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Tolerization of recent thymic emigrants is required to prevent RBC-specific autoimmunity

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Abstract

Autoimmune hemolytic anemia (AIHA) leads to accelerated destruction of autologous red blood cells (RBCs) by autoantibodies. AIHA is a severe and sometimes fatal disease. While there are several therapeutic strategies available, there are currently no licensed treatments for AIHA and few therapeutics result in treatment-free durable remission. The etiology of primary AIHA is unknown; however, secondary AIHA occurs concurrently with lymphoproliferative disorders and infections. Additionally, AIHA is the second most common manifestation of primary immunodeficiency disorders and has been described as a side effect of checkpoint inhibitor therapy. Given the severity of AIHA and the lack of treatment options, understanding the initiation of autoimmunity is imperative. Herein, we utilized a well-described model of RBC biology to dissect how RBC-specific autoreactive T cells become educated against RBC autoantigens. We show that, unlike most autoantigens, T cells do not encounter RBC autoantigens in the thymus. Instead, when they leave the thymus as recent thymic emigrants (RTEs), they retain the ability to positively respond to RBC autoantigens; only after several weeks in circulation do RTEs become nonresponsive. Together, these data suggest that any disruption in this process would lead to breakdown of tolerance and initiation of autoimmunity. Thus, RTEs and this developmental process are potential targets to prevent and treat AIHA.

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Author Contributions

AW and KEH conceptualized and designed experiments. All authors participated in performing experiments and data collection. AW and KEH analyzed data and wrote the paper. All authors read the paper, provided comments, and approved the final version of the manuscript.

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Keywords

tolerance; red blood cell; autoimmunity; autoimmune hemolytic anemia; recent thymic emigrants

1. Introduction

Red blood cells (RBCs) are essential for life and are critically important for tissue oxygenation. As such, reduced RBC lifespan or production have profound consequences for the organism. One mechanism by which RBCs are destroyed is by antibody-mediated mechanisms that result in hemolysis. For example, autoimmune hemolytic anemia (AIHA), caused by pathogenic RBC-specific autoantibodies, is a severe and sometimes fatal disease[1; 2]. Patients with AIHA present with pallor, fatigue, decreased hematocrit, hemoglobinuria, and splenomegaly[3]. Treatment strategies have variable success and relapse rates can be as high as 50%[4; 5; 6]. Moreover, supportive care using RBC transfusions is challenging, because most autoantibodies recognize ubiquitous RBC antigens; thus, virtually all donor units are crossmatch incompatible[7; 8].

The etiology of AIHA is unknown. Although some cases are secondary and are associated with infections or lymphoproliferative disorders, >50% of AIHA cases are idiopathic[8; 9; 10]. Interestingly, in observational studies, ~0.1% of asymptomatic blood donors have detectable, non-hemolytic autoantibodies bound to their RBCs; the prevalence is even higher in hospitalized patients (i.e., up to 8%)[11; 12]. In addition, in patients with primary immunodeficiencies, such as those with a defect in the *FAS* apoptosis gene in autoimmune lymphoproliferative syndrome (ALPS), the rate of AIHA is increased to 30%[13; 14]. Moreover, in recent studies evaluating checkpoint inhibitors for cancer treatment, AIHA was an adverse effect of this immunotherapy[15; 16; 17]. Thus, although failure of humoral tolerance to RBC autoantigens occurs frequently, it is still unknown which tolerance programs are initiated in response to RBCs and how these checkpoints become compromised.

In murine AIHA models, autoreactive B cells are incompletely tolerized and are susceptible to activating signals that promote autoantibody secretion; this is similar to several other autoimmune mouse models and observations in humans[18]. As such, most studies have focused on T cells and their tolerization and regulatory functions. For example, in the HOD mouse model, in which transgenic expression of the HOD antigen (a triple fusion protein consisting of hen egg lysozyme, ovalbumin (OVA), and the human Duffy blood group antigen) is restricted to RBCs, RBC-specific autoreactive CD4⁺ T cells are detectable, but are functionally anergic and do not expand upon immunization[19; 20]. Nonetheless, RBC-specific autoantibodies can be generated in HOD mice by circumventing T cell tolerance through adoptive transfer of T cell receptor (TCR) transgenic OTII CD4⁺ T cells, which recognize the OVA epitope in the HOD antigen. Thus, T cell tolerance is required to prevent RBC autoimmunity.

Herein, we use the HOD mouse model to examine RBC-autoreactive T cell development in the thymus and throughout maturation, as recent thymic emigrants (RTEs) differentiate into functional, mature, naïve T cells. In this setting, unlike for most other autoantigens, the

thymus is dispensable for tolerance to RBC autoantigens. However, multiple mechanisms of peripheral tolerance are initiated to prevent autoreactive T cell activation. In particular, RBC-specific autoreactive T cells are both deleted and diverted into regulatory T cells in the periphery. Perhaps most strikingly, there was a narrow window for tolerization of autoreactive T cells. Thus, T cells that had recently egressed from the thymus were capable of proliferating in response to RBC autoantigens, but, upon their maturation into fully-functional, mature, naïve T cells, they lost that capacity. This functional gradient was associated with upregulation of anergy and exhaustion transcription factors and of relevant cell surface markers, particularly co-expression of CD73 and FR4. Taken together, these data demonstrate that tolerization of newly-minted T cells is required to prevent AIHA and imply that both incomplete and/or ineffective tolerization of RTEs would lead to RBC-specific autoantibody production.

2. Materials and Methods

Mice

C57BL/6 (B6), B6.Thy1.1 (B6.PL-Thy1^a/CyJ; stock #000406), and OTII (B6.Cg-Tg(TcraTcrb)425CBn/J; stock #004194) mice were purchased from the Jackson Laboratory. OTII.Rag2^{-/-} mice (B6.129S6- Rag2^{tm1Fwa}Tg(TcraTcrb)425Cbn, stock #1896) were purchased from Taconic Biosciences. OTII.Rag2p-GFP mice were a gift from Dr. Fink (University of Washington)[21]. TCR75 mice were a gift from Dr. Bucy (University of Alabama Birmingham)[22]. HOD mice were developed and described previously[20]. All transgenic mice were genotyped by PCR and phenotyped by flow cytometry. Mice were maintained in a pathogen-free environment on standard rodent chow and water in a light and temperature-controlled environment. Protocols were approved by the Institutional Animal Care and Use Committees at Bloodworks NW and Columbia University Irving Medical Center.

In vivo functional studies

Splenocytes were harvested from HODxOTII F1 and HODxOTII.Rag2p-GFP mice and processed into single cell suspensions. CD4⁺ T cells were enriched using the MagniSort Mouse CD4 T cell Enrichment Kit (ThermoFisher). **HODxOTII F1.** Recipient B6.Thy1.1 mice were adoptively transferred with 10×10⁶ total cells of a 1:1 mix of CellTrace-Far Red labeled HOD⁻OTII⁺ T cells and CellTrace-CFSE labeled HOD+OTII⁺ T cells followed the next day by a HOD RBC transfusion (100μL packed RBCs at 20% hematocrit). CellTrace dilution was assessed in CD4⁺Va2⁺Vb5⁺Thy1.2⁺ OTII T cells 3 days post-transfusion. **HODxOTII.Rag2p-GFP.** Enriched CD4⁺ T cells were stained with antibodies against surface antigens and CD8⁻Thy1.2⁺CD62L^{hi}CD44^{lo} T cells were sorted based on GFP expression (GFP^{hi}, GFP^{int}, GFP^{lo}) with a BD FACSAria[23]. At least 150,000 T cells were adoptively transferred into B6.Thy1.1 animals, followed the next day by a transfusion with 100μL of packed HOD RBCs. Splenocytes were harvested 3 days post-transfusion and CD4⁺Va2⁺Vb5⁺Thy1.2⁺ OTII T cells were evaluated for intracellular expression of Ki-67 (FoxP3 transcription factor staining buffer set, ThermoFisher), per the manufacturer's instructions. Flow cytometric analysis was performed on a BD LSRII and data were analyzed with FlowJo (TreeStart).

Bone marrow (BM) chimera generation

4–8 week old B6.Thy1.1/1.2 and HOD.Thy1.1/1.2 BM recipients received 2 doses of 600 cGy irradiation (RS 2000 Biological Irradiator, Rad Source Technologies) spaced 2–4 hours apart. Recipients received 20×10^6 bone marrow cells consisting of 10% OTII.Rag2^{-/-}.Thy1.2 + 10% TCR75.Thy1.1 + 80% of BM recipient genotype (either B6.Thy1.1/1.2 or HOD.Thy1.1/1.2) 24 hours post-irradiation and were placed on 2mg/mL neomycin-containing water for up to 6 weeks post-transplant. BM reconstitution was evaluated 4–6 weeks post-transplant by staining peripheral blood leukocytes with antibodies against CD4, Vb8.3, Va2, Thy1.1, Vb5, CD45, Thy1.2, and CD8 (ThermoFisher) to assess engraftment.

Leukocyte Staining

Thymi and spleens were harvested from HODxOTII F1 mice (8–12 weeks old) and BM chimeric mice (4–6 weeks post-transplant) and processed into single cell suspensions. Cells were stained with Va2, Vb5, CD4, Thy1.1, Thy1.2, and CD8 (ThermoFisher) to delineate the stages of T cell development and identify OTII and TCR75 T cells. In some experiments, cells were stained with antibodies specific for surface expression of CD25, CD200, PD1, FR4, CD73, CD62L, and CD44 (ThermoFisher). Intracellular expression of transcription factors NFAT2, Helios, Egr2, FoxP3, and Ki67 was assessed with the FoxP3 transcription factor staining buffer set (ThermoFisher) per manufacturer's instructions. Live cells were determined by viability dye exclusion (ThermoFisher) and cell counts were calculated using a hemocytometer. Cell analysis was performed on a BD Biosciences LSRII flow cytometer and data analyzed with FlowJo (TreeStar).

In vitro activation assays

Splenocytes were harvested from HODxOTII F1 mice, CD4⁺ T cells were enriched using the MagniSort Mouse CD4 T cell Enrichment Kit, and 2×10^5 T cells were cultured *in vitro* in complete RPMI 1640 supplemented with phorbol myristate acetate (PMA; 50ng/mL) and ionomycin (1μg/mL) for 48 hours. Brefeldin A and monensin were added for the last 5 hours. Production of intracellular Interleukin (IL)-2 was assayed with the Intracellular Fixation & Permeabilization Buffer Set (ThermoFisher) per the manufacturer's instructions. Samples were run on a BD LSRII.

Statistics

Comparison of means between two groups was analyzed with an unpaired t-test. For experiments with 3 or more groups, individual experiments with each repetition were compiled and analyzed with a 2-way ANOVA or mixed model analysis with Sidak's comparisons post-test. Statistics were performed with GraphPad Prism. A p-value of 0.05 was considered significant and *p 0.05, **p 0.01, ***p 0.001, ****p 0.0001.

3. Results

3.1 Thymic central tolerance is not required for tolerization of RBC-autoreactive CD4⁺ T cells

To address how tolerance specific to RBC autoantigens is established, we utilized a murine model that expresses the transgenic RBC-restricted HOD antigen. In previous studies with these mice, T cell tolerance was the stopgap to RBC autoimmunity, which could be circumvented by adoptive transfer of CD4⁺ OTII T cells (which react to the OVA epitope in the HOD antigen)[19]. As such, HOD and OTII mice were bred to generate HOD⁺OTII⁺ autoreactive mice and HOD⁻OTII⁺ littermate controls. Thymi were collected from F1 mice and stages of thymocyte development were evaluated for evidence of central tolerance, including negative selection and generation of regulatory T cells (Tregs)[24]. No significant differences in the number of total thymocytes were observed (Figure 1A). Consistent with the properties of CD4 TCR transgenic animals, most single positive (SP) thymocytes cells expressed CD4; however, there were no significant differences between the absolute number or percentage of CD4 SP thymocytes when comparing autoreactive HOD⁺OTII⁺ and control HOD⁻OTII⁺ mice (Figure 1B–C), suggesting that autoreactive CD4⁺ thymocytes did not undergo negative selection in the thymus of HO⁺OTII⁺ mice. Similar expression of Va2 and Vb5, the TCR gene combination associated with OTII T cells, in CD4 SP thymocytes was observed in F1 mice (Figure 1C, right panels). CD4 SP thymocytes were also stained with CD25 and FoxP3, a phenotype consistent with Tregs. However, no significant differences in the absolute number or percentages of Tregs were seen when comparing autoreactive HOD⁺OTII⁺ mice to their HOD⁻OTII⁺ littermate controls (Figure 1D and Supplemental Figure 1). To exclude the possibility that the TCR transgene itself prevented deletion and/or Treg generation in this model, F1 mice were injected intravenously with soluble OVA. In this case, significant deletion of CD4 SP thymocytes was observed in both autoreactive HOD⁺OTII⁺ and control HOD⁻OTII⁺ mice (Supplemental Figure 1). Moreover, similar frequencies of Tregs were generated in F1 mice (Supplemental Figure 1). Together, these data suggest that the thymus is dispensable for CD4⁺ T cell tolerance to RBC autoantigens.

It is plausible that negative selection was not evident in the OTII TCR transgenic mice due to background TCR rearrangement or expansion of T cells lacking Va2⁺Vb5⁺ co-expression[25]. Moreover, normal T cell development may not be reflected due to high clonal frequencies in the TCR transgenic mice. To circumvent these limitations, mixed bone marrow (BM) chimeras were generated with reduced frequencies of CD4⁺ OTII T cells; these also used OTII.Rag2^{-/-}.Thy1.2 donors to prevent potential background TCR rearrangement. Briefly, B6.Thy1.1/1.2 and HOD.Thy1.1/1.2 recipients were lethally irradiated and reconstituted with 10% of TCR75.Thy1.1 (a CD4⁺ TCR transgenic with a different specificity), 10% OTII.Rag2^{-/-}.Thy1.2, and 80% of the recipient BM genotype (B6.Thy1.1/1.2 or HOD.Thy1.1/1.2, respectively) (Figure 1E). Using this approach, thymi were analyzed from autoreactive HOD.Thy1.1/1.2 and control B6.Thy1.1/1.2 BM chimeras. For controls, some mice received irradiation and BM transplants with 100% of their genotype to control for background antibody staining in BM chimeras. No significant differences were observed in total thymocyte numbers between BM recipients (data not shown). In contrast to HODxOTII F1 mice, the precursor frequency of CD4⁺ OTII T cells

was markedly reduced in BM chimeras (Figures 1C compared to 1F and 1G). Determination of CD4⁺Va2⁺Vb5⁺ OTII.Thy1.2 T cell frequencies revealed no significant differences between reconstitution into HOD.Thy1.1/1.2 and control B6.Thy1.1/1.2 BM recipients (Figure 1F and 1G). Additionally, no significant differences were observed between the percentages of non-specific CD4⁺Vb8.3⁺ TCR75.Thy1.1 T cells between HOD.Thy1.1/1.2 and control B6.Thy1.1/1.2 BM chimeras. Of the individual OTII.Thy1.2 and TCR75.Thy1.1 T cell populations identified within the BM chimeras, no statistically significant differences were observed in percentages or numbers of CD4⁺CD25⁺FoxP3⁺ Tregs (Supplemental Figure 2 and data not shown). Taken together, these data generated with the HODxOTII F1 and BM chimeric mice demonstrate that RBC-autoreactive CD4⁺ OTII T cells are neither deleted nor selected into a regulatory T cell compartment in the thymus. As such, central tolerance mechanisms do not play a pivotal role in tolerizing RBC-specific autoreactive CD4⁺ T cells, suggesting that peripheral tolerance mechanisms are responsible for the profound tolerance observed in HOD mice.

3.2 Autoreactive CD4⁺ T cells are susceptible to deletion and are prompted to become Tregs in the periphery

Given that autoreactive OTII T cells were not removed during central tolerance, we next investigated whether deletion occurred in the periphery. Analysis of splenocytes from HODxOTII F1 mice showed no differences in overall splenocyte counts, but did identify a significant reduction in CD4⁺ T cells and CD4⁺Va2⁺Vb5⁺ OTII T cells in autoreactive HOD⁺OTII⁺ mice, as compared to HOD⁻OTII⁺ controls (Figures 2A–C and flow plots in 2D). Moreover, of the CD4⁺ T cells detected in HOD⁺OTII⁺ mice, there was a significant reduction in the number and percentage of T cells co-expressing Va2 and Vb5, as compared to control HOD⁻OTII⁺ animals, suggesting either TCR downregulation and/or expansion of non-OTII T cells (due to the potential for background rearrangement) (Figure 2D). Additionally, there was a significant increase in Tregs detectable in HOD⁺OTII⁺ mice (Figures 2D and E). In parallel, we also analyzed splenocytes from BM chimeras for evidence of peripheral tolerance. Unexpectedly, there were no observed differences in the absolute numbers of total splenocytes, CD4⁺ T cells, OTII.Thy1.2 T cells, or TCR75.Thy1.1 T cells in HOD.Thy1.1/1.2 recipients, as compared to control B6.Thy1.1/1.2 BM chimeras (Figures 2F–I). However, there were significant increases in the percentage and number of OTII.Thy1.2 Tregs in HOD.Thy1.1/1.2 BM chimeras (Figures 2I and J). Analysis of peripheral blood showed a significant deletion of OTII.Thy1.2, but not TCR75.Thy1.1, T cells in HOD.Thy1.1/1.2 BM chimeras, as compared to controls (Figure 2K). Taken together, these data demonstrate that, in response to the HOD RBC autoantigen, both deletion and Treg generation occurred in the periphery.

3.3 Non-deleted autoreactive T cells are functionally impaired

Although deletion removed a significant number of splenic OTII T cells in HOD⁺OTII⁺ mice, approximately 50% of the autoreactive OTII T cells remained (see Figure 2C). To test whether these remaining T cells were functional, CD4⁺ T cells were isolated from HODxOTII F1 mice, labeled with lipophilic dyes, and co-transferred into B6.Thy1.1 mice. The next day, recipients were transfused with HOD RBCs or control PBS. Proliferation of adoptively transferred T cells was then assessed 3 days post-transfusion. No dilution of

proliferative dyes was observed in cohorts that received a PBS infusion. However, despite similar numbers of both OTII populations utilized for adoptive transfer, there was a marked reduction in the frequency of OTII T cells from autoreactive HOD⁺OTII⁺ spleens detectable after 3 days, suggesting a competitive disadvantage for survival. In response to HOD transfusion, CD4⁺ T cells from HOD⁻OTII⁺ donors proliferated robustly, whereas T cells from HOD⁺OTII⁺ donors failed to expand (Figure 3A). Taken together, these data suggest that TCR-dependent activation may be inhibited. To assess whether TCR-independent signals could prompt T cell activation, CD4⁺ T cells from HODxOTII F1s were purified and stimulated *in vitro* with PMA and ionomycin; in this case, fewer CD4⁺ T cells from HOD⁺OTII⁺ mice produced IL-2, and at lower levels, as compared to HOD⁻OTII⁺ animals (Figures 3B and 3C). In aggregate, these data suggest that RBC autoreactive T cells were functionally anergic.

OTII T cells from HODxOTII F1 mice were also evaluated for expression of markers associated with anergy and antigen experience. By *ex vivo* analysis of OTII T cells from HOD⁺OTII⁺ mice, several anergy-associated markers (e.g., transcription factors NFAT2 and Egr2) were upregulated, and there was a significant increase in the percentage of cells that co-expressed CD73 and FR4, as compared to control animals (Figures 3D and 3E)[26; 27; 28]. The exhaustion-associated transcription factor, Helios, and the inhibitory receptors CD200 and PD1, were also elevated in autoreactive OTII T cells (Figure 3D)[29; 30]. To assess whether autoreactive T cells had encountered their cognate antigen, Ki-67, CD62L, and CD44 expression were evaluated. In HOD⁺OTII⁺ animals, there was increased expression of Ki-67 within TCRb-expressing cells, particularly in OTII T cells, as compared to control mice (Figures 3F and 3G). In contrast, similar frequencies of Ki-67 expression were detected in non-T cell populations in HODxOTII F1 mice. Additionally, autoreactive OTII T cells from HOD⁺OTII⁺ mice had increased frequencies of memory CD62L^{lo}CD44^{hi} CD4⁺ OTII T cells, as compared to controls. In addition, in contrast to autoreactive T cells from HOD⁺OTII⁺ mice, most of the OTII T cells from HOD⁻OTII⁺ mice expressed a naïve CD62L^{hi}CD44⁻ phenotype (Figures 3H). In aggregate, these data demonstrate that deletion-resistant autoreactive T cells encounter their cognate antigen and are subsequently rendered tolerant upon further TCR-dependent and TCR-independent re-stimulation.

3.4 Tolerance to the HOD RBC autoantigen is initiated in recent thymic emigrants

Thus far, the data indicate that tolerance of autoreactive OTII T cells to an RBC-specific autoantigen does not require the thymus, but does involve peripheral tolerance. In previous studies, the youngest peripheral T cells (i.e., those that have recently left the thymus) are more responsive to tolerance induction in the absence of inflammation, as compared to mature T cells[31]. These RTEs differ phenotypically and functionally from mature T cells and represent a post-thymic maturation state that occurs as RTEs exit the blood circulation and enter secondary lymphoid organs[32]. To investigate whether tolerance against the HOD RBC-autoantigen occurred in RTEs or in mature T cells, OTII.Rag2p-GFP mice were bred with HOD animals. OTII.Rag2p-GFP mice express GFP downstream of the recombination activating gene 2 (*Rag2*) promoter, allowing RTEs to be distinguished from mature T cells based on higher GFP expression. To this end, purified CD4⁺ T cells from HODxOTII.Rag2p-GFP mice were sorted based on GFP expression into GFP^{neg}, GFP^{int} and

GFP^{hi} populations (Supplemental Figure 3A) and adoptively transferred into B6.Thy1.1 recipients, followed by HOD RBC transfusion. Proliferative responses, measured by Ki-67 staining, were determined at 3 days post-transfusion. Similar to previous observations (see Figure 3A), mature autoreactive CD4⁺ T cells (i.e., GFP^{neg}) had diminished proliferative capacity in response to HOD RBCs (Figure 4A and Supplemental Figure 3B). In contrast, RTEs (i.e., GFP^{hi}) from the same donors robustly proliferated following HOD transfusion, indicating that these cells had not yet been tolerized. These differential responses to HOD RBCs based on maturation stages in HOD⁺OTII.Rag2p-GFP⁺ mice were not observed in CD4⁺ OTII T cells from HOD⁻OTII.Rag2p-GFP⁺ animals, as all GFP^{neg}, GFP^{int}, and GFP^{hi} CD4⁺ T cells had similar proliferation profiles in response to a HOD RBC transfusion. Thus, these data suggest that tolerance mechanisms are activated as RTEs are functionally maturing into naïve T cells.

To investigate the molecular basis behind the differential proliferation of GFP^{hi} vs. GFP^{neg} autoreactive CD4⁺ OTII T cells from HOD⁺OTII.Rag2p-GFP⁺ mice, the sorted cells were stained for the anergy- and exhaustion-associated markers that were upregulated on OTII T cells from HOD⁺OTII⁺ animals (See Figures 3D and 3E). The most striking observation was the increasing frequency of autoreactive OTII T cells co-expressing FR4^{hi}CD73^{hi} as the T cells matured in HOD⁺OTII.Rag2p-GFP⁺ mice (Figure 4B). And, although NFAT2, Egr2, and Helios were upregulated in autoreactive T cells in HOD⁺OTII.Rag2p-GFP⁺ mice, there were no consistent trends upwards or downwards throughout the CD4⁺ T cell maturation process (Supplemental Figure 3C). Taken together, these data demonstrate that RTEs are not tolerized and maintain their capacity to proliferate in response to their cognate RBC antigen. However, as RTEs mature, they express markers associated with anergy and lose the ability to respond to antigenic stimulation. These data also suggest that incomplete tolerization of RTEs, or failure of the relevant tolerance mechanisms, lead to autoreactive T cell proliferation and subsequent autoantibody production.

4. Discussion

Failure of tolerance to RBCs can induce AIHA, a human disorder accompanied by significant morbidity and mortality. The mechanisms leading to the breakdown of tolerance, and the production of pathogenic autoantibodies, are unknown. By using murine models, we provide evidence that RTEs play a key role in establishing tolerance to RBCs. Importantly, our data show that thymic central tolerance mechanisms are not required to establish tolerance. As such, peripheral tolerance mechanisms, such as deletion and Tregs, are essential to prevent autoimmunity. Although these mechanisms lead to functional anergy of mature naïve T cells, the youngest RTEs are still able to respond to the RBC autoantigen; nonetheless, this functionality wanes upon maturation. Correlating with their maturation, RTEs upregulate transcription factors and cell surface antigens associated with anergy and exhaustion. Taken together, these data suggest that incomplete or inefficient tolerization of RTEs can predispose individuals to develop RBC-specific autoimmunity.

Unexpectedly, the thymus was not required for T cell education against RBC-specific autoantigens. Data presented herein suggest that thymic negative selection does not occur in autoreactive animals as similar numbers and frequencies of thymocytes and CD4⁺ SP cells

are comparable to those in control mice. One limitation to these studies is the inability to rule out different rates of thymic T cell differentiation and apoptosis between autoreactive and control animals. However, both deletion and increased frequencies of Tregs are observed upon infusion of soluble OVA into HOD⁺OTII⁺ animals (Supplemental Figure 1), demonstrating that negative selection can occur in autoreactive mice. Together, these data suggest that RBC antigens are not presented, or not efficiently presented, in a way that prompts significant reduction in autoreactive thymic T cell numbers. Tissue-restricted antigens are expressed in the thymus by medullary thymic epithelial cells (mTECs), mediated by the *AIRE* transcription factor, no data exist regarding RBC-specific antigens. However, in several instances, autoantigens presented in the thymus do not initiate tolerance induction. For example, autoantigens may be presented at concentrations too low, or of a different isoform (as compared to the same antigen expressed on the tissue-specific cell), to mediate deletion[33; 34; 35]. Thus, it is plausible that RBC antigens are not presented, or are inefficiently presented, by mTECs to initiate central tolerance mechanism. Moreover, if we posit that *AIRE* does control thymic expression of RBC antigens, then patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED, e.g., autoimmune polyglandular syndrome type 1 (APS1)), who have *AIRE* mutations, would have associated AIHA. Nonetheless, despite the correlation between AIHA and underlying primary immunodeficiencies, very few patients with APECED have secondary AIHA[36; 37; 38]. Thus, it is likely that the thymus and *AIRE* play minor roles, if any, in tolerizing RBC-autoreactive T cells.

Similar to observations with naïve HOD mice, RBC-autoreactive T cells in both HOD⁺OTII⁺ and HOD.Thy1.1/1.2 BM chimeric mice persist in the periphery. However, some of these autoreactive T cells have downregulated their T cell receptor, expressed Treg cell surface markers, and/or upregulated transcription factors associated with anergy and/or exhaustion. However, one T cell population, the youngest RTEs, was not tolerized to HOD RBC autoantigen. Indeed, GFP^{hi} RTEs retained the capacity to proliferate in response to this autoantigen. Nonetheless, as RTEs matured in the periphery, they became functionally impaired. This transition correlated with upregulation of the CD73 and FR4 anergy-associated cell surface markers. CD73^{hi}FR4^{hi} anergic T cells, together with Tregs, were shown to limit expansion and activation of autoreactive T cells in autoimmunity and transplant models[39; 40; 41]. Progression toward tolerization is not definitively determined, though, because concurrent inflammation is associated with development of effector T cells and immunopathology[31]. Thus, taken together, these data identify a narrow window of tolerization for RBC-autoreactive CD4⁺ T cells, suggesting that if tolerance is not established during this time frame, for any reason, then AIHA may develop.

In summary, we used well-defined mouse models to determine how T cell tolerance is established to an RBC-specific autoantigen. Our data show that, unlike most self-antigens, tolerization to RBC autoantigens does not require the thymus. Instead, RTEs are progressively tolerized during post-thymic maturation. Importantly, we pinpoint a developmental time frame during which RBC-autoreactive T cells encounter antigen and become anergic. Thus, given that deletional tolerance is incomplete, subsequent reversal of anergy or inefficient tolerization of RTEs may cause the onset of AIHA. Finally,

understanding these underlying mechanisms will suggest new therapeutic approaches for preventing and/or treating AIHA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. The thymus is dispensable for tolerization of RBC-specific autoreactive T cells.
2. Newly emigrated autoreactive RTEs can proliferate in response to antigen.
3. Tolerized RTEs upregulate anergy and exhaustion markers and are nonresponsive.
4. Incomplete or inefficient tolerization of RTEs would lead to RBC-specific autoantibodies.

Figure 1: Thymic central tolerance is dispensable for RBC-autoreactive CD4⁺ T cells

To evaluate central tolerance mechanisms and thymocyte development, thymi were harvested from 8–12 week old HODxOTII F1 mice. The total numbers of (A) thymocytes and (B) CD4 single positive (SP) thymocytes was calculated. (C, left) The percentage of CD4 SP, CD4⁺CD8⁺ double positive (DP), and CD4⁻CD8⁻ double negative (DN) of total CD3⁺ thymocytes was determined. Additional characterization of CD4 SP thymocytes included (C, right) expression of TCR Va2 and Vb5 and (D) calculation of total numbers of CD25⁺FoxP3⁺ Tregs. (E) Schematic for generating mixed BM chimeras. B6.Thy1.1/1.2 and HOD.Thy1.1/1.2 recipients were irradiated and reconstituted with a mix of BM from OTII.Rag2^{-/-}.Thy1.2, TCR75.Thy1.1, and either B6.Thy1.1/1.2 or HOD.Thy1.1/1.2. (F) Thymocytes were analyzed at 6–8 weeks post-transplant and the CD4 SP thymocytes were identified and assessed for expression of Thy1.2, Thy1.1, Va2, and Vb5 expression. (G) The percentage of CD4⁺Va2⁺Vb5⁺ OTII.Thy1.2 and CD4⁺Vb8.3⁺ TCR75.Thy1.1 T cells were determined for BM recipients B6.Thy1.1/1.2 and HOD.Thy1.1/1.2. Data shown are representative of 3 independent experiments with 2–5 mice per group. Data were analyzed with a 2-way ANOVA with Sidak's multiple comparisons post-test.

Figure 2: Peripheral tolerance mechanisms lead to deletion and regulatory T cell generation of autoreactive OTII T cells.

Splenocytes were collected from HODxOTII F1 mice and the absolute numbers of (A) splenocytes, (B) CD4⁺ T cells, and (C) CD4⁺Va2⁺Vb5⁺ OTII T cells were determined. (D) Flow plots of gating strategy shown for determining percentages of CD4 T cells, co-expression of OTII TCR Va2 and Vb5, and expression of Treg markers CD25 and FoxP3. (E) The absolute number of splenic OTII Tregs. Filled circles represent splenocytes from HOD⁻OTII⁺ mice, whereas open circles represent HOD⁺OTII⁺ animals. Data are representative of 3 independent experiments with 2–5 mice per group. Data were analyzed with an unpaired T-test. To assess peripheral tolerance in BM chimeras, splenocytes were

collected and numbers of (F) splenocytes, (G) CD4⁺ T cells, and (H) reconstituted OTII.Thy1.2 and TCR75.Thy1.1 cells were enumerated. (I) Flow plots of gating strategy for determining Thy1.1 and Thy1.2, OTII.Thy1.2 expression of TCR Va2 and Vb5, and co-expression of CD25 and FoxP3. (J) Absolute numbers of OTII.Thy1.2 Tregs in BM chimeras. (K) Percentages of OTII.Thy1.2 and TCR75.Thy1.1 T cells of total CD4⁺ T cells in peripheral blood. Data are representative of 4 independent experiments with 3–5 mice per group, except in Panel K where peripheral blood mononuclear cells (PBMC) data are representative of 1 of 3 BM chimera cohorts with 10–15 recipients per genotype per cohort. Data with 2 groups was analyzed with an unpaired T-test whereas 3 or more groups were analyzed with a 2-way ANOVA with Sidak's multiple comparisons post-test.

Figure 3: Autoreactive OTII T cells from HOD⁺OTII⁺ mice are functionally impaired. CD4⁺ T cells were enriched from HODxOTII F1 spleens. (A) Recipient B6.Thy1.1 mice received 5×10^6 of CD4⁺ T cells from both HOD⁺OTII⁺ CellTrace-Far Red (FR) and HOD⁺OTII⁺ CellTrace-CFSE donors. Proliferation was assessed 3 days after PBS infusion or HOD RBC transfusion. For analysis, events shown were pre-gated on live cells with phenotype Thy1.2⁺CD4⁺Va2⁺Vb5⁺. (B) Enriched CD4⁺ T cells were co-cultured in vitro with PMA and ionomycin for 48 hours and assessed for IL-2 production by intracellular cytokine staining. The percent of IL-2⁺CD4⁺ T cells was determined and (C) a representative flow plot of IL-2 produced is shown. The flow plot was pre-gated on Thy1.2⁺CD4⁺ live cells. To determine expression of anergy-associated transcription factors and cell surface markers, splenocytes from HODxOTII F1 mice were collected and stained with antibodies against (D) NFAT2, Helios, Egr2, CD200, and PD1, and (E) CD73 and FR4. For analysis, events shown were pre-gated on Thy1.2⁺CD4⁺Va2⁺Vb5⁺CD25⁺FoxP3⁺ live cells. (F) Intracellular Ki-67 staining profiles for TCRb⁺ and TCRb⁺ cells and (G) frequency of Thy1.2⁺CD4⁺Va2⁺Vb5⁺CD25⁺FoxP3⁺ OTII T cells that express Ki-67 and (H) co-express CD44 and CD62L. Data shown are representative of 1 of 3 independent experiments with 3 mice per group. Data were analyzed with a mixed models test with Sidak's multiple comparisons post-test.

Figure 4: Tolerization to the HOD RBC-autoantigen occurs during the RTE developmental stage.

To determine whether RTEs are tolerized to RBC autoantigens, spleens were harvested from HODxOTII.Rag2p-GFP mice and processed into single cell suspensions. CD4⁺ T cells were enriched and CD8⁻ Thy1.2⁺CD62L^{hi}CD44^{lo} T cells were sorted based on GFP expression (GFP^{hi}, GFP^{int}, GFP^{lo}). (A) Sorted T cell populations were adoptively transferred into B6.Thy1.1 mice, followed by a HOD RBC transfusion or PBS control infusion. Proliferation of Thy1.2⁺CD4⁺Va2⁺Vb5⁺ OTII T cells was assayed 3 days post treatment by staining for Ki-67. (B) Sorted T cells were stained with antibodies against CD73 and FR4. Data shown are representative of 1 of 3 independent experiments with 3 mice per experimental group.