Published in final edited form as:

Transfusion. 2014 November ; 54(11): 2880–2891. doi:10.1111/trf.12683.

Immunological characterization suggests reduced alloimmunization in a murine model of thalassemia intermedia

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Abstract

Background—Transfusion therapy remains a mainstay of treatment for patients with thalassemia major and to a lesser extent for the less anemic patients with thalassemia intermedia. We have previously reported a role for regulatory T cells (Tregs) in the control of antibody responses in wildtype C57BL/6 (WT) mice exposed to allogeneic RBC transfusions. As an initial step to study and characterize immune regulation in thalassemias, we performed an immunological cell type characterization of C57BL/6 Hbb^{th-1}/Hbb^{th-1} mouse model of thalassemia intermedia (Thal) in steady state as well as following transfusions with allogeneic blood.

Study Design and Methods—The myeloid and lymphocyte compartments including Tregs and T helper (Th) responses were analyzed in transfusion naïve Thal and WT mouse spleens. The effect of allogeneic transfusions on Treg and global Th responses was also measured.

Results—We found elevated levels and activity of splenic Tregs in Thal mice with lower T helper (Th)1/Th2 ratios pre- as well as post-transfusion. Furthermore, pre-transfused Thal mice had altered ratios of the splenic myeloid compartment with increased proportion of macrophages but lower frequency of conventional dendritic cells. Surprisingly, transfusions resulted in lower alloimmunization levels in Thal as compared to WT mice.

Conclusion—These data suggest that this experimental model of thalassemia intermedia has an intrinsic alteration in splenic immunoregulation with an increased resistance to alloimmunization, raising the possibility studying this animal model may help to identify potential immunoregulatory networks to inhibit alloimmunization.

Keywords

thalassemia; experimental model; Tregs; Th2; DC; alloimmunization

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The authors report no potential conflicts of interests.

Authorship

W.B. performed research, analyzed data, drafted the manuscript, H.Z. performed research and analyzed data, edited the manuscript and K.Y. designed, directed and wrote the paper.

Introduction

β -thalassemia is a congenital hemolytic anemia caused by defects in β -globin chain synthesis. Since the α -globin production is not affected, excess free α -globin chains accumulate in the erythroid precursor and mature red blood cells (RBCs), causing membrane damage accompanied by reduced survival of erythroid precursors and RBCs¹. Ineffective erythropoiesis as a result of destruction of erythroid precursors characterizes this hemoglobinopathy². In patients with thalassemia major, who have severe anemia, the disease is treated by lifelong red cell transfusions. Patients with thalassemia intermedia, who have less severe form of the disease and are not chronically transfused, will nevertheless require RBC transfusions intermittently for the management of complications related to hemolytic anemia during childhood and as adults^{3,4}. However, transfusions can cause complications including development of antibodies against the transfused RBCs that may result in potentially life-threatening hemolytic reactions. In addition, finding compatible units for patients with alloantibodies can be difficult and identifying and characterizing the antibodies can be costly, time-consuming and laborious, causing transfusion delays. In regularly transfused patients with thalassemia major, alloimmunization rates as high as 30% have been reported^{5–10}. In a recent publication from the Thalassemia Clinical Research Network Investigators, no significant differences in alloimmunization frequencies between regularly transfused patients with thalassemia major and patients with thalassemia intermedia who were intermittently transfused were reported,¹¹ suggesting that alloimmunization is also a relatively common observation even in patients with thalassemia intermedia.¹² RBC antigenic differences between the donor and the recipient are the major trigger for alloantibody production, although additional factors¹³ including the clinical state of the recipient¹⁴, their genetic predisposition¹⁵, and number of transfused units¹⁶ appear to play a role in alloimmunization risk. Since not all transfused recipients develop alloantibodies even in regularly transfused patients with hemoglobinopathies¹⁷, we have hypothesized that host immune regulatory factors are critical to alloimmunization pathogenesis^{17–19}. Indeed, we showed that key immune response regulators, CD4⁺ regulatory T cells (Tregs) expressing FoxP3, control the magnitude of alloimmunization in wildtype C57BL/6 (WT) mice^{18;19}. Understanding the regulation of immune responses to transfused RBCs may help in future design of therapeutic interventions to prevent alloimmunization. As an initial step to study and characterize immune regulation in thalassemias, we studied the splenic immunological compartment including Treg status in C57BL/6 Hbb^{th-1/th-1} mouse model of beta-thalassemia intermedia, known to exhibit mild anemia, reticulocytosis and hepatosplenomegaly.²⁰

Materials and Methods

Mice and transfusions

All experimental protocols were approved by the Institute Animal Care and Use Committee of the New York Blood Center. Transgenic mice expressing human glycoprotein A (huGPA)²¹ were in the FVB background. C57BL/6 Hbb^{th-1/th-1} mice²⁰ (referred to in this study as Thal mice) were kindly provided by Dr. Mary Fabry. The mice are homozygous for a deletion of the murine β -major gene, and had been bred from a mixed genetic background

parental stock (Jackson Laboratories) and backcrossed for more than 10 generations onto a C57BL/6 background at the Albert Einstein Medical School by Dr. Mary Fabry and her colleagues. Male or female 12-week-old Thal and their WT littermate controls were transfused intravenously with 50 μ L RBCs, equivalent to 1 to 2 packed RBC units, from mice transgenic for huGPA that had been ficoll-treated to remove WBCs (Supplemental Fig. 1).¹⁸ Transfusions were administered with or without 50 μ g unmethylated CpG-containing oligonucleotides (CpG) for the first week and then on a weekly basis for 3 additional weeks with RBCs alone.¹⁸ Alloantibody levels were measured by incubating diluted plasma with GPA transgenic RBCs followed by the indirect antiglobulin test and analysis by flow cytometry.¹⁸ For post-transfusion RBC survival studies (Supplemental Fig. 1), ficoll-treated, WBC reduced huGPA RBCs were labeled with a fluorescent linker (PKH-26, Sigma-Aldrich) before transfusion. Transfused mice were bled shortly thereafter (within 1 min post-transfusion) and at different time points after that, and the blood was analyzed by flow cytometry. The percentage of labeled cells at the initial bleed for each mouse was set at 100% and the fraction of cells remaining at later time points was calculated accordingly.

Analysis of myeloid and lymphoid compartments

Splenic myeloid compartment was analyzed using a previously published protocol to distinguish DCs, monocytes, macrophages, and granulocytes²². Single spleen cell suspensions were prepared by perfusing the spleens with Collagenase D (final concentration 400 U/ml, Roche Diagnostics Corporation, Indianapolis, IL) and DNaseI (final concentration 50 μ g/ml, Roche Diagnostics Corporation) solution, followed by teasing the spleen with a syringe into small pieces, and further incubating at 37°C for 25 min. The reaction was stopped by neutralizing with EDTA (final concentration 5mM) for 5 mins. The cell suspension was then removed of red cells using lysis buffer (BD Biosciences, San Jose, CA) for 30 seconds. Cell viability was assessed by incubation in the amine-reactive dye Aqua [(Invitrogen, Eugene, OR) (1:500) dilution in Ca²⁺ and Mg²⁺ free PBS)] for 30 min in the dark at room temperature, followed by a single wash in 1x PBS. For all experiments, cells were incubated in 0.5 μ g Fc Block (BD Biosciences) for 10 min at room temperature. Surface staining (Supplemental Table 1) was performed in the dark for 30 min at 4°C in staining buffer.

T-cell phenotypic analysis was performed in the spleen and lymph nodes using fluorescently conjugated antibodies against mouse CD4 and CD25 (all from BD Biosciences). FoxP3 expression was measured by intracellular staining with anti-Foxp3 following the manufacturer's instructions (eBioscience, San Diego, CA, USA). For intracellular cytokine expression studies, splenocytes were resuspended (2×10^8 cells/mL) in media containing 10% fetal calf serum, 1% L-glutamine, and antibiotics (all from Invitrogen Life Technologies) before stimulation with PMA (20 ng/mL; Sigma-Aldrich), ionomycin (2000 ng/mL; Sigma-Aldrich), and GolgiPlug (Brefeldin A; BD Biosciences) plus GolgiStop (monensin; BD Biosciences) for 5 hours. After CD4⁺ or CD8⁺ T-cell surface staining, cytoplasmic IFN- γ , IL-2, TNF- α , IL-17, IL-4 and IL-10 expression were analyzed using a Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions and staining with anti-mouse antibody (all from BD Biosciences except anti-IL-17 from eBioscience) or an isotype-matched control. CD4⁺ cells expressing IFN- γ , IL-4 and IL-17

were referred to as Th1, Th2 and Th17 cells, respectively whereas CD8+ cells expressing IFN- γ and IL-4 were defined as Tc1 and Tc2, respectively.^{23;24} The ratios of Th1/Th2 and Tc1/Tc2 were calculated based on relative expression of IFN- γ as compared to IL-4 expressing CD4+ or CD8+ cells, respectively. The ratio of Th1/Th17 was calculated based on the ratio of IFN- γ as compared to IL-17 expressing CD4+ cells.

Treg suppressive assay

CD4+ T cells were isolated from adult (12 week old) splenocytes by depletion of non-CD4+ T cells (negative selection, Miltenyi Biotec, Auburn, CA, USA). Cells were stained with anti-mouse CD4 PE and anti-mouse CD25APC antibody and subjected to cell sorting (purity > 95%) using either MoFlo (Beckman Coulter, Indianapolis, IN) or Aria (BD Biosciences) instruments. CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells (5×10^4 cells/well) were cultured in duplicates alone or together at various ratios in the presence of anti-CD3 (clone 2C11, 1 μ g/mL; BD Biosciences) together with 5×10^4 /well of irradiated splenocytes from WT mice as antigen presenting cells for 72 hours as previously described.¹⁸ The cultures were pulsed with 1 μ Ci [3H] thymidine for the last 16 hours and uptake of labeled thymidine was measured by liquid scintillation (PerkinElmer, Waltham, MA). Percent proliferation was determined as (cpm incorporated in the coculture)/cpm of CD4⁺CD25⁻ cells alone) $\times$ 100.

Statistical analysis

All data are presented as mean $\pm$ standard deviation. Statistical analyses were performed using the Mann-Whitney *U* test for all comparisons described in the study, and differences were considered significant at $P < 0.05$.

Results

Altered splenic immune profiles in Thal mice

As a first step to better characterize the immunological responses of Thal mice, we compared the relative proportion of various splenic immune cell types between Thal and WT mice. Splenic B220+ B cell lymphocyte frequencies were comparable in the 2 strains (Table 1). CD3+ T cell lymphocyte subset was 30% lower in Thal (n=12) vs WT (n=12) mice ($p=0.009$), although the relative proportion of CD4+ or CD8+ within the CD3+ lymphocytes was similar in both strains of mice (Table 1). We also examined splenic T cell cytokine expression profiles in short-term cultures activated with PMA/ionomycin. We found a higher frequency of IL-2+, IL-17+ and IL-4+ expressing splenic CD4+ cells in Thal as compared to WT mice ($p<0.05$). Frequencies of splenic CD8+ cells expressing IFN- γ +, TNF- α +, IL-2+ and IL-4+ levels were also significantly higher in Thal than WT mice (Table 1). Altogether, the ratio of Th1/Th2 was lower in Thal ($5.1 \pm 1.1\%$) than WT ($8.8 \pm 5.9\%$) mice ($p=0.01$), but the relative ratios of Th1/Th17 and Tc1/Tc2 were comparable in the 2 groups (Table 1).

Classical DCs (CD11c^{hi}), which are most effective in antigen presentation and T cell stimulation²⁵, were less abundant in the spleens of Thal as compared to WT mice (Fig. 1A and B, $p=0.01$). Murine CD11c+ DCs in the spleen and lymph nodes can be further divided into CD8+ and CD8- subpopulations with the former subset more effective in inducing CD8

cytotoxic responses and cross-presentation whereas the CD8⁺ subset more efficient in processing and presenting antigen via MHC class II and inducing Th2 responses ²⁶. Despite the overall reduction of CD11c^{hi} population, we did not detect any differences in the frequencies of CD8⁺ or CD8⁺ subsets within the CD11c^{hi} DC population between Thal and WT mice. Similarly, we found comparable frequencies of the plasmacytoid DCs (Fig. 1B), which are known as the major producers of type I IFNs required for pathogen clearance ²⁷. Red pulp macrophage (CD11c^{dim}CD11b^{dim}F4/80^{high}) and granulocyte (CD11c^{dim}CD11b^{high}Ly6G⁺) frequencies were higher in Thal mice whereas the monocyte (CD11c^{dim}CD11b^{high}Ly6G⁻CD115⁺) percentages lower (Fig. 1B). We did not perform careful measurements to determine the absolute numbers of any of the immune subsets in transfusion naïve mice, although total number of nucleated cells that included both WBCs and nucleated erythroblasts was calculated to be more than 3 times higher in Thal as compared to WT mouse spleens.

Thal mice have increased Treg frequency and activity

Despite an overall lower T cell frequency in splenocytes in Thal mice, the proportions of CD4⁺CD25⁺ and Tregs (CD25⁺FoxP3⁺ in the CD4⁺ cells) were significantly higher (about 30%) in splenic Thal as compared to WT mice (Table 2). Interestingly, we found no difference in the proportions of CD4⁺, CD4⁺CD25⁺ and Tregs in the lymph nodes between the 2 strains (Table 2).

Since the splenic Treg frequency was higher in Thal mice, we next examined whether the functional activity of the Tregs was equally higher. In vitro suppression of T-cell receptor-stimulated proliferation of other T cells is a commonly used assay for assessing Treg suppressive function ²⁸. We therefore measured the immunosuppressive effects of Thal and WT Tregs on proliferation of autologous and allogeneic effector (CD4⁺CD25⁻) T cells (n=3, involving 3–5 mice per group). Sorted Tregs (CD4⁺CD25⁺) from both groups were equally hyporesponsive to polyclonal stimulation with anti-CD3 and WT antigen-presenting cells (3H incorporation for Thal 188±27 cpm and WT 198±65 cpm; *P*>0.5), indicating that Tregs from both cohorts of mice do exhibit the characteristic anergic Treg phenotype. On cocultures with sorted CD4⁺CD25⁻ cells at different ratios, Tregs from Thal mice and WT suppressed proliferation of autologous CD4⁺CD25⁻ T cells at 1:1, 1:2 and 1:4 ratios in a dose-dependent manner. However, Tregs from Thal mice were better suppressors as indicated by lower proliferation rates at different Treg to T effector ratios (Fig. 2A). This was further confirmed by cross-mixing experiments in which Tregs from Thal mice showed a similar suppressive activity when Thal or WT effector T cells were assayed (Fig. 2B, compare white and light grey columns). In addition, Tregs from WT mice were equally effective in suppressing Thal and WT effector T cells (Fig. 2C, compare white and dark grey columns).

Transfusion studies in Thal mice

We next compared the immunomodulatory effects of transfusion in WT and Thal mice. We transfused hu-GPA RBCs, following removal of WBCs by ficoll (Supplemental Fig. 2), for 4 consecutive weeks without or with injection of the Toll-like receptor (TLR)-9 agonist, CpG included with the first allogeneic transfusion ¹⁸. Transfused mice produced

alloantibodies not only against the huGPA moiety but also against FVB determinants on RBCs (Supplemental Fig. 2), although the antibody specificities were not characterized. Mouse RBCs express MHC class I molecules³¹ and since there is MHC-mismatch between huGPA donor mice (H2^q haplotype because of FVB background) and the recipient C57BL/6 mice (H2^b haplotype), it is likely that the specificity of the alloantibodies include anti-H2^q produced against donor RBCs and/or contaminating WBCs, the latter situation similar to patients developing anti-HLA antibodies despite transfusion of leukoreduced RBCs.^{29;30} Transfusion of mice without CpG resulted in some alloreactivity with surprisingly slightly lower levels in Thal as compared to WT mice (Fig. 3). When CpG was included with the first transfusion, alloimmunization levels increased although the levels were significantly lower in Thal as compared to WT mice (Fig. 3), suggesting that Thal mice are more resistant to alloimmunization than wildtype mice. Using transfusion-naïve mice, we also compared the survival of PKH-labeled huGPA RBCs in WT and Thal mice, and found a similar survival at 48 hours post-transfusion (n=2 per group). Interestingly, however, survival of transfused cells at day 10 post-transfusion was considerably better in non-alloimmunized Thal (n=6) as compared to non-alloimmunized WT mice (n=4, 96±9% versus 60±9%, p=0.01).

The frequency of IFN- γ (Fig. 4A) and IL-4 (Fig. 4B) expressing CD4⁺ and CD8⁺ cells were increased while IL-10 levels (Fig. 4C) were decreased following transfusion in both Thal and WT mice. In fact, Th1/Th2 and Tc1/Tc2 cytokine response ratios were reduced following transfusion as compared to non-transfused mice, regardless of the strain of mice albeit more significantly in Thal mice. Nevertheless, as we had observed in transfusion naïve mice (Table 1), splenic Th1/Th2 (Fig. 4D, p=0.02) and Tc1/Tc2 (Fig. 4E, p=0.04) were lower in Thal as compared to WT mice following transfusion. On the other hand, the ratio of Th1/Th17 cytokine responses increased following transfusion (Fig. 4F, p<0.01) due to a higher frequency of IFN- γ +CD4⁺ cells (Fig. 4A). As with transfusion naïve mice, Th1/Th17 ratios did not differ between transfused Thal and WT mice (Fig. 4F, p=1).

Similar to transfusion-naïve mice (Table 2), we found a lower frequency of splenic CD4⁺ (Fig. 5A, p=0.04), and a higher frequency of CD4⁺CD25⁺ (Fig. 5B, p=0.002), and Tregs (Fig. 5C, p=0.002) in Thal as compared to WT mice following transfusion (Fig. 5). CD4⁺CD25⁺ and Treg subsets were higher after transfusion in both mouse strains, although the increase in Tregs in Thal mice was more evident than in WT mice (Fig. 5C).

Discussion

In this study, we have performed a comparative analysis of immunologic profiles of an experimental model of thalassemia with that of WT mice. Surprisingly, we found a reduced level of alloimmunization in Thal as compared to WT mice, although it should be stressed that we did not characterize the specificity of the alloantibodies and that our transfusion model does not distinguish between alloantibodies initially induced by RBCs vs. WBCs. Frequency as well as suppressive function of splenic Tregs, which we have shown to inhibit alloimmunization,^{18;19} was increased in Thal mice, and there was a skewing of their global Th1/Th2 responses toward Th2. As compared to WT mice, Thal mice also exhibited a higher frequency of splenic Th17 cells, known for their role in tissue inflammation³².

Furthermore, we identified altered myeloid populations in the spleens of Thal mice, including reduced frequency of classical DCs, but an increased proportion of macrophages and granulocyte subsets. The observed higher frequency of Thal splenic granulocytes may reflect the increased inflammatory state, and may further exacerbate the pathologic splenic tissue. Post-transfusion RBC survival studies at day 10 using non-alloimmunized mice indicated a better survival of circulating transfused RBCs in Thal as compared to WT mice, suggesting that despite an increase in F4/80+ macrophage subset, their phagocytic capability in Thal mice may be compromised, possibly due to increased RBC destruction and splenic overload. Additional studies are needed to explore this possibility and to test whether RBC survival and alloimmunization using other allogeneic RBCs are similarly affected in this thalassemia model. RBC transfusion resulted in an expansion of T effector cells such as those expressing IFN- γ , and IL-4 as well as frequencies of Tregs. Since these frequencies were altered in both WT and Thal mice, it suggests that blood transfusion may stimulate expansion of T effector cells but that as a counter-regulatory response regulatory cells such as Tregs are also expanded to dampen any potential collateral damage by effector cells. Even following transfusion, Thal mice displayed a higher frequency of Tregs and therefore a stronger immune regulatory phenotype as compared to WT mice. In contrast to the Thal mouse studies, in our analysis of peripheral blood of a cohort of regularly transfused splenectomized adult patients with thalassemia, we did not find altered Treg frequency,³³ although Bozdogan et al found increased peripheral Treg frequency regardless of splenectomy in their cohort of mostly pediatric thalassemia patients on regular transfusions³⁴. The difference between the mouse and human thalassemia Treg data may be due to inherent differences between human and mouse model studied (see below). However, the source material used to perform the analysis differed in the 2 studies (peripheral blood in the human studies as compared to spleens in the mouse studies) and there are differences in the transfusion protocols with respect to the number of transfusions and donor RBC exposures between the human and mouse studies (see below).

We have previously reported a role for Tregs in the control of the magnitude of alloimmunization;^{18;19} the possibility remains that the expanded Treg compartment in Thal mice is responsible for lower levels of alloimmunization. In our analysis of transfusion-naïve adult sickle cell disease (SCD) (n=5) and control (n=5) mice, a higher frequency of CD25⁺FoxP3⁺ Tregs was also detected in the peripheral blood³⁵ and spleens of SCD group (14.8%±2% versus 9.3%±1.6%, p=0.008). Splenic Treg frequency was 30% higher in a different thalassemia intermedia mouse model, C57BL/6 Hbb^{th-3/+}, as compared to WT mice, and similarly, transfused thalassemia major mice (C57BL/6 Hbb^{th-3}/Hbb^{th-3}, n=2) had higher splenic Tregs as compared to transfused WT mice (14.4±0.4% vs 11.5±1.6%). Since Tregs play a key role in limiting excessive immunopathology in chronic inflammatory diseases,³⁶ the increased Treg percentages in these mouse models of hemoglobinopathies may represent a compensatory counter regulatory mechanism to dampen the ongoing chronic inflammation associated with stress erythropoiesis/increased RBC destruction. This raises the question as to whether Tregs in the spleens of transfusion-naïve human thalassemia or SCD are also expanded. It should be noted that inflammation such as provision of TLR agonists prior to transfusion can increase alloimmunization levels as seen in our WT and Thal mice, although this is more akin to an acute inflammation, such as the

immune response to an infection, altering the balance of Tregs/Th cells toward higher effector T cells,^{37;38} which then drive alloimmunization. Interestingly, unlike the spleen, Treg percentages in lymph nodes of the Thal mice were not elevated, suggesting that the inflammation potentially responsible for expansion of Treg compartment is restricted to the spleen. Since Tregs are expanded in the spleens, but not in the lymph nodes, the prediction will be that Thal mice may have normal responses to antigens that are normally presented in the lymph nodes. Future studies are needed to address whether these mice are more susceptible to infections as compared to WT mice, and whether transfusions alter their risk of infections.

Regularly-transfused patients with hemoglobinopathies including thalassemia and SCD have a higher alloimmunization rates (>30%) as compared to the general hospital population (<3%)⁵⁻⁹. However, in this study, we found a reduced magnitude of alloimmunization in a mouse model of thalassemia as compared to WT mice. Similarly, in mouse models of SCD, increased levels of alloimmunization following transfusion of allogeneic filter-leukoreduced RBCs were not observed⁴⁰. The difference between the mouse and human studies may be due to different transfusion protocols used. Our transfusion protocol in Thal mice involved 4 weekly transfusions and in the study Hendrickson et al. using SCD models,⁴⁰ the mice were only given a single transfusion. Since the number of transfused units appears to play a role in alloimmunization risk in the human studies,^{16;41} it may be that alloimmunization levels in Thal or SCD mice will be higher with increasing numbers of transfusions. Intrinsic differences in the splenic microenvironment of humans and mice may also account for alloimmunization discrepancies between human and mouse models. For example, the splenic marginal zone whose role is to trap and present antigens from the circulation to resident immune cells is structurally distinct in humans and mice.⁴² Such spatial differences in juxtaposition antigen presenting cells and T cells may be more affected in Thal mice which have expanded red pulp areas due to extramedullary erythropoiesis⁴³. In fact, the mouse spleen is the primary hematopoietic organ under stress conditions possibly due to limited bone marrow space in rodents,⁴⁴ whereas the bone marrow is considered the primary hematopoietic organ in humans, despite the known contribution of the spleen as a site of extramedullary erythropoiesis in chronic pathologies including thalassemia major.⁴⁵ This is best reflected in the spleen weight of Thal mice which is almost 5 times more than that of WT mice ($0.348 \pm 0.005\text{g}$ vs $0.072 \pm 0.011\text{g}$)⁴³. Thus, the murine spleen which has a dual role as the site of RBC destruction and production in Thal mice may favor a cytokine/cellular environment that is more resistant to alloimmunization. For example, the erythroid differentiation stage when erythrocyte maturation is arrested differs in human and mouse thalassemia⁴⁶, and as a result the levels of soluble factors/cytokines produced by the erythroid precursors that accumulate in the two species are different⁴⁷, raising the possibility that the cytokine environment in erythroid niches may be different. Such differences may also limit Treg expansion in the spleens of human thalassemia. Better characterization of human thalassemia spleens are clearly needed to address this, ideally comparing spleens without and with extramedullary erythropoiesis. In addition, by comparing the magnitude of alloimmunization without and with splenectomy, we may be able to address the role of the spleen in thalassemic or SCD mouse alloimmunization, although the interpretation of such experiments may be difficult since splenectomy in at

least in one mouse model resulted in failure to mount a T cell dependent alloantibody response against transfused RBCs ³⁹.

In conclusion, we have found an expansion of the splenic Treg compartment accompanied by a lower global Th1/Th2 response as well as altered myeloid subsets in an experimental model of thalassemia intermedia. As this experimental mouse is less susceptible to alloimmunization, our data suggest that immune subversion mechanisms may be responsible for altered alloantibody production. By understanding the factors that alter regulatory mechanisms in this mouse model before and during alloimmunization, intervention can aim to promote regulatory arm of the immune response during RBC transfusions with the goal that it would lead to suppress alloimmunization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by National Heart, Lung, and Blood Institute grant R21HL097350 (K.Y.). The authors would like to thank Petra Pham (Head of laboratory of Flow Cytometry, NYBC) for assistance with flow cytometric analysis. We are deeply grateful to Dr. Stefano Rivella and his team members Dr. Carla Casu and Dr. Laura Breda (all Weill Cornell Medical College) for giving us spleen preparations from their *Th3/+* and *Th3/Th3* mice.

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Fig. 1. Splenic myeloid profiles of WT and Thal mice

(A) The gating strategy was followed based on the report by Idoyaga et al.²² After excluding debris, doublets and nonviable cells from splenocytes, the population of CD19⁻, Ter119⁻, CD3⁻, and CD49b⁻ were selected and further analyzed for expression of CD11c and CD11b. Classical DCs were identified by high CD11c integrin expression and were subdivided into CD11b^{high}CD8⁻ and CD11b^{low}CD8⁺ subsets. In addition, the CD11c^{dim}CD11b^{dim} subpopulation was selected to identify red pulps macrophages as the cells with high expression of F4/80, as well as plasmacytoid DCs expressing PDCA-1 and B220 (PDCA-1⁺, B220⁺, CD11c^{int}, CD11b^{low}). Similarly, by gating on CD11c^{dim}CD11b^{high} population, granulocytes were identified as Ly6G⁺ subset (CD11b^{high} Ly6G⁺) whereas Ly6G⁻ cells were further analyzed using CD115 expression to identify monocytes (CD11b^{high}, CD115⁺Ly6G⁻). (B) Frequencies (mean±SD) of these subsets in transfusion-naïve WT and Thal mice (5 mice per group) are shown. Statistically significant differences are indicated by * next to the p values.

Fig. 2. Treg suppressive activity of Thal and WT mice

Sorted population of splenic CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were cultured alone (not shown) or together at various ratios and stimulated with anti-CD3 and irradiated splenocytes from WT mice as antigen presenting cells for 72 hours as previously described.¹⁸ The cultures were pulsed with tritiated thymidine for the last 16 hours and uptake of labeled thymidine was used to measure proliferation. Percent proliferation was calculated from measurements of proliferation in the cocultures relative to proliferation of effector T (CD4⁺CD25⁻) cells. **(A)** Percent proliferation in cocultures of T effector (“T eff”) with autologous Tregs from WT (black bars) and Thal (white bars) mice. With dose-dependent decrease in the ratio of Tregs to T effectors, percent proliferation increases, indicating release of suppression. **(B)** Percent proliferation in cocultures of T effector (“T eff”) from

WT mice with Tregs from Thal mice (light grey bars). For comparison, autologous proliferative responses of WT (black bars) and Thal (white bars) from (A) is shown. (C) Percent proliferation in cocultures of T effector ("T eff") from Thal mice with Tregs from WT mice (dark grey bars). Here too, autologous proliferative responses of WT (black bars) and Thal (white bars) from (A) is shown for comparison.

Fig. 3. Lower levels of alloimmunization in Thal mice

Mice were given intravenous transfusion of ficoll-treated huGPA RBCs (to remove WBCs, Supplemental Fig. 2) equivalent to 1–2 packed units without (open or filled circles) or with (open or filled triangles) CpG adjuvant, followed by weekly transfusions of red cells alone for another 3 weeks. The presence of IgG-specific alloantibodies against huGPA RBCs in plasma from mice was then measured by an indirect anti-globulin test using flow cytometry as described previously.^{18;19} As a control, the background reactivity of huGPA RBCs with plasma from non-transfused mice was also tested (filled diamonds, “No transfusion”). (A) Representative histograms showing RBC fluorescence; the numbers indicate relative mean fluorescent activity (MFI). In these experiments, background fluorescence of mouse huGPA RBCs was higher (MFI 181) than in the experiment shown in Supplemental Fig 2 (MFI 31), probably due to small fluctuations in laser voltages of the flow cytometer. (B) Data analysis of MFI of alloreactivity is shown on y-axis. Statistically significant differences between the transfused groups are indicated by p values. If we exclude from the analysis the one WT mouse transfused “without CpG” which has strongly reactive alloantibody (MFI>1000), the p value reaches 0.057 between the transfused WT and Thal mice without CpG.

Fig. 4. Altered T cell cytokine expression following RBC transfusions

Thal and WT Mice were transfused with huGPA together with CpG, followed by 3 additional transfusions with huGPA alone given once per week as in Fig. 2. One week after the last transfusion, the mice were sacrificed and the splenocytes stimulated with PMA/ionomycin (5 hr) in presence of the Golgi inhibitors. Frequencies of CD4+ and CD8+ cells expressing cytoplasmic (A) IFN- γ , (B) IL-4 and (C) IL-10 were measured by flow cytometry according to the representative gating strategy using the appropriate isotype control antibody. The relative ratios of (D) Th1/Th2, (E) Tc1/Tc2 and (F) Th1/Th17 in

splenocytes from the same transfused mice are shown. For comparison, cytokine frequencies of transfusion naïve mice from Table 2 are also shown. Statistically significant differences between groups (5 mice per group) are indicated by an asterisk. (“p=n.s.” means not significant)

Fig. 5. Treg compartment characteristics following RBC transfusion

Frequencies of (A) CD4+, (B) CD4+CD25+, and (C) CD25+Foxp3+ lymphocyte population in the spleen of WT and Thal transfused mice (5 mice per group) was analyzed by flow cytometry according to the gating strategy shown and as comparison, the frequencies in transfusion naïve mice from Table 2 are also shown.

Table 1

Splenic lymphocytic profiles of WT and Thal mice.

		WT (n 5)	Thal (n 5)	p value
B cell lymphocytes	B220+	47.4±7.6	46.8±7.9	0.9
	CD3+	45.1±9.5	30.8±7.6	0.009*
T cell lymphocytes	CD4+	62.2±4.3	62.6±2.7	0.9
	CD8+	32.4±3.9	30.8±3.5	0.4
	CD4+	8.3±2.0	9.4±2.8	0.1
	IFN- γ	12.1±2.8	15.0±4.0	0.04*
	IL-2	41.2±8.8	41.0±10.4	0.9
	TNF- α	0.41±0.11	0.55±0.20	0.04*
	IL-17	1.21±0.60	1.91±0.93	0.007*
	IL-4	8.8±5.9	5.1±1.1	0.01*
	Th1/Th2	20.9±4.1	18.6±7.1	0.2
	Th1/Th17	19.8±4.9	28.0±8.5	0.006*
	CD8+	4.4±1.6	7.6±2.3	0.0005*
	IFN- γ	21.9±4.7	25.9±6.1	0.03*
	TNF- α	0.16±0.13	0.16±0.13	0.9
	IL-17	0.1±0.12	0.2±0.12	0.04*
	IL-4	254±165	211±190	0.3
	Tc1/Tc2			

Table 2

CD4⁺, CD4⁺CD25⁺ and Treg frequencies in spleen and lymph nodes of WT and Thal mice.

	<u>WT (n 5)</u>	<u>Thal (n 5)</u>	<u>p value</u>
Spleen			
CD4 ⁺	20.5±2.9	17.7±2.8	0.004*
CD4 ⁺ CD25 ⁺	10.9±1.9	14.9±2.2	< 0.0001*
CD25 ⁺ Foxp3 ⁺	10.1±1.3	13.2±1.2	< 0.0001*
Lymph node			
CD4 ⁺	33.5±2.8	33.5±3.4	0.8
CD4 ⁺ CD25 ⁺	13.3±1.6	13.4±1.2	0.9
CD25 ⁺ Foxp3 ⁺	12.1±1.7	12.1±1.3	0.9