Identification of multiple genetic loci associated with red blood cell alloimmunization in mice

Humoral immunization to red blood cell (RBC) alloantigens can represent a barrier to ongoing transfusion therapy. Humans have a range of tendencies to become alloimmunized. On the extremes, some patients become immunized to multiple alloantigens after a single transfusion whereas others make no detectable alloantibodies after hundreds of transfusions.[1] Although an oversimplification of a complex trait, humans have been described as being "responders" *versus* "non-responders" based upon their tendency to become alloimmunized.[1] Alloimmunization against multiple antigens can complicate obtaining sufficient compatible units, leading to insufficient or delayed treatment, and mortality in severe cases. In addition to transfusion, engineered RBC are now being developed to induce immunity (e.g., vaccines[2] and tumor immunotherapy[3]), or to suppress immunity (e.g., treatment of autoimmune disease).[4] Thus, understanding factors that regulate immune responses to RBC is important for multiple therapeutic approaches. Numerous murine studies have shown that transfused RBC do not induce a significant alloantibody response unless recipients are first inflamed or transfused RBC are damaged. These findings have translated into humans and constituted considerable progress in understanding environmental factors that regulate responder/non-responder status. However, recipient genetics likely also play a role. Several human loci of interest have been identified,[5-7] but achieving sufficient statistical power has been a challenge. Application of murine models to exploration of recipient genetics has not been carried out, since RBC are only weakly immunogenic in the reported mouse models. Importantly, existing murine studies have almost exclusively used mice on a C57BL/6 (B6) background as recipients. We hypothesized that B6 mice represent a model of "non-responders" and that the tendency to become alloimmunized after transfusion would vary across genetically distinct inbred strains. Herein, we demonstrate a continuum of humoral RBC alloimmunization tendency in different inbred strains of mice and identify six genetic loci that are associated with RBC alloimmunization through a genome-wide association study (GWAS).

Thirteen different strains of mice, chosen to represent different phylogenetic arms of inbred mice, were each transfused with RBC expressing either a model alloantigen (HOD)[8] or the human K2 alloantigen at low copy number (KEL-K2$_{Lo}$).[9] Blood was collected, transfused, and IgG responses were measured as previously described.[9] HOD RBC have repeatedly been shown to induce little to no IgG in otherwise untreated B6 mice (i.e., in the absence of recipient inflammation). KEL-K2$_{Lo}$ RBC have been reported to not induce IgG in B6 mice even in the presence of inflammation, but rather lead to tolerance.[9] Consistent with published findings, neither HOD nor KEL-K2$_{Lo}$ RBC induced considerable IgG in B6 recipients (Figure 1). In contrast, there was wide variability of responsiveness across other strains, with similar (but not identical) patterns between HOD and KEL-K2$_{Lo}$ RBC.

HLA differences in humans can regulate RBC alloimmunization to specific antigens based upon the differential ability of MHCII to present peptides from RBC alloantigens. However, differences in RBC alloimmunization across murine strains were not simply due to variant MHCII, since 129 mice consistently responded more strongly than B6 mice, and both have the same MHC haplotype (H-2^b). Also, DBA2 mice have significantly higher average responses than seen in B6 mice that are congenic for the DBA2 H-2^d MHC haplotype and are homozygous for H-2^d on a B6 background (B6.H2^d). Finally, all tested strains have the same amino acid sequence of murine orthologues of lysozyme (contained in HOD) and KEL, ruling out orthology to alloantigen as an independent variable.

Alloantibody responses and high-resolution single nucleotide polymorphisms (SNP) genotyping profiles were generated on 156 F2 mice from a 129S1xB6 F2 cross. F2 mice had a range of responses between the means of parental strains (Figure 2A). Quantitative trait locus (QTL) analysis (reviewed in [10]) was performed on the F2 mice using alloantibody levels as a trait. At least six different QTL were identified (false discovery rate [FDR] <0.01), on chromosomes 1, 7, 9, 11, 12, and the X chromosome, respectively (Figure 2B). An additional QTL on chromosome 17 approached the cutoff for significance. The QTL on chromosome 1 appeared to indicate two, and potentially three different loci.

As a control for the QTL process, leukocytes from each mouse were also tested for expression of NK1.1 by flow cytometry. NK1.1 is expressed by B6 but not 129 mice and is encoded by the *Klrb1c* gene on chromosome 6 at position 129755448-128765604. QTL analysis mapped the *NK1.1* gene product to the correct position on chromosome 6 with extreme statistical significance ($P < 10^{-22}$) (Figure 2C), validating the QTL approach as well as the F2 cohort.

Together, the findings reported herein demonstrate genetic variability across inbred strains of mice regarding the tendency to mount a humoral immune response to a foreign antigen on transfused RBC. This observation demonstrates that the common narrative that RBC are intrinsically non-immunogenic in mice is an error born from studying only a single inbred recipient strain (i.e., B6). The current data
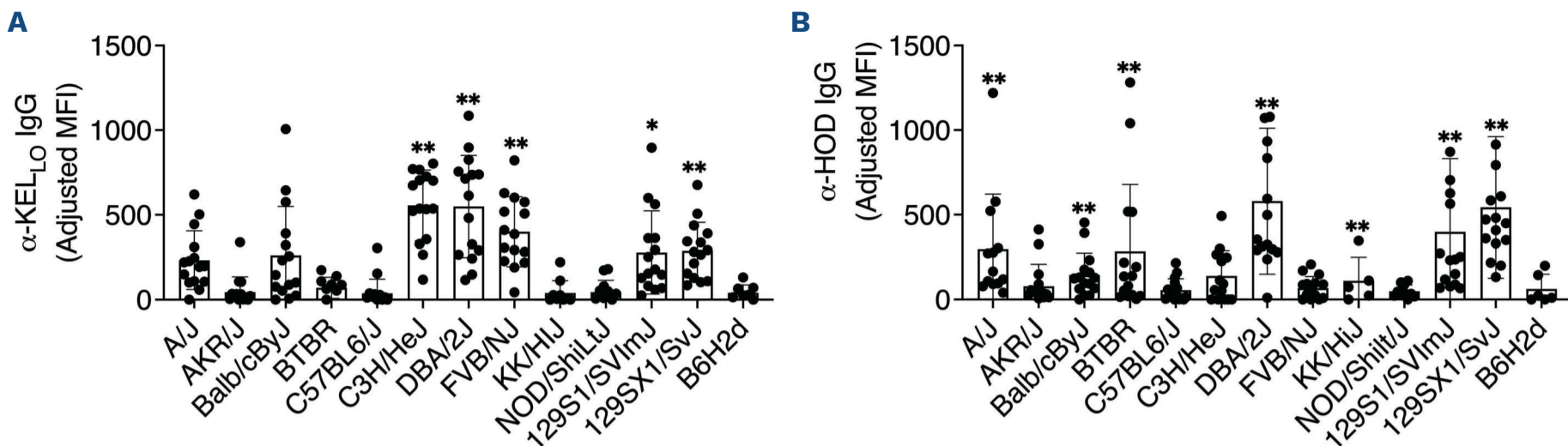
Figure 1. **Genetically distinct mouse strains have different tendencies towards red blood cell alloimmunization.** Each of the indicated strains were transfused with HOD (A) or KEL-K2$_{Lo}$ (B) red blood cells (RBC) and IgG responses were tested in serum at 21 days after transfusion. Serum was incubated with alloantigen expressing RBC followed by a fluorescently labeled secondary antibody and median fluorescence intensity (MFI) was determined by flow cytometry. As both alloantigen transgenic mice are on a B6 background, serum was also incubated with B6 RBC and the MFI subtracted from values of alloantigen expressing target cells. Major histocompatibility complex (MHC) haplotypes of the mice used are [A/J (H-2^a), AKR/J (H-2^k), Balb/cByJ(H-2^d), BTBR(H-2^b), C57BL6/J(H-2^b), C3H/HeJ(H-2^k), DBA/2J(H-2^d), FVB/NJ(H-2^q), KK/HiJ(H-2^b), NOD/ShiLtJ(H-2^{g7}), 129S1/SVImJ(H-2^b), 129SX1/SvJ(H-2^b)]. No antibodies to background antigens on B6 RBC were detected and the MFI of B6 RBC was subtracted from the MFI of target RBC expressing the indicated alloantigen. Negative values for IgG were determined to be non-biologically relevant and were thus set to a value of zero. The combined results of 3 different experiments are shown, with samples sizes ranging from 13-15 mice. Statistical significance is defined as a Sidak-Bonferroni adjusted $P<0.05$ in pairwise comparisons estimated from a two-part model (logit & log-linear links) adjusted for experiment – each strain is compared to B6.H2d. Statistical significance is indicated by (*P=<0.05) and (**$P<0.001$). No experimental results that were obtained with this approach that were excluded from this figure. HOD and KEL-K2$_{Lo}$ mice were bred in the University of Virginia vivarium, all recipient mice were purchased from The Jaxson Laboratory (Bar Harbor, ME) and all procedures were carried out in compliance with approved IACUC protocols.

demonstrate that, similar to humans, recipient genetics affect the tendency to become alloimmunized to RBC transfusion in mice.

Interestingly, one of the few papers that reported transfused RBC as being immunogenic, was an early report by Campbell-Lee *et al.*, which used CBA mice as recipients.[11] Although CBA mice are not in the current panel, they are closely related to C3H animals – the significance of these early findings regarding recipient genetics has likely been overlooked. Similarly, papers in RBC engineering that showed strong antibody responses used BALB/c or B6D2F1 mice.[2,12,13] Thus, careful attention to recipient strain may help to resolve apparent contradictions in the literature, as well as leading to new understanding of the genetic regulation of RBC alloimmunization. This will be a fundamental consideration in translating RBC-based immunotherapies into humans; murine studies should pay careful attention to the recipient strain being studied and arguably test a range of recipient strains to understand the landscape of potential responses.

Genetic variation in immune responses to various antigens is not itself new. However, it is well understood that genetic determinants of immune response differ with regards to the nature of the antigen.[14] Genetic variation of different murine strains in magnitude of antibody responses to sheep red blood cells (SRBC) have previously been reported; however, QTL were not identified.[14] More importantly, SRBC are a strong xenoantigen which is fundamentally different than alloimmunization within the same species. To the best of our knowledge, the current study is the first analysis of immunogenetics of response to RBC alloantigens in mice.

Another potentially serious issue raised by the current findings is that the vast majority of knockout mice have been made on a 129 background and then backcrossed to B6. As we (and others) have documented, this approach is susceptible to mistaking the effects of traits inherited from 129 mice for effects of the knocked out gene.[15] Even with many generations of backcrossing, genes flanking the knockout gene typically maintain genetics from the donor strain, in this case 129. Because 129 mice tend to respond to RBC antigens, any data from using knockout mice generated with 129 ES cells should consider the proximity of the knocked out gene to the QTL defined herein.

Identification of the precise genetic elements that regulate alloimmunization will require additional refinement of the QTL and experimentation through genetic modification. Although highly significant, the QTL generated by this approach are broad (i.e., 880 genes in QTL11). As such, more precise mapping and/or congenesis will be required to identify precise genetic elements. Potential translation into humans will require focused analysis of human genomes associated with alloimmunization; however, it is worth noting that QTL 7 contains ARAP1/STARD10, which was identified as associating with alloimmunization in human patients with sickle cell disease.[5] Variation in human HLA correlates with alloimmunization to some RBC alloantigens

and the QTL approaching statistical significance on chromosme 17 may represent variation in mouse MHC. However, as above, the MHC seems neither necessary nor sufficient to regulate RBC alloimmunization as mice with the same H-2 haplotype have significantly different average immune responses to RBC transfusion. Likewise, donor RBC having a different MHC than recipient mice does not seem determinative, as there is a wide range of average responses for A/J, AKR/J, Balb/cByJ, C3H/HeJ, DBA/2J, FVB/NJ, and

NOD/ShiLTJ strains even though each see the H-2^b on HOD and KEL-K2$_{Lo}$ RBC as foreign. Importantly, the QTL need not indicate immunoregulatory genes, as they could indicate variant amino acids in RBC proteins that are processed and presented in MHCII between donor and recipient strains. Due to linked recognition, such epitopes could come from proteins other than the transgenic alloantigens being studied.

In addition to generating new genetic regions of interest in
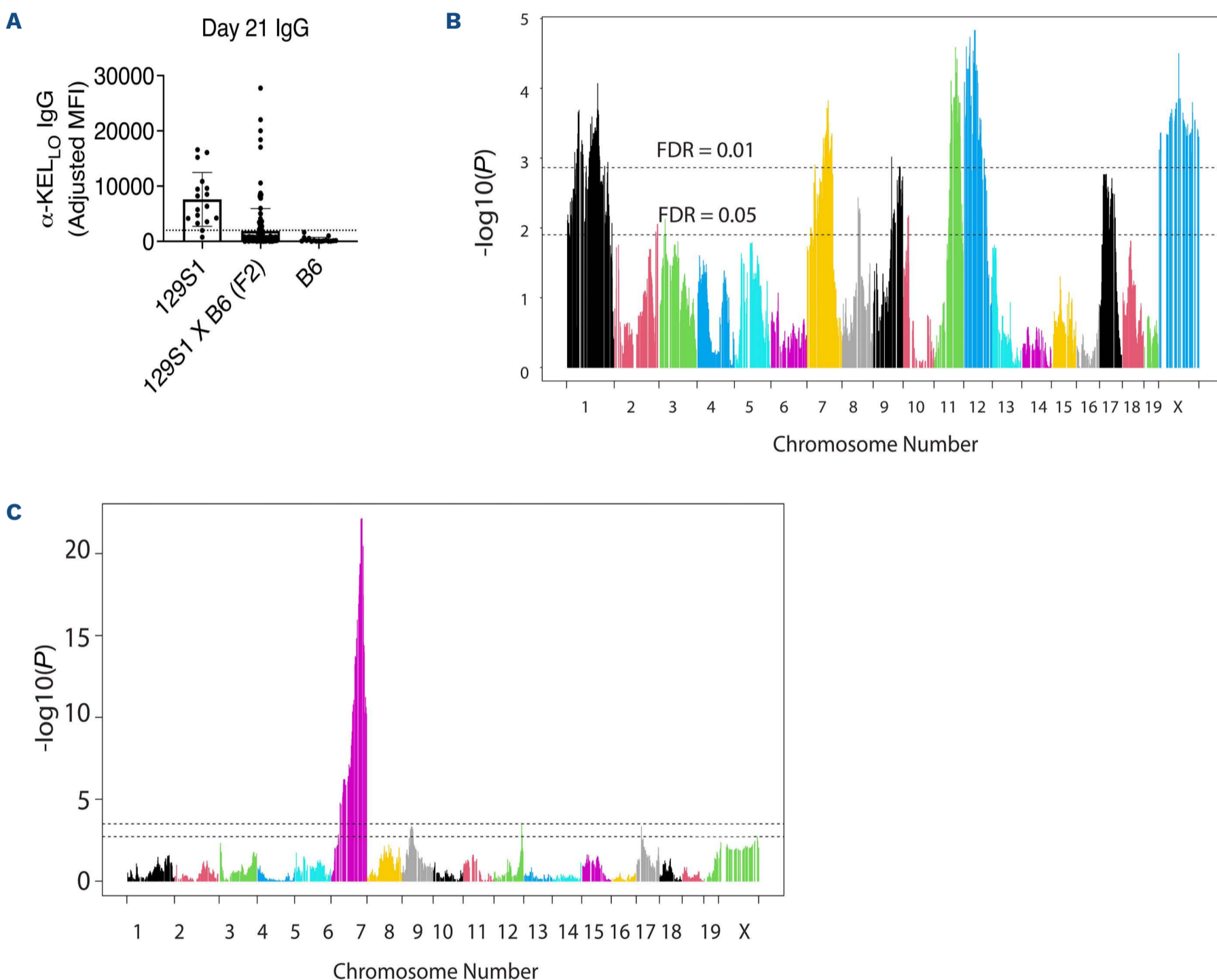
Figure 2. Identification of multiple genetic loci that associate with red blood cell alloimmunization. 156 F2 mice were each transfused with KEL-K2$_{Lo}$ red blood cell (RBC) and alloantibodies were measured at 21 days post transfusion (A). For each animal, expression of NK1.1 was determined on splenocytes by flow cytometry (data not shown). Quantitative trait locus (QTL) analysis was carried out using alloimmunization to RBC (B) or NK1.1 expression (C) as the trait. Starting with a total of 11,125 single nucleotide polymorphisms (SNP), 3,220 differed between the B6 and 129 strains, with 3,118 remaining after filtering for informative markers. Genome-wide association study (GWAS) analysis used a linear model with a transformation y =log$_e$(Pheno1 + 60) applied prior to data analysis and fitted using the lm function in R. From each SNP model fitted, the P value form the F-test was extracted, and adjusted P values (false discovery rate [FDR]) were calculated using the P.adjust function in R. Manhattan plots were constructed with thresholds taken as FDR =0.05 (suggestive) and FDR =0.01 (significant). Back-transformed means for each SNP genotype (AA, AB and BB) along with their standard errors were calculated using the emmeans package in R. For chromosomes where at least one SNP with FDR <0.05 was found, the most significant SNP in the chromosome was identified. In order to assess if any of the other 'significant SNP' in the chromosome had any effect in addition to the most significant one, a linear model was fitted with the most significant SNP as well as the SNP to be tested. No formal adjustment for multiple tests was undertaken for this analysis.

alloimmunization (and immunology in general), the data contained herein widen our gaze of the immunogenic properties of RBC, indicating that they are neither intrinsically immunogenic nor non-immunogenic, and their effects are contextual with regards to recipient genetics. Understanding the genetics of RBC alloimmunization, and how it translates into humans, will be essential both to predicting and controlling RBC alloimmunization during transfusion therapy, as well as precision medicine in the use of modified RBC as a cellular therapy with regards to recipient genetics.

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Disclosures
JCZ is a co-founder and CSO of Svalinn Therapeutics, neither of which were involved with the current studies. All other authors have no conflicts of interest to disclose.

Contributions
AJ and AMH performed the research. JCZ, CJL, and KEH conceived the work and JCZ supervised the work. HLH processed genomics data. SJR and MS carried out statistical analysis, and PCT performed the QTL analysis. All authors were involved in data interpretation. JCZ wrote the manuscript with editorial input from other authors.

Data-sharing statement
Genomics and alloimmunization data available upon request.

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