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Bridging channel dendritic cells induce immunity to transfused red blood cells

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Red blood cell (RBC) transfusion is a life-saving therapeutic tool. However, a major complication in transfusion recipients is the generation of antibodies against non-ABO alloantigens on donor RBCs, potentially resulting in hemolysis and renal failure. Long-lived antibody responses typically require CD4+ T cell help and, in murine transfusion models, alloimmunization requires a spleen. Yet, it is not known how RBC-derived antigens are presented to naive T cells in the spleen. We sought to answer whether splenic dendritic cells (DCs) were essential for T cell priming to RBC alloantigens. Transient deletion of conventional DCs at the time of transfusion or splenic DC preactivation before RBC transfusion abrogated T and B cell responses to allogenic RBCs, even though transfused RBCs persisted in the circulation for weeks. Although all splenic DCs phagocytosed RBCs and activated RBC-specific CD4+ T cells in vitro, only bridging channel 33D1+ DCs were required for alloimmunization in vivo. In contrast, deletion of XCR1+CD8+ DCs did not alter the immune response to RBCs. Our work suggests that blocking the function of one DC subset during a narrow window of time during RBC transfusion could potentially prevent the detrimental immune response that occurs in patients who require lifelong RBC transfusion support.

Chronic RBC transfusion therapy is essential for patients with hematological disorders and bone marrow failure syndromes, such as sickle cell anemia and myelodysplastic syndrome. Further, bone marrow transplantation is not possible without ancillary transfusion support. However, a major complication of RBC transfusion is the development of non-ABO alloantibodies (Vamvakas and Blajchman, 2010). Induction of alloantibodies to blood group antigens present on donor RBCs, but absent on recipient RBCs, affects nearly 5% of general patients and up to 30% of chronically transfused patients (Vichinsky et al., 1990; Tormey et al., 2008). RBC alloimmunization can induce acute or delayed hemolytic transfusion reactions and can increase the risk of hemolytic disease of the newborn; both conditions are potentially fatal. With the exception of the prophylactic use of anti-D immunoglobulin during pregnancy, no therapeutic interventions currently exist to prevent RBC alloimmunization, other than avoiding transfusion of RBCs with specific antigens (Casas et al., 2015).

Despite the fundamental role that blood group antigen characterization by Landsteiner and Levine (1928) had on the emergence of immunology as a field, few immunologists study or even recognize the phenomenal diversity of, and immune responses to, human RBC antigens. Thus, we have a limited understanding of what immune signals or cells dictate when alloimmunization occurs. A primary unanswered question is how RBC-derived antigens are presented to lymphocytes. In contrast to carbohydrate RBC antigens (e.g., in the ABO system), most protein alloantigens require CD4+ T cell help to generate alloantibodies (Stephen et al., 2012); therefore, it is not surprising that one genetic risk factor for development of RBC alloantibodies is a recipient’s human leukocyte antigen (HLA) type, specifically MHC II (Chioroni et al., 2006; Stephen et al., 2012). Nonetheless, which APCs present RBC-derived antigens on MHC II is unknown.

As mechanistic studies in humans are not possible, we developed a murine transfusion model to study the response to RBC alloantigens and to identify which splenic APCs present these antigens to CD4+ T cells. Mice have a poorly
understood system of blood group antigens and do not express the same minor antigens as human RBCs. Therefore, we developed transgenic mice expressing well-defined foreign antigens on RBCs. HOD encodes a triple fusion integral membrane protein only on RBCs under control of the β globin promoter; it contains the model polypeptides hen egg lysozyme (HEL) and chicken OVA fused to the human Duffy blood group antigen (Desmarets et al., 2009; Fig. 1 A).

Our previous work demonstrated that both splenic DCs and macrophages phagocytose alloantigenic murine RBCs in vivo, but did not address antigen presentation by these cells (Hendrickson et al., 2007).

Given the dominant role of splenic macrophages in phagocytosing aged RBCs and original work demonstrating that sheep RBC-stimulated macrophages could activate T cells in vitro, it has been assumed that macrophages play a primary role in alloimmunization (Swierkosz et al., 1978). Studies using parasite-infected or sheep RBCs suggested that DCs are the primary APC in the spleen (Yi and Cyster, 2013; Borges da Silva et al., 2015). Conventional DCs in the spleen can be divided into two broad categories based on ontogeny, cell surface marker expression and predilection for CD4+ vs. CD8+ T cell activation (Dudziak et al., 2007). The antibody 33D1 marks one of these subsets, which expresses the C-type lectin receptor DCIR2 (DC-inhibitory receptor 2; Dudziak et al., 2007; Yi and Cyster, 2013). 33D1+ DCs are known to preferentially capture transfused sheep RBCs (Yi and Cyster, 2013), and the inability of sheep CD47 to engage murine SIRPα on 33D1+ DCs has been shown to stimulate an inflammatory response (Yi et al., 2015).

However, unlike allogeneic RBCs, xenogeneic sheep RBCs are completely cleared from the circulation immediately after transfusion, possibly secondary to opsonization by naturally occurring antibodies or secondary to the missing CD47 inhibitory signal. We have previously shown that CD47 is not significantly altered on allogeneic murine RBCs during processing despite being immunogenic (Gilson et al., 2009). Therefore, although it is a useful model to study germinal center responses, sheep RBC transfusion is unlikely to mimic APC handling of allogeneic RBCs. Using a murine model of RBC alloimmunization, we dissected the mechanism by which RBC-specific CD4+ T cell help is generated for alloantibody induction after transfusion.

Although many cells in the spleen with antigen-presenting capability phagocytosed transfused RBCs, only DCs...
activated RBC-specific T cells in vitro. In vivo, transient depletion of conventional DCs (cDCs) abrogated the adaptive immune response to allogeneic RBCs. However, not all cDC subsets could induce RBC alloimmunization; indeed, only one splenic DC subset, the bridging channel 33D1+ DC, was required for T cell–dependent B cell responses to transfused RBCs. In addition, preactivation of DCs 24 h before transfusion blocked alloimmunization by preventing RBC uptake, despite ongoing circulation of allogeneic RBCs for weeks.

RESULTS AND DISCUSSION

RBC alloimmunization requires CD4+ T cells

When HOD RBCs are processed similarly to human RBCs (leukoreduced to remove >99% of white cells and stored in blood bank anticoagulant/preservative solution for 12–14 d), they induce an initial innate immune response followed by a delayed alloantibody response in transfused WT mice (Hod et al., 2010; Hendrickson et al., 2011; Fig. 1 A). The nature of the inflammatory stimulus on RBCs with a storage lesion remains unknown. Consistent with our previous findings, stored RBCs, regardless of alloantigen expression, circulate for >2 wk in recipients after an initial rapid clearance within the first few hours after transfusion (Hendrickson et al., 2011; Fig. 1 B). Although significant evidence suggests that antibodies to protein-based RBC alloantigens are CD4+ T cell dependent, we directly tested the requirement for CD4+ T cells in our murine alloimmunization model. Antigen–specific antibodies in serum were identified using ELISA-based quantification of HEL-specific antibodies (Fig. 1 C) and flow cytometric cross matching, which measures antibody bound to antigen–expressing but not native RBCs (analogous to blood bank cross matching; Fig. 1 D). After CD4+ T cell depletion with antibodies or using MHC II knockout recipients, we found a complete lack of alloantibody induction to transfused HOD RBCs (Fig. 1, C and D). Therefore, this RBC alloimmunization model is CD4+ T cell dependent and, accordingly, requires antigen presentation on MHC II. This is consistent with human studies of antibodies to Kell and Rh(D) group antigens (Boctor et al., 2003; Stephen et al., 2012).

Multiple MHC II-expressing cell types in the spleen phagocytose transfused RBCs

Although the site in humans of adaptive immune responses to transfused RBCs remains controversial (Ryder et al., 2014), splenectomized mice fail to mount a robust T or B cell response to transfused RBCs expressing a protein alloantigen (Hendrickson et al., 2009). Therefore, we used transfusion of leukoreduced GFP-expressing RBCs to identify which MHC II-expressing splenocytes phagocytose RBCs (Fig. 2, A and B). In contrast to xenogeneic sheep RBCs, which localize solely to the marginal zone (Yi and Cyster, 2013), the majority of transfused murine RBCs were in the red pulp, presumably returning to circulation (Fig. 2 B). The extent of RBC phagocytosis by APCs 30 min after transfusion was quantified by gating on surface markers for cells of interest and assessing GFP fluorescence; RBC lysis buffer and Ter-119 staining were used to exclude surface-attached RBCs. B cells and monocytes contained few transfused GFP+ RBCs; in contrast, a significant fraction of splenic macrophages and DCs ingested transfused RBCs (Fig. 2 A). These results suggest that splenic DCs and macrophages are the primary APCs for alloreactive T cells.

To identify which splenocytes are capable of activating RBC-specific CD4+ T cells, these four cell populations were sorted 8 h after HOD RBC transfusion and used to stimulate OT-II CD4+ TCR transgenic cells in vitro. Only DCs presented RBC-derived antigens ex vivo to RBC-specific CD4+ T cells; in contrast, B cells, macrophages, and monocytes induced little T cell activation (Fig. 2 C). Therefore, not all RBC+ APCs (Fig. 2 A) could process and present RBC-derived antigens to CD4+ T cells in vitro (Fig. 2 C). Interpretation of in vitro antigen presentation experiments must be done with caution (Itano and Jenkins, 2003); this assay only confirms antigen uptake and processing capability. As such, we tested the in vivo requirement for DCs during RBC alloimmunization.

Conventional DCs are required for T and B cell responses to transfused allogeneic RBCs

A model commonly used to test the in vivo role of DCs in immunity involves diphtheria toxin (DT) injection into mice expressing the DT receptor (DTr) under the control of the CD11c promoter, resulting in selective killing of CD11c-expressing cells (Jung et al., 2002). Because tissue DC turnover is rapid, with continual reseeding of bone marrow DC precursors, DT injection produces only transient deletion, on the order of days (Fig. 2 D). Using this system, CD11c-DTr mice injected with DT failed to mount an alloantibody response or induce CD4+ T cell proliferation following HOD RBC transfusion, suggesting that the relevant APC for T cell priming was deleted (Fig. 2 E). However, certain macrophage and monocyte populations are also deleted in the CD11c-DTr mice (Jung et al., 2002; Meredith et al., 2012). Multiple types of myeloid cells express CD11c without acting as a classical or conventional DC; cDCs are derived from a specific DC precursor, require the zinc finger transcription factor Zbtb46 for development (Meredith et al., 2012; Satpathy et al., 2012a) and act almost exclusively as APCs. In contrast, plasmacytoid DCs and monocyte-derived cells both express CD11c but develop independently of Zbtb46 and have immunological functions distinct from cDCs (Jung et al., 2002; Meredith et al., 2012; Satpathy et al., 2012a,b; Schlitzer et al., 2013). The role of cDCs in initiating CD4+ T cell priming to RBC alloantigens was tested using Zbtb46-driven DTr expression (Meredith et al., 2012). Transient deletion of cDCs alone at the time of transfusion abrogated the T and B cell responses to allogeneic RBCs (Fig. 2, F and G). Therefore, cDCs, a minor subset of splenic APCs, are responsible for initiating T cell–dependent RBC alloimmunization in vivo.
Figure 2. The presentation of RBC antigens to CD4+ T cells requires conventional DCs. (A) Cell populations in the spleen that phagocytose GFP+ RBCs 30 min after transfusion as compared with untransfused (naive) mice. n = 2–3 mice/group. Representative of four independent experiments. (B) Fluorescent image of a spleen from a mouse transfused with GFP-expressing RBCs 6 h prior. Bar, 100 µm. RP, red pulp; WP, white pulp. Representative image from one of three mice. (C) The same populations as in A were sorted from spleens of mice transfused with HOD RBCs 6–8 h prior and used to stimulate OT-II CD4+ T cells in vitro for 3 d. Proliferation measured by CFSE dilution of stimulated (open histogram) versus unstimulated (shaded histogram) T cells. (D) Percentage of DCs in the spleen in CD11c-DTr mice injected i.p. with DT on day 0. Gated from TCRβ−B220− cells. (E) WT and CD11c-DTr mice were treated with DT and received 10^6 CFSE-labeled CD45.1+ CD4+ OT-II cells. 48 h after T cell transfer, mice were transfused with HOD RBCs and proliferation in the spleen was
A single subset of DCs in the spleen is required for alloimmunization

There are two major subsets of cDCs in the spleen, recently termed cDC1 (XCR1⁺) and cDC2 (33D1⁺) based on divergence in ontogeny (Satpathy et al., 2012b; Guilliams et al., 2014). The cDC1 BATF3-dependent XCR1⁺CD8α⁺ DC subset readily cross presents phagocytosed antigen on MHC I to CD8⁺ T cells and includes DCs that express DEC-205 or CD103. The cDC2 IRF4-dependent 33D1⁺ DC subset preferentially primes CD4⁺ T cells and almost all express CD11b and SIRPα (Fig. 3 A; Lewis et al., 2011) and includes all CD4⁺ DCs. We examined whether one or both cDC subsets phagocytose transfused RBCs in vivo and present RBC-derived antigens to CD4⁺ T cells ex vivo. DCs from both subsets are positioned in the marginal zone of the spleen, although in different subdomains, and therefore should be exposed to transfused RBCs (Fig. 2 B; Idoyaga et al., 2009; Yi and Cyster, 2013). 33D1⁺ cDCs selectively express SIRPα, which is involved in the clearance of senescent RBCs and has been recently shown to regulate the inflammatory response to sheep RBCs; this suggests that 33D1⁺ DCs might preferentially phagocytose RBCs (Yi et al., 2015). Although 33D1⁺ DCs are the dominant subset in the spleen (Fig. 3 A), transfused GFP⁺ RBCs were ingested by both subsets (Fig. 3 B), resulting in roughly equivalent numbers of 33D1⁺ and XCR1⁺ DCs carrying RBCs. We analyzed which cDC subsets, when removed from the spleen 8 h after HOD RBC transfusion, could activate RBC-specific CD4⁺ T cells in vitro. Both XCR1⁺ and 33D1⁺ DCs induced OT-II T cell division in vitro, although that induced by 33D1⁺ DCs was more extensive (Fig. 3 B). This is consistent with known differences in MHC II antigen processing by the two subsets (Dudziak et al., 2007), but suggests that either or both DC subsets could initiate RBC alloimmunization.

We tested the impact of eliminating either the XCR1⁺ or 33D1⁺ DC subset on RBC alloimmunization in vivo using BATF3⁻ or IRF4⁻deficient mice, respectively (Suzuki et al., 2004; Bachem et al., 2012). As IRF4 is a transcription factor required for the effector function of many immune cells, these mice were generated by crossing a floxed IRF4 mouse to a CD11c-Cre strain (Schlitzer et al., 2013; Williams et al., 2013; Vander Lugt et al., 2014). We confirmed previous reports of impaired development of the CD4⁺ DC lineage and checked that 33D1⁺ DCs were concomitantly impaired. Of all nonlymphocytes in the spleen, CD4⁺ DCs were reduced by 91% (0.73% in Cre⁻ to 0.07% in Cre⁺), and 33D1⁺ DCs were reduced by 85% (0.38% in Cre⁻ to 0.06% in Cre⁺). Given known off-target IRF4 deletion in Cre⁻ cell lineages, we excluded mice in which >25% of lymphocytes were GFP⁺ (IRF4-deleted) for all experiments (Klein et al., 2006; Schlitzer et al., 2013).

Deletion of XCR1⁺ DCs using BATF3 knockout mice did not impact the adaptive immune response to transfused RBCs (Fig. 3 C). In contrast, loss of 33D1⁺ DCs abrogated RBC alloantibody induction as measured by cross matching (Fig. 3 D) or HEL-specific ELISA (unpublished data). Therefore, although both DC subsets phagocytose transfused RBCs and activate RBC-specific CD4⁺ T cells in vitro, only the 33D1⁺ DCs are required for HOD RBC alloimmunization in vivo. This suggests that 33D1⁺ cDCs have a unique capacity to present RBC-derived antigens to CD4⁺ T cells within the spleen. Ongoing work will determine how this preference is established.

Disrupting DC function during a narrow pretransfusion time window prevents alloimmunization

Having identified the APC responsible for RBC alloimmunization, we asked whether we could use this insight to prevent alloimmunization based on known DC biology. When DCs are activated, typically by an innate immune stimulus, they lose their capacity to present subsequently encountered antigens (Young et al., 2007). Further, the lifespan of most DCs after activation is ~1–2 d, necessitating replacement by incoming DC precursors, which can eventually present newly encountered antigens (De Smedt et al., 1996; Kamath et al., 2000). Although how transfused allogenetic RBCs activate innate immune pathways remains unknown, we predicted that RBCs are only immunogenic immediately after transfusion, given the dramatic loss of alloimmunization observed after transient DC depletion in CD11c-DTr and Zbtb46-DTr mice (Fig. 2, E–G), despite continued circulation of HOD RBCs for weeks (Fig. 1 B). We infused a well-known innate immune stimulus, LPS, 24 h before transfusion to test whether preactivating DCs before RBC alloantigen exposure prevents antigen presentation to T cells. LPS is a bacterial-derived molecule that activates TLR4 and matures DCs. After maturation, the percentage of splenic DCs remains constant over the first day, but 2 d after LPS activation, >50% of splenic DCs are lost (unpublished data), consistent with previous work (De Smedt et al., 1996; Kamath et al., 2000). Therefore, we introduced RBCs 1 d after LPS injection to give time for DCs to mature but before significant DC loss.

Pretransfusion LPS injection prevented both in vivo CD4⁺ T cell proliferation measured on day 3 (Fig. 4 A), as measured by CFSE dilution (left). Anti-RBC antibody in the sera of mice 21 d after HOD RBC transfusion (naive mice did not receive HOD RBCs; right). n = 2–9 mice/group. Representative of three independent experiments. **, P < 0.002. (F) Zbtb46-DTr BM chimeras were left untreated or treated with DT and transferred with CFSE-labeled OT-II cells. 3 d after DT treatment, mice were transfused with HOD RBCs and proliferation in the spleen was measured by CFSE dilution 3 d later (left). Anti-RBC antibody levels in the sera of Zbtb46-DTr chimeric mice at different time points after HOD RBC transfusion (right). ***, P = 0.0002; ****, P < 0.0001. (G) Representative flow cytometry plots of class-switched germinal center B cells (B220⁺ GL7⁺ IgG1⁺) 8 d after HOD RBC transfusion in Zbtb46-DTr BM chimeric mice treated or not treated with DT. n = 2–10 mice/group. Representative of three independent experiments.
well as alloantibody induction measured 3 wk after HOD RBC transfusion (Fig. 4 B), and remained undetectable after >5 wk (not depicted). Indeed, DC preactivation by LPS impaired GFP+ RBC uptake by splenic DCs (Fig. 4 C). We obtained similar results using other pathogen-associated molecular pattern, including the TLR2 agonist Pam2CSK4 (unpublished data). Therefore, DC preactivation by multiple kinds of innate stimuli 1 d before RBC transfusion impairs the ability of the splenic DCs to present alloantigens and stimulate adaptive immunity. As expected, impaired T cell activation to an antigen delivered to a preactivated DC is not RBC specific; if we use the same experimental layout as that shown in Fig. 4 A and use a soluble protein antigen instead of RBCs, we again see failure of OT-II CD4+ T cell proliferation in LPS pretreated mice (not depicted; Young et al., 2007). To test whether DC maturation before RBC transfusion induced immune tolerance or ignorance to the alloantigens, we rechallenged mice in which LPS had blocked the initial alloantibody response with a second transfusion of HOD RBCs (Fig. 4 D). Mice that had failed to respond to HOD transfusion 3 wk before due to DC preactivation by LPS were indeed capable of producing alloantibodies upon retransfusion, in fact making a level of antibody almost equivalent to the non–LPS-treated mice after the first transfusion. Mice that initially produced alloantibodies without LPS pretreatment boosted the level of antibodies in the sera after the second transfusion. Altogether this indicates that the requisite CD4+ T cells had not been tolerized to the alloantigen. Consistent with this finding, we were also able to elicit alloantibodies in DC-depleted Zbtb46-DTr mice after DT was discontinued and DCs returned to the spleen (unpublished data). Thus, in the absence of splenic DC function, the adaptive immune system remains ignorant to the initial immunogenic transfusion of allogeneic RBCs.

The blockade of RBC alloantibodies in LPS-pretreated mice is striking in light of the known circulatory lifespan of transfused alloantigen-expressing RBCs for >2 wk (Fig. 1 B). Therefore, the mere exposure of 33D1+ DCs to allogeneic RBCs is not sufficient to induce T and B cell activation. Either the subsequent wave of new DCs cannot capture or present the recirculating allogeneic RBCs or the initial transfusion is inflammatory, but the innate immune stimulus rapidly dissipates even as the transfused RBCs continue to circulate. In the latter case, DCs would not receive adequate activation stimuli to induce T cell priming. Further work must be done to parse out these two possibilities. As inflammatory events can also promote alloimmunization, our finding raises the interesting possibility that the dramatically different alloimmunization rates observed under different clinical scenarios might in part be related to the timing of inflammatory insult and the RBC transfusion (Fasano et al., 2015).

Identifying the APC that regulates the immune response to blood group antigens is the first step toward understanding, and ultimately preventing, the immune reaction.
elicited after RBC transfusion. Deletion of conventional DCs for 3–4 d after transfusion was sufficient to prevent activation of T cells specific for RBCs that circulated for weeks. Yet the two cDC subsets do not have equal abilities to induce alloimmunization in vivo, despite equivalent RBC phagocytosis and antigen presentation to T cells in vitro. Only 33D1+ DCs were essential for inducing an alloantibody response to transfused HOD RBCs. In contrast, deletion of the other major DC subset, marked by XCR1 and CD8αα, did not alter alloimmunization. Future work will address how this division of labor between the two cDC subsets dictates the T cell–dependent B cell response in vivo. The work presented here identifies the cells responsible for driving alloimmunization and, because equivalent DC subsets exist in humans (CD1c+ splenic DCs; Mittag et al., 2011), suggests new therapeutic targets for immunomodulation in chronically transfused patients that might avoid long term or widespread immunosuppression.

MATERIALS AND METHODS

Mice. C57BL/6 mice were purchased from Charles River. HOD mice were generated as previously described (Desmaret et al., 2009; Hendrickson et al., 2011); UBC-GFP, BATF3 KO, CII TA KO, CD11c-Cre, and IRF4fl/fl mice were purchased from The Jackson Laboratory. OT-II mice were purchased from the The Jackson Laboratory and bred with wild-type CD45.1 mice in our facility. All protocols used in this study were approved by Yale Institutional Animal Care and Use Committee.

RBC transfusion model. RBCs were collected from HOD transgenic mice in 12% CPDA-1 (citrate phosphate dextrose adenine) anticoagulant (Desmaret et al., 2009) and leukoreduced using either a Pall neonatal filter or a murine adapted Pall Acrodisc PSF 25-mm WBC filter with Leukosorb Media, followed by 4°C storage for 12 d; this mimics processing and storage conditions in human transfusion prac-
tice. Before transfusion, the RBCs were washed by centrifugation and the packed RBCs were diluted 1:2 with sterile PBS; 200 µl of diluted blood was transfused i.v. into recipient mice (the equivalent of 1–2 human RBC units). A dilution of 1:2 was used for alloantibody induction or ex vivo antigen presentation and 1:20 for in vivo OT-II proliferation.

**CD4⁺ T cell depletion.** To assess the requirement of CD4⁺ T cells in the formation of anti-HOD antibodies, C57BL/6 and MHC class II knockout (CIITA KO) recipients were transfused with 12-d stored HOD RBCs. In addition, some C57BL/6 recipients were depleted of CD4⁺ T cells by intraperitoneal injection of an anti-CD4-depleting monoclonal antibody (clone GK1.5; BioXcell) at 4 and 2 d before transfusion.

**Serum analysis.** Serum from transfused mice was collected 3 wk after RBC transfusion. To identify the presence of alloantibodies, sera were added to 96-well plates coated either with 5 µl of HOD⁺ RBCs or with 5 µl of HOD⁻ RBCs (negative control). After 30 min of incubation, plates were washed and stained with anti-Ig conjugated with APC for 30 min. The stained samples were washed and the total IgGs were analyzed by flow cytometry. Anti-RBC antibodies in all figures indicate anti-HOD antibodies detected this way with the adjusted MFIs shown (arbitrary unit). Where indicated, anti-HEL–specific IgG1 antibodies were detected in sera (starting dilution 1:50) measured by ELISA as described previously (Hendrickson et al., 2007). Anti-IgG1 (clone A85-1) served as detection antibody and HEL–specific IgG1 (clone 4B7) was used as the reference standard.

**Clearance of transfused RBCs.** At different time points after HOD–RBC transfusion, 20–30 µl of blood was collected in anticoagulant. The collected blood was processed and the percentage of transfused RBCs was determined using anti-Fy3 antibody (the anti-Fy3 antibody, provided by the NY Blood Center [New York, NY], recognizes a specific portion of the human Duffy, molecule expressed on the surface of transfused RBCs).

**GFP-RBC uptake.** GFP⁺ RBCs were collected from UBC-GFP mice, leukoreduced, and stored as described for HOD RBCs. 0.5 h after transfusion, the spleen was harvested and processed to obtain a single-cell suspension. The uptake of GFP-RBCs was analyzed by flow cytometry in B cells (TCRβ⁺B220⁻), DCs (TCRβ⁺B220⁻MHC II⁺CD11c⁻), monocytes (TCRβ⁺B220⁻Ly6G⁻Ly6C⁺), and macrophages (TCRβ⁺B220⁻Ly6G⁻Ly6C⁺F4/80⁻).

**Immunofluorescence microscopy.** Fresh splenic tissue was dehydrated through sequential exposure to solutions of 10, 20, and 30% sucrose, mounted in a cryomold with O.C.T. Compound (Tissue-Tek; Sakura), and stored at ~−80°C before sectioning. GFP was acquired with the Eclipse Ti microscope (Nikon) using 20× objectives.

**Ex vivo T cell proliferation.** Wild-type mice were transfused with HOD transgenic RBCs and, 8 h later, spleens were harvested and processed to obtain single-cell suspensions. Splenic T cells were depleted using anti-Thy1 antibody. Splenic B cells were purified by positive selection kit using biotinylated CD19, followed by anti-biotin MicroBeads (Miltenyi Biotec). Splenic DCs (Ly6G⁻Ly6C⁻CD11c⁻), monocytes (Ly6G⁺Ly6C⁺F4/80⁻), and macrophages (Ly6G⁺Ly6C⁺F4/80⁺) were sorted with FACS Aria (BD). After sorting, the desired cell populations were co-cultured with OT-II CFSE⁺ CD4⁺ T cells. 3 d later, proliferation was assessed by measuring CFSE dilution by flow cytometry.

**In vivo T cell proliferation.** 1 million transgenic CD45.1⁺ CD4⁺ T cells, isolated from OT-II mice, were purified with the CD4⁺ negative isolation kit (Miltenyi Biotec), CFSE labeled, and adoptively transferred i.v. into recipient mice. RBCs were transfused 24 h later, and 3 d after transfusion spleens were collected and the CFSE dilution of transgenic CD4⁺ T cell was determined by flow cytometry.

**DT.** DT was purchased from Sigma-Aldrich and titrated in Zbtb46-DTr BM chimeric mice (due to variability between different lots). For transient DC depletion in Zbtb46-DTr bone marrow chimeric mice, 60 ng of DT/gram of body weight was injected IP on day 0 followed by a second dose of 40 ng of DT/g on day 2. For transient DC depletion in CD11c-DTr, the same protocol was used except the day 0 injection was 5 ng DT/g on day 0 and 2.5 ng/g on day 2. For CD4⁺ T cell adoptive transfer experiments, transgenic T cells were adoptively transferred into mice on day 1. For either T cell adoptive transfer experiments or alloantibody experiments (without T cell transfer) HOD RBCs were transfused on day 3.

**BM chimera generation.** Wild-type mice were irradiated with two doses of 650 rad 3 h apart. 2 h after the second treatment, 10⁵ bone marrow cells from Zbtb46-DTr mice were adoptively transferred by i.v. injection into wild type recipient mice. All experiments with BM chimeric mice were performed 7–10 wk after bone marrow transplant.

**Antibodies.** Single-cell suspensions of spleen were acquired either with LSR II (BD), or MACSQuant (Miltenyi Biotec) flow cytometers and analyzed using FlowJo software (Tree Star). The following antibodies were used for staining different cell subsets (all from BioLegend): TCRβ (H57-597), B220 (RA3-6B2), MHC II (M5/114.15.2), CD11c (N418), 33D1 (33D1), XCR1 (ZET), Va2 (B20.1), CD4 (GK1.5), Ly6C (HK1.4), Ly6G (1A8), F4/80 (CI:A3-1), CD11b (M1/70), and polyclonal anti–mouse Ig (BD).

**Statistical analysis.** All statistical analyses were performed using GraphPad Prism software. Data were analyzed with the unpaired Student’s t test.
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