [12,13]. Moreover, this model has demonstrated a role for MHC alleles in the predisposition to develop AIHA; NZB mice (which normally express MHC H-2^d) that have been genetically modified to express MHC H-2^b exhibit delayed onset of AIHA with lower autoantibody titers [14,15]. Additional analysis has revealed more risk factors for AIHA onset, including a shift in cytokine production, dysregulation of B and/or T lymphocytes, and pathogenic infections [16]. Studies in the NZB model have also led to the identification of common RBC autoantibody targets (e.g., Band 3, Band 4.1) and allowed for autoantibody cloning, which, in turn, allowed for mechanistic analysis of the relationship between autoantibody subtype and RBC hemolysis [17–19]. These antibodies have been further utilized in passive immunization experiments to evaluate the role of Fc receptors (FcRs) and complement in RBC:autoantibody immune complex clearance and pathology [20,21].

Recent findings: oxidative stress may be a risk factor for autoimmune hemolytic anemia

More recent studies with the NZB murine model have focused on the correlation between oxidative stress and AIHA. Analysis of RBCs from NZB mice have demonstrated elevated levels of intracellular reactive oxygen species (ROS) compared to nonautoimmune prone mice [22,23]. Intriguingly, as NZB RBCs age, levels of intracellular ROS production increase, and erythrocytes display higher levels of methemoglobin and lipid peroxidation – metrics associated oxidative stress. The degree of RBC oxidative stress was directly correlated with autoantibody production and anemia. Moreover, prevention of AIHA in NZB mice was achieved through overexpression of superoxide dismutase 1 (SOD1), an enzyme that breaks down free radicals such as ROS, thereby demonstrating a direct role of oxidative stress and AIHA onset [22,24,25,26]. These studies lend additional insight and importance to the role of oxidative stress and RBC survival. Moreover, these findings complement observations in human blood storage biology that show some individuals have RBCs that exhibit oxidative stress markers upon storage, which, in turn, may

negatively impact posttransfusion RBC survival [27]. Thus, given the emergence of mass spectrometry analysis in transfusion medicine research, metabolomics and blood storage biology may provide key knowledge into AIHA pathogenesis (and autoimmunity in general) and provide a foundation to evaluate ways to prevent harmful oxidative damage and reverse the course of autoimmunity.

PLAYFAIR AND MARSHALL-CLARKE MODEL: EXPERIMENTALLY INDUCED AUTOIMMUNE HEMOLYTIC ANEMIA

Summary of previous findings

Autoimmune hemolytic anemia can be induced in mice by weekly intraperitoneal injection of rat RBCs, commonly referred to as the Playfair and Marshall-Clarke model [28]. Erythrocyte autoantibodies are detectable within 5–6 weeks and correlate with a shortened RBC lifespan and a significant drop in hematocrit and hemoglobin [29]. Similar to observations with the NZB murine model, predisposition to AIHA involves genetics, gender, and dysregulation of cytokines [30–33]. Distinctly, findings in this model have elucidated that T cell immune responses to RBC-derived antigens are cross-reactive between species and that linked recognition of foreign rat RBC-derived epitopes provide help to murine RBC autoreactive B cells and can induce AIHA [34–36]. Additionally, this model of AIHA has been instrumental in demonstrating a key role of regulatory T cells in modulating RBC autoimmunity [37,38].

Recent findings: pathogenic antierythrocyte autoantibodies target reticulocytes

Most recently, the use of the Playfair and Marshall-Clarke model has been utilized to study how pathogenic autoantibodies affect RBCs. Saxena *et al.* has developed a technique called double in-vivo biotinylation (DIB) to delineate erythrocyte age, which has been instrumental in elucidating age-related changes and clearance. Incorporation of DIB in multiple models of anemia has demonstrated that clearance patterns of RBC precursors, reticulocytes, and mature RBCs are distinct, depending on how anemia is induced (e.g., heavy metal, autoantibodies) [39,40]. With regard to the Playfair and Marshall-Clarke model, reticulocytes have more autoantibodies bound to their surface, produce more ROS, and are preferentially cleared from circulation [41]. Moreover, injection of pathogenic autoantibodies (from rat RBC-induced AIHA mice) into DIB-treated naïve mice leads to an accumulation of

autoantibodies and phosphatidylserine exposure on reticulocytes, thereby demonstrating that autoantibody binding induces changes in the RBC membrane, which ultimately affect clearance [42]. Thus, the DIB technique has allowed for additional insight into AIHA sequelae and demonstrate that reticulocytes are most susceptible to the effects of pathogenic autoantibodies. Furthermore, these studies demonstrate interconnectivity between the effects of pathogenic autoantibody binding of RBCs, FcR-mediated erythrophagocytosis, and AIHA pathogenesis; additional extension of studies with the DIB technique may help parse out how different autoantibody subtypes modulate RBC membranes, clearance, and hemolysis.

TRANSGENIC MODELS OF AUTOIMMUNE HEMOLYTIC ANEMIA: HEAVY AND LIGHT (HL) AND HEN EGG LYSOZYME, OVALBUMIN, DUFFY (HOD) MICE

The first murine transgenic model of autoimmune hemolytic anemia, HL mice

Okamoto and colleagues generated HL transgenic mice, in which all B cells express the same B cell receptor comprises the heavy and light chain (HL) from monoclonal antibody 4C8, derived from NZB mice [43]. 4C8 has been described as a pathogenic immunoglobulin M (IgM) antibody that recognizes Band 4.1, and upon passive immunization, causes anemia and agglutination of RBCs in the spleen and liver [20]. Transgenic HL mice experience a range of AIHA symptoms from tolerance to severe anemia, depending on the state of inflammation and housing conditions [44,45]. Analysis of HL mice has revealed that B cell tolerance is abnormal; conventional RBC autoreactive B cells undergo deletion or anergy, whereas anti-RBC innate B-1 B cells in the peritoneal cavity and lamina propria are enriched [46]. Additionally, there is a correlation between deletion-resistant B-1 B cells and production of antierythrocyte antibodies. Polyclonal activation of peritoneal B cells with lipopolysaccharide prompts B cell proliferation, an increase in autoantibody production, and exacerbates anemia [44]. In contrast, intraperitoneal injection of deionized water or autoantigen-expressing RBCs initiate B-1 B cell apoptosis and lead to amelioration of AIHA symptoms [47–49]. Thus, data from this model supports a pool of autoreactive anti-RBC B cells that, by existing in extramedullary spaces, has escaped tolerance mechanisms and maintain capacity to secrete autoantibodies. The HL model provided the foundation for the prevailing paradigm of B cell tolerance to RBC autoantigens for almost two decades.

HOD model of red blood cell tolerance and autoimmunity

The AIHA models described above have generated significant knowledge and understanding of AIHA pathogenesis; however, there are several limitations to using these models as a platform to study the disease initiation and broader concepts of tolerance and autoimmunity to erythrocyte antigens. The biggest challenge is the absence of tools and reagents specific for these models such that we can extract more mechanistic understanding. To overcome these limitations, a new murine model for RBC tolerance and autoimmunity was developed: the HOD mouse (Fig. 1a). HOD mice express an RBC-restricted triple fusion protein consisting of hen egg lysozyme (HEL), ovalbumin (OVA), and human blood group molecule, Duffy (HOD) [50]. The combination of these proteins takes advantage of the currently available tools and reagents that enable the dissection of the immune response, including

(1) well described proteins with B and T cell antigens (HEL and OVA, respectively)

- (2) OVA-specific tetramers which allow for identification of OVA-reactive T cells
- (3) tetramerized HEL to identify HEL-specific B cells
- (4) Transgenic animals: OTII (CD4 T cells that recognize OVA presented by I-A^b), 3A9 (CD4 T cells that recognize OVA presented by I-A^k), OTI (CD8 T cells that recognize OVA presented by K^b), IgHEL (also called MD4, B cells that recognize HEL), SwHEL (B cells that recognize HEL)
- (5) monoclonal antibodies that recognize HEL, OVA, and Duffy

Analysis of naïve HOD mice has demonstrated that tolerance to RBC-specific antigens is robust; immunization with either HEL or OVA proteins emulsified in CFA is not sufficient to break tolerance and lead to autoantibody production [51]. This observation is consistent with those of the Playfair and Marshall–Clarke model as induction of anemia is dependent on cross-reactivity of foreign and self-epitopes and, possibly, linked recognition. Moreover, autoreactive lymphocytes are detectable in HOD mice. Autoreactive B cells persist despite central and peripheral tolerance mechanisms and, upon

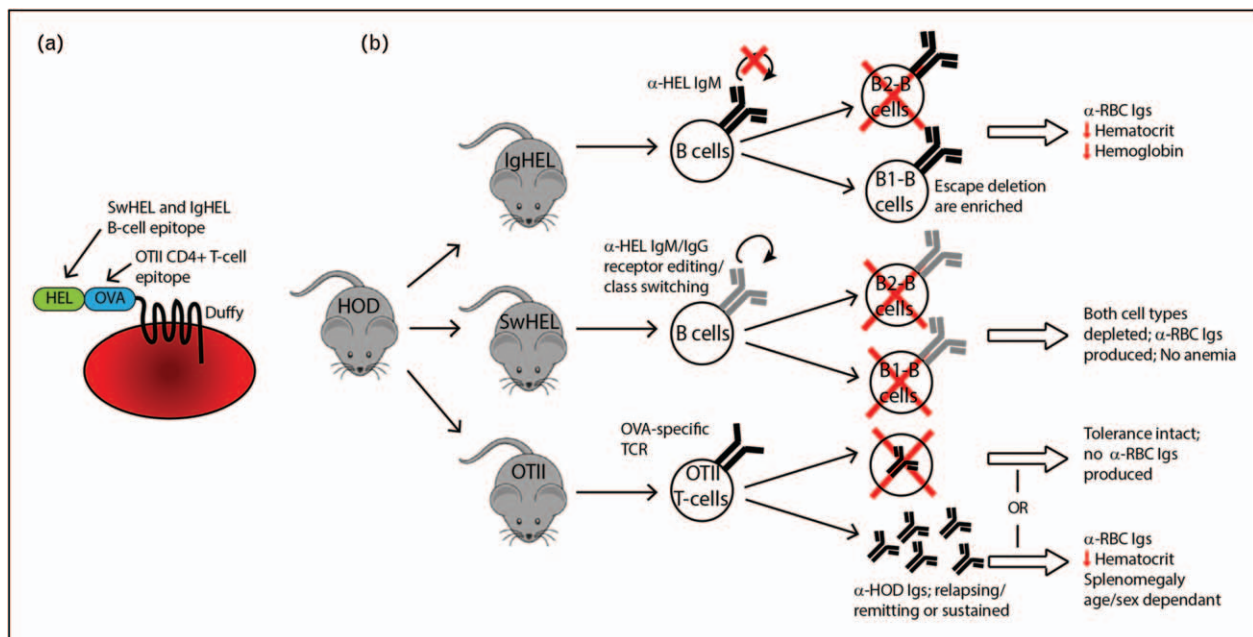


FIGURE 1. The HOD model of erythrocyte autoreactivity. (a) The HOD mouse expresses an RBC-restricted triple fusion protein consisting of hen egg lysozyme (HEL), ovalbumin (OVA), and human blood group Duffy. (b) HOD crossed to IgHEL, SwHEL, and OTII to generate new models of AIHA. In HOD+IgHEL+ mice (top), IgM HEL-specific B cells cannot undergo receptor editing or class-switching, which leads to a deletion of B-2 B cells and an enrichment of peritoneal B-1 B cells. HOD+SwHEL+ mice (middle) produce B cells that can undergo receptor rearrangement and class-switching. In this model, deletion of B-1 and B-2 B cell subsets is observed. Both HOD+IgHEL+ and HOD+SwHEL+ develop anti-RBC autoantibodies, however only HOD+IgHEL+ mice develop decreased hematocrit and hemoglobin. HOD+OTII+ mice (bottom) have CD4 T cells that are specific for OVA. Upon aging, a subset of HOD+OTII+ mice develop erythrocyte autoantibodies, which correlated with splenomegaly and anemia. Similar to other autoimmunity models and observations in humans, autoimmune mice were mostly female.

adoptive transfer of HOD-reactive CD4 T cells, are stimulated to secrete autoreactive antibodies [51]. These findings also align with observations made with NZB, Playfield and Marshall–Clarke, and HL models of AIHA as all mice make detectable autoantibodies, which demonstrate that erythrocyte-specific autoreactive B cells are present in the periphery and retain the capacity to secrete autoantibodies. Thus, T cell tolerance is a stopgap to autoimmunity. Indeed, while autoreactive CD4 T cells are detectable in the periphery of HOD mice, they are nonfunctional. Intriguingly, and in contrast to the Playfield and Marshall–Clarke model, despite the presence of autoreactive T cells expressing a CD4+CD25+FoxP3+ regulatory phenotype, depletion of Tregs in HOD mice did not lead to autoimmunity [52[■]]. Thus, the HOD model shares common themes with currently described models of AIHA and provides additional evidence to support the hypotheses that linked recognition and dysregulation of lymphocyte activation are risk factors for AIHA.

Based on the observations made with all AIHA models, it has become clear that tolerance to RBC antigens, especially those restricted to RBCs, is not complete; autoreactive lymphocytes are detectable in the periphery, receptive to activating signals, and can perpetuate an autoimmune response. These data are consistent with observations made in humans as tolerance to RBCs breaks down frequently; 0.1% of asymptomatic blood donors have detectable autoantibodies on their erythrocytes and this frequency increases to 7% in hospitalized patients [53–55]. Thus, given the functional importance of RBCs (e.g., tissue oxygenation and hemostasis), how are lymphocytes tolerized to erythrocyte antigens?

Are B cells educated against red blood cell-restricted antigens?

Innate B-1 B cells represent a distinct population of B cells; in general, they are polyreactive, express/secrete (mostly) IgM, utilize restricted variable, diversity, joining (VDJ) genes for their B cell receptor (BCR), and are positively selected for during development by strong BCR:self-antigen signals [56]. NZB and HL models of AIHA implicate B-1 B cells as the source of RBC autoantibodies, whereas conventional autoreactive B-2 B cells undergo deletion or anergy [46]. However, these results have recently been challenged by use of the HOD model in which HOD mice were crossed to two different BCR transgenic (Tg) mice (IgHEL and SwHEL) [57[■]]. Both BCR Tg mouse lines express the same paratope specific for HEL but the IgHEL mouse was generated

via a random transgenic approach in which the IgHEL is expressed as an IgM and with allelic suppression of background immunoglobulin G (IgG) recombination, whereas the SwHEL mouse was made with VDJ knock-in technology allowing normal class switching [58,59]. As such, the IgM-restricted IgHEL mouse cannot undergo BCR rearrangement, receptor editing, or class-switching. Analysis of bone marrow and splenocytes of autoreactive HOD+IgHEL+ mice have shown that B-2 B cells are deleted in the lymphoid organs, B-1 B cells are enriched in the peritoneum, and mice had detectable IgM autoantibodies that correlated with mild anemia. These observations are consistent with those made with HL transgenic mice, which are (like IgHEL) cannot class switch. In contrast, while HOD+SwHEL+ mice make HOD-specific autoantibodies, both B-1 and B-2 B cell subsets undergo significant deletion in this model. These findings demonstrate that B cells are educated against RBC self-antigens early during development, as evidenced by deletion of autoreactive B cells. Moreover, they suggest that B cells require receptor rearrangement and editing (available in SwHEL but not IgHEL or HL BCR Tg mice) for tolerance mechanisms to be effective. It is worth noting, however, B cell tolerance is incomplete as autoantibodies are present in both HOD+IgHEL+ and HOD+SwHEL+ mice. These studies highlight the importance of how BCR Tg mice are constructed, as very different results are obtained in each cross. Importantly, these data challenge the existing paradigm that B-1 B cells are solely responsible for erythrocyte autoantibodies.

How are T cells tolerized to red blood cell-specific autoantigens?

Evidence from analysis of naïve HOD mice has demonstrated that T cells are the stopgap to autoimmunity in the HOD model. Wong *et al.* recently described a model of AIHA using the F1 of HOD and OTII mice (OTII mice are CD4 T cell receptor transgenic mice that recognize OVA contained within the HOD triple fusion RBC antigen) (AABB oral abstract; 2017). Analysis of these mice has shown that autoreactive thymic T cells are neither deleted nor diverted into Tregs, indicating that central tolerance to an RBC-specific antigen (HOD) is not present in these animals and suggesting that RBC autoantigens are not presented in the thymus. Younger HOD+OTII+ mice do not have detectable autoantibodies; however, upon aging, a subset of mice displayed symptoms of AIHA with detectable HOD-specific autoantibodies, decreased hematocrit, splenomegaly, and increased frequency of

RBC precursors. Additionally, these studies mirror characteristics observed in human patients with AIHA and autoimmunity in general – female predominance, age-related onset, and two groups of autoantibody producers: relapse-remitting and low-level sustained. Thus, these findings show that T cells can be tolerized to erythrocyte antigens; and, although peripheral tolerance mechanisms may be more critical than thymic central tolerance, tolerance can break down and lead to AIHA.

Advantages and limitations to the HOD model

The HOD model of erythrocyte autoreactivity has proven to be a powerful platform to dissect how the immune system responds to antigens on RBCs and will likely continue to provide fruitful information. Similar to HL Tg mice, the autoantibody target in HOD mice is RBC-restricted, making both models relevant to AIHA studies as the most frequently targeted antigens in human patients are those with limited expression patterns (e.g., the Rh complex, Band 3). By crossing HOD mice with BCR and TCR Tg mice, it has been possible to recapitulate the HL Tg model whereby autoreactive lymphocytes are present and AIHA symptoms develop. However, the advantage of the HOD model over the HL Tg model is that the HOD transgene is not a critical component to RBC structure or function; thus, it is possible to allow for characterization of Tg B and T cells in the absence of their autoantigen (i.e., HOD-OTII⁺ or HOD-IgHEL⁺ or HOD-SwHEL⁺ mice). In contrast, HL Tg mice express a BCR specific against Band 4.1 and, while Band 4.1 knockout mice have been generated, they develop AIHA and their RBCs are unstable. Thus, analysis of the BCR Tg in the absence of their autoantigen could not be accomplished. As such, the observation that IgM-restricted peritoneal B-1 B cells were responsible for antierythrocyte autoantibodies is a direct result of how the BCR Tg mouse was constructed. Moreover, despite autoantibody generation in the HOD model, HOD mice experience only mild anemia (like the vast majority of autoantibodies to RBCs in humans); thus, studying how tolerance breaks down and the subsequent mechanism of RBC clearance and immunity is easier to study as there is no confounding pathology associated with chronic hemolysis. Finally, by crossing the HOD mouse with BCR and TCR Tg animals, it is now possible to dissect how autoreactive lymphocytes are tolerized to RBC antigens thereby allowing for identification of when and where RBC autoantigens are encountered.

There are limitations to the HOD model, however. Akin to many other RBC antigens, some antibodies generated against the HOD antigen do not induce hemolysis but promote antigen-modulation. While this provides an opportunity to study the mechanisms of antigen-modulation and may allow for insight into the key characteristics of an antibody that promotes antigen-modulation versus hemolysis; it also confounds the observed results. Of greater concern with use of the HOD model is that, when combined with the BCR and TCR Tg mice, the autoreactive lymphocytes have a strong affinity to their cognate antigen; thus, these studies could represent the extreme spectrum of immune responses. This can be remedied in future experiments by altering the sequences of HEL and/or OVA in the HOD molecule, such that affinity is less. Nonetheless, the HOD model of erythrocyte autoreactivity provides an opportunity to elucidate the mechanisms of tolerance and autoimmunity to RBC autoantigen that may inform future therapies for AIHA patients.

FUTURE PERSPECTIVES

The past 2 decades have seen rapid advancement in genomics, proteomics, and metabolomics. The application of these approaches to study RBC biology will likely continue to provide significant information about how RBCs respond to environmental cues (e.g., exposure to ROS), provide insight into potential therapies to reverse and/or mitigate the harmful effects of oxidative stress on RBCs, and contribute considerable knowledge to understanding how RBCs are affected by autoantibody binding. Moreover, there is potential to extend these findings into RBC storage biology and alloimmunization. Given our recent technological advances, it is now possible to explore the relationship between AIHA onset and microbiome composition, lymphocyte tolerance mechanisms, and autoantibody sequence and functional characteristics that will provide answers to many outstanding questions:

- (1) What are the mechanisms of T cell tolerance employed against RBC antigens?
- (2) How does RBC-specific T cell tolerance break down? Can it be prevented or reversed?
- (3) Does RBC antigen density or expression pattern play a role in tolerance breakdown?
- (4) Can the microbiome predispose an individual to develop AIHA?
- (5) How does RBC hemolysis affect the microbiome?
- (6) Why do some autoantibodies induce hemolysis whereas others do not?

CONCLUSION

Autoimmune hemolytic anemia is a severe, and sometimes fatal, disease. Treatment options are limited and sometimes ineffective. And, while much knowledge is known about the destructive effects of pathogenic autoantibodies, there is still much to learn about why and how autoantibodies are generated. Additional studies with new transgenic murine models in RBC transfusion will provide additional insight into how tolerance to RBCs is established, maintained, and broken down; these studies will lead to new understanding into AIHA initiation and provide a foundation to generate new, more effective, treatment options.

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Conflicts of interest

There are no conflicts of interest.

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- of special interest
- of outstanding interest

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