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Characterization of Kell Glycoprotein Transgenic Mice: Murine Model for Immune Response to Transfusion

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B.S. in Biology, Brandeis University, 2009

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An abstract of
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Abstract
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Mouse models are useful tools to study the generation and effects of immunity to alloantigens on transfused and transplanted tissues. However, mouse models are much more relevant to the particulars of human biology when they express the human form of the alloantigens of interest. Our laboratory generated two strains of transgenic mice, each of which expresses one of two antithetical antigens (Cellano and Kell epitopes) from the Kell glycoprotein blood group system that differ by a single amino acid (Methionine-Threonine at position 193). The Kell glycoprotein red blood cell antigen is one of the most clinically significant antigens in blood transfusions after ABO and RhD red blood cell antigens. These Kell glycoprotein transgenic mice, named Cellano or Kell based on the variant that they express, are useful models to study immune responses to transfusion. Here we describe our initial characterization of these Kell glycoprotein transgenic mice.

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Introduction:

Human experimentation with blood transfusion dates back to the 1600's. However, adverse reactions to transfusions due to transfusing mismatched blood were not fully appreciated until Landsteiner's discovery of the five main blood groups (A, B, O in 1901, AB in 1902 and Rh in 1941), and the agglutination reaction that occurred as a consequence of exposing serum of one blood group with red blood cells (RBCs) of another.[1,2] In an effort led by Dr. Ludvig Hektoen in 1907, patients were tested prior to transfusion for antibodies against the donor red blood cells (RBCs).[1] These antibodies present in the patient against the donor RBCs are responsible for the agglutination reaction initially observed by Landsteiner. Detection of patient antibodies specific for donor RBC antigens defines the donor RBCs as incompatible for the patient. As there are now over 300 blood group antigens that have been identified, incompatibility is not limited to antibodies present against the main RBC antigens: A, B, and RhD.[3] Typing for incompatibility is important because transfusion of incompatible blood can lead to adverse reactions such as: acute hemolytic transfusion reactions, delayed hemolytic transfusion reactions and transfusion-related acute lung injury.[4] These adverse side effects range from mild to severe and hemolysis of blood can lead to hypotension, organ failure, coagulopathy, shock and death.[5] Furthermore, transfusion of compatible (i.e. no antibodies specific to the donor RBCs are present) but unmatched blood carries the risk of alloimmunization against the donor RBC antigens. Alloimmunization occurs in 2-6% of "healthy" patients who received RBC transfusions, and it can reach rates as high as 36% in patients with hematological disorders such as Sickle Cell Disease.[6] Although hemolysis is a clinical emergency when it occurs, it is important to note that even in events in which incorrect blood components were transfused into patients, the majority of the patients (3439/3593) had minor or no morbidity as a consequence.[4] This indicates that more times than not, little hemolysis of blood occurs.

Hemolysis of transfused RBCs, even in the presence of antibodies specific for donor RBC antigens, is rare; many RBC specific antibodies are clinically insignificant, i.e. do not lead

to transfusion reactions.[7] Furthermore, a clinically observed phenomenon called antigen loss provides an alternative pathway to hemolysis of incompatible RBCs. Antigen loss is defined as undetectable levels (or significantly reduced but still detectable levels) of antigen on the surface of the cell in presence of alloantibodies or autoantibodies to the antigen. Moreover, antigen loss excludes the possibility of being masked by antibody binding because direct antiglobulin tests (DATs), which probe for bound antibodies or bound complement, are negative.[8] Antigen loss has been observed clinically for red blood cell antigens and in particular has been observed with the Kell blood group system.

In one clinical case report, a patient presented with Immune Thrombocytopenic Purpura (ITP), an autoimmune disease in which antibodies are made against platelet antigens, leading to clearance of platelets and potential bleeding. The presence of these autoantibodies is suggestive of a break in B cell tolerance (and perhaps T cell tolerance, depending on the subset of B cells responsible for the antibody production). Furthermore, the patient presented with low hemoglobin levels and difficulty breathing. Yet there was no immediate evidence of hemolysis. However, the patient had detectable levels of antibodies against the Kell glycoprotein in his serum. This was suggestive of an alloantibody because the anti-Kell glycoprotein antibodies were non-reactive to the patient's own RBCs (and the patient's own RBCs typed as lacking several common antigens in the Kell system), suggesting the person was Kell null. A Kell null individual has a congenital defect in production of the Kell glycoprotein due to a homozygous mutation and does not produce the Kell glycoprotein. However, in the referenced patient, upon aggressive treatment for the anti-platelet antigen autoantibodies, the anti-Kell glycoprotein antibodies also resolved. Upon resolvation of the antibodies, the patient's RBCs were cross-reactive from serum taken previously, containing anti-Kell antibodies and the patient's RBCs now typed as Kell positive by reagent typing antibodies. This indicated that the patient was not Kell null and properly coded and produced the Kell glycoprotein. Therefore, these anti-Kell antibodies were autoantibodies.

Moreover, the loss of antigen on the RBCs that was observed prior to treatment was confirmed by western blot, eliminating a mechanism of antigen masking by antibodies or C3.[9]

The aforementioned case study indicates that the Kell glycoprotein is capable of undergoing antigen loss. The Kell glycoprotein contains 12 antithetical antigens, each antithetical pair differing by a single amino acid, (e.g. Kell and Cellano are antithetical antigens (Methionine-Threonine) at amino acid position 193 of the Kell glycoprotein). Antithetical antigens that are not present in a recipient can be immunogenic and can lead to transfusion reactions and hemolytic disease of the newborn.[9] On the basis of these possible responses to transfusion, the human Kell glycoprotein is an ideal molecule for the study of immune responses to transfusion in murine systems. The Zimring laboratory engineered transgenic mice to express the Kell glycoprotein. The transgenic mice were constructed using a pronuclear injection approach and pups were screened for founders that expressed the type II transmembrane protein on their red blood cells. Antithetical Kell glycoprotein transgenics were made, those expressing the Cellano epitope and those expressing the Kell epitope. These transgenics can be utilized to model the aforementioned responses to incompatible transfusion but furthermore to study immune responses to single amino acid differences.

In this paper we report that the transgenic Kell glycoprotein is expressed on red blood cells in varying degrees in our different transgenics, and that it is absent on peripheral blood leukocytes, and platelets. Moreover, expression of the transgenic protein is present during early differentiation of bone marrow (BM) into the erythroid lineage. Furthermore, the antigen is stable upon transfusion of transgenic RBCs into MuMT agammaglobulinemic recipients but becomes undetectable or present in reduced levels upon transfusion of transgenic RBCs into C57BL/6 (B6) mice. These B6 mice mount immune responses to the transgenic RBCs as determined by positive DATs for complement and IgM as early as day 3 post-transfusion. These responses are absent in MuMT recipients. We also report enhanced immune responses to these transgenic RBCs by

administering polyinosinic:polycytidylic acid [poly(I:C)] and multiple transfusions. This demonstrates a more generalized principle for effects of inflammation (albeit an inflammatory response that mimics response to viral infection)on immunization against RBC antigens as previously described with synthetic RBC antigens.[10,11,12]

Methods:

Characterization of Kell Glycoprotein Transgenic Red Blood Cells (RBCs):

Kell glycoprotein transgenic mice were bled retro-orbitally into 20-40uL of acid citrate dextrose (ACD) BD (Franklin Lakes, NJ). 5uL of RBCs were pipetted into 96 well plates and washed 3x with fluorescence activated sorting buffer (FACS buffer) comprised of 1L phosphate buffered solution from HyClone (Logan, Utah) with 0.9g ethylenediaminetetraacetic acid tetrasodium salt hydrate (EDTA) from Sigma-Aldrich, Co. (St. Louis, MO) and 2g bovine serum albumin (BSA) from Sigma-Aldrich (St. Louis, MO). Plates were centrifuged at 1200 rpm for 2 minutes at 20^{o} Celsius. RBCs were then stained with MIMA8 (3.6mg/mL), MIMA9 (4.8m/mL), and MIMA23 (4.44mg/mL) or anti-Cellano IgM from Alba Bioscience Limited (Newtown, PA). MIMA8, MIMA9 and MIMA23 were used at 1:100 dilutions. ALBA anti-Cellano was diluted 1:10. The primary antibodies were incubated with the RBCs at room temperature (RT) for 30 minutes. The RBCs were washed 3x as described above and then those stained with MIMA8,9 or 23 were incubated with 1:100 dilution of allophycocyanin-labeled goat anti- mouse immunoglobulins (anti-Igs- APC) from BD Pharmingen (San Jose, CA) for 30 minutes at room temperature. RBCs stained with ALBA anti-Cellano were incubated with a goat anti-mouse IgM-APC from Jackson ImmunoResearch Lab, Inc. (West Grove, PA) for 30 minutes at room temperature. RBCs were washed again as described above and resuspended in 100uL of FACS buffer. 10uL of this solution was resuspended in 300uL of FACS and analyzed by flow cytometry on a FACScan.

RBCs were also analyzed using polyclonal anti-sera against the Kell glycoprotein

generated in female C57BL/6 (NCI) mice that were immunized 3+ times with poly(I:C) and Cellano B blood. 5uL of RBCs were incubated with a 1:10 dilution of the polyclonal anti-sera under the previously described conditions, washed 3x, and then incubated with anti-Igs-APC as described. RBCs were washed, resuspended, and diluted as described and analyzed via FACS Can with a minimum of 100,000 RBC events.

Characterization of Kell Transgenic White Blood Cells (WBCs):

Kell glycoprotein transgenic mice were sacrificed and their spleens and peripheral blood were harvested for analysis. The splenocytes were isolated using frosted microscope slides from Fisher Scientific (Location?) and collected in HyClone RPMI-1640 Medium (1x) from Thermo Scientific (Logan, UT). Splenocytes were strained and washed 1x with RPMI-1640 Medium at 1200 rpm for 10 minutes at 20^{o} Celsius. The supernatant was decanted and then the pelleted cells were resuspended in 2mL of red blood cell lysis buffer from Sigma-Aldrich (St. Louis, MO) for 5 minutes and then washed with RPMI as previously described, decanted and washed with PBS. The remaining WBCs were then resuspended in 4mL of FACS Buffer and 200uL were plated in a 96 well plate. Cells were washed 3x with FACS Buffer at 1200rpm for 2 minutes at 20^{o} Celsius. Cells were then Fc blocked (BD) for 20 minutes at room temperature. Cells were washed as described above and then incubated with 1:10 dilution of polyclonal anti-sera (from IgHa, congenic C57BL/6 female mice immunized 3+ times with poly(I:C) and Cellano B blood) for 15 minutes at room temperature. Cells were washed 3x and then stained with anti-IgG1a conjugated to biotin from BD Pharmingen (San Jose, CA) at 1:100 for 15 minutes at room temperature. Cells were washed and then incubated with a cocktail containing 1:100 streptavidin-APC, 1:50 fluorescein isothiocyanate (FITC)-labeled anti-Ter119 (Ter119 -FITC), and 1:100 dilution of phycoerythrin(PE)- labeled anti -CD45 (CD45-PE). Cells were labeled for 15 minutes at room temperature in the dark and then washed. Cells were resuspended in 100uL FACS buffer and diluted to 500uL total volume. Cells were analyzed on a FACScan. A minimum of 100,000 WBC

events were collected. CD45+ Ter119- events were analyzed.

Peripheral blood was analyzed in a similar fashion in which whole blood was collected in 20uL ACD. 10uL of whole blood was washed 3x with 100uL of FACS buffer at 1200rpm for 2 minutes at 20^{o} Celsius. Blood was then blocked with Fc block as mentioned above and washed 3x. Blood was incubated with polyclonal anti-sera and anti-IgG1a-biotin as mentioned above for splenocytes. Blood was washed and then incubated for 15 minutes in the dark with a cocktail containing 1:100 CD45-PE streptavidin-APC, and 1:25 Ter119 –FITC, all from BD Pharmingen (San Jose, CA). Cells were washed and resuspended in 100uL of FACS Buffer. 10uL of resuspended RBCs were then diluted in 300uL FACS Buffer and analyzed on FACScan. A minimum of 1,000 CD45+ Ter119- events were collected.

Characterization of Kell Transgenic Platelets:

Platelets from Kell transgenic mice were analyzed by collecting peripheral blood into 20uL of ACD. Platelets were assayed for expression of Kell glycoprotein expression using the same methods as mentioned above for peripheral blood with the exception of all washes being performed at 3,000 rpm and CD45-PE substituted with 1:100 CD41-PE from BD Pharmingen (San Jose, CA). 10,000 CD41+ Ter119- events were the criteria for collection on the FACS Can.

Characterization of Kell Glycoprotein Expression on Red Blood Cell Precursors:

BM from femur and tibia was harvested from Kell glycoprotein transgenic mice into HyClone RPMI-1640 Medium. The bone marrow cells were strained and then washed with RPMI at 1200 rpm for 10 minutes at 20^{o} Celsius. Supernatant was decanted and cells were then resuspended in 2mL of red blood cell lysis buffer for 5 minutes at room temperature. Cells were then washed with RPMI, aspirated and then washed with PBS. Cells were resuspended in 2mL of FACS buffer and 200uL was plated in 96 well plate. BM cells were washed 3x with FACS buffer for 2 minutes at 1200rpm. Cells were then Fc blocked as previously described and stained with

polyclonal anti-Kell glycoprotein antisera and anti- IgG1a- biotin as previously described. Cells were washed and then incubated with a cocktail containing 1:100 anti-CD71-PE (CD71-PE), 1:50 Ter119-FITC, and 1:100 Streptavidin-APC, (all from BD Pharmingen) in the dark for 15 minutes. Cells were then washed and resuspended in 100uL of FACS buffer and then diluted into a total volume of 500uL. A minimum of 100,000 BM cells were collected on the FACS Can.

Characterization of Kell Glycoprotein by Western Blot:

COS-1 cells were transfected with expression vectors for GFP and Kell (pIRES-EGFP DNA and pcDNA-Kell) with FuGene 6 Transfection Reagent from Roche (Mannheim, Germany) in order to express these proteins for selection of transfected cells and to use as a positive control. Once cells were analyzed via flow cytometry for the presence of GFP via fluorescence on FL1 and for expression of the Kell glycoprotein via fluorescence on FL4 after staining with 1:100 MIMA23 and 1:100 anti-Igs-APC, plates were lysed with 27microliters of RIPA Buffer for 15 minutes on ice while shaking and then scraped. The cells and buffer were collected and centrifuged at 13,000 rpm for 15 minutes. Supernatant was collected and stored.

RBCs from the transgenic mice were collected in 3 heparinized tubes into 40uL ACD. Blood was washed 1x with PBS at 13,000 rpm for 7 minutes at room temperature. Supernatant was removed and RBCs were lysed with hypotonic lysis (5mM pH 7.5 Na_3PO_4 buffer). Membranes were isolated by centrifugation and were washed 5x. The membrane pellet was then resuspended in 0.3mL of RIPA Buffer and shaken on ice for 15 minutes. Preparation was then centrifuged for 15 minutes at 13,000rpm and supernatant (lysate) was collected and stored.

Solubilized membranes were used for SDS-PAGE analysis. Lysate was prepared under reducing conditions with 15uL NuPAGE LDS Sample Buffer from Invitrogen (Carlsbad, CA), 5uL of NuPAGE Sample Reducing Agent 10x from Invitrogen, and 30uL of lysate. Preparations were incubated at 70^{o} Celsius for 10 minutes and 20uL was loaded/well in a NuPAGE 4-12% Bis-Tris Gel from Invitrogen. Gels were run under reducing conditions with NuPAGE

Antioxidant from Invitrogen for 40 minutes at 200V. Gels were transferred under reducing conditions to a nitrocellulose membrane at 30V for one hour. Membranes were blocked over night at 4^{o} Celsius with 5% fat free instant milk in PBS. Membranes were then treated with 5uL/mL anti-Kell glycoprotein antibody from Abcam (Cambridge, MA) for one hour at room temperature while shaking. Membrane was washed 3x for 10 minutes each with PBS and 0.01% Tween20. Membrane was then incubated with 1:1000 dilution of peroxidase-conjugated goat anti- mouse IgG, Fcy chain specific, from Jackson ImmunoResearch Lab, Inc. (West Grove, PA) for one hour at room temperature while shaking. Membrane was washed 3x as previously described and developed using HyGLO from Denville Scientific, Inc. (Metuchen, NJ).

Labeling of RBCs with DiI and DiO:

RBCs were collected in ACD (~120uL ACD/1mL of peripheral blood). RBCs were washed 3x with PBS at 1350rpm for 10 minutes at 20^{o} Celsius. RBCs were then labeled with DiO or Cell- Tracker CM-DiI from Invitrogen (Eugene, OR). DiO was prepared by weighing out 0.001g DiO and adding it to 1mL of 50^{o} Celsius dimethylformide (DMF). 100uL of DiO was added per 1mL of packed blood in 10mL of PBS. CM-DiI was prepared by resuspending 4 DiI aliquots in 50uL DMSO and adding 200uL CM-DiI to 1mL of packed RBCs in 10mL of PBS. Blood was incubated for 30 minutes at 37^{o} Celsius, washed 3x as described above and the two labeling reactions were then mixed in a 2:1 DiI:DiO labeled blood ratio in a total volume of 500uL. Mixed blood was transfused into C57BL/6 and MuMT mice.

Immunization of C57BL/6 with Cellano B blood and poly(I:C):

C57BL/6 mice and C57BL/6 mice congenic for IgHa were given 99.6 ug of polyinosinic: polycytidylic acid (poly(I:C)) via intraperitoneal (i.p.) injection in a total volume of 500uL or were given 500uL of PBS i.p. Four hours later mice were transfused with 100uL of packed RBCs in a total volume of 500uL PBS intravenous (i.v.). This was repeated once a week for at least 3 weeks. Each week mice were bled retro-orbitally with heparinized capillary tubes without ACD.

Serum was collected by centrifuging whole blood at 13,000rpm for 15 minutes and collecting the supernatant for analysis.

Analysis of Transfused RBCs:

RBCs were collected in 20uL of ACD and washed with FACS buffer prior to analysis. RBCs were stained with anti-Igs-APC alone or anti-IgM-APC alone to analyze RBCs for direct immunoglobulin binding. Expression of the Kell glycoprotein was assayed by incubating with primary antibodies (either MIMA23 or anti-Cellano Albaclone) and corresponding secondary antibodies under the previously mentioned conditions for characterization of RBCs. Binding of complement was determined by incubating RBCs with 1:100 biotin labeled anti-C3 (C3-biotin) antibody from Cedar Lane Laboratories, Inc. for 30 minutes at room temperature. RBCs were washed 3x with FACS buffer and then incubated in the dark with 1:100 streptavidin-APC for 30 minutes at room temperature. Positive control for complement staining was made by incubating mouse RBCs collected in ACD (washed 9x with FACS buffer)with 3:100 uL of rabbit anti-mouse RBCs at for 1 hour at room temperature. The cells were washed 3x with FACS buffer and then 45uL of mouse serum (collected as described above) for 45 minutes to 1 hour at 37^{o} Celsius. Cells were washed 3x with FACS and then treated the same as the rest of the cells being assayed for complement binding. Cells were assayed via FACScan. A minimum of 1,000 DiO+ RBC events were collected.

Analysis of Serum:

Mice transfused with Kell glycoprotein transgenic RBCs were bled retro-orbitally without ACD. The whole blood was centrifuged at 13,000rpm for 15 minutes to clot and the supernatant was removed and stored. Serum was then used with 3uL of RBC targets for cross-match analysis at 1:10 and 1:100 dilutions in a total volume of 10uL for 30 minutes at room temperature. RBC targets were washed and then stained 1:100 with anti-Igs-APC or anti-IgM-APC for 30 minutes at room temperature. RBCs were washed and resuspended in FACS

buffer and analyzed on a FACS Can. A minimum of 100,000 RBC events were collected.

Results:

Kell glycoprotein expression on RBCs, RBC precursors, splenocytes, peripheral blood leukocytes, and platelets

In order to determine the levels of expression of the Kell protein in the transgenic mice, RBCs from each transgenic line were collected and stained with Kell glycoprotein specific antibodies: MIMA8, MIMA9, MIMA23, anti-Cellano and poly- clonal antisera (Figure 1). MIMA8 is a mono- clonal antibody generated in BALB/c mice and is specific for the Kp^b epitope on the Kell glycoprotein. MIMA9 is a monoclonal antibody generated similarly and is specific for the Js^b epitope. MIMA23 is a monoclonal antibody specific for the Kell epitope on the Kell glycoprotein. Anti-Cellano is a monoclonal antibody which recognizes the Cellano epitope of the Kell glycoprotein. The polyclonal antisera was generated in C57BL/6 which were immunized via poly(I:C) treatment and transfusion with Cellano B whole blood. The antithetical Kell glycoprotein constructs (those expressing Kell epitope and Cellano epitope) can be compared directly to each other using their common epitopes, Jsb and Kpb. Using the anti-Jsb and anti-Kpb antibodies, we directly compared the expression of the Kell glycoprotein on the different transgenic RBCs. Cellano A RBCs have the highest level of expression of the Kell glycoprotein and a bimodal distribution. The majority of the Cellano A RBCs have a higher level of expression, with a smaller subset having a lower level of expression of the Kell glycoprotein. Cellano B RBCs express lower levels of Kell protein than Cellano A. Cellano B RBCs' expression of the Kell glycoprotein also has a bimodal distribution. The majority of the RBCs from the Cellano B have a lower level of expression in comparison to the Cellano A RBCs. However, a small subset has a higher level of expression of Kell glycoprotein. The Cellano D RBCs have the lowest level of expression of the Cellano, with a single population of RBCs, which all have a modest level of expression of the Kell glycoprotein in comparison to Cellano A

and Cellano B. Kell A has the highest level of expression of the transgenic mice expressing the Kell epitope. It has a bimodal distribution, similar to Cellano A and B, with the majority of the cells having a lower level of expression than a small subset with expression similar to Cellano D RBCs. Kell B has the lowest level of expression as demonstrated by very little binding of anti-Jsb and undetectable binding of anti-Kpb above a B6 background. In Kell B, the majority of RBCs are negative, but the small percentage of positive cells has staining of equivalent intensity as Kell A.

Staining with anti-Kell epitope antibodies further confirms that Kell A has a higher level of Kell glycoprotein expression than Kell B. There is very little cross-reactivity of this anti-Kell antibody with mice expressing the Cellano epitope, but it is detectable above B6 back- ground staining; greater shifts are consistent with RBCs with higher levels of Kell glycoprotein expression. Anti-Cellano staining of the transgenic RBCs reveals the same pattern as noted by the anti-Kpb and anti-Jsb staining: Cellano A has the highest level of expression, followed by Cellano B and then Cellano D. The anti-Cellano antibody has specificity for the Cellano epitope with some cross-reactivity for the Kell epitope.

The use of the polyclonal anti-sera is to eliminate cross-reactivity with B6 RBC proteins because the polyclonal anti-sera were generated in B6 mice which are tolerant to their own proteins. As a consequence, we can differentiate between Kell glycoprotein positive cells and negative cells. Using the polyclonal antisera, all RBCs in Cellano A, B, and D transgenic mice are positive for the Kell glycoprotein. Kell A and Kell B appear to have cells negative for the Kell glycoprotein as determined by the polyclonal anti-sera.

Red blood cell precursors in the bone marrow were harvested and analyzed with polyclonal anti-sera from B6 mice congenic for IgH^a. Using anti-CD71 antibodies and anti-Ter119 antibodies, RBC precursors were identified at different stages during erythropoiesis (Figure 2). (CD71 is a transferrin receptor which is present on stromal bone marrow cells.

(Ter119 is a glycophorin associated red blood cell marker.) These stages are designated as RII, RIII, and RIV. Non-RBC precursors are designated as non-erythroid. RII is CD71 high, Ter119 high. RIII is CD71 intermediate and Ter119 high. RIV is CD71 low/negative and Ter119 high. Once identified, the RBCs in different stages of development were analyzed for expression of the Kell glycoprotein using the anti-sera from the IgH^a immunized mice. Using these means for analysis, Cellano B has the greatest level of expression during development. It peaks at RII and has decreased levels of expression of the Kell glycoprotein as the cells prepare to exit the bone marrow into the periphery. Cellano A follows Cellano B with next highest level of expression. The highest level of expression is during RII of the Cellano A RBCs but a dramatic change is not seen between the different stages (RII, RIII, and RIV), although there is a dramatic difference between erythroid lineages and non-erythroid. Cellano A RBC precursors maintain a higher level of Kell glycoprotein expression during development than any of the other transgenic RBC precursors. This is consistent with Cellano A RBCs in the periphery having the highest level of expression. Kell A has the third highest level of Kell glycoprotein expression during development with a peak in expression during RII, and the lowest level of expression in the erythroid lineage by RIV. Cellano D has the second lowest level of Kell glycoprotein expression. RII, RIII and RIV's levels of expression are all very similar in the Cellano D mouse. These subsets do have a noticeable shift from the background expression on non-erythroid cells. Finally, Kell B has the lowest level of expression on cells in the bone marrow which coincides with its diminutive expression on RBCs in the periphery.

In order to determine if expression of the Kell glycoprotein was restricted to red blood cells as was predicted due to use of the β-globin promoter, splenocytes (Figure 3), peripheral blood leukocytes (Figure 4), and platelets (Figure 5) were analyzed for expression of the Kell glycoprotein. Platelets were identified by virtue of being CD41+ and Ter119-. (CD41 is a platelet marker.) Splenocytes and peripheral blood leukocytes were identified as being CD45+ and

Ter119-. (CD45 is a marker for hematopoietic cells that is absent on RBCs, platelets, and plasma cells.) While the splenocytes were red blood cell lysed, the peripheral blood fractions used for analysis of platelets and peripheral blood leukocytes were not lysed as to minimize fragmented RBCs binding to the platelets and leukocytes. Furthermore, platelets and leukocytes were analyzed for expression of the Kell glycoprotein using polyclonal anti-sera generated against Cellano B RBCs in B6 mice congenic for IgH^a. Using this approach, neither platelets nor peripheral blood leukocytes express detectable levels of the Kell glycoprotein above B6 background controls. Similarly, splenocytes from Cellano A, B, and D transgenic mice do not express the Kell glycoprotein as determined by this assay. Kell A and B transgenic mice have a population of cells which appear positive for the Kell glycoprotein. These populations are being further investigated, but initial studies suggest that they are dead cells as determined by forward and side-scatter parameters.

Stability of Kell Glycoprotein:

In order to study the stability of the Kell glycoprotein on the RBCs in the periphery, one must be able to exclude new erythropoiesis as a means of expression of protein. Therefore, we transferred DiO labeled transgenic RBCs into agammaglobulinemic mice (MuMTs). As these mice cannot make antibodies against the foreign protein on the transgenic RBCs, we predicted the RBCs would continue circulating for approximately 50 days in the recipient MuMT mice. (55 days is the average lifespan of murine RBCs.) In three separate experiments, DiO labeled Cellano B RBCs maintained detectable levels of expression of the antigen in MuMT mice up to at least day 42 post-transfusion. In a single experiment in which expression of the Kell glycoprotein on Cellano A, Cellano D, and Kell A RBCs was analyzed post- transfusion in MuMT mice, there was detectable levels of expression at day 42 post-transfusion in all strains tested with the exception of Cellano D (in which n=1) (Figure 6).

Immunogenicity of Kell Glycoprotein Transgenic RBCs

B6 mice transfused with Kell glycoprotein transgenic RBCs mount an immune response to the Kell expressed on the RBCs. In 3 individual experiments, DiO labeled Cellano B RBCs led to antibody production specific for the Kell glycoprotein, as determined by cross-match analysis in which 3uL of Cellano B RBCs were incubated with serum from MuMT and B6 mice transfused one time with DiO labeled Cellano B RBCs and DiI labeled B6 RBCs. Serum was taken after 45 days post-transfusion (Figure 7). Antibodies were specific to the Cellano B RBCs and not B6 RBCs. There were no detectable antibodies produced by MuMT mice, as predicted (data not shown). Furthermore, when DiO labeled transgenic Kell glycoprotein RBCs are transfused into B6 recipients, there is detectable IgM bound to the Cellano A, B, and Kell A transgenic RBCs as early as day 3 post-transfusion. IgM bound to Cellano D transfused RBCs is detectable by day 5 (Figure 8). By day 3, Cellano A, B, and Kell A transfused RBCs have complement bound preferentially over their B6 transfused counterparts (Figure 9). Complement binding to Cellano A RBCs and Kell A peaks at day 5, day 7 for Cellano D, and day 12 for Cellano B RBCs. Complement binding remains detectable on the Cellano A, B, D, and Kell A transgenic RBCs and undetectable on the syngeneic RBCs through day 12 post-transfusion. It remains detectable on Cellano A RBCs and B RBCs until at least day 21. Furthermore, over time the levels of detectable Kell glycoprotein expression are reduced post-transfusion in B6 mice (Figure 10).

Enhanced Immunogenicity of Kell Glycoprotein Transgenic RBCs:

Given the previously above data regarding baseline immunogenicity of the Kell glycoprotein in B6 mice, we hypothesized that we could enhance the immune response with i.p. injections of poly(I:C) to induce inflammation in the recipients, in accordance with previous experiments done with other RBC model systems.[12] In 3 independent experiments, we observed that poly(I:C) treatment significantly increases antibody staining intensity to Cellano B RBCs in

comparison to PBS, and in one experiment we show that the response increases with number of immunizations with Cellano B RBCs (as opposed to a temporal increase). This response is specific to the Kell glycoprotein as cross-match analysis with sera from immunized mice to B6 RBCs does not produce a shift (Figure 11).

Discussion:

During the engineering of the Kell glycoprotein transgenic mice, the LCB vector was used as the backbone in which to insert the Kell glycoprotein gene for expression. This LCB construct contains a locus control region (LCR), the β-globin promoter, and an intron and enhancer. Unfortunately, during the construction process, after the generation of both the Cellano A and B lines through pronuclear injection, it was noticed that there was a cryptic translation start site upstream of the desired start site for the Kell glycoprotein. If the Kell glycoprotein gene was translated in two of three reading frames, this would not be an issue. However, if the third reading frame was employed during transcription, there would be the potential that during translation additional amino acids would be added to the amine terminus of the protein. As the amine terminus is cytoplasmic and may not participate in the immune response (unless the additional amino acids are T cell epitopes), we continued use of these transgenic mice at this time. Furthermore, in order to avoid this potential complication in other transgenic mice, a stop codon was entered downstream of the cryptic start site but before the desired start site in subsequent LCB constructs used for pronuclear injection. Ergo, the Cellano D, Kell A, and Kell B mice are not predicted to have additional amino acids from the LCB construct on their amino terminus. Analysis using western blots to detect the Kell glycoprotein could not detect a difference in molecular weight from Cellano A and Cellano B mice versus Cellano D, Kell A, and Kell B mice (Figure 12). Further analysis would need to be conducted using 2-D gel analysis to have a more sensitive assay for detection of additional amino acids. Furthermore, the addition of a translation stop codon may have affected the interaction of the ribosome with the mRNA and resulted in

reduced translation of the Kell glycoprotein. This interpretation is supported by analysis of membrane-bound Kell glycoprotein. After analysis of the Kell glycoprotein on the RBCs from germ line transgenic mice, it is evident that the different transgenic mice have varying levels of expression on their RBCs. This was determined using antibodies against Js^b and Kp^b, as both of these epitopes are expressed by both Cellano transgenic and Kell transgenic mice. Thus, by directly comparing them we have concluded that the order of expression of the Kell glycoprotein on RBCs in the peripheral blood is as follows: Cellano A, B, D, Kell A, and Kell B. As Cellano D, Kell A and Kell B have an additional translation stop codon, their reduced levels of Kell glycoprotein expression could be due to the ribosome disassociating with the mRNA, followed by a failure to re-attach and produce a full length protein. However, the differences in expression could also be due to differences in the site of integration of the transgene, as they were not targeted integrations.

Polyclonal anti-sera against the Kell glycoprotein was generated in C57BL/6 female mice (either of IgH^a or IgH^b allotype) using poly(I:C) to induce inflammation and transfusion of Cellano B blood. As C57BL/6 mice will be tolerant to their own proteins, it was predicted that there would be no binding of this anti-sera against C57BL/6 RBCs. Therefore, we could assess if any of the RBCs from the transgenic mice were negative for expression of the Kell glycoprotein. Implementing this approach, 100% of the Cellano RBCs had expression of the Kell glycoprotein above B6 RBCs. However, the two mouse lines with Kell RBCs had cells that overlaid with B6 RBCs stained with the same anti-sera, suggesting some of the cells may be negative for expression of the Kell glycoprotein. However, as the anti-sera was generated against RBCs containing the Cellano epitope, it cannot unequivocally be ruled out that a polyclonal anti-sera generated against the Kell glycoprotein containing the Kell epitope would cause a staining pattern in which all of the cells would be positive.

As erythropoiesis occurs in the bone marrow, we were interested in the level of

expression of the Kell glycoprotein throughout red blood cell development and the stage at which expression first starts in the Kell glycoprotein transgenic mice. Red blood cells develop in four stages, designated as RI, RII, RIII, and RIV. These cells are denoted by their expression of CD71 and Ter119. Other cells in the bone marrow of non-erythropoietic origin are termed "nonerythroid", as they lack the red blood cell marker Ter119. We analyzed the level of Kell glycoprotein expression on our transgenic cells in RII, RIII, RIV and non-erythroid cells in comparison to B6 controls. We employed polyclonal antisera generated in IgH^a mice to analyze the bone marrow. In doing so noted that in those mice in which the red blood cell precursor stages differed in levels of expression, the lowest level of Kell glycoprotein expression occurred in the RIV stage, the stage immediately prior to release of RBCs into the periphery. This is consistent with observations of other RBC transgenic mouse models in our laboratory in which RIV has the lowest level of expression for erythroid precursors. However, the level of expression (at least for Cellano A mice) is greater during RIV in comparison to cells in the periphery. This could be due to instability of the protein on the surface of the red blood cells due to lack of the human KX (a protein known to heterodimerize with the Kell glycoprotein in humans, encoded by the XK gene). The human KX protein is thought to be required for expression of the human Kell glycoprotein because in patients with congenital defects in the XK gene have reduced levels of Kell glycoprotein expression on their red blood cells. An alternative interpretation of these results is that as the red blood cell precursors transition to an anucleate stage, transcription of genes ceases as a consequence of lacking a nucleus. It would follow that there were would be reduced levels of mRNA and less translation into protein. In order to test this, reticulocytes (newly emigrated peripheral RBCs which still contain mRNA) can be analyzed for their Kell glycoprotein expression levels; although, this may not definitively resolve this controversy. Moreover, in experiments in which Kell glycoprotein transgenic RBCs were DiO labeled and transfused into MuMT mice with syngeneic DiI labeled controls, at day 42 post-transfusion the Kell glycoprotein

was detectable on DiO RBCs over DiI B6 controls from Cellano A, B, and Kell A RBCs out of those tested (Cellano A, B, D, and Kell A). This suggests even if the protein is slightly unstable as hypothesized above, it is not completely lost from the membrane over the lifespan of the RBC.

Moreover, as these mice will be used in the study of blood transfusions, non-erythroid cells present in the peripheral blood are of immense interest. Therefore, we analyzed white blood cells and platelets in the peripheral blood and splenocytes. While RBCs from the spleens were lysed during processing, RBCs in the peripheral blood fractions were not lysed. The overwhelming number of RBCs in the peripheral blood relative to white blood cells and platelets would potentially allow for RBC fragments lacking Ter119 (used to exclude RBCs from analysis) to bind to the platelets and white blood cells. This could produce a positive signal for the presence of the Kell glycoprotein which not be recapitulated in the B6 controls because the B6 RBCs do not have Kell glycoprotein positive fragments to attach to white blood cells and platelets. Using these methods and a polyclonal anti-Kell glycoprotein serum isolated from IgH^a mice, we were capable of investigating the expression of Kell glycoprotein on white blood cells (including B cells) and platelets. In our analysis we concluded that platelets and peripheral blood leukocytes in all of the Kell glycoprotein transgenic mice lack the detectable presence of the Kell glycoprotein. Furthermore, the splenocytes in the Cellano transgenic mice also lack detectable amounts of the Kell glycoprotein. However, there appears to be some expression on the splenocytes in the Kell transgenic mice. However, these positive populations are not detectable in the peripheral blood, although the number of cells analyzed is diminutive compared to the number of splenocytes analyzed. Furthermore, upon backgating the Kell glycoprotein positive splenocytes appear to be "dead" cells as determined by SSC and FSC. However, further analysis using 7AAD will need to be performed to test this hypothesis. With the current results and using caution against any biased interpretations, the Cellano transgenic mice have restricted expression of the Kell glycoprotein to RBCs; Kell transgenic mice do not have sole expression of the Kell glycoprotein on RBCs (in

regards to the cell types tested).

In addition to analyzing the expression of the Kell glycoprotein on the peripheral RBCs, during RBC development in the BM, and expression on other cells such as platelets, peripheral blood leukocytes and splenocytes, the transgenic mice were assessed for immunogenicity by transfusing into B6 recipients. During transfusion of these DiO labeled transgenic RBCs (Cellano A, B, D, and Kell A), immune responses were detectable against the transgenic RBCs in comparison to syngeneic B6 controls. The immune response was detected as positive direct anti-globulin tests (DAT) with antibodies recognizing IgM and C3 bound to the transgenic cells but not to the B6 syngeneic cells. There was a detectable immune response as early as day 3 post-transfusion for Cellano A, B, and Kell A, with Cellano D following behind at day 5. These responses increased over time for about the course of 1 week to 2 weeks and then became undetectable on the transfused cells in most recipients. The loss of positive DATs coincided with loss of detectable antigen on these cells. When serum was taken from the once transfused animals at day 42, antibodies specific for the Kell glycoprotein were isolated from mice transfused with Cellano B blood in a cross-match analysis. During the initial cross-match analysis Cellano RBCs or B6 RBCs were incubated with serum from the transfused mice and anti-Igs was used to detect binding of antibodies to the RBC. Employing this method antibodies were not detectable in mice transfused with Cellano A, D, or Kel A blood. However, when an anti-IgM secondary was used to detect binding, mice transfused with Cellano A blood showed a detectable response to the transfused RBCs. These data indicate that these Kell glycoprotein RBC transfusions are capable of initiating an immune response. Moreover, the antibody binding to the transgenic RBCs as early as day 3 suggests that B1 cells which do not require T cell help may be involved or “natural” serum antibodies are responsible. As naïve mouse sera incubated with Kell glycoprotein transgenic RBCs do not have detectable titers against the transgenic protein, we favor the interpretation that B1 B cells are involved. We could test these two alternative hypotheses by

transferring sera from naïve mice into MuMT recipients and analyzing transfused RBCs 3 days post- transfusion. Also, we could deplete B cells in B6 recipients prior to transfusion. However, only certain outcomes would be interpretable; if B cell depletion did not eradicate antibody binding at day 3, incomplete B cell depletion could not be excluded as an explanation for the result. Furthermore, if transfer of sera into MuMT recipients did not result in antibody binding, it could not be excluded that insufficient amounts of antibody specific for the protein were transferred. These data also suggest the lack of detectable antibodies at day 45 post-transfusion in B6 mice transfused singly with Cellano D and Kell A blood may be indicative of an abortive immune response. Differences in levels of expression of the transgenic protein may be responsible for differences seen in immune responses to single transfusions. It should be noted that DATs are using the transfused cells and because the transfused cells have different levels of expression, the DATs cannot be directly compared. Moreover, immune response as determined by cross-match analysis from sera from immunized mice does not allow for the comparison between Cellano and Kell transgenic mice, as different targets are used. Alternatively, as the half-life of antibodies is typically around 3 weeks, this time point may be too late to detect the antibodies in the sera because, as previously mentioned the RBCs lose detectable antigen during the course of circulation post-transfusion. While these experiments mimic a RhD antigen response (i.e. an immune response in which the whole protein is foreign to the recipient), experiments are underway to study transfusion of Kell RBCs into Cellano recipients in which the Cellano recipients express a human MHC II protein (specifically, DRB 04014) which has be known to be capable of expressing the single amino acid difference between the Kell and Cellano epitopes.

Regardless of how these data are interpreted, it is clear that an immune response does occur in response to transfusion with the Kell glycoprotein RBCs. Therefore, we were interested in determining if we could enhance the immune response to the Kell glycoprotein (specifically

through Cellano B RBC transfusions) with or without poly(I:C), with single or multiple transfusions. We noted in three individual experiments that poly(I:C) enhances immunization over that observed with transfusion and PBS alone. Moreover, we saw an enhanced response with 3 immunizations over time in comparison to singly transfused mice over time (in a single experiment). The difference between the PBS treated and poly(I:C) treated group was significant. Further experiments in which other Kell glycoprotein transgenic RBCs are used for transfusion may produce varying results.

In summation, these data demonstrate our successful generation of antithetical Cellano and Kell transgenic mice. Furthermore, we have shown restriction of expression to red blood cells (at least in the cell types tested) and red blood cell precursors in the bone marrow as early as the RII stage. Furthermore, we have shown stability of the antigen on red blood cells post-transfusion in MuMT mice and reduction of stability when transfused into B6 mice. B6 mount an immune response that is detectable on the transfused cells via DAT for complement and IgM. Immunization is enhanced by administration of poly(I:C).

FIGURES

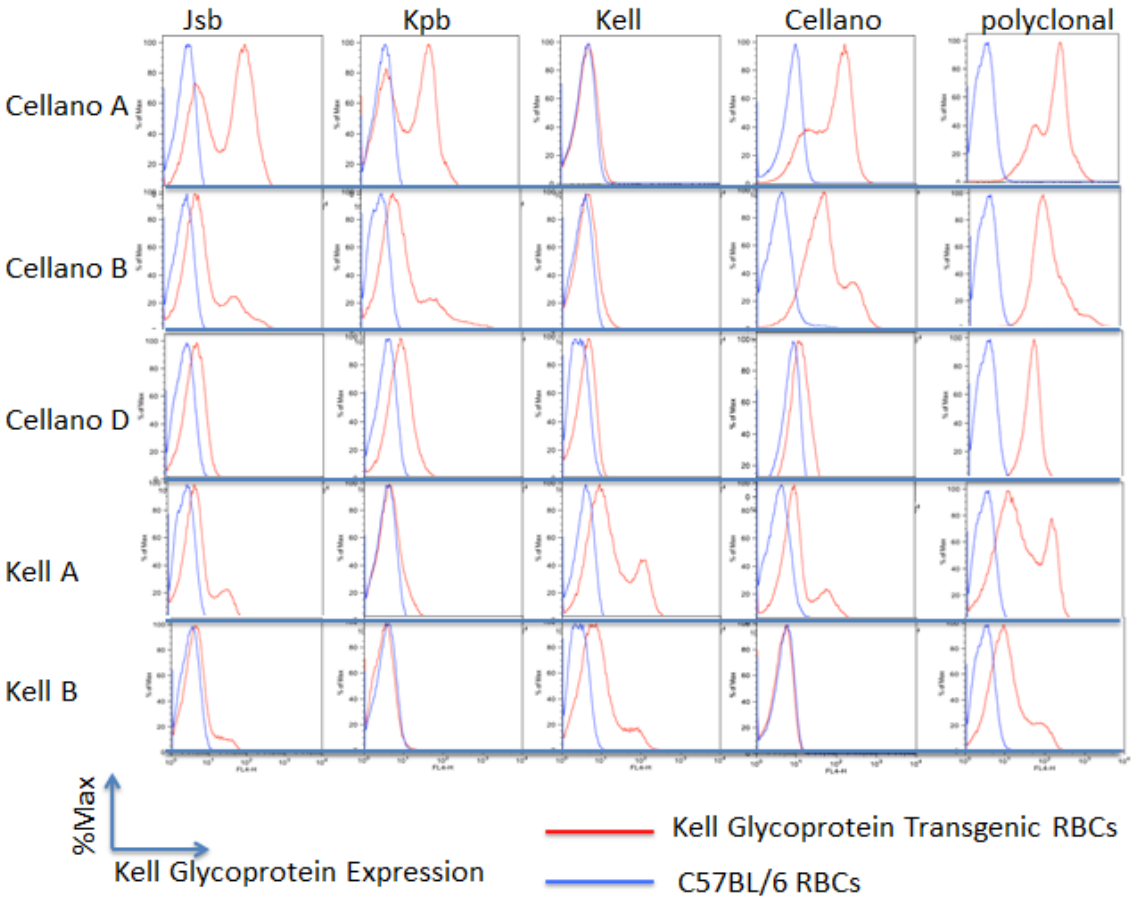


Figure 1: Characterization of Kell Glycoprotein Transgenic Mice RBCs

Using antibodies against Js^b, Kp^b, Kell, Cellano epitopes and a polyclonal anti-sera against the Kell glycoprotein (from left to right), the Kell glycoprotein transgenic RBCs (Cellano A, B, D, Kell A, and B from top to bottom)were characterized. The level of expression is demonstrated above in terms of histograms representing the percent of maximum cells. Red histograms represent the transgenic RBCs in comparison to blue histograms representing B6 RBCs stained with the same antibodies. Secondary antibodies for anti-Js^b, anti-Kp^b, anti-Kell and the polyclonal antisera were anti-Igs-APC. The secondary antibody for anti-Cellano was anti-IgM-APC. 100,000 RBC events were collected based on FSC and SSC.

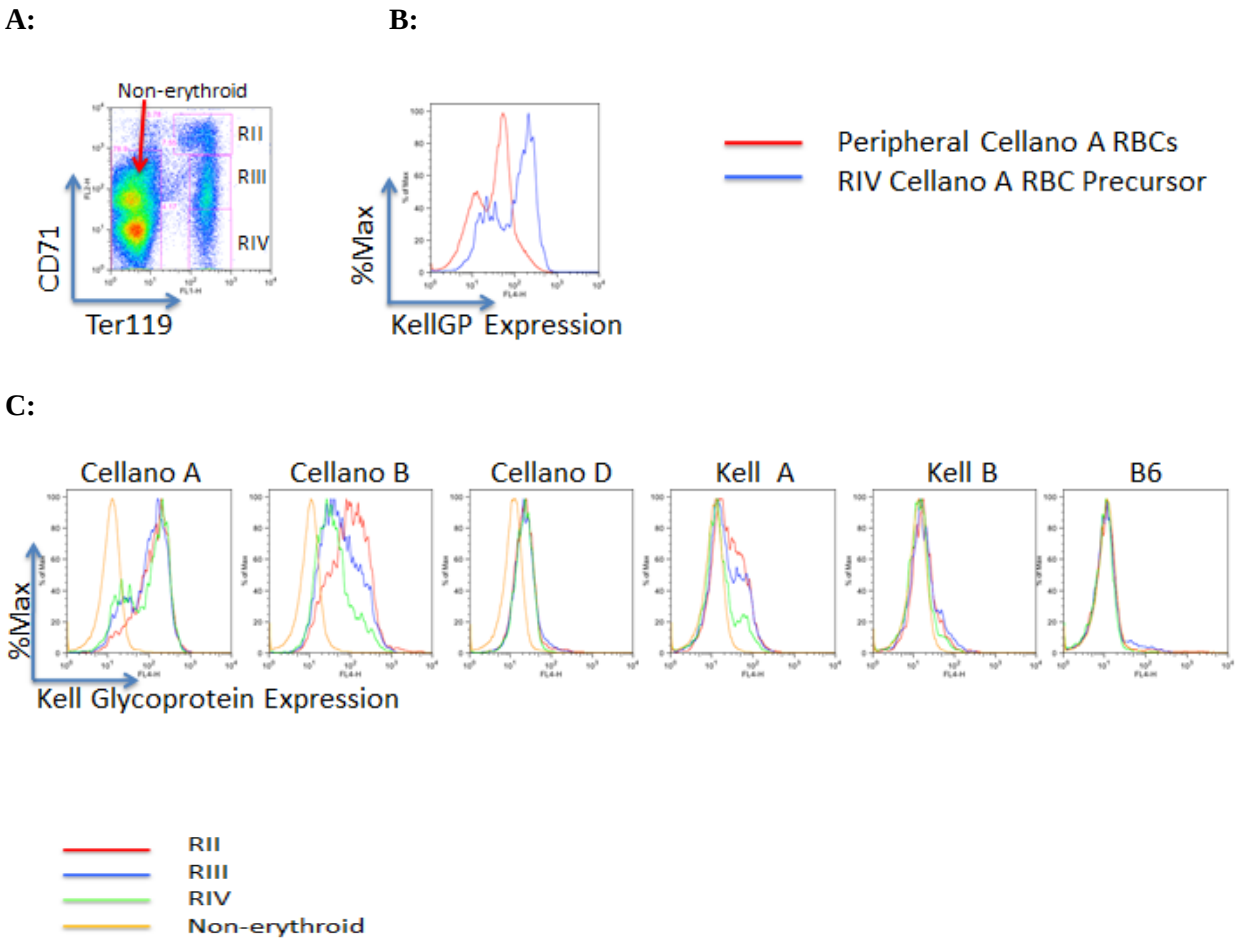
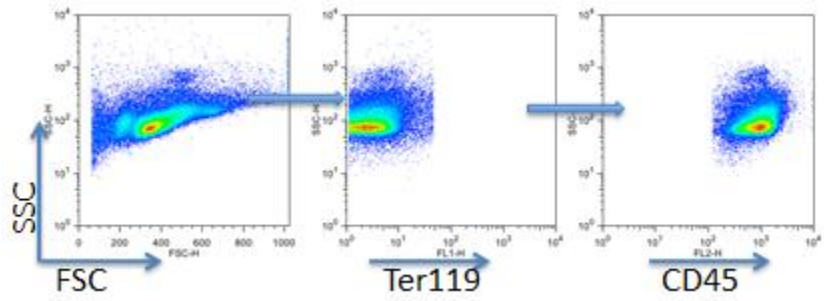


Figure 2: Characterization of Kell Glycoprotein Expression on RBC Precursors

The gating strategy is shown in (A) for analysis of RBC precursors in the BM of the transgenic Kell glycoprotein mice. BM cells were stained with anti-CD71 and anti-Ter119 in order to designate cells as RBC precursors and cells of non-erythroid lineage. Ter119+ $CD71^{hi}$ cells are termed "RII" (the second stage in erythropoiesis). Ter119+ $CD71^{intermediate}$ cells are termed "RIII" (the third stage in erythropoiesis). Ter119+ $CD71^{low}$ cells are termed "RIV" (the fourth stage of erythropoiesis, the stage prior to entrance in the periphery). Cells negative for Ter119 are not cells of the erythroid lineage and are termed "non-erythroid". The cells in each of these groups are then analyzed as shown in (C) for Kell glycoprotein expression by staining with polyclonal anti-sera against the Kell glycoprotein. Peripheral Cellano A RBCs (shown in red) are overlaid with "RIV" Cellano A RBC

precursors (shown in blue) in (B). RII (red), RIII (blue), RIV (green), and non-erythroid (orange) cells are overlaid from each of the transgenic mice and B6 controls to assess differences in level of expression during development. 100,000 BM events were collected for BM cells. 100,000 RBC events were collected for peripheral RBCs.

A:



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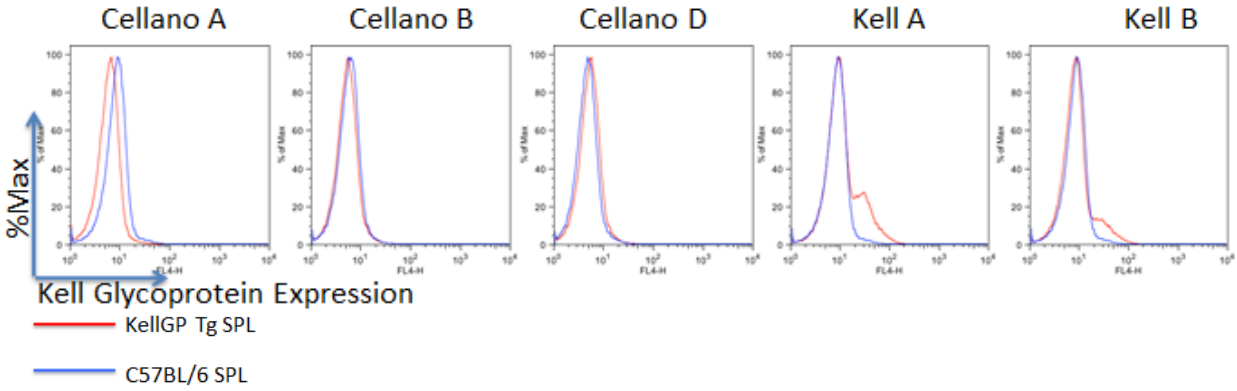


C:

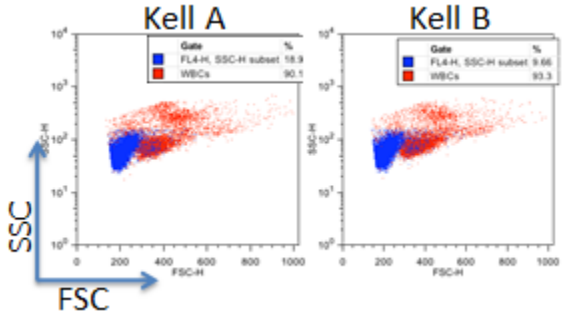
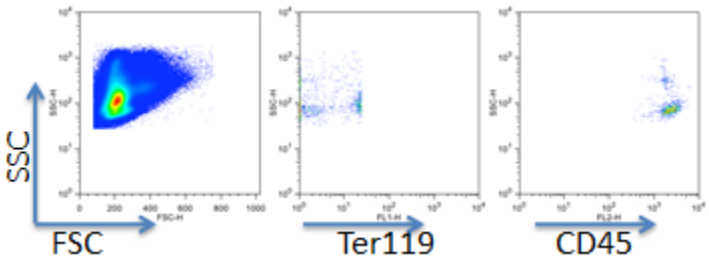


Figure 3: Characterization of Kell glycoprotein expression on splenocytes

The gating strategy is shown in (A). Splenocytes from the transgenic mice and B6 controls were gated on Ter119- events. The Ter119- events were then selected for CD45+ events. CD45+ events were then assessed in (B) for Kell glycoprotein expression. The red histograms represent the transgenic Kell glycoprotein splenocytes (KellGP Tg SPL) and the blue histogram represents B6 splenocytes (C57BL/6 SPL) controls. Using a back gating strategy, the Kell glycoprotein positive

cells (above B6 background) observed from the Kell A and Kell B splenocytes were analyzed via FSC and SSC to elucidate if they were a specific leukocyte population. The blue dots represent the Kell glycoprotein positive cells and the red dots represent the remaining Kell glycoprotein negative splenocytes. 100,000 CD45+ Ter119- cells were collected.

A:



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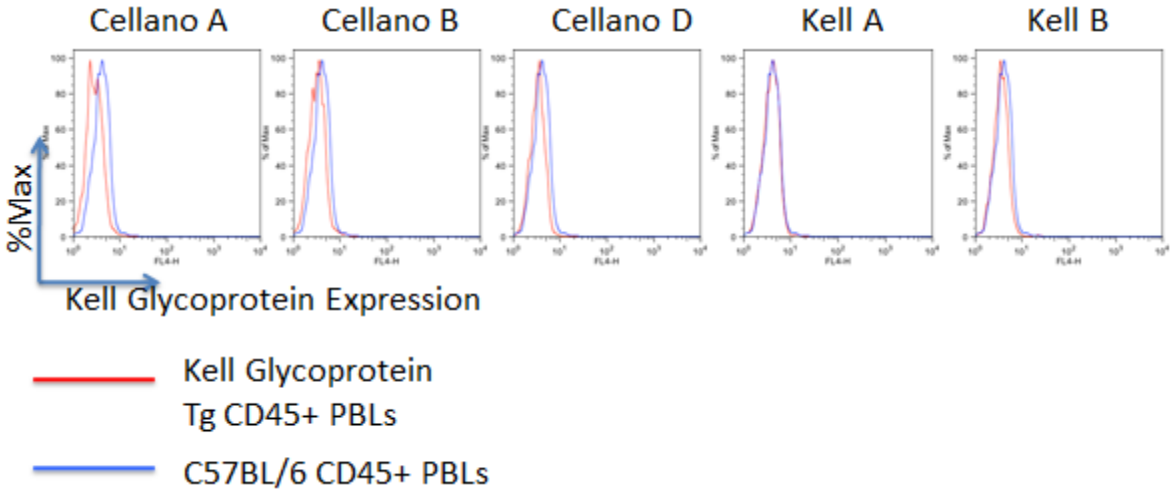
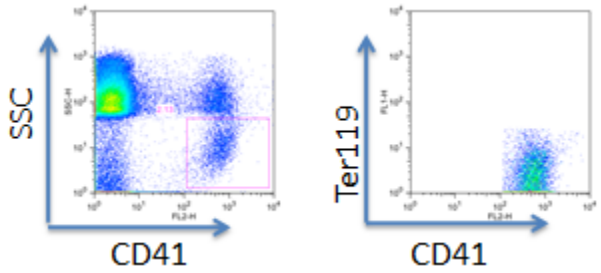


Figure 4: Characterization of Kell Glycoprotein Transgenic Peripheral Blood Leukocytes

Similarly to figure 3, the gating strategy is shown in (A). Peripheral blood leukocytes (PBLs) cannot be distinguished from red blood cells by FSC and SSC parameters. Therefore, we gated on Ter119- cells and then on CD45+ cells. These cells were assessed for Kell glycoprotein expression in (B). Kell glycoprotein transgenic (Tg) PBLs are shown in red and C57BL/6 controls are shown in blue. 1,000 CD45+ Ter119- events were collected from the peripheral blood.

A:



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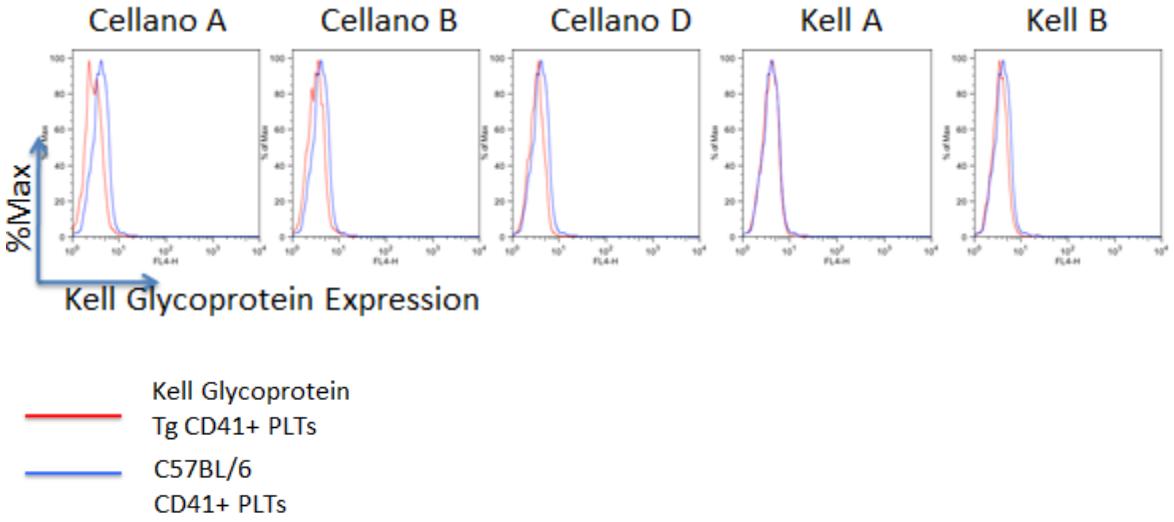
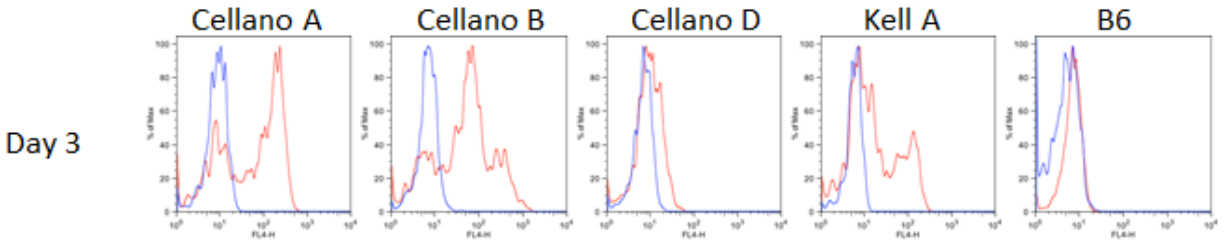


Figure 5: Characterization of Kell Glycoprotein Expression on Platelets

The gating strategy is shown in (A). CD41+ SSC^{low} events are gated on to isolate platelets from RBC events. These events were then selected on Ter119- CD41+ events. Platelets from transgenic mice are the red histograms (Kell glycoprotein Tg CD41+ PLTs) and the blue histograms are platelets from B6 mice.

A:



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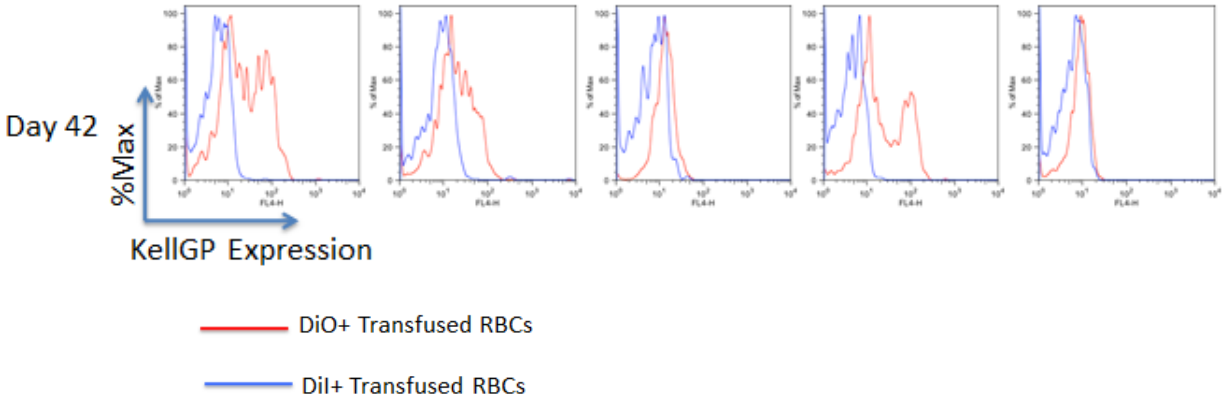


Figure 6: Stability of Kell Glycoprotein Post-Transfusion in MuMT Mice

MuMT mice were transfused with DiO-labeled and DiI-labeled RBCs. The recipient mice were bled at day 3 (A) and day 42 (B). The DiO+ and DiI+ cells from the recipient mice were stained for Kell glycoprotein expression. The histograms in red (from left to right) are DiO+ Cellano A, Cellano B, Cellano D, Kell A, and B6 RBCs in MuMT mice. The histograms in blue are DiI+ B6 RBCs from MuMT mice.

A:

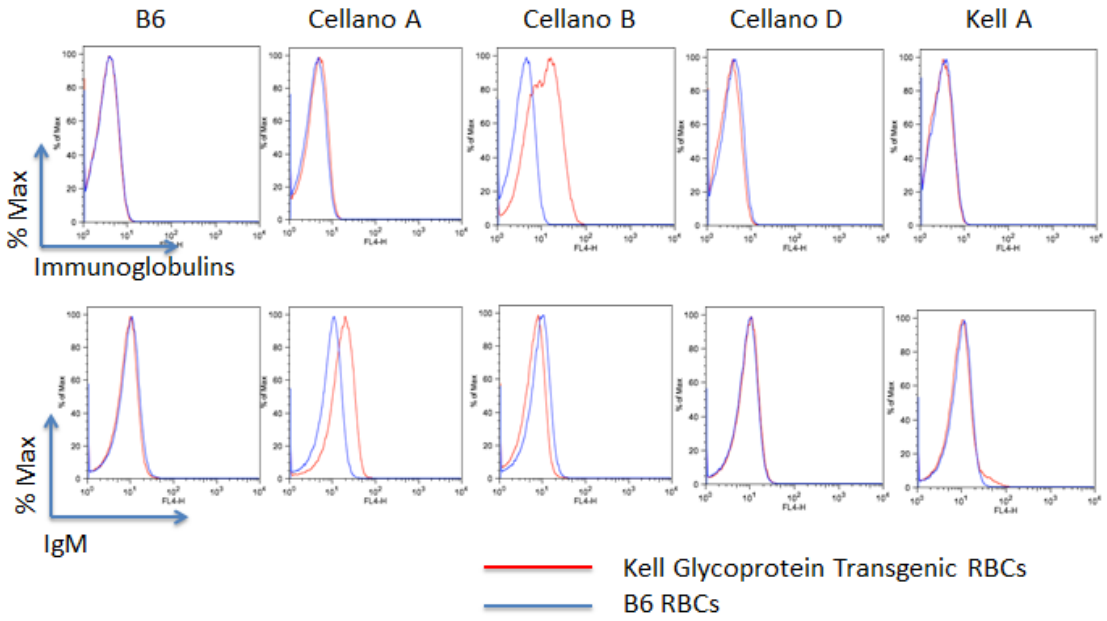


Figure 7: Serum Analysis from B6 Mice Transfused One Time with Kell Glycoprotein Transgenic RBCs or B6 RBCs

The top panel is cross-match analysis histograms of serum from B6 mice transfused with RBCs from B6,Cellano A, Cellano B, Cellano D, and Kell A (left to right)using an anti-immunoglobulin secondary. The bottom panel is a cross-match analysis of serum from B6 mice transfused with RBCs from B6, Cellano A, Cellano B, Cellano D or Kell A(left to right) using an anti-IgM secondary. The blue histograms are B6 RBC targets. The red histograms are serum cross-matched to transgenic RBCs. (Mice transfused with Cellano A, B and D were cross-matched against Cellano A target RBCs; Mice transfused with Kell A were cross-matched against Kell A target RBCs.)The Cellano A and Kell A figures in the bottom panel are an example of detectable IgM. However, some mice demonstrated this response and some had no detectable response.

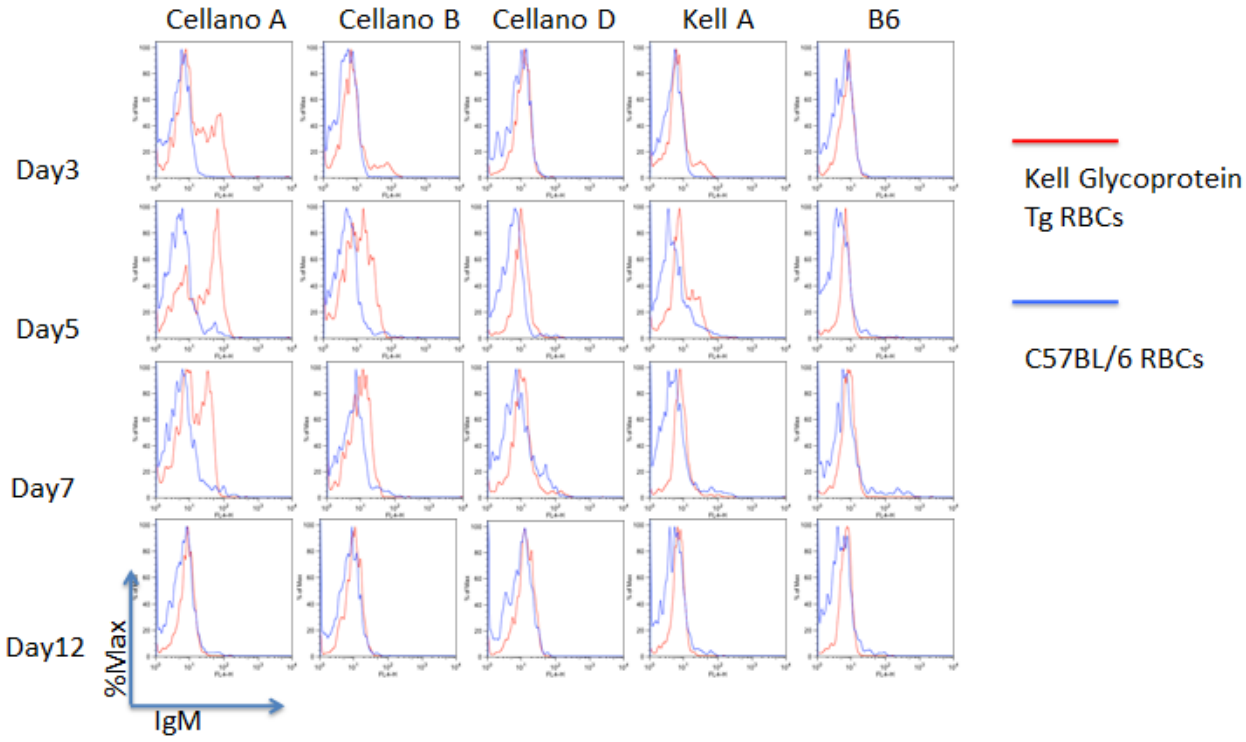


Figure 8: Antibody Preferential Binds to Transgenic RBCs versus B6 RBCs Post- Transfusion in B6 Mice

DATs are shown from DiO+ RBCs (red histograms; Cellano A, B, D, Kell A, and B6 from left to right) taken from B6 recipients at days 3, 5, 7, and 12. Shown in blue are histograms from DiI+ B6 RBC controls post-transfusion into the same B6 recipients.

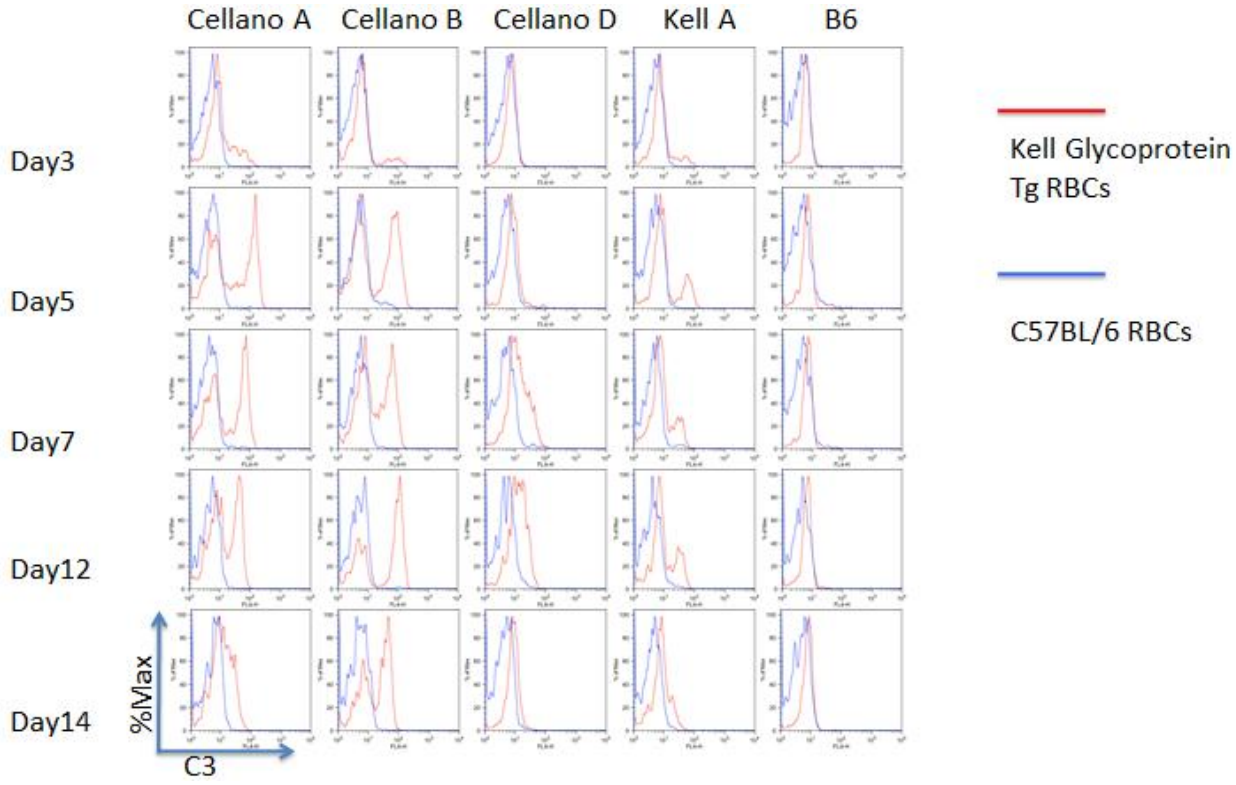


Figure 9: Complement Preferentially Binds to Transgenic RBCs versus B6 RBCs Post-Transfusion in B6 Mice

DATs are shown for C3 binding DiO+ RBCs (shown in the red histograms; Cellano A, B, D, Kell A, B6 from left to right) at days 3, 5, 7, 12, and 14 post-transfusion into B6 recipients. Blue histograms are DiI+ B6 RBCs at the same time points post-transfusion into the same B6 recipients.

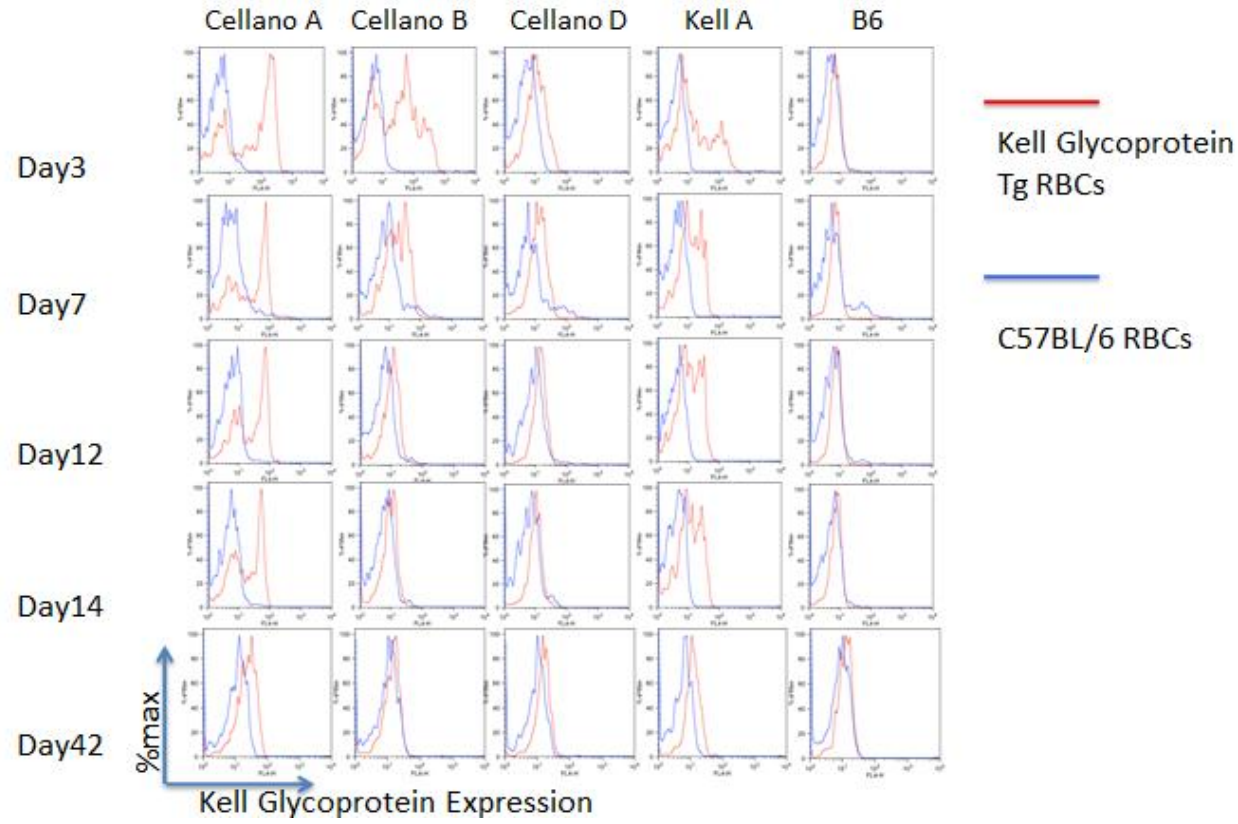
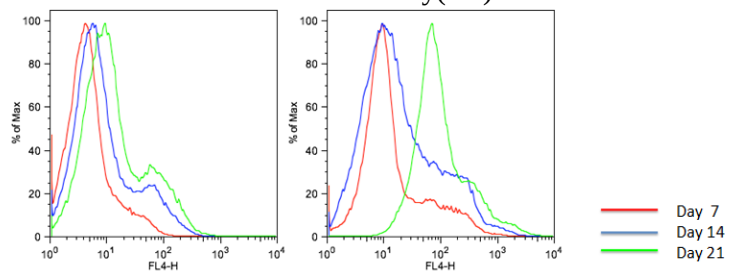


Figure 10: Kell Glycoprotein Expression is Reduced Post-Transfusion into B6 Mice

Expression of the transgenic Kell glycoprotein was assessed using an anti-Cellano antibody for Cellano A, B or D RBCs at days 3, 7, 12, 14 and 42 post-transfusion into B6 mice. Expression of the transgenic Kell glycoprotein was assessed using an anti-Kell antibody for Kell A RBCs at days 3, 7, 12, 14, and 42 post-transfusion into B6 recipients. The red histograms represent DiO+ RBCs (Cellano A, B, D, Kell A, and B6 RBCs from left to right) and the blue histograms represent DiI+ B6 RBCs.

A: PBS Treated Poly(I:C) Treated



B:

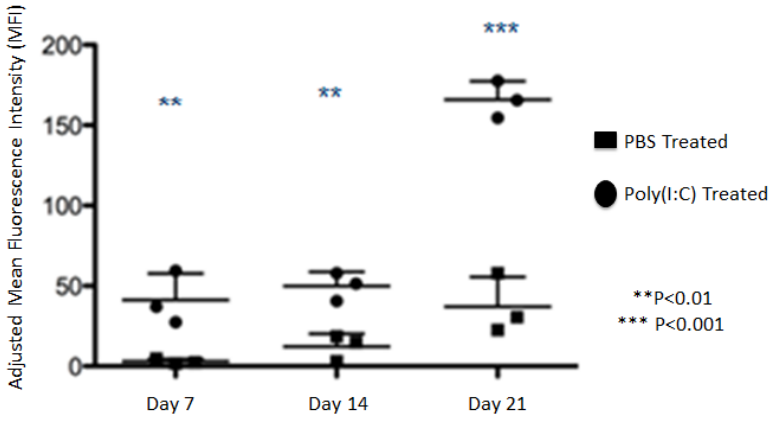


Figure 11: Poly(I:C) vs. PBS Treated Mice Antibody Responses to Cellano B Transfusions

A: Mice were injected i.p. with either PBS (left) or poly(I:C) (right) 3x and transfused 4 hours later for 3 consecutive weeks. Serum was taken on days 7, 14, and 21 post- transfusion. Serum was then cross-matched using transgenic RBCs or B6 controls (not shown). The red histograms are results from serum taken at day 7 post-transfusion, blue from day 14, and green from day 21.

B: The graph shows adjusted mean fluorescence intensity (MFI) calculated from a representative experiment comparing serum from mice treated with poly(I:C) or PBS prior to three weekly transfusions. Serum was analyzed at a 1:10 dilution. The squares are from PBS treated mice and the circles are poly(I:C) treated mice. The P values between the two groups (PBS versus poly(I:C)) are shown.

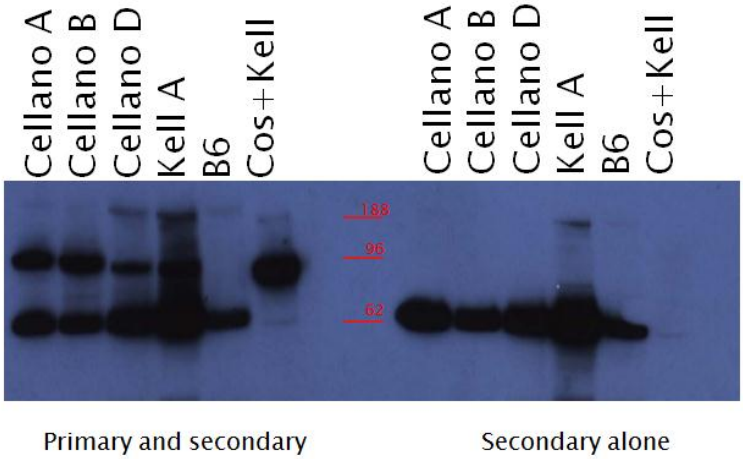


Figure 12: Detection of Kell Glycoprotein via Western Blot under Reducing Conditions

Membrane preparations from transgenic RBCs and B6 RBCs were made in RIPA Buffer. COS-1 cells were transfected using FuGene Transfection reagent to express the Kell glycoprotein. Membrane preparations were made from the COS-1 cells expressing the Kell glycoprotein (Cos+Kell) as a positive control. Shown above is the western blot with the 6 left-most lanes containing: Cellano A, Cellano B, Cellano D, Kell A, B6 and Cos+Kell membrane preparations (left to right) that were incubated with primary antibody (anti-Kell glycoprotein IgG) and secondary antibody (peroxidase-conjugated goat anti-mouse IgG). The red markings indicate the molecular weight (kDa). The six right-most lanes contain membrane preparations Cellano A, Cellano B, Cellano D, Kell A, B6 and Cos+Kell (left to right) that were incubated with secondary antibody only. This western blot was prepared under reducing conditions.

References

1.Red Gold: The epic story of blood. Available at www.pbs.org/wnet/redgold. (Accessed May 17, 2011).

2.Landsteiner, K., and Wiener, A.S. (1941). *Studies on an Agglutinogen (Rh) in Human Blood Reacting with an Anti-Rhesus Sera and with Human Isoantibodies.* Journal of Experimental Medicine. 74(4): 309-320.

3.Daniels, G. (2009) *The molecular genetics of blood group polymorphism.* Human Genetics. 126:729-742.

4.The Serious Hazards of Transfusion (SHOT) Steering Group (2009). *Serious Hazards of Transfusion, Annual Report Summary 2009.* Manchester: SHOT Office. Manchester Blood Centre. Available from http://www.shotuk.org/wp-content/uploads/2010/06/SHOT-2009-Summary.pdf. (Accessed May 17, 2011).

5.Hillyer, C., Shaz, B., Zimring, J., and Abshire, T.: *Transfusion Medicine and Hemostasis: Clinical and Laboratory Aspects.* Burlington, MA: Elsevier; 2009.

6.Hillyer, C.: *Blood Banking and Transfusion Medicine: Basic principles and practice.* Ed 2. Philadelphia: Churchill Livingstone/ Elsevier; 2007.

7.Zimring, J., Hair, G.A., Chadwick,T.E., Deshpande, S.S., Anderson, K.M., Hillyer, C.D., and Roback, J.D. (2005) *Nonhemolytic antibody-induced loss of erythrocyte surface antigen.* Transfusion Medicine. 106:1105-1112.

8.Williams, L.M., Poole, J., Redman, C., Clark, N., Liew, Y.W., Russo, D.C., Lee, S., Reid, M.E., and Black, A.J.(1993) *Transient loss of proteins carrying Kell and Lutheran red cell antigens during consecutive relapses of autoimmune thrombocytopenia.* British Journal of Haematology. 87: 805-812.

9.Westhoff, C.M. and Reid, M.E. (2004) *Review: the Kell, Duffy, and Kidd blood group systems.* Immunohematology. 20:37-49.

10.Hendrickson, J.E., Roback, J.D., Hillyer, C.D., Easley, K.A., and Zimring,J.C.(2008) *Discrete Toll-like receptor agonists have differential effects on alloimmunization to transfused red blood cells.* Transfusion. 48: 1869-1877.

11.Hendrickson, J.E., Chadwick, T.E., Roback, J.D., Hillyer, C.D., and Zimring, J.C.(2007) *Inflammation enhances consumption and presentation of transfused RBC antigens by dendritic cells.* Blood. 110: 2736-2743.

12.Hendrickson, J.E., Desmarets, M., Deshpande, S.S., Chadwick, T.E., Hillyer, C.D., Roback, J.D., and Zimring, J.C. (2006) *Recipient inflammation affects the frequency and magnitude of immunization to transfused red blood cells.* Transfusion. 46: 1526-1536.