Transfused platelets enhance alloimmune responses to transfused KEL-expressing red blood cells in a murine model

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Background. Factors influencing the development of alloantibodies against blood group antigens on transfused red blood cells are poorly defined. We hypothesised that transfused platelets may act as a danger signal to recipients and affect humoral immune responses to transfused red blood cells.

Materials and methods. Platelet-rich plasma prepared from wild-type C57BL/6 or CD40L knock-out donors was transfused into wild-type or CD40L knock-out recipients. Leucoreduced red blood cells from transgenic donors expressing high levels of the human KEL glycoprotein in an erythrocyte-specific manner (KEL^{hi} donors) were transfused after the platelets, and anti-KEL responses were measured longitudinally. In some experiments, recipients were treated with poly (I:C), monoclonal CD40L-blocking antibody, or CD4-depleting antibody prior to transfusion.

Results. Transfusion of wild-type C57BL/6 platelets or treatment with poly (I:C) prior to KEL^{hi} red blood cell transfusion led to an anti-KEL alloimmune response in wild-type recipients. Transfusion of platelets from wild-type but not CD40L knock-out donors prior to KEL^{hi} red blood cell transfusion led to an IgG anti-KEL alloimmune response in CD40L knock-out recipients; unexpectedly, transfusion of platelets from CD40L knock-out donors prior to KEL^{hi} red blood cell transfusion led to a robust anti-KEL alloimmune response in wild-type recipients. Recipient treatment with MR1 CD40L-blocking antibody or CD4-depleting antibody prevented KEL alloimmunisation altogether.

Discussion. Transfused platelets serve as an adjuvant in this T-dependent murine model of anti-KEL red blood cell alloimmunisation, with CD40/CD40L interactions being involved to some degree but with additional mechanisms also playing a role. These findings raise questions about the role that transfused or endogenous platelets may play in other innate/adaptive immune responses.

Keywords: red blood cells, platelets, antibodies.

Introduction

Transfusion of red blood cells (RBCs) is the most common procedure during hospital admissions[1], and development of alloantibodies against non-ABO RBC antigens is one serious complication of transfusion[2]. Induced antibodies can cause haemolytic transfusion reactions, and such reactions are one of the leading causes of transfusion-associated deaths[3]. RBC alloantibodies can also make compatible RBCs difficult to locate and can lead to haemolytic disease of the foetus and newborn.

Only a fraction of transfusion recipients will ever develop RBC alloantibodies, with factors affecting antibody formation being poorly defined. We and others have shown that the recipient inflammatory status at the time of exposure to RBCs[4,5], as well as underlying autoimmunity[6,7], are important variables in the predisposition of humans to develop RBC alloantibodies. Similar findings have been observed in murine models, with recipient inflammation around the time of RBC exposure affecting humoral immune responses[8]. Polyinosinic-polycytidylic acid (poly [I:C]), a double-stranded RNA, is one adjuvant that enhances RBC alloimmunisation in numerous murine models[9]; the adjuvant CpG has also been shown to enhance alloimmunisation[10].

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Platelets are best known for their roles in haemostasis and thrombosis. However, they are also involved in innate and adaptive immunity[11]. Platelets interact with antigen-presenting cells, T cells, and B cells, and are capable of affecting cellular and humoral immune responses[12-17]. It has been shown that activated platelets can not only recruit T cells, but can also initiate a feedback through which T cells stimulate platelets to secrete cytokines that then recruit additional T cells[18-20]. At least one model has been described in which platelets affect humoral immune responses to a virus[21,22]. However, the potential role of platelets in affecting humoral immune responses to transfused blood products, including RBCs, has never been previously studied.

The transplantation and immunology literature has extensively described the importance of co-stimulation in immunity[23], and we have previously observed that co-stimulatory CD40/CD40L interactions are important in a T-cell-dependent murine model of RBC alloimmunisation to the human glycophorin A antigen[24]. Given the constitutive expression of CD40 on platelets and the elaboration of CD40L expression on the platelet surface after activation[18,25], in combination with the emerging appreciation of the inflammatory capabilities of platelets, we hypothesised that platelets may affect RBC alloimmune responses in part through the CD40/CD40L co-stimulatory axis. Here, we describe the contribution of transfused wild-type platelets to RBC alloimmune responses and discuss potential translational implications of these findings.

Materials and methods

Mice

C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA, USA). CD40L knock-out (KO) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Transgenic mice expressing the KEL glycoprotein (previously referred to as KEL2B and referred to as KEL^{hi} in this manuscript) were bred in Yale University's animal facilities. Donor and transfusion-recipient mice were 8-12 weeks of age. All animal protocols were approved by the Yale Institutional Animal Care and Use Committee.

Inflammation-induced alloimmunisation and blood transfusion

In some experiments, recipient mice received intraperitoneal (i.p.) injections with 100 μg of poly(I:C) (Invivogen, San Diego, CA, USA) 3 to 4 hours prior to RBC transfusion and in some experiments recipient mice were treated with CD40L blockade (MR1, Bioxcell, West Lebanon, NH, USA) or an isotype-matched control prior to transfusion. Platelet-rich plasma was generated by centrifuging peripheral blood at 80×*g* for 10 minutes, as previously described[26], and platelets from one donor were transfused into four recipients; as such the data points shown are not fully independent. Peripheral blood from KEL^{hi} donors was collected in 12% citrate phosphate dextrose adenine (CPDA-1, Jorgensen Labs, Melville, NY, USA), leucoreduced with a Pall syringe filter (East Hills, NY, USA), and washed with phosphate-buffered saline to remove residual citrate. Recipient mice were transfused (intravenous injection) in the lateral tail vein with the equivalent of 1 unit of human RBC (75 mL of packed RBCs in phosphate-buffered saline). In some experiments, RBCs were labelled with the lipophilic dyes chloromethylbenzamido 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) or 3,3′-dihexadecyloxacarbocyanine perchlorate (DiO) according to the manufacturer's instructions (Molecular Probes, Eugene, OR, USA) as previously described[27], to track post-transfusion RBC recovery.

Characterisation of transfused components

Peripheral blood, platelet-rich plasma, and splenocytes were stained with fluorescently conjugated anti-TER119, anti-CD41, and/or anti-CD45 antibodies (Biolegend, San Diego, CA, USA). Polyclonal anti-KEL, generated after transfusion of KEL^{hi} RBCs into C57BL/6 recipients in the presence of poly (I:C) or monoclonal anti-Jsb (generously provided by the New York Blood Center) were used for detection of the KEL glycoprotein. Anti-mouse immunoglobulin G (IgG) (Jackson ImmunoResearch, West Grove, PA, USA) was also used as a detection reagent.

CD4 depletion and CD40 ligand blockade

For CD4-depletion experiments[28], mice were given two i.p. injections of 200 μg anti-mouse CD4 monoclonal antibody (clone: GK1.5, BioXcell, West Lebanon, NH, USA), saline, or an isotype-matched control 2 days apart; a third dose was given 7 days after the transfusion. For CD40 ligand blockade experiments[29], mice were given i.p. injections of 250 μg of anti-mouse CD40 ligand (CD154) monoclonal antibody (clone: MR1, BioXcell, West Lebanon, NH, USA), saline, or an isotype-matched control every other day for a total of seven doses.

Detection of alloantibodies

Antibodies produced against the KEL glycoprotein, referred to throughout this manuscript as anti-KEL IgG, were measured by flow-cytometric crossmatch longitudinally after RBC transfusion. Peak antibody values are typically observed 28 days after transfusion[30]. The adjusted mean fluorescence intensity (adjusted MFI) was calculated by subtracting the reactivity of serum incubated with syngeneic wild-type RBCs from the reactivity of serum incubated with KEL^{hi} RBCs; as such, the adjusted MFI represents the anti-KEL

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specific signal. Flow cytometry was completed using an eight-colour MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analysed using FlowJo software (Tree Star, Ashland, OR, USA).

Germinal centre evaluation

Seven days following a KEL^{hi} RBC transfusion boost, spleens in some experiments were evaluated for germinal centres by flow cytometry and/or by immunofluorescence. Antibodies used included B220 (RA3-6B2) from Ebioscience (San Diego, CA, USA); TCRβ (H57-597), IgD (11-26c.2a), GL7 (GL7), and Streptavidin from Biolegend (San Diego, CA, USA); CD95 (Jo2) from BD Biosciences (San Jose, CA, USA); and peanut agglutinin (PNA) from Vector Labs (Burlingame, CA, USA). Immunofluorescence was completed as previously described[24,31]; in brief, splenic tissue was dehydrated through sequential exposure to solutions of 10%, 20% and 30% sucrose, mounted in a cryomould with O.C.T. Compound (Tissue-Tek, Sakura, CA, USA), and stored at −80 °C prior to sectioning (7 μm) and staining.

Statistics

Statistical analyses were performed using Graph Pad Prism software (San Diego, CA, USA). The data were investigated for normality using the D'Agostino and Pearson test. Statistical significance between two groups of non-parametric data was determined using a Mann-Whitney U test, and statistical significance between three or more groups of non-parametric data was determined using the Kruskal-Wallis test with Dunn's post-test.

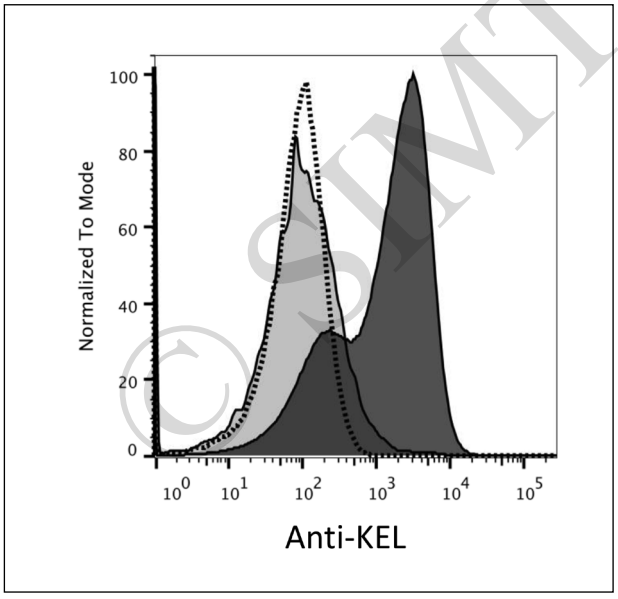


Figure 1 - KEL glycoprotein expression on haematopoietic cells.
KEL glycoprotein expression from a representative KEL^{hi} donor, as detected using monoclonal anti-Jsb antibody on TER119+ red blood cells from peripheral blood (dark shaded histogram), CD41+ platelets from a platelet-rich plasma preparation (dotted line with white histogram), and CD45+ splenocytes (light-shaded histogram).

Results

KEL^{hi} transgenic mice express the human KEL glycoprotein in an erythrocyte-specific manner

Multiple transgenic animals expressing the KEL glycoprotein have previously been created and characterised. For the current studies KEL^{hi} transgenic animals, which express high levels of the KEL glycoprotein relative to other KEL transgenic mice, were utilised as RBC donors. KEL^{hi} animals were briefly described in the initial publication on the first KEL transgenic mice and, at that time, the animals were referred to as KEL2A as they express KEL2 (also known as Cellano) instead of KEL1 (also known as KEL) as part of their KEL glycoprotein[32].

Prior to initiation of the current experiments, KEL glycoprotein expression was characterised. Peripheral blood, platelet-rich plasma, and splenocytes were used for analysis of RBCs, platelets, and white blood cells, respectively. Polyclonal anti-KEL sera was used for staining, as was a monoclonal anti-Jsb antibody. KEL expression was observed on TER119+ RBCs but not CD41+ platelets or CD45+ white blood cells (Figure 1); RBCs, platelets, and white blood cells from control wild-type C57BL/6 animals did not have detectable KEL expression. This erythroid-specific pattern of KEL glycoprotein expression has been observed on all KEL transgenic strains described to date as would be predicted, given that these animals were created using a β-globin promoter.

Transfusion of platelets from wild-type C57BL/6 donors prior to KEL^{hi} red blood cell transfusion enhances the anti-KEL alloimmune response

Platelets are well-known mediators of haemostasis and coagulation. However, their role in serving as adjuvants to other transfused blood products is not known. To test the potential ability of platelet transfusion to affect RBC alloantibody responses, whole blood from wild-type C57BL/6 donors (that do not express the human KEL glycoprotein) was collected, centrifuged at a slow speed, and processed as previously described[26] into platelet-rich plasma. The platelet-rich plasma was characterised by flow cytometry and confirmed to have a significant enrichment of platelets compared to whole blood (Figure 2A). Each platelet transfusion contained approximately 1×10^8 total platelets, which is a murine equivalent of the number of platelets (3×10^{11}) transfused to a human in a given apheresis-derived platelet transfusion[26].

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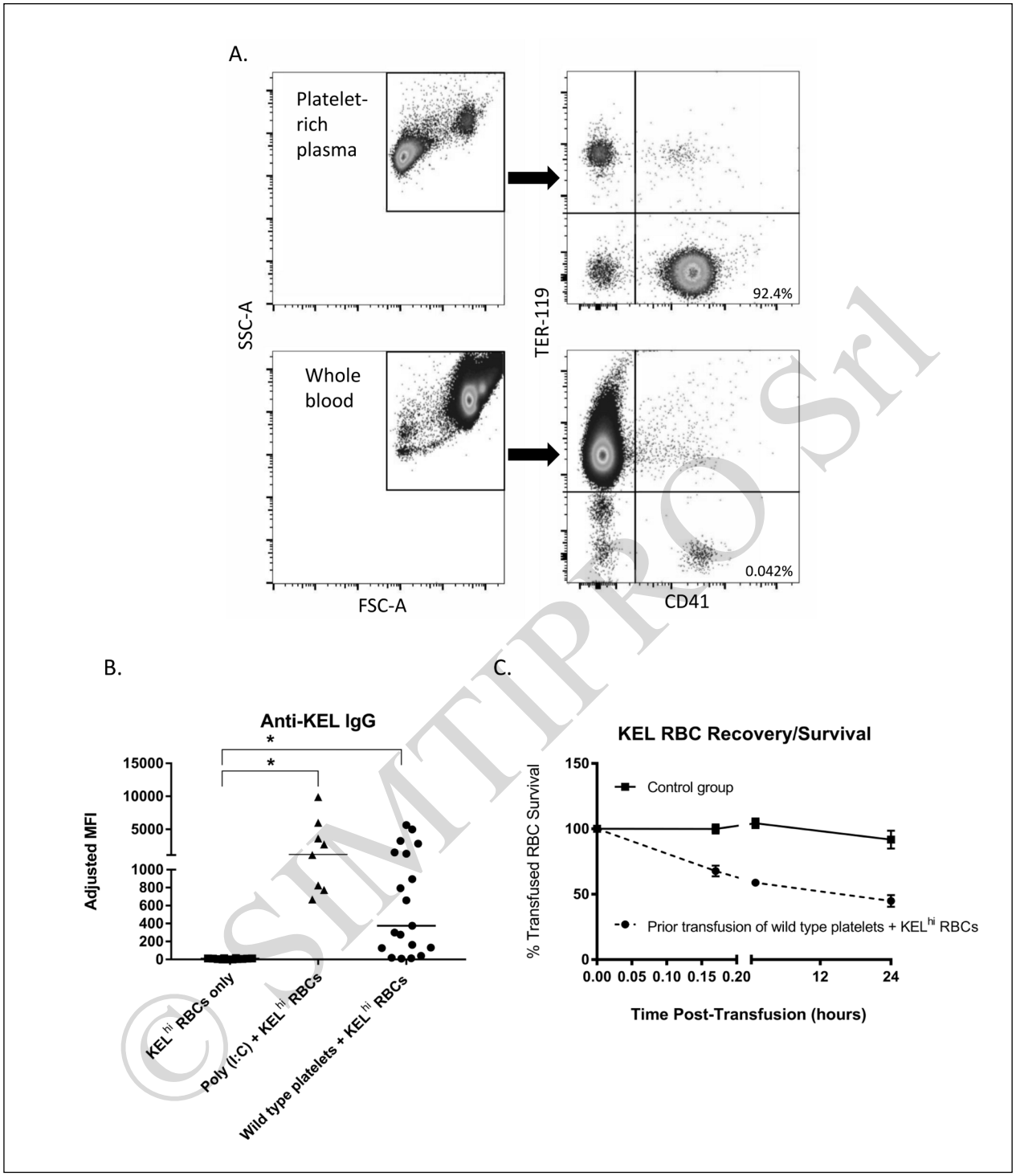


Figure 2 - The impact of wild-type C57BL/6 platelet transfusion on alloimmune responses to KEL^{hi} red blood cells. (**A.**) The composition of platelet-rich plasma (top panel) as compared to whole blood (bottom panel), with TER-119 staining RBCs and CD41 staining platelets. (**B.**) Anti-KEL IgG responses after the transfusion of leucoreduced KEL^{hi} RBCs into wild-type C57BL/6 recipients treated with no adjuvant and transfused with no additional platelets (squares), into recipients pre-treated with the adjuvant poly (I:C) prior to RBC transfusion (triangles), and into recipients transfused with wild-type C57BL/6 platelets prior to RBC transfusion (circles); *p<0.0001 between controls and poly (I:C)-treated group; *p<0.001 between controls and platelet-transfused group; p=non-significant between poly (I:C)-treated and platelet-transfused groups; pooled data from four experiments are shown. (**C.**) Post-transfusion recovery and survival of transfused KEL^{hi} RBCs compared to co-transfused C57BL/6 RBCs, in control animals (solid line) and in animals previously transfused with C57BL/6 platelets and KEL^{hi} RBCs (dashed line). IgG: immunoglobulin G; RBCs: red blood cells; poly [I:C]: polyinosinic-polycytidylic acid.

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Animals were transfused with these platelets from wild-type C57BL/6 donors 2-3 hours prior to the transfusion of leucoreduced KEL^{hi} RBCs. Initial experiments involved transfusing a second dose of platelets 24 hours after exposure to the RBCs, but this repeat transfusion was stopped after similar results were observed with just a single platelet transfusion. In a total of four experiments, with three to five mice per group per experiment, 15/19 (79%) of animals transfused with wild-type platelets prior to KEL^{hi} RBC transfusion became alloimmunised against KEL (Figure 2B); the adjusted MFI shown is the anti-KEL specific response. In contrast, transfusion of KEL^{hi} RBCs in the absence of any adjuvant failed to result in a detectable anti-KEL response. As it has been observed in other murine models of alloimmunisation[33], pretreatment of recipient animals with the double-stranded RNA poly (I:C) prior to RBC transfusion resulted in a robust anti-KEL IgG response in 100% of recipients.

To determine whether the anti-KEL generated in the animals transfused with platelets and then RBCs was clinically significant, KEL^{hi} RBCs were labelled with the lipophilic dye DiO and co-transfused with wild-type C57BL/6 RBCs labelled with the lipophilic dye DiI. Preferential clearance of labelled KEL^{hi} RBCs was observed in the animals previously transfused with platelets and KEL^{hi} RBCs, with no significant KEL^{hi} RBC clearance observed in control recipients (Figure 2C).

Germinal centres are detected by flow cytometry but not by immunofluorescence in animals transfused with platelets from wild-type C57BL/6 donors prior to KEL^{hi} red blood cells

To investigate whether the anti-KEL response in the platelet-transfused group involved germinal centres, a secondary immune response was induced by transfusing these recipients with KEL^{hi} RBCs again, 42 days after the initial RBC exposure. Seven days later, serum was evaluated for anti-KEL, and splenic germinal centres were evaluated by flow cytometry and immunofluorescence. Increased anti-KEL after this secondary RBC exposure was measured in the animals initially transfused with wild-type platelets prior to KEL^{hi} RBCs *(data not shown)*, and an increased number of B220+GL7+CD95+ cells was observed in these animals as compared to those initially transfused with KEL^{hi} RBCs alone (Figure 3A, 3B). However, germinal centres could not be detected by immunofluorescence in these alloimmunised animals, using either PNA or GL-7 (Figure 3C).

Recipient animals lacking CD40L become alloimmunised after co-transfusion with wild-type platelets and KEL^{hi} red blood cells, and platelets from CD40L knock-out donors enhance anti-KEL responses in wild-type recipients

We hypothesised that platelet CD40L expression may play a role in alloimmunisation. Recipient animals

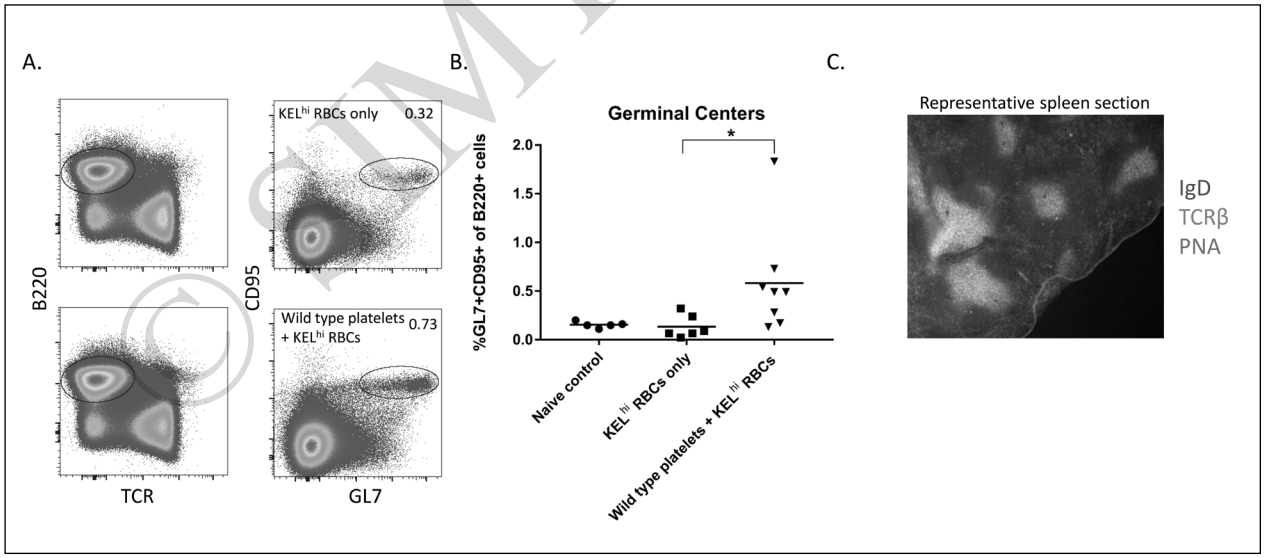


Figure 3 - Recipient germinal centre formation after wild-type C57BL/6 platelet transfusion and KEL^{hi} red blood cell transfusion. (**A.**) General spleen gating strategy, with B220+, TCRβ– cells evaluated for CD95 and GL7 expression. (**B.**) Comparative data of these GL7+CD95+ germinal centre B cells, 7 days after a repeat KEL^{hi} RBCs transfusion, in animals initially transfused with KEL^{hi} RBCs in the presence or absence of wild-type platelets (as compared to naïve controls). (**C.**) Representative immunofluorescence image of a spleen from a re-transfused animal initially transfused with KEL^{hi} RBCs in the presence of wild-type platelets, stained with immunoglobulin D (IgD; red), T-cell receptor beta (TCRβ; blue) and peanut agglutinin (PNA; green). *p<0.02; pooled data from two experiments are shown.
RBCs: red blood cells; IgD: immunoglobulin D; TCRβ: T cell receptor beta chain; PNA: peanut agglutinin.

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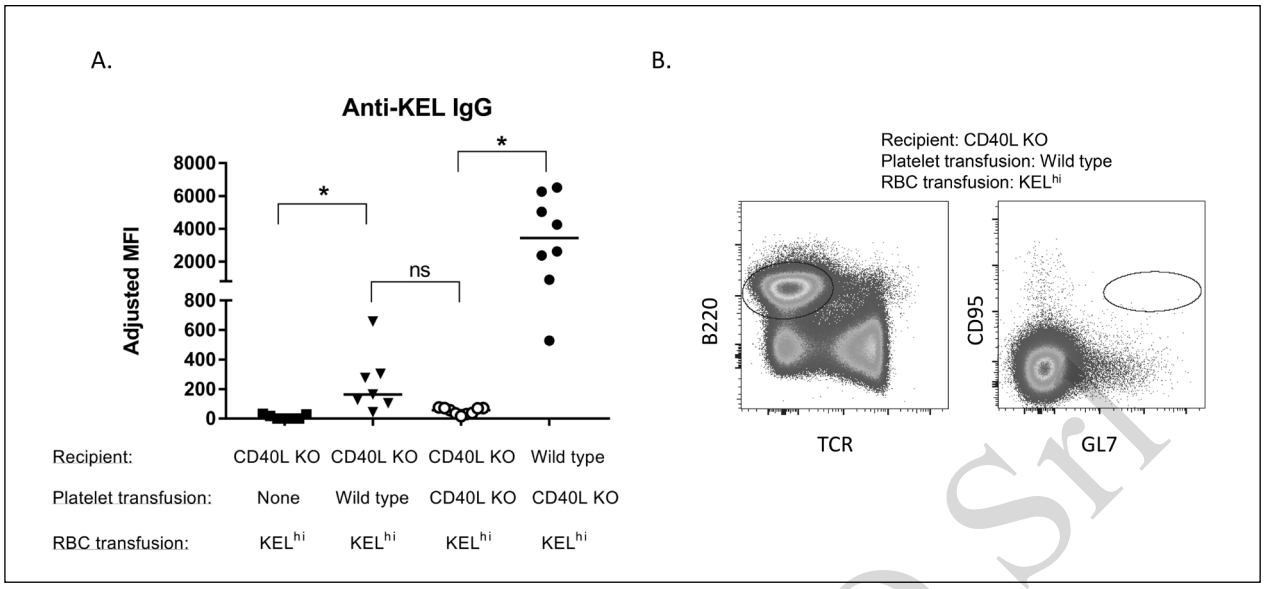


Figure 4 - Impact of CD40L in platelet donors or in transfusion recipients on anti-KEL alloantibody responses. (**A.**) Anti-KEL alloantibody responses 28 days after transfusion as measured by flow cytometric crossmatch, in CD40L knock-out (KO) recipients transfused with KEL^{hi} RBCs alone, in CD40L KO recipients transfused with wild-type C57BL/6 platelets followed by KEL^{hi} RBCs, in CD40L KO recipients transfused with CD40L KO platelets followed by KEL^{hi} RBCs, and in wild-type recipients transfused with CD40L KO platelets followed by KEL^{hi} RBCs. *p<0.05 between CD40L KO recipients transfused with KEL^{hi} RBCs alone compared to CD40L KO recipients transfused with wild-type C57BL/6 platelets followed by KEL^{hi} RBCs; p<0.05 between CD40L KO recipients transfused with CD40L KO platelets followed by KEL^{hi} RBCs compared to wild-type recipients transfused with CD40L KO platelets followed by KEL^{hi} RBCs; p=non-significant between CD40L KO recipients transfused with wild-type platelets followed by KEL^{hi} RBCs and CD40L KO recipients transfused with CD40L KO platelets followed by KEL^{hi} RBCs. Pooled data from two experiments are shown. (**B.**) Flow cytometric plot for splenic germinal centres in a representative CD40L KO recipient transfused with wild-type C57BL/6 platelets followed by KEL^{hi} RBCs. No germinal centres were visible in any CD40L KO recipient evaluated, regardless of prior transfusion exposure or alloantibody response. RBCs: red blood cells.

genetically lacking CD40L (CD40L KO) were transfused with KEL^{hi} RBCs or platelets from wild-type C57BL/6 donors prior to transfusion with KEL^{hi} RBCs. In both of two experiments the wild-type C57BL/6 platelets induced a detectable anti-KEL response in the CD40L KO recipients, with CD40L KO recipients transfused with KEL^{hi} RBCs alone not becoming alloimmunised (Figure 4A, left-most groups). However, the alloantibody response in the platelet-transfused CD40L KO recipient group was notably lower than that typically observed in C57BL/6 recipients and none of the alloimmunised CD40L KO recipients had splenic germinal centres detectable by flow cytometry (Figure 4B).

To investigate whether CD40L expression on wild-type C57BL/6 donor platelets augments immune responses to transfused KEL^{hi} RBCs, platelets from CD40L KO donors were transfused into wild-type or CD40L KO recipients, before KEL^{hi} RBCs were transfused. Whereas platelets from CD40L KO donors were not capable of significantly enhancing the humoral immune response to transfused KEL^{hi} RBCs in CD40L KO recipients, in both of two experiments platelets from CD40L KO donors were capable of resulting in robust responses to transfused KEL^{hi} RBCs in wild-type recipients (Figure 4A, right-most groups). These data, which were unexpected, suggest that CD40L expression is not the sole way in which transfused platelets can affect the recipient humoral immune response to antigens on transfused RBCs.

Recipient CD40L blockade or CD4 depletion prevents anti-KEL IgG alloantibody responses

To investigate whether check-point blockade of the CD40/CD40L interaction may impact alloimmunisation, animals were infused prior to transfusion with a monoclonal antibody (MR1) that binds to CD40L and blocks the CD40/CD40L interaction. Following blockade or treatment with isotype-matched control, the animals were treated with poly (I:C) and transfused with KEL^{hi} RBCs; CD40L blockade was continued every other day for a total of seven doses[24]. As shown in Figure 5A**,** all control mice became alloimmunised, whereas none of the mice that received the CD40L blockade showed a response. These data suggest that durable immune responses to transfused KEL^{hi} RBCs rely on CD40/CD40L interactions.

CD40/CD40L interactions regulate T-cell-dependent responses. To determine whether the anti-KEL

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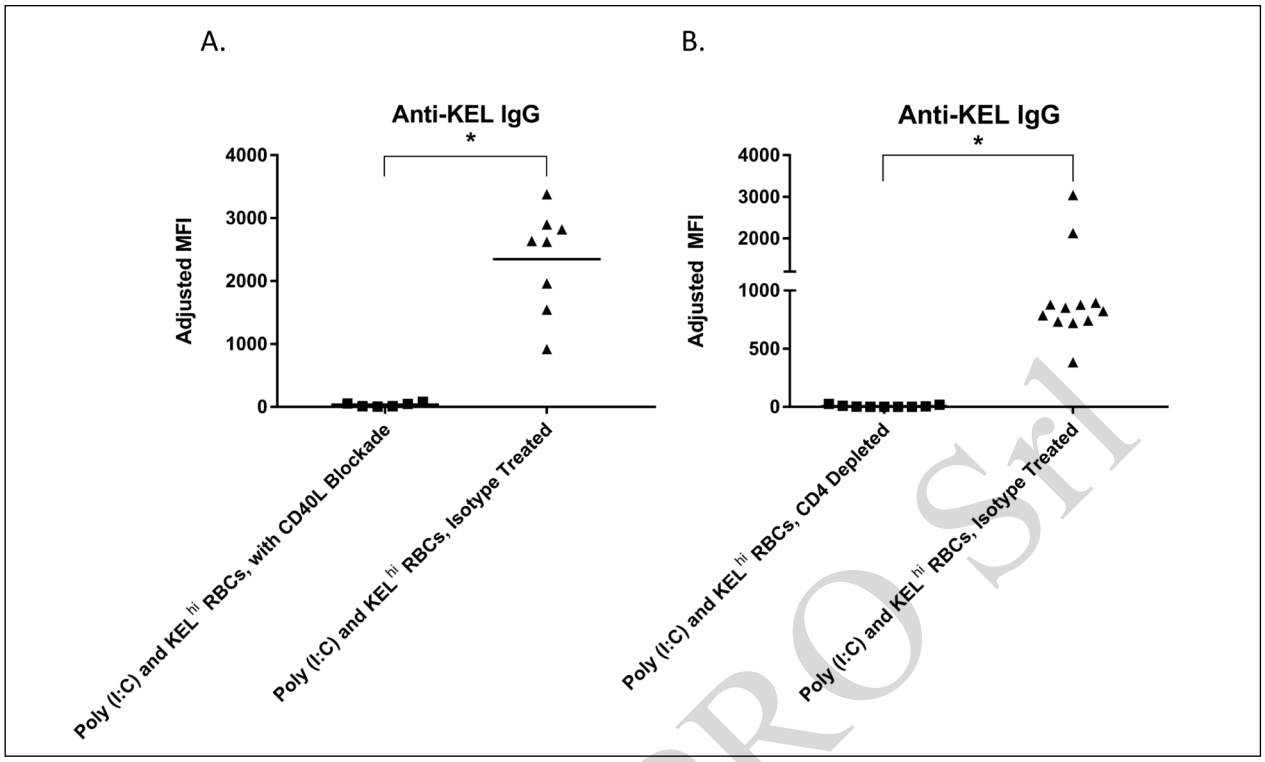


Figure 5 - Anti-KEL alloantibody responses after treatment of recipient animals with CD40L blockade or CD4 depletion. (**A.**) Anti-KEL alloantibody responses 28 days after transfusion as measured by flow cytometric crossmatch, in wild-type C57BL/6 recipients treated with CD40L blockade or isotype-matched control antibody, all treated with poly (I:C) prior to KEL^{hi} red blood cell (RBC) transfusion. *p<0.001; two combined experiments are shown. (**B.**) Anti-KEL alloantibody responses 28 days after transfusion as measured by flow cytometric crossmatch, in wild-type C57BL/6 recipients depleted of CD4 T cells or treated with isotype-matched control, all treated with poly (I:C) prior to KEL^{hi} RBC transfusion. *p<0.0001; pooled data from two experiments are shown.
IgG: immunoglobulin G; RBCs: red blood cells; poly [I:C]: polyinosinic-polycytidylic acid.

alloimmune response was indeed dependent on T cells, wild-type C57BL/6 recipients were depleted of CD4+ T cells using the monoclonal antibody GK1.5; we and others have previously characterised the complete depletion of CD4+ T cells that occurs after treatment with GK1.5[24,28]. CD4+ T-cell-depleted animals were treated with poly (I:C) and transfused with KEL^{hi} RBCs. Whereas 100% of wild-type animals treated with an isotype-matched control antibody or saline developed robust anti-KEL responses, no recipient animals lacking CD4+ T cells became alloimmunised (Figure 5B). Taken in combination with the CD40L blockade data, these CD4 depletion data suggest that antigen-presenting cell/CD4+ T-cell interactions or CD4+ T-cell/B-cell interactions are critically important in the alloantibody response to transfused KEL^{hi} RBCs.

Discussion

We have shown that transfused platelets from wild-type donors enhance recipient humoral immune responses to transfused RBCs expressing the human KEL glycoprotein in a murine model, turning recipients who would otherwise be "non-responders" to transfused RBCs into "responders." The anti-KEL RBC alloimmune response is, like other T-cell-dependent responses[24], fully prevented by recipient CD40L blockade. We posit that CD40L expression on the transfused wild-type platelets contributes, at least in part, to the immune responses observed, as platelets from wild-type but not CD40L KO donors result in detectable anti-KEL alloantibodies following RBC transfusion into CD40L KO recipients. However, platelets lacking CD40L are still able to enhance the response to transfused RBCs in wild-type recipients, suggesting the relevance of alternative immune activating pathways.

An enlarging body of literature points to "inflammation" as being associated with an increased risk of becoming alloimmunised after RBC transfusion. Prior human studies have shown an increase in frequency and magnitude of alloimmune responses to RBC antigens in the presence of an inflammatory stimulus or inflammatory disease[4,5]. Furthermore, previous murine studies have also shown that recipient inflammation has a critical role in determining whether a transfused recipient will become alloimmunised[9,10,33].

To our knowledge, this is the first study examining

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transfused platelets as a source of "inflammation" that dictates humoral immune responses to transfused RBCs, although other studies have evaluated the role of transfused platelets on anti-viral responses[21,25,34]. Platelets are increasingly appreciated for the role(s) they play outside of coagulation, including in innate and adaptive immunity[11]. Containing α-granules, δ granules, lysozymes, and microvesicles, platelets and their secreted molecules are now known to interact with most immune-cell types[35-37]. Platelets constitutively express CD40 and, upon activation, export CD40L from α-granules to their surface. This CD40L induces an auto-amplification loop through tumour necrosis factor receptor associated factor 2 (TRAF2) after binding to CD40 on the platelet surface, and can also be secreted in soluble form[18]. CD40L expression by platelets activates dendritic cells to be more potent antigen-presenting cells[21], and these antigen-presenting cells have previously been shown to be critically important in RBC alloimmune responses[38]. Platelet CD40L expression has been shown to facilitate B-cell activation and differentiation during *in vitro* studies, with down-regulation of surface IgD, increased CD86/CD27 expression, and memory B-cell formation[39]. Beyond CD40 and CD40L, platelets release many other mediators of immune function, including P-selectin, interleukin-1β, RANTES, CXCL4, ADP, and transforming growth factor-β, among others[35].

Our observation that wild-type transfused platelets can serve as an adjuvant to RBCs is not surprising, when considering the immune contributions of platelets. However, the described findings raise some important questions. This study evaluated transfused platelets but what role, for example, might the recipient's own platelets play in determining RBC alloimmune responses? Patients with sickle cell disease, who have amongst the highest RBC alloimmunisation prevalence of any population of patients, also have thrombocytosis and extremely high circulating plasma levels of soluble CD40L[40,41]. A recent study found an association between soluble CD40L levels and a history of acute chest syndrome[41], and RBC exposure around the time of an episode of acute chest syndrome is associated with the development of RBC alloantibodies[4]. Could RBC alloimmunisation be associated with recipient CD40L levels, or with other platelet-associated cytokines[42]? Furthermore, patients with systemic lupus erythematosus, who are also highly RBC alloimmunised[43,44], have increased CD40L expression on platelets due in part to circulating immune complexes[45].

Another interesting but unanswered question, which could link the current data with prior studies completed in our laboratory, revolves around the relationship between CD40L expression and type 1 interferon (interferon α/β). Recent studies by Gibb *et al.* have shown that type 1 interferon sensing is critical to determining whether a RBC alloimmune response will develop in transfused murine recipients. Animals lacking type 1 interferon receptors (IFNAR KO mice) do not become alloimmunised despite exposure to KEL-expressing RBCs in the presence of an adjuvant, and exogenous type 1 interferon administration can turn what would otherwise be a "non-responder" into a "responder"[9,46]. Since CD40L expression on platelets in patients with systemic lupus erythematosus has been shown to increase type 1 interferon secretion from plasmacytoid dendritic cells[45], it is plausible that type 1 interferon is released by recipient cells in response to transfused platelets; future studies will address this possibility. Studies in mice prone to developing systemic lupus erythematosus suggest that treatment with a P2Y(12) antagonist, known to decrease platelet CD40L expression, may mitigate disease severity[45]; these studies raise a question of whether anti-platelet drugs[47] could have an expanded role in other disease entities.

The fact that low levels of anti-KEL antibodies were formed in CD40L KO recipients that were incapable of forming germinal centres is notable. Furthermore, the fact that a small number of germinal centres could be detected by flow cytometry but not by immunofluorescence after secondary KEL^{hi} RBC exposure suggests that the germinal centre response is not robust even in wild-type recipients. Ongoing studies are investigating the broader question of whether alloantibody responses to certain antigens on transfused RBC are germinal centre-dependent, or whether they may be extrafollicular in nature.

Limitations to our studies must be considered. These studies are largely descriptive, and follow-up studies will be needed to investigate possible mechanism(s) in more detail. The fact, for example, that CD40L KO donor platelets are immunogenic following transfusion into wild-type but not into CD40L KO recipients potentially suggests that CD40L expressed by host cells may be able to induce an auto-amplification loop on transfused platelets leading to the release of cytokines. These data also suggest, however, that it is important to investigate pathways beyond CD40 and CD40L in the future in order to understand the role of platelets in RBC alloimmunisation. Another consideration is the activation status of the donor platelets at the time of transfusion. It is notable that a wide range of anti-KEL responses were observed in each individual experiment involving wild-type recipients despite each recipient receiving platelets from the same collected "unit". Past studies using the same platelet-rich plasma isolation technique showed intact aggregation[26], although any manipulation of platelets inevitably leads to some degree of baseline activation. It is not clear from the

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studies completed to date whether soluble CD40L from platelets is adequate to induce alloimmunisation to transfused RBCs, or whether intact platelets have to be transfused; it is also not clear whether residual white blood cells in the platelet preparation may have played a role in the observed responses. Furthermore, these studies were completed using the equivalent of one or two units of transfused human platelets, and as such the minimum number of platelets required to induce a response is not known at this time. This question is relevant when considering the fact that some human RBC leucoreduction filters are more "platelet-sparing" than others[48] and thus RBC transfusion recipients are exposed to variable numbers of platelets. It should also be mentioned that human studies have shown that both apheresis and whole blood-derived platelets expose transfusion recipients to relatively large amounts of soluble and membrane-bound CD40L[49,50].

Conclusions

In conclusion, our data show the importance of transfused platelets in influencing recipient humoral immune responses to transfused RBCs expressing the KEL glycoprotein in a murine model. These studies provide the foundation for further investigations of the underlying mechanism of platelet involvement, either transfused or endogenous, as related to general immune activation and RBC alloimmunisation in animal models and in humans. These studies also raise awareness about the "immune continuum[11]" surrounding platelets.

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Authorship contributions

DJM, PN, and JEH designed and planned the experiments completed by DJM, MS, JL, DL, PN, DB, CM, and JEH. All Authors made suggestions regarding the experiments and helped with interpreting the data. DJM wrote the initial draft of the manuscript, and all Authors edited the manuscript.

The Authors declare no conflicts of interest.

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