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# MHC II on Transfused Murine Blood is Not Required for Alloimmunization Against MHC I

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## Abstract

**Background and Objectives—**Transfusion of allogeneic platelet products can result in antibodies against donor MHC I antigens, leading to a refractory state to subsequent platelet transfusions. However, there is disagreement in the field regarding the molecular mechanisms of humoral alloimmunization. One hypothesis states that donor MHC II is a requirement for alloimmunization. However, other studies have suggested that donor MHC I is alone sufficient and MHC II is not required.

**Materials and Methods—**We utilized a mouse model of anti-MHC I alloimmunization to transfused blood, which employed donors with a complete deletion of all MHC II genes. BALB/c (H-$2^d$) recipients were transfused with blood from either C57BL/6 (H-$2^b$) or MHC II null donors on a C57BL/6 background. Anti-MHC I alloimmunization was monitored by indirect immunofluorescence.

**Results—**Recipients of either wild type or MHC II null blood produced equivalent humoral responses against donor MHC I antigens. However, there was variation in the relative amounts of IgG subclasses.

**Conclusion—**These data reject the hypothesis that donor MHC II expression is required for alloimmunization to MHC I antigens.

### Keywords

Transfusion; alloimmunization; MHC class II

## Introduction

The major histocompatibility complex (MHC) I is both highly polymorphic and expressed on a wide variety of hematopoietic cells. Thus, it is not surprising that transfusion frequently results in humoral alloimmunization in the form of anti-HLA antibodies. Leukoreduction of platelet products has substantially decreased HLA alloimmunization. In one study, the transfusion of allogeneic platelet products to acute myelogenous leukemia patients induced

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anti-HLA antibodies in approximately 45 percent of recipients, while leukoreduction decreases the incidence to 18 percent [1]. The most obvious therapeutic sequelae of anti-HLA antibodies is platelet refractoriness, which can result in difficulty or even the inability to treat thrombocytopenia by transfusion of platelets in patients immunized to multiple allotypes [1–3]. In addition, anti-HLA antibodies can promote chronic rejection of transplanted organs, including decreasing survival of transplanted lungs [4], hearts [5], and kidneys [6].

Significant investigation has been carried out regarding the cellular and molecular mechanisms involved in HLA alloimmunization. The substantial decrease in HLA alloimmunization by filter leukoreduction of platelets is not due to elimination of the offending antigen, as platelets themselves account for roughly 70% of the total MHC I molecules found in whole blood [7]. In the context of leukoreduction efficacy, it has been concluded that leukocytes are more immunogenic than platelets regarding MHC I antigens. Because MHC II is expressed on leukocytes, but generally absent from platelets, it has been hypothesized that MHC II expression is responsible for the increased immunogenicity of leukocytes.

The co-expression of MHC II along with MHC I on donor cells has been widely circulated as a requirement for alloimmunization and stated as fact in the platelet transfusion immunology literature [8–11]. However, there are conflicting data as to the validity of this conclusion. Kao et al. used a mouse model to demonstrate that induction of MHC I antibodies by transfusion of leukocytes was profoundly diminished after depletion of MHC II positive cells from the donor cells[11]. These data serve as the most definitive experimental basis for concluding that MHC II expression is responsible for the increased immunogenicity of leukocytes. However, Semple et al. have reported that recipient antigen presenting cells (APCs) can phagocytose, process, and present donor cell derived MHC I molecules on recipient MHC II. These APCs can then activate recipient $CD4^+$ T cells [12,13]. The capacity of the indirect pathway to induce immunity does not directly test a role for donor MHC II, but it does raise the question, if recipient APCs can present donor MHC I antigens on their own MHC II, then by what mechanisms would donor MHC II be required?

In aggregate, the separate observations of Kao and Semple appear mutually exclusive in the context of the normal paradigms of humoral alloimmunization; indeed, the data seem to necessitate the generation of a hypothesis by which donor MHC II is required through mechanisms other than activation of $CD4^+$ T cells. Alternatively, the reported data are not generalizable phenomena outside the confines of the experimental systems used to generate them. Kao et al. utilized highly purified and manipulated leukocyte populations that do not necessarily reflect the composition or status of transfused leukocyte populations. To address this issue we carried out alloimmunization experiments with MHC II null donors, using minimally manipulated blood products for transfusion.

Herein, we report that anti-MHC I alloantibody responses were of equal magnitude comparing transfusion of blood from wild type vs. MHC II null donors. This observation indicates that whatever is responsible for the increased immunogenicity of leukocytes regarding MHC I alloantigens, it is not co-expression of MHC II. Together, these data address an apparent paradox in the field by rejecting the current belief that donor MHC II is responsible for increased immunogenicity of donor leukocytes.

## Materials and Methods

### Mice

6 to 8 week old B6.129S2-H2$^{dlAb1\text{-}Ea}$/J (MHC II null, H-2$^b$) [14], C57BL/6 (H-2$^b$), BALB/c (H-2$^d$), and BALB.B (H-2$^b$) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). H2-K$^{b-/-}$D$^{b-/-}$ (K$^b$D$^b$ null, H-2$^b$) mice were generously provided by Dr. Aron Lukacher (Emory University, Atlanta, GA) [15]. All studies and procedures were carried out in accordance with Emory University's Institutional Animal Care and Use Committee guidelines.

### Blood preparation and transfusion

For each experiment, fresh blood from two donor mice was collected by retroorbital enucleation into 200μL of acid citrate dextrose solution and washed twice with Dulbecco's phosphate buffered saline (DPBS). Recipient animals were transfused with 100μL of washed whole blood resuspended to 500μL in DPBS by tail vein injection.

### Indirect antibody test and Ig subclass determination

Serum was collected at the indicated time points and frozen at −80°C until use. To quantify the anti-donor antibodies and their Ig subclass, sera were diluted as indicated and incubated with $1\times10^6$ splenic target cells for 30 minutes at 4°C. The cells were washed in FACs buffer (DPBS supplemented with 0.5% bovine serum albumin, 1mM EDTA and 50mM Hepes buffer, pH 7.2) and then incubated with FITC-anti-CD19, PE-anti-CD3, and either allophycocyanin-goat anti-mouse Ig, allophycocyanin-anti-$IgG_1$ (BD Pharmingen, San Jose, CA) or allophycocyanin-anti-IgM (Southern Biotech, Birmingham, AL) for 30 minutes at 4°C. Additional secondary antibodies included horseradish peroxidase (HRP) conjugated goat anti-mouse $IgG_{2a}$, $IgG_{2b}$, or $IgG_3$ (Bethyl Laboratories, Montgomery, TX) followed by Cy5 conjugated goat anti-HRP (Jackson ImmunoResearch, West Grove, PA). The IgG subtype specific reagents undergo extensive solid phase absorption so as to eliminate crossreactivity to common epitopes shared by other IgG subtypes. Determination of bound anti-donor antibodies utilized a $CD3^+$ $CD19^-$ parent gate to avoid interference from anti-Ig binding to B cells or Fc receptor binding. Samples were acquired by a FACScan flow cytometer (BD Pharmingen) and analyzed using FlowJo (Treestar, Ashland, OR).

### Statistical Analysis

Statistical significance was determined by two-way ANOVA with a Bonferroni post-test at an alpha level of 0.05. Analysis was performed using the GraphPad Prism Software Suite (GraphPad Software, Inc., La Jolla, CA).

## Results

### Absence of donor MHC II does not decrease the humoral response to allogeneic MHC I

Washed whole blood from wild type C57BL/6 or MHC II null donors (B6.129S2-H2$^{dlAb1\text{-}Ea}$/J) was transfused into BALB/c recipients. The B6.129S2-H2$^{dlAb1\text{-}Ea}$/J strain has a complete genetic deletion of the entire MHC II locus, resulting in the absence of MHC II. The phenotype of the MHC II null mice was confirmed by flow cytometry with antibodies to MHC II; no MHC II was present on blood cells from B6.129S2-H2$^{dlAb1\text{-}Ea}$/J mice (data not shown).

The anti-donor antibody response was quantified by indirect immunofluorescence against donor targets and syngeneic control targets (Figure 1A). The adjusted mean fluorescent intensity (MFI) reported was the fluorescence signal from experimental sera minus the

background signal from naïve BALB/c control sera. The difference in the anti-donor antibody response was not statistically significant between transfusion of wild type (average MFI of 415.1 ± 77.5 SEM) and MHC II null blood (average MFI of 514.7 ± 23.4 SEM) from day-21 post-transfusion sera (Figure 1B). Furthermore, titration of sera from day 21 showed essentially overlapping curves (Figure 1C).

A panel of splenocyte targets was used to analyze the specificity of the antibody response. Antibody binding was similar using either C57BL/6 or BALB.B targets, congenic for the C57BL/6 MHC haplotype H-$2^b$ on a BALB/c genetic background (Figure 2). Because the BALB.B targets are genetically identical to the recipient mice, with the exception of encoding the donor C57BL/6 MHC genes, these data indicate that most of antibodies recognized antigens encoded by the donor MHC. To further characterize the specificity, targets were used from C57BL/6 donors with a complete deletion of MHC I genes ($K^dD^b$ null mice [15]). The majority of the antibody binding was lost with MHC I null targets, indicating that the alloantibodies were predominantly specific for MHC I (Figure 2). However, low levels of fluorescence above that seen with syngeneic targets suggest some minor binding to elements outside of the MHC I locus, possibly minor antigenic variants between the donor and recipients. Together, these data indicate that recipient mice make quantitatively similar antibody responses specific for MHC I antigens regardless of whether the donor cells express MHC II.

### Qualitative analysis of alloimmunization to MHC II null and wild type whole blood

Kinetics and Ig subtypes were analyzed by testing serum from 3, 7, 14, and 21 days post-transfusion with a panel of secondary reagents. Consistent with the above data, total Ig and IgM responses were similar for recipients transfused with either wild type or MHC II null blood (Figure 3). However, analysis of IgG subtypes revealed that the relative levels of $IgG_1$ predominated for responses against wild type blood whereas $IgG_{2a}$, $IgG_{2b}$, and $IgG_3$ predominated for responses against blood from MHC II null donors (Figure 3).

## Discussion

Our data demonstrate that transfusion of blood from donor mice with a complete genetic deletion of MHC II stimulates an anti-MHC I alloantibody response of similar magnitude to transfusion of blood from wild type donors. While these findings do not assess the requirement for donor MHC II in all settings under which transfusion may be carried out, they do reject the hypothesis that donor MHC II expression is a general requirement for recipient anti-MHC I alloantibody formation. Our findings are in disagreement with similar studies previously reported by Kao et al. [11]. The exact reasons are unclear, but there were substantial methodological variations between the two studies. Kao et al. transfused highly purified and manipulated leukocytes; however, these cells were not clearly characterized as to their composition prior to transfusion and may have lacked significant immunogenic subsets [16–18]. Furthermore, the viability of the cells transfused was not reported. Purification and manipulation can lead to apoptosis of leukocytes, and apoptotic cells are known to be poorly immunogenic, and in some cases tolerogenic [19]. Kao et al. did define conditions under which MHC II null leukocyte transfusions are not immunogenic. However, the positive immunological responses in the current study reject the hypothesis that it is a general biological principle that donor MHC II is a requirement for transfusion induced alloimmunization to MHC I.

Although the current findings are in contradiction to the studies by Kao et al., our findings are consistent with the logical prediction of published data in reductionist systems that suggest the indirect pathway is alone sufficient for alloimmunization to donor MHC I [12,13]. Semple et al. demonstrated that allogeneic platelets phagocytosed *ex vivo* by recipient

adherent splenic macrophages could stimulate the proliferation of previously sensitized splenocytes under certain cell culture conditions [12]. Further studies by this group reported that these *ex vivo*-manipulated macrophages were sufficient to initiate an anti-donor antibody response when adoptively transferred to naïve recipients[13]. Although these data do not address the requirements of donor MHC II in the process of alloimmunization to donor MHC I, they nevertheless provide a biological pathway (Indirect recognition, see Figure 4), which is a plausible scenario for immunization that does not involve donor MHC II.

Semple et al. also demonstrated a relationship between allogeneic platelets and contaminating leukocytes in modulating the recipient immune response, showing that platelet transfusions remain immunogenic even with extreme leukoreduction [20]. The donor mice used in this study were of a severe combined immunodeficiency, or SCID, phenotype. Lymphocytes were absent, including MHC II positive B cells, however the authors showed that MHC II expressing monocytes persist in SCID donors at twice the frequency as wild-type mice. Although this study assessed the requirements and regulatory roles of lymphocytes, it did not specifically address the requirement of donor MHC II. In contrast, the current study formally tests and rejects the hypothesis that donor MHC II is required as a modality of alloimmunization to MHC I.

While our data demonstrate that donor MHC II is not required, this does not necessarily mean that it is not affecting alloimmunization. We observed significant differences in the relative amounts of IgG subtypes in mice responding to wild type vs. MHC II null donors. The increase in $IgG_1$ suggests a $T_H2$-like polarization when MHC II was present on donor cells while the increase in $IgG_{2a}$, $IgG_{2b}$ and $IgG_3$ suggests a $T_H1$-like polarization in its absence. This may be due to bystander effects from the direct pathway contributing cytokines such as IL-4 and TGF-β to the local milieu. However, the altered IgG subtypes may also be an epiphenomenon of the relative absence of $CD4^+$ T cells and regulatory T cells in the MHC II null transfusion.

One might question the use of unfractionated blood as opposed to leukoreduced units of platelets in the current experiments. However, this design was utilized because the hypothesis being tested was not concerned with platelets themselves, as platelets do not express MHC II; the hypothesis was focused on the requirements for MHC II expression on leukocytes contained in a transfused blood product. So as to avoid unnecessary manipulation, which may lead to alterations in biology, unfractionated blood was utilized. Due to this approach, it is essential to note that the current data have no bearing on the question of whether or not leukocytes are required for alloimmunization to transfused platelets, only the extent to which expression of MHC II is required. The current data indicate that if leukocytes are required, their necessity is not a function of expression of MHC II inducing the direct pathway of $CD4^+$ T cell activation (Direct recognition, see Figure 4), as is widely held in the transfusion literature [8–11]. There are a large number of alternate properties held by leukocytes and not platelets, including secretion of certain cytokines/chemokines, trafficking to peripheral lymph nodes, and proliferative capacity which may be responsible for the immunogenicity of leukocytes compared to platelets.

The data contained herein provide a clear answer in the context of a mouse model; however, caution must be taken in extending these findings to the clinical setting as murine and human biology may differ. Of course, the antecedent studies arguing in favor of an MHC II requirement are likewise mouse studies. Thus, the current findings lay the rational basis for new skepticism to the claim that donor MHC II is required for alloimmunization to MHC I, and highlight the need to re-examine the role of donor MHC II in human alloimmunization to MHC I.

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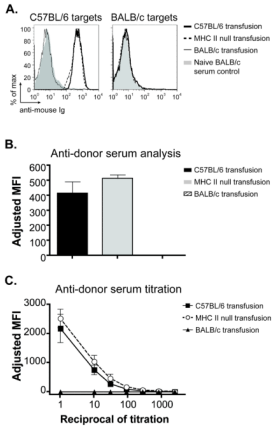


**Figure 1. Quantification of the anti-donor antibody response following transfusion from wild type or MHC II null donors**

**(A)** Representative histograms of the anti-donor antibody response by indirect immunofluorescence at a serum dilution of 1:30 using C57BL/6 allogeneic targets (left panel) or BALB/c syngeneic targets (right panel). **(B)** Total Ig anti-donor antibody response by indirect immunofluorescence using C57BL/6 targets. **(C)** Titration of day-21 sera against C57BL/6 targets. Combined data from 3 independent experiments with 5 mice per group (n=15). Error bars indicate standard error of the mean.

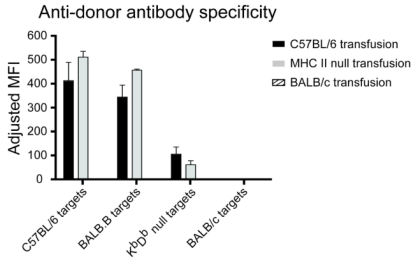


**Figure 2. Anti-donor antibody epitope specificity**

Sera from BALB/c recipients were diluted 1:30 and incubated against a panel of splenocyte targets. Combined data from 3 independent experiments with 5 mice per group (n=15). Error bars indicate standard error of the mean.

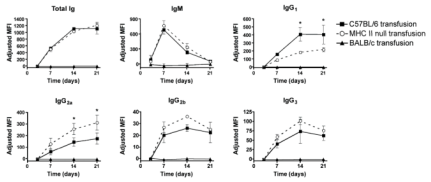


**Figure 3. Kinetics and Ig subclass determination of the anti-donor antibody response**

Sera from BALB/c recipients were diluted 1:30 and incubated against C57BL/6 targets. Ig specific secondary reagents were then used to determine the particular Ig subclass of the bound antibody at the indicated time points. Combined data from 3 independent experiments with 5 mice per group (n=15). Error bars indicate standard error of the mean. (* $p < 0.05$)

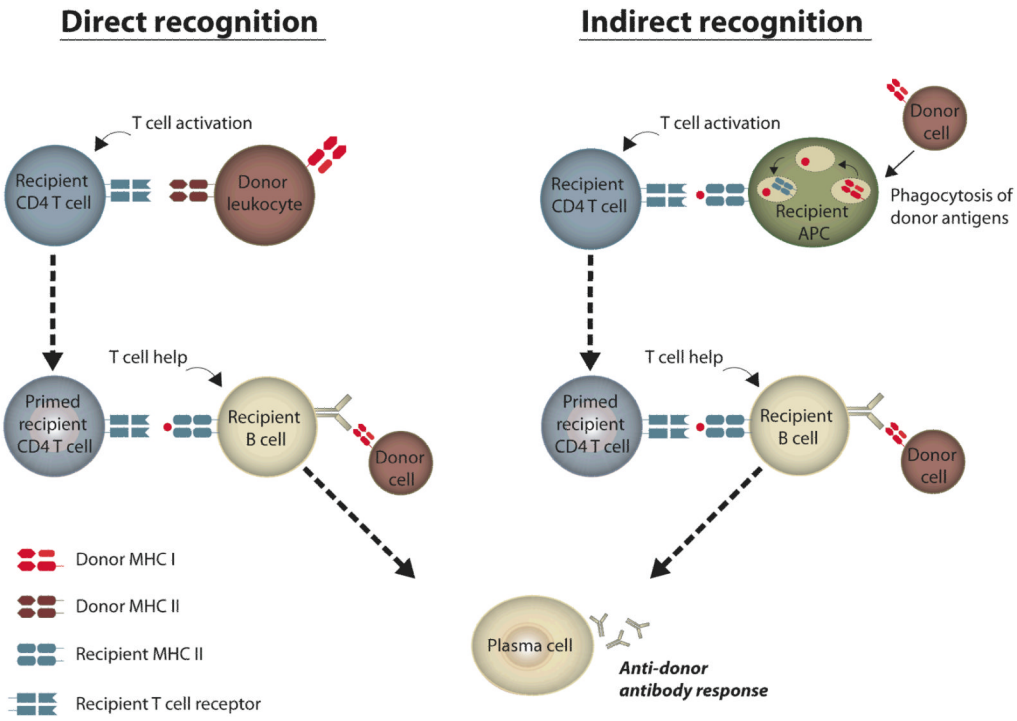


**Figure 4. Direct and indirect antigen presentation pathways in the context of whole blood transfusion**

For direct recognition, transfusion of MHC II expressing donor leukocytes (shown in maroon) as contaminates in the platelet unit can interact with and activate recipient $CD4^+$ T cells (shown in blue), which then may provide help to recipient B cells (shown in yellow) specific for anti-donor antigens. With indirect recognition, donor MHC I molecules (shown in red) from donor cells, i.e. platelets, red blood cells, leukocytes, etc., can be phagocytosed by recipient APCs (shown in green) then processed and presented on recipient MHC II. These recipient APCs can then activate a $CD4^+$ helper T cell response to provide signals to B cells for the production of anti-donor antibody.