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Innate and adaptive immunity to transfused allogenic red blood cells in mice requires MyD88

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Abstract

Red blood cell (RBC) transfusion therapy is essential for the treatment of anemia. A serious complication of transfusion is the development of non-ABO alloantibodies to polymorphic RBC antigens, yet mechanisms of alloantibody formation remain unclear. Storage of mouse RBCs prior to transfusion increases RBC immunogenicity through an unknown mechanism. We previously reported that sterile, stored mouse RBCs activate splenic dendritic cells (DCs), which are required for alloimmunization. Here we transfused mice with allogeneic RBCs to test whether stored RBCs activate pattern recognition receptors (PRRs) on recipient DCs to induce adaptive immunity. Toll-like receptors are a class of PRRs that regulate DC activation, which signal through two adaptor molecules, MyD88 and TRIF. We show that the inflammatory cytokine response, DC activation and migration, and the subsequent alloantibody response to transfused RBCs require MyD88 but not TRIF, suggesting a restricted set of PRRs are responsible for sensing RBCs and triggering alloimmunization.

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Introduction

Chronic red blood cell (RBC) transfusion therapy is a mainstay of treatment for patients with hematological disorders and bone marrow failure syndromes such as sickle cell anemia, thalassemia major, and myelodysplastic syndrome (1). However, a major complication of RBC transfusion is the development of non-ABO alloantibodies. Unlike natural IgM antibodies against A and B carbohydrate blood group antigens, non-ABO alloantibodies target polymorphic minor antigens on donor RBCs following exposure via transfusion or pregnancy (2). RBC alloimmunization affects 3–10% of transfused patients and up to 50% of chronically transfused groups (3–5). As more than 12 million units of RBCs are transfused in the U.S. every year, the number of patients with alloantibodies is significant. RBC alloimmunization can result in hemolytic transfusion reactions, which are potentially fatal and can also delay the time to transfusion while compatible blood is sought (1, 6–8). Other than attempting to avoid transfusion altogether, there are limited therapeutic options to prevent RBC alloimmunization. Despite its clinical significance, the mechanism of alloantibody formation in response to RBC transfusion remains unclear.

In the United States, a typical sterile red cell unit for human transfusion is collected in anticoagulant, processed to remove leukocytes, and stored in special solutions to preserve RBC function at 1–6°C for up to 42 days at a hematocrit of approximately 60% (9). The average storage age of blood used for transfusions in the United States is estimated to be 18 days (10). During storage, RBCs can accumulate various types of damage, collectively termed “storage lesions”, which in mice has been shown to contribute to immunogenicity through an unknown mechanism (11). Our previous work and others have shown that transfusion of RBCs stored in this manner for as little as 12 days induces an inflammatory response in recipient mice and promotes subsequent alloantibody formation (12). Units of stored RBCs contain many potentially inflammatory components including iron, free heme, extracellular ATP, and modified lipids (13, 14). Human studies suggest that transfusion in the presence of recipient inflammation may be associated with enhanced RBC alloimmunization (15, 16), but the timing of innate immune stimulation may matter for this effect by regulating the dendritic cell (DC) response to RBCs (17). The specific role of storage itself on RBC alloimmunization is unclear in humans given the difficulty in identifying when alloimmunization occurs, potential use of immunosuppressive drugs, and lack of knowledge regarding recipient HLA type (18–21). Therefore, we used mouse models to test the hypothesis that damage to the RBC itself during storage is essential for triggering innate immune pathways that induce non-ABO alloantibodies.

To activate naïve T cells and B cells, antigen-presenting cells, such as DCs, must be activated through innate immune receptors termed pattern recognition receptors (PRRs); this is a crucial checkpoint in regulating T-dependent B cell antibody responses (22). Indeed, our previous work demonstrated that although transfused sterile RBCs are consumed by and activate numerous antigen presenting cells in the spleen, only type 2 conventional dendritic cells (cDC2s) were required for the generation of alloantibodies (17), likely due to a defect in T cell priming (23). How sterile RBC transfusion activates these DCs is not known (13). Prior work from others has similarly shown that the same bridging channel cDC2s are responsible for T-dependent B cell responses to sheep RBCs; for these xenogeneic RBCs,

DC activation is induced, at least in part, though species differences in CD47 (24, 25). As CD47 is not significantly altered by storage nor induces DC activation during allogenic transfusion (26, 27), we searched for other innate immune pathways activated by RBC transfusion. We hypothesized that the damage accumulated by RBCs during storage might stimulate an initial innate immune response through the ligation of one or several of PRRs on cDC2s.

PRRs sense both foreign motifs expressed by pathogens termed pathogen associated molecular patterns (PAMPs) and self-molecules released by or expressed on damaged host cells termed damage associated molecular patterns (DAMPs) (28). Our previous work demonstrated that NOD-like receptor (NLR) PRRs were not required for alloantibody formation, nor was the presence of the IL-1 family cytokines (IL-1 and IL-18) (13). Murine studies demonstrate that recipient exposure to various PAMPs such as Poly(I:C), CpG and influenza virus infection prior to transfusion of RBCs enhances alloimmunization to multiple alloantigens (29–31). These PAMPs are ligands for another family of PRRs, the Toll-like receptors (TLRs). Ten TLRs have been identified in humans and 13 in mice, which can be activated by both PAMPs and DAMPs and signal through the adapter proteins TRIF and/or MyD88 (32).

Using mice with RBCs expressing a model alloantigen, we tested the hypothesis that stored RBCs activate TLRs on DCs to induce adaptive immunity (12, 33). Here we show that MyD88, but not TRIF, is required for activation of splenic DCs by transfusion of stored RBCs. These results suggest that one or more TLRs dictate whether transfused sterile RBCs induce an inflammatory response capable of promoting RBC alloimmunization. Further we found that storage of C57BL/6 RBCs makes them intrinsically immunogenic, rendering them capable of inducing DC maturation even in the absence of foreign epitopes. Therefore, RBCs become immunogenic under normal storage conditions, even in the absence of foreign epitopes, and activate immune responses through the adapter protein MyD88. These findings identify key immune signaling pathways contributing to RBC alloimmunization that could ultimately lead to identification of RBC-associated ligands necessary to initiate alloimmunization. Such an understanding is a critical step towards developing targeted therapies to mitigate RBC alloimmunization.

Materials and Methods

Mice.

WT C57BL/6 mice were purchased from Charles River. HOD mice were generated as previously described (32). *Trif*$^{-/-}$ mice were purchased from The Jackson Laboratory (stock # 005037). *Myd88*$^{-/-}$ mice were purchased from The Jackson Laboratory (stock # 009088) and bred in our facility to generate *Myd88*$^{-/-}$ and *Myd88*$^{+/-}$ littermate controls to facilitate breeding, to create MyD88 homozygous and heterozygous littermate controls, and to control for microbiota driven effects. *Trif*$^{-/-}$ *Myd88*$^{-/-}$ mice were generated by breeding mice purchased from The Jackson Laboratory in our facility.

RBC Transfusion Model.

Red blood cells (RBCs) were collected from HOD or C57BL/6 mice into sterile 13% Citrate Phosphate Dextrose Adenine anti-coagulant (final volume) in a polystyrene round bottom 5 mL tube and leukoreduced using a murine adapted Pall Acrodisc PSF 25mm WBC filter with Leukosorb Media. Leukoreduced blood was adjusted to a hematocrit of approximately 75% as previously described (14, 25). We have validated that RBCs processed in this way are free from LPS contamination (21). RBCs were then transfused immediately or stored for 12–14 days at 4°C in 700 uL Eppendorf tubes (21). 100uL of RBCs were transfused retro-orbitally into recipient mice. For transfusion of supernatant or RBCs diluted in sterile PBS, leukoreduced blood was centrifuged at 1500×g for 10 minutes in a polypropylene Falcon round bottom 5 mL tube. Supernatant was pipetted into a new polypropylene Falcon tube and volume was measured. RBCs were then resuspended in an equal volume of sterile PBS. 100uL of supernatant or diluted RBCs were transfused retro-orbitally into recipient mice.

Enzyme-linked Immunosorbent Assay (ELISA).

Serum samples were analyzed by ELISA for CCL2 (MCP-1), CXCL1 (KC), or IL-6. MCP-1, KC, and IL-6 were detected with respective mouse cytokine ELISA kits (R&D) according to enclosed protocol.

Flow cytometry analyses.

Spleens were manually digested, washed in 2% FBS in PBS, and incubated with fluorescent antibodies for 30 minutes at 4°C. Cells were analyzed on either a Cytoflex (Beckman Coulter) or MACSQuant (Miltenyi) flow cytometer and analyzed using FlowJo software (BD Biosciences). The following antibodies were used for staining different cell subsets (Biolegend unless otherwise noted): F4/80 (BM8), CD11c (N418), CD86 (GL 1), B220 (RA3–6B2), Ly6C (HK1.4), CD8a (53–6.7), TCRb (H57–579), CD11b (M1/70), MHC II (M5/114.15.2), CD23 (B3B4), CD19 (6D5), CD21/35 (7E9), NK1.1 (PK136), 33D1 (33D1), CD169 (3D6.112), and goat anti-mouse Ig (BD Biosciences Cat #550826).

Immunofluorescence microscopy.

Spleens were harvested and then dehydrated for an hour in successive solutions of 10, 20, and 30 percent sucrose. Spleens were mounted in a cryomold with O.C.T. Compound (Tissue-Tek, Sakura) and frozen at −80°C before sectioning into 7 micron slices. All images were acquired with the Nikon eclipse Ti scope using 10x objectives.

Statistics.

All statistical analyses were performed using GraphPad Prism software. Data were analyzed with Welch’s unpaired t-test or one-way ANOVA with Tukey test.

Serum analysis.

Transfused mice were terminally bled via cardiac puncture or by retro-orbital bleed 21 days post RBC transfusion for anti-HOD alloantibody measurements or 2 hours post transfusion for acute serum cytokine measurements. 21 days is the peak of the alloantibody response

based on our previous work (21). Blood was left to clot, the clot was removed, and then the remaining blood was centrifuged at 1,500 × g for 10 minutes. The liquid serum was pipetted off and frozen at −20°C. To identify anti-HOD alloantibodies, sera were plated in a 96-well U bottom plate, incubated with 3uL of HOD RBCs for 30 minutes, washed, and incubated with APC anti-Ig antibodies for 30 more minutes. Sera were washed again and then run on the cytometer to detect total anti-HOD Igs. Anti-RBC antibodies in figures are all anti-HOD total Igs and were quantified by mean fluorescence intensity (MFI) in arbitrary units.

Study approval.

All protocols were approved by the Institutional Animal Care and Use Committee of the Yale Animal Resource Center.

Results

We utilized a transgenic mouse to model human blood transfusions in which RBCs express a triple fusion protein containing Hen egg lysozyme (HEL), Ovalbumin (OVA), and $Duffy_b$ (a human minor erythrocyte antigen), denoted the “HOD” mouse (Figure 1A) (12, 33). This model allows us to dissect the specific receptors and pathways required for the generation of detrimental alloimmunity during transfusion. Storing HOD RBCs under similar conditions that are used in hospitals for human RBC transfusion allows for examination of the innate and adaptive immune response to a model RBC antigen (12, 26, 33). Sterile HOD RBCs processed and stored for 12 days at 4°C induced a robust anti-HOD alloantibody response post transfusion into wild type (WT) C57BL/6 mice. In contrast, the transfusion of fresh (non-stored) HOD blood induced minimal alloantibody production, at levels similar to un-transfused naïve mice (Figure 1B).

Our prior work demonstrated that a subset of splenic conventional DCs identified by staining with the antibody 33D1, known as type 2 conventional DCs (cDC2s), are required for T-dependent alloantibody production to stored RBCs (17). Surprisingly, we and others have not been able to recapitulate the RBC-DC interaction in vitro that is observed in vivo (data not shown). Therefore, we use CD86 expression as a functional marker of DC activation in vivo, as it is induced by multiple types of innate immune stimuli. While type 1 conventional DCs (cDC1s) also become activated in response to stored blood transfusion (Figure S1A), we previously showed that cDC2s, and not cDC1s, are necessary for alloimmunization to stored RBCs (17); therefore we focused on regulators of cDC2 activation. We found that transfusion of stored, but not fresh, HOD RBCs into C57BL/6 recipients induced CD86 expression on splenic cDC2s at 6 hours post transfusion (Figure 1C, S2). This time point was selected based on our previous work demonstrating the maximal innate immune response (17, 34). We next sought to determine whether DC activation by stored RBCs requires alloantigens on the RBCs. Similar to HOD RBCs, we demonstrate that syngeneic stored, but not fresh, C57BL/6 RBCs transfused into C57BL/6 recipients similarly induced CD86 expression on splenic cDC2s (Figure 1D), indicating that innate immune activation of cDC2s does not require alloantigen to be present on transfused RBCs. Further, we found that transfusion of fresh RBCs three weeks post stored RBC transfusion also did not activate cDC2s, demonstrating that the HOD alloantigen is not sufficient to cause cDC2 activation

even after HOD alloantibodies are induced (Figure S1C). Finally, stored RBCs themselves, not the associated supernatant, contains the relevant innate immune stimulus as transfusion of stored RBCs diluted in sterile PBS but not transfusion of isolated supernatant produced after RBC storage activated cDC2s in recipient C57BL/6 mice (Figure 1E).

In both mice and humans, the cellular architecture of the spleen is highly organized and dictates its function (35). cDC2s reside in specialized zones between the red and white pulp, called bridging channels, where they can readily sample the blood for antigens. Upon activation, cDC2s migrate into the T cell zone (TCZ) of the white pulp to provide stimulatory signals to CD4+ T cells (34). We observed that transfusion of stored RBCs, regardless of alloantigen presence, resulted in cDC2 migration from bridging channels into the white pulp TCZ; this was not seen at steady state or in mice transfused with fresh RBCs (Figure 1F–G, S1D). Both DC migration into T cell zones and CD86 expression are necessary for subsequent T cell activation to occur, indicating that stored RBCs are capable of inducing requisite DC activation for adaptive immune responses, even in the absence of foreign antigen (35, 36).

As our previous work had ruled out relevant NLRs as receptors for these mediators (13), we considered whether TLRs serve as receptors facilitating the immunogenicity of stored RBCs. All TLRs utilize TRIF and/or MyD88 as adapter proteins (32). We therefore used a *Trif*$^{-/-}$ *Myd88*$^{-/-}$ mouse to determine whether TLRs play a role in the recognition of stored RBCs. In contrast to WT mice, *Trif*$^{-/-}$ *Myd88*$^{-/-}$ mice had significantly decreased serum levels of pro-inflammatory cytokines and chemokines IL-6, CXCL1 (also known as KC), and CCL2 (also known as MCP-1) two hours post transfusion (Figure 2A). Six hours post transfusion, *in vivo* splenic cDC2s from *Trif*$^{-/-}$ *Myd88*$^{-/-}$ mice failed to upregulate CD86 in response to stored RBC transfusion (Figure 2B). Moreover, *Trif*$^{-/-}$ *Myd88*$^{-/-}$ mice also demonstrated an impaired alloantibody response post transfusion, with anti-HOD antibody titers similar to those of naïve mice (Figure 2C).

We next sought to differentiate the effects of MyD88 and TRIF deficiency independently using *Myd88*$^{-/-}$ or *Trif*$^{-/-}$ mice. Different PRRs signal through unique downstream pathways utilizing distinct adaptor proteins (28, 37). Through the use of *Trif*$^{-/-}$ and *Myd88*$^{-/-}$ mice, we aimed to narrow down the number of candidate receptors that stored RBC products can stimulate. MyD88 is a pleiotropic adaptor molecule and plays an important role in both innate and adaptive immune signaling pathways (38–42). Unlike WT mice, *Myd88*$^{-/-}$ mice failed to produce the pro-inflammatory chemokine CCL2 two hours following stored RBC transfusion (Figure 3A). However, *Trif*$^{-/-}$ mice secreted CCL2 at levels similar to WT mice (Figure 3B). This suggests that MyD88, but not TRIF, plays an important role in the early innate immune response to stored RBC products. Notably, *Myd88*$^{-/-}$ mice were also unable to mount an alloantibody response to HOD RBCs post RBC transfusion (Figure 3C). In contrast, *Trif*$^{-/-}$ mice demonstrated anti-HOD alloantibody levels comparable to WT mice (Figure 3D). Therefore, receptors that require signaling through the adaptor protein TRIF to function, such as TLR3, are not required for the alloimmune response to stored donor HOD RBCs.

We next addressed which cell type requires MyD88 signaling for RBC-induced activation. The spleen is responsible for filtration of the blood and monitoring of the circulation for antigens (35). To determine responsiveness to stored RBCs, we examined upregulation of the activation marker CD86 on different splenic immune cell populations *in vivo* six hours following transfusion. Using this readout, stored RBCs did not directly activate follicular B cells or marginal zone B cells (Figure S3A–B). We also examined splenic resident innate immune cells including cDC subsets, macrophages, monocytes, and plasmacytoid DCs (pDCs). Consistent with our previous findings, both subsets of cDCs were strongly activated in response to stored transfusion (Figure 4A). Upregulation of CD86 by cDCs was completely abrogated in *Myd88*$^{-/-}$ mice, indicating that functional MyD88 signaling is required for cDC activation to stored RBCs (Figure 4A). In contrast, monocytes and pDCs did not become activated in response to stored RBCs (Figure 4A, S3A). CD69 is often used to gauge pDC activation (43); however when we stained CD69 expression on pDCs, we saw no difference between naïve WT mice and WT mice transfused with stored HOD blood (Fig S1B), indicating that pDCs do not become significantly activated following stored RBC transfusion. Although macrophage CD86 upregulation in response to RBC transfusion was statistically significant between control and knockout mice, it is of questionable biological significance given the low level of activation relative to cDCs (Figure 4A). It is also likely that our method of digesting does not capture large numbers of macrophages because the manual digest is optimized to extract cDCs, making this macrophage activation data difficult to interpret. Our previous work demonstrated that cDC2s are the primary DC subset responsible for alloantibody production in response to RBC transfusion in part by selectively migrating to the CD4+ T cell region of the white pulp (17, 34). We found that cDC2 migration into the white pulp of the spleen was impaired in *Myd88*$^{-/-}$ mice six hours after stored RBC transfusion relative to spleens from control mice, supporting the conclusion that cDC2 activation to RBCs requires MyD88 (Figure 4B).

Discussion

Taken together, these data show that stored but not fresh RBCs activate cDCs through a MyD88 dependent but TRIF independent pathway. This innate immune pathway has not been previously associated with the immune response to transfused RBCs, but it has been associated with alloimmunity in the skin (44, 45). This raises the possibility that common PRRs mediate adaptive immune responses in the context of foreign but non-pathogen derived alloantigens. Further we found that storage of non-allogeneic C57BL/6 RBCs also renders them able to induce DC maturation. This demonstrates that alloantigen itself is not required for innate immune stimulation by RBCs but rather damaged or altered self-molecules trigger innate immunity. We propose a model in which storage causes damage to RBCs that acts as an adjuvant to activate the innate immune response, leading to cDC2 activation, T cell priming and promotion of RBC alloantibodies. Indeed, recent work suggests that TLRs, such as TLR4, TLR7, and TLR9, either sense DAMPs released from RBCs or enable RBCs to scavenge DAMPs, which could ultimately promote the innate immune response (46–48). Our preliminary work, however, indicates that loss of TLR4, TLR7, or TLR9 does not impair RBC alloimmunization (data not shown). Previous work from our collaborators has shown that transfusion of stored RBC lysate, supernatant derived

from stored RBCs, or stored RBC ghosts do not trigger the release of innate cytokines such as IL-6, CXCL1, and CCL2, suggesting that the innate stimulus triggering the alloimmune response is not hemoglobin or heme and thus that TLR4 is unlikely the relevant TLR. As multiple other TLRs signal through MyD88 and can react to DAMPs, we aim in future work to identify which TLR is in fact required for the response to RBCs.

Determining whether MyD88 is acting in a DC-intrinsic versus -extrinsic manner will provide mechanistic insights into the process of RBC alloimmunization and might help narrow down candidate receptors (e.g., particular TLRs) through which RBC alloimmunization is initiated. Although we have demonstrated that cDCs are activated during the course of transfusion, it is possible that additional accessory cells in the spleen, such as the multiple macrophage populations in and around the bridging channel, are activated through MyD88 to provide DC activating signals. Other cells in the spleen might also influence DC-dependent T cell priming to allogenic RBCs. While no difference in marginal zone B cell activation was observed following RBC transfusion, recent studies demonstrate that RBC alloimmunization likewise requires marginal zone B cells, suggesting that cDCs may work in concert with marginal zone B cells to facilitate alloimmunization (49–51).

Understanding which innate immune receptors are responsible for the initiation of the RBC alloimmune response will help identify possible immunogenic compounds released by or bound to RBCs that enhance immunogenicity and therefore risk for alloantibody production. Therefore, work aiming to identify whether such an effect also occurs during storage of human RBCs will be important. Interestingly, some blood donors demonstrate better RBC storage characteristics than others; in parallel, some transfusion recipients are considered “responders” whereas others are “non-responders”, in terms of alloantibody production. More work is needed to identify the immunologic underpinnings of these donor and recipient effects on alloimmunization. We posit that defining the innate immune pathways that regulate the alloimmune response is the first step towards understanding and ultimately preventing RBC alloimmunization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Key Findings

Normal storage conditions increase immunogenicity of sterile red blood cells (RBCs).

MyD88 but not TRIF is required for induction of alloantibodies to transfused RBCs.

MyD88 is required for transfused RBCs to activate dendritic cells.

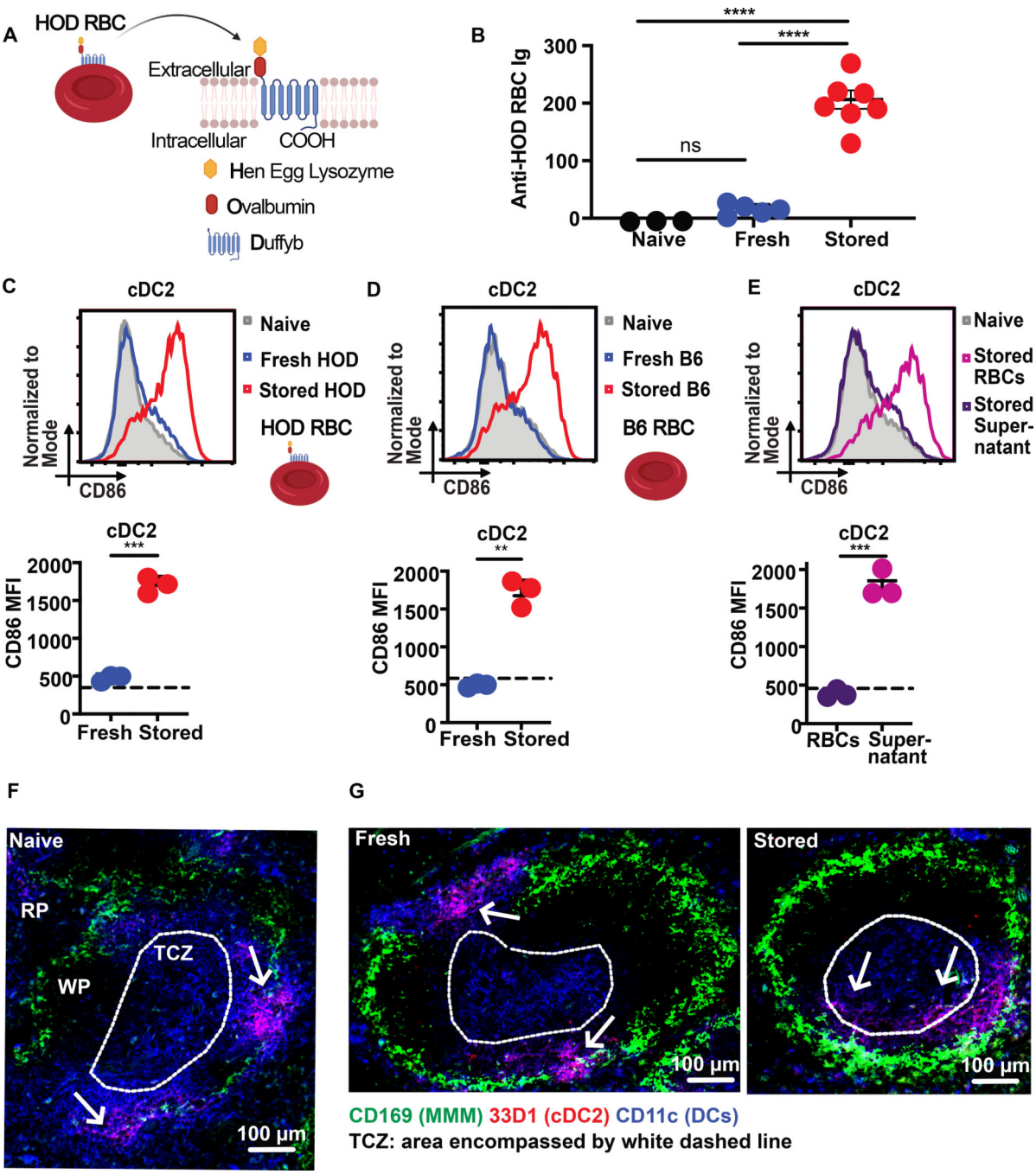

Figure 1. RBC storage but not alloantigen presence activates splenic cDC2s.

(A) Model of HOD alloantigen containing Hen Egg Lysozyme (B cell epitope), Ovalbumin (T cell epitope), and Duffyb (Human RBC antigen). Mice were transfused with freshly harvested RBCs or RBCs that had been stored for 12–14 days. (B) Anti-HOD RBC alloantibodies measured by flow cytometric crossmatch 21 days post fresh or stored RBC transfusion. (C) Flow cytometric analysis of splenic cDC2 CD86 expression 6h post fresh or stored HOD RBCs. Representative histogram of surface CD86 expression with accompanying quantification. Dashed line indicates value of naïve control. (D)

Flow cytometric analysis of splenic cDC2 CD86 expression 6h post fresh or stored B6 RBCs without HOD alloantigen. Representative histogram of surface CD86 expression with accompanying quantification. Dashed line indicates value of naïve control. (E) Flow cytometric analysis of splenic cDC2 CD86 expression 6h post stored supernatant or RBCs diluted in sterile PBS. Representative histogram of surface CD86 expression with accompanying quantification. Dashed line indicates value of naïve control. (F) Immunofluorescence (IF) microscopy of naïve spleen with arrows indicating localization of cDC2s in the bridging channel; (G) IF microscopy of spleen 6h post fresh or stored RBC transfusion with arrows indicating localization of cDC2s in the bridging channel after fresh transfusion and movement of cDC2s into the TCZ after stored RBC transfusion. TCZ = T-cell zone, RP = red pulp, WP = white pulp. Bar, 100 um. Error bars represent SEM for 3–6 mice per group. Groups were compared using Welch's t-test. **P<0.01, ***P<0.001, ****P<0.0001; ns, not significant. Representative data are shown for two to four independent experiments with n=3–7 mice per group.

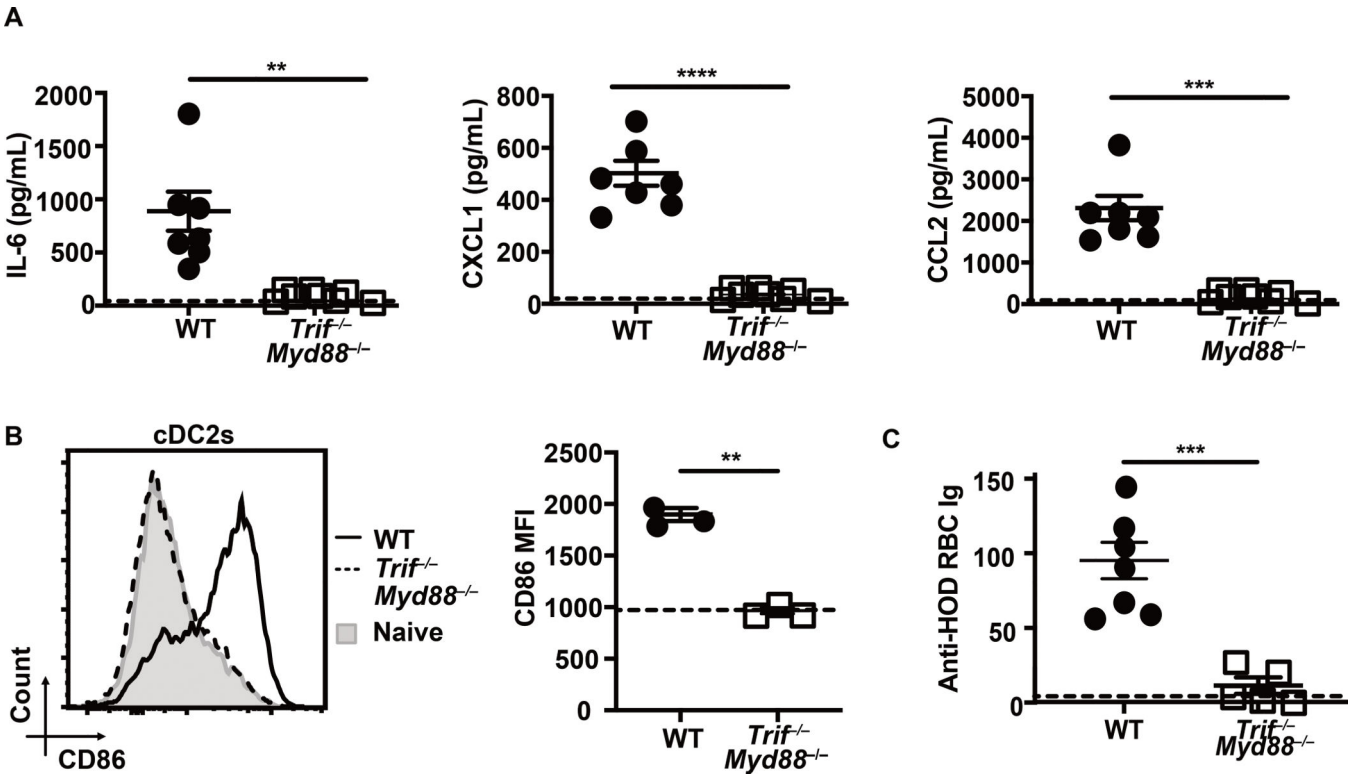

Figure 2. Loss of TLR signaling leads to impaired innate and adaptive immune responses to stored RBC transfusion.

WT or *Trif*$^{-/-}$ *Myd88*$^{-/-}$ mice were transfused with RBCs that had been stored for 12–14 days. (A) ELISA quantification of IL-6 (left), CXCL1 [also known as KC] (middle), or CCL2 [also known as MCP-1] (right) in recipient serum 2h post transfusion. Dashed line indicates value of naïve control. (B) Flow cytometric analysis of splenic cDC2 CD86 expression 6h post stored RBC transfusion into WT or *Trif*$^{-/-}$ *Myd88*$^{-/-}$ mice. Representative histogram of surface CD86 expression with accompanying quantification. Dashed line in quantitation graph indicates value of WT naïve control. (C) Anti-HOD RBC alloantibodies measured by flow cytometric crossmatch 21 days post stored RBC transfusion. Dashed line indicates value of naïve control. Error bars represent SEM for n=3–7 mice per group. Groups were compared using Welch's t-test. **P<0.01, ***P<0.001, ****P<0.0001.

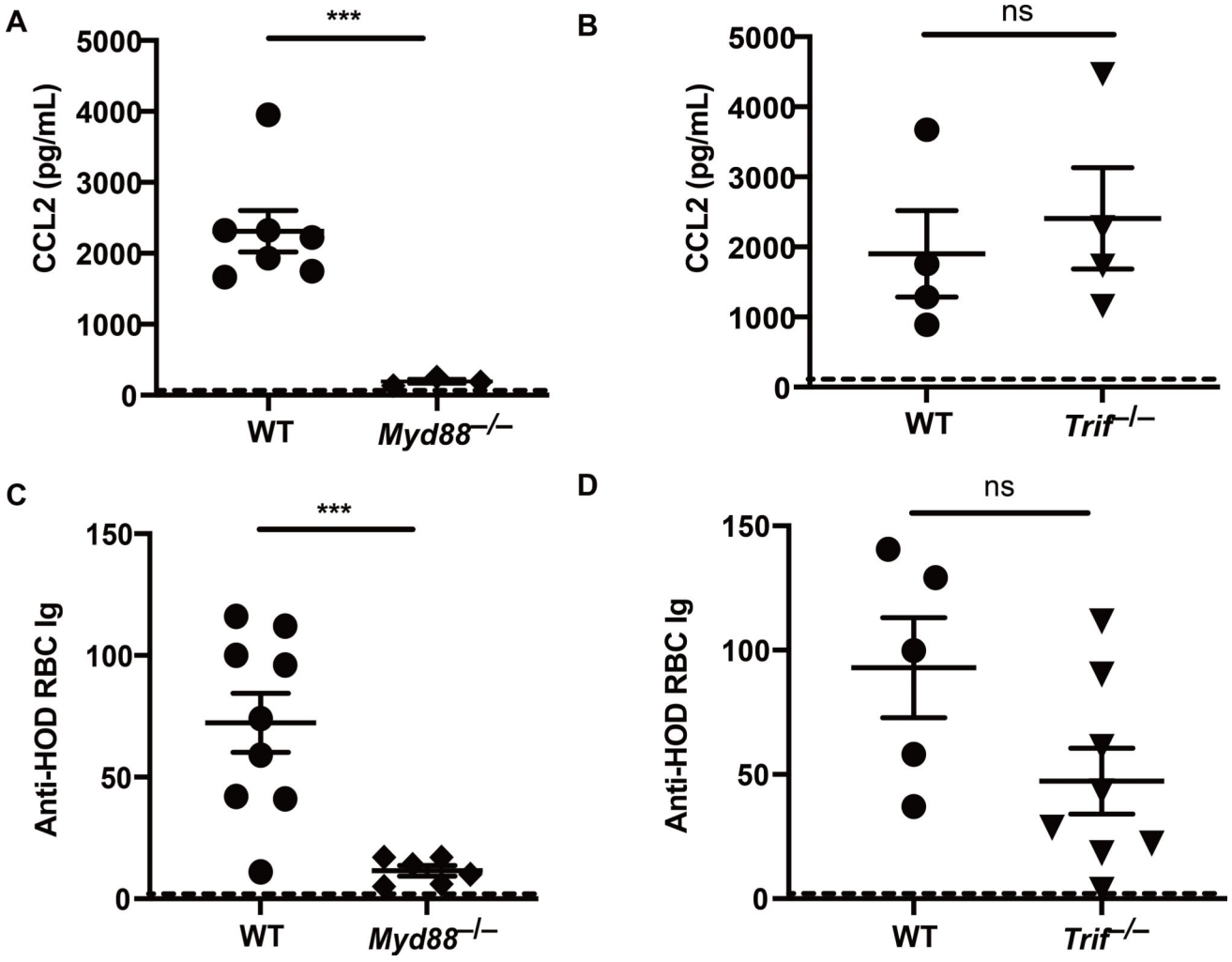

Figure 3. MyD88 but not TRIF is required for the innate and adaptive response to stored RBC transfusion.

Myd88$^{-/-}$*,Trif*$^{-/-}$, or WT mice were transfused with HOD RBCs that had been stored for 12–14 days. (A) ELISA quantification of CCL2 in *Myd88*$^{-/-}$ recipient serum 2h post transfusion. Dashed line indicates value of naïve control. (B) ELISA quantification of CCL2 in *Trif*$^{-/-}$ recipient serum 2h post transfusion. Dashed line indicates value of naïve control. (C) Anti-HOD RBC alloantibodies in *Myd88*$^{-/-}$ recipients measured by flow cytometric crossmatch 21 days post stored RBC transfusion. Dashed line indicates value of naïve control. (D) Anti-HOD RBC alloantibodies in *Trif*$^{-/-}$ recipients measured by flow cytometric crossmatch 21 days post stored RBC transfusion. Dashed line indicates value of naïve control. Error bars represent SEM for n=3–9 mice per group. Groups were compared using Welch's t-test. ***P<0.001; ns, not significant.

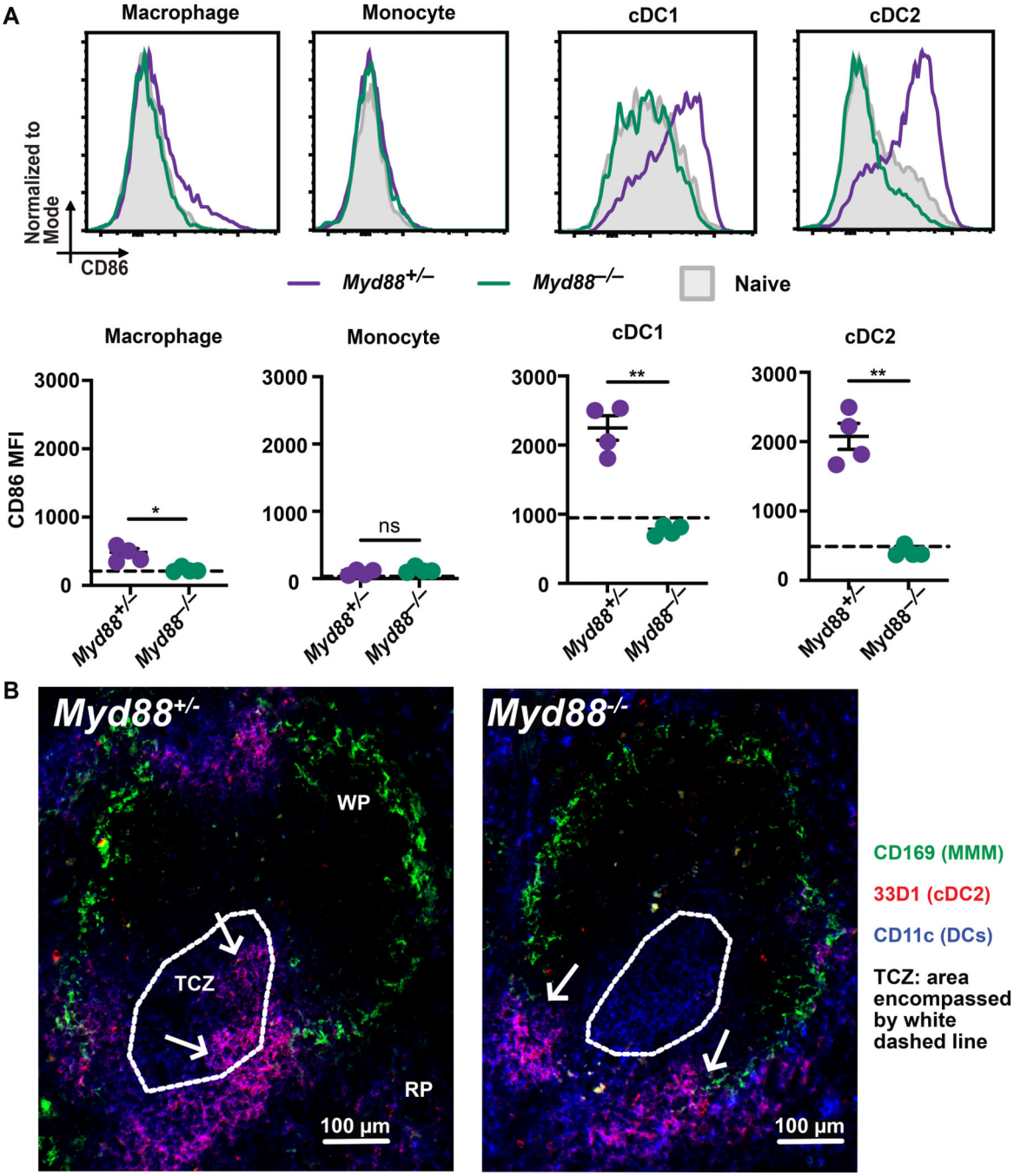

Figure 4. Stored RBC transfusion activates conventional DCs through a MyD88 dependent signaling pathway.

Myd88$^{+/-}$ or *Myd88*$^{-/-}$ mice were transfused with C57BL/6 RBCs that had been stored for 12–14 days. (A) Flow cytometric analysis of CD86 on splenic macrophage, monocyte, cDC1, and cDC2 (gating shown in Supplement 2) 6h post stored RBC transfusion into *Myd88*$^{+/-}$ (purple) or *Myd88*$^{-/-}$ (green) mice. Representative histogram of surface CD86 expression (top) with accompanying quantification (bottom). Dashed line indicates value of naïve control. Error bars represent SEM for n=4 mice per group. *Myd88*$^{+/-}$ or *Myd88*$^{-/-}$ groups were compared using Welch's t-test. **P<0.01; ns, not significant.

(B) Immunofluorescence microscopy of *Myd88*$^{+/-}$ or *Myd88*$^{-/-}$ spleen 6h post stored RBC transfusion with arrows indicating localization of cDC2s in the bridging channel in *Myd88*$^{-/-}$ and movement of cDC2s into the TCZ in *Myd88*$^{+/-}$. Bar, 100 um.