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Reduced MHC Alloimmunization and Partial Tolerance Protection With Pathogen Reduction Of Whole Blood

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Abstract

BACKGROUND—Allogeneic blood transfusion can result in an immune response against major histocompatibility complex (MHC) antigens, potentially complicating future transfusions or transplants. We have previously shown that pathogen reduction of platelet-rich plasma (PRP) with riboflavin and UV light (UV+R) can prevent alloimmunization in mice. A similar pathogen reduction treatment is currently under development for the treatment of whole blood using riboflavin and a higher dose of UV light. We sought to determine the effectiveness of this treatment in prevention of alloimmunization.

STUDY DESIGN AND METHODS—BALB/c mice were transfused with untreated or UV+R treated allogeneic C57Bl/6 whole blood with or without leukoreduction. Mice were evaluated for donor specific antibodies and *ex vivo* splenocyte cytokine responses, as well as for changes in the frequency of regulatory T (T_{reg}) cells.

RESULTS—UV+R treatment blocked cytokine priming and reduced anti-MHC alloantibody responses to transfused whole blood. Leukoreduction reduced alloantibody levels in both the untreated and UV+R groups. Mice transfused with UV+R treated whole blood had reduced alloantibody and cytokine responses when subsequently transfused with untreated blood from the same donor type. This reduction in responses was not associated with increased T_{reg} cells.

CONCLUSIONS—Pathogen reduction of whole blood with UV+R significantly reduces, but does not eliminate the alloimmune response. Exposure to UV+R treated whole blood transfusion does appear to induce tolerance to alloantigens resulting in reduced anti-MHC alloantibody and cytokine responses to subsequent exposures to the same alloantigens. This tolerance does not appear to be driven by an increase in T_{reg} cells.

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Keywords

pathogen reduction; murine model; alloimmunization; UV; leukoreduction; whole blood

INTRODUCTION

Allogeneic blood transfusion can expose recipients to a range of foreign antigens capable of stimulating an immune response, and alloimmunization towards donor MHC antigens found on donor white blood cells (WBCs) and platelets is common following transfusion.[1–10] These immune responses against foreign MHC can be problematic for patients resulting in complications such as platelet refractoriness and transplant rejection.[6,11–15] Efforts to remove the WBCs from transfused products has significantly reduced rates of alloimmunization, but even with leukoreduction, alloimmunization remains a problem.[5,8–10,16–19]

Pathogen reduction, in which blood products are irradiated with UV light in the presence of a photosensitizer (either riboflavin or a psoralen), was designed to reduce the risk of transfusion-transmission of infectious disease by inactivating pathogens. Donor WBCs are also effectively killed by this treatment, helping to reduce the risk of graft-versus-host disease.[20,21] Surprisingly, UV treatment also appears to render these donor WBCs non-immunogenic *in vitro*.[22] This is likely the result of down-regulation of surface adhesion molecules, which prevents cell conjugation between treated donor cells and host T cells, blocking direct recognition of donor alloantigens.[23] In mice, the treatment of allogeneic PRP with UV+R virtually eliminates alloimmunization to primary exposure and significantly reduces alloantibody responses to repeated exposure.[24,25] Leukoreduction also significantly reduces the levels of antibody in these mice, with the best protection from repeated exposure seen by combining leukoreduction and UV+R treatment.[24,25] Furthermore, when splenocytes collected from mice transfused with UV+R treated PRP are exposed to untreated donor cells *ex vivo*, they have naïve profile of cytokine cytokine production, suggesting a lack of priming *in vivo*. When mice given UV+R treated PRP are subsequently transfused with untreated PRP from the same donor type, these naïve *ex vivo* cytokine profiles are maintained, suggesting some form of induced tolerance to these alloantigens. This “tolerance” does not extend to antibody generation as these mice generate antibodies at the same levels as mice given only the single untreated PRP transfusion.[24]

The effects of UV-based pathogen reduction on MHC alloimmunization in the clinical setting have been difficult to assess as currently approved products are only available for platelets and plasma, and most recipients are also exposed to untreated allogeneic red cell units as well, making it difficult to evaluate changes in alloimmunization that might occur with treatment. In spite of these other alloexposures, UV treatment of allogeneic platelets has been shown to offer some protection from MHC alloimmunization.[1,12,26] In addition, transfusion with UV-treated cells has been shown to have immunomodulatory effects in other settings, such as with extracorporeal photopheresis (ECP). For ECP, autologous WBCs are collected by apheresis, treated with a psoralen and UV light and returned to the patient, and have been shown to down-modulate damaging immune responses such as those seen in

autoimmune disease and graft rejection.[27–30] This immunomodulatory effect appears to result from induction of a tolerogenic dendritic cell phenotype and regulatory T (T_{reg}) cells.[31,32] All of these UV-based treatments use different doses and wavelengths of UV light, which in addition to the use of different photosensitizers, may impact alloimmunization outcomes.

Currently, there are no approved methods for pathogen reduction of red cells or whole blood in the U.S., but two approaches are under development. One uses a chemical compound to cross-link nucleic acids in the absence of UV, and the other uses the same UV+R combination as has been used for the treatment of platelets and plasma but with an increased dose of UV to compensate for UV light absorption by hemoglobin. This UV+R approach was CE-marked (approved for sale in the Europe) at the end of 2015. The hope with these new approaches would be to close the protective gap left by transfusion of untreated red cell units alongside treated platelet and plasma components. Both treatments have been shown to be effective at killing pathogens *in vitro*,[33–41] and more recently, UV+R pathogen reduction has been shown to effectively prevent transmission of malaria in whole blood transfusion.[36,42] The need for pathogen reduction technology (PRT) for whole blood has been recently underlined by the Zika virus outbreak in Puerto Rico, which temporarily halted blood collection on the island.[43]

The treatment of whole blood would provide an additional opportunity to treat prior to separation into components as well as provide protection where whole blood is currently transfused. While blood component therapy predominates in the developed world, whole blood transfusion is currently used in some military settings[44–46] and many developing countries,[47–50] and some have argued that whole blood is preferable for a subset of patients.[45,51,52] In the case of the military, fresh whole blood is used where transportation and storage of components is not feasible or is insufficient to keep up with demand, the same settings where comprehensive blood screening is not available.[44,52] While military personnel are all screened for or immunized against many bloodborne pathogens and some rapid tests are available, there is still an elevated risk of transmission in this population.[53,54] Many developing countries that lack the infrastructure and resources for blood component therapy, leukoreduction, and screening also have higher risks of infection in the donor population. A single treatment for whole blood that could neutralize a wide range of pathogens as well as leukocytes may provide a more feasible approach to protect whole blood transfusion recipients.

Little is known about the impact of whole blood pathogen reduction on alloimmunization. A recent study using UV+R pathogen reduction found that it did not prevent alloimmunization against red cell antigens in mice.[55] Responses to MHC alloantigens may be different for a number of reasons, including the possibility of direct recognition by T cells and expression on WBCs and platelets rather than red cells, which are impacted differently by treatment. WBCs have been shown to be inactivated by this treatment.[33,56]

In this study, we have utilized a murine model to determine the impact of UV+R pathogen reduction of whole blood on MHC alloimmunization. Specifically, we have evaluated alloantibody generation and WBC priming in recipients of treated versus untreated

allogeneic whole blood transfusions. In addition, we have assessed how these exposures impact responses to subsequent allogeneic transfusions.

MATERIALS AND METHODS

Mice

Female BALB/c mice between 9–11 weeks old were used as recipients and female C57Bl/6 mice between 2–6 months old were used as donors. All mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and were maintained in a specific-pathogen-free vivarium under barrier conditions at Blood Systems Research Institute (San Francisco, CA). All research was performed with approval and oversight of the Institutional Animal Care and Use Committee at PMI Preclinical, LLC (San Carlos, CA) under Animal Welfare Assurance A3367-01.

Donor blood collection

Whole blood was collected via orbital enucleation from multiple donor mice into a single tube containing citrate-phosphate-dextrose (CPD) anticoagulant (Sigma, St. Louis, MO) at 14% final volume and gently mixed between collections. For experiments involving leukoreduction, filtration was done using neonatal leukoreduction filters (Purecell NEO, Pall, Port Washington, NY) under sterile conditions prior to any UV+R treatment. Blood was UV+R treated or not, then stored at room temperature until use within 4 hours of collection.

UV+R treatment

Riboflavin was added to whole blood for a final concentration of 6.6%, then plated in 12 well plates with 0.5mL/well. Plate lids were replaced with a sheet of bag material cut from a riboflavin and UV light PRT Illumination/Storage bag and a quartz cover to mimic conditions of treatment for typical large volumes. The plate was then treated with a scaled dose of UV light determined by volume and hematocrit of 100 J/mL$_{RBCs}$. Blood was then transferred to sterile tubes for transfusion.

Sample collection and processing

Recipient mice were euthanized 2 weeks after their final transfusion by exsanguination via orbital enucleation under inhalation anesthesia (isoflurane), followed by spleen collection. Serum was separated from collected blood by centrifugation, then aliquotted and frozen for future use. Spleens were minced between sterile frosted glass slides, strained through a 100 μm cell strainer (BD Falcon, Franklin Lakes, NJ), and centrifuged over a ficoll layer to separate light density cells. These cells were washed and counted and used fresh or transferred to a freezing media containing 7.5% DMSO in fetal bovine serum and frozen in liquid nitrogen for future use.

Alloantibody detection

Antibodies were measured as previously described.[25] Briefly, C57Bl/6J splenocytes (donor-type target cells) were preincubated with an anti-FcR blocking antibody, then incubated with

the serum to be screened, and washed. Cells were then stained with a biotin-conjugated anti-Igk antibody to detect bound antibody along with antibodies against B220 as a B cell marker and TCRβ as a T cell marker. Cells were then washed and stained with streptavidin allophycocyanin (APC), washed and fixed, and run on a flow cytometer (LSRII, BD Biosciences, San Jose, CA) with ≥10,000 T cell events ($TCR\beta^+$, $B220^-$) acquired. Median fluorescent intensity (MFI) of Igκ staining for this population is reported. All antibody data shown in this paper were generated in a single screening assay using the same pool of target cells to allow for direct comparisons between samples collected in different experiments. Flow data analysis was performed with flow data analysis software (FlowJo, L.L.C., Ashland, OR).

Whole blood stain for MHC

Whole blood was collected from C57Bl/6J and BALB/cJ mice into EDTA tubes and diluted in PBS. Blood was stained with anti-Ter119 (red blood cell marker), anti-CD61 (platelet marker), and anti-CD45 (white blood cell marker), along with either anti-H2Db (Class I MHC expressed by C57Bl/6J, but not BALB/cJ mice), or anti-H2Dd (Class I MHC expressed by BALB/cJ, but not C57Bl/6J mice). Samples were run on a flow cytometer (LSRII, BD Biosciences, San Jose, CA) with sufficient events collected to provide ~5,000 WBCs for analysis. Cell populations were identified based on expression of the above markers and forward and side scatter gating, and MHC expression was analyzed (FlowJo, L.L.C., Ashland, OR). To compare expression pre- and post-treatment with UV+R, C57Bl/6J blood was collected into 14% CPD, and a portion was treated with UV+R as described above. A second portion was diluted equivalently with PBS in place of riboflavin and left untreated for comparison. MFIs were compared against stains of BALC/cJ blood run at the same time to normalize values. Stains were done in Trucount tubes (BD Biosciences, San Jose, CA), and cell concentrations were calculated based on the number of beads per tube, the volume of blood added, and the ratio of beads to each cell type. Cells were stained, but not fixed, and samples were run between 2–3 hours after UV+R treatment (equivalent to timing of transfusion post-treatment).

Cytokine detection

Freshly isolated splenocytes were cultured either alone or with mitomycin C treated C57Bl/6J splenocytes (donor-type stimulator cells). After 48 hours, culture supernatants were collected and frozen. Samples were later thawed and assayed using the Milliplex Map Mouse Cytokine/Chemokine Premixed 25 Plex kit (Millipore, Billerica, MA). Cytokines were measured with the Luminex 100 platform (Luminex, Austin, TX) with BioManager Software (BioRad, Hercules, CA) used for analysis as previously described.[24]

T_{reg} detection

Frozen splenocytes were thawed and washed for staining. Cells were first stained with a LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, MA) and washed before surface staining. Surface stain included anti-CD4 Alexa Fluor 700 and anti-TCRβ phycoerythrin-cyanine 5 (PE-Cy5). Cells were washed, fixed and permeabilized, stained with anti-FoxP3 phycoerythrin-cyanine 7 (PE-Cy7), washed, and run on a flow cytometer (LSRII, BD Biosciences, San Jose, CA). Mean splenocyte viability was 94.5% (+/

− S.D. of 2.15%). Flow data analysis software (FlowJo, L.L.C., Ashland, OR) was used to analyze the results.

Statistical Analysis

Prism 5.0f (GraphPad Software, Inc., La Jolla, CA) was used for analysis and to generate plots of all data. Groups were compared using 1-way ANOVA. Individual comparisons were done using either Dunnet's post-test (when comparing multiple groups to a single control sample), or Tukey's post-test (when comparing all groups to each other pairwise) as indicated in figure legends.

RESULTS

BALB/c mice were given no transfusion or transfusions of allogeneic C57Bl/6 whole blood (Figure 1). Four different transfusion regimes were tested: 1) a single transfusion with non-leukoreduced blood, 2) a single transfusion with non-leukoreduced blood that had been pathogen reduced using UV+R, 3) transfusion with UV+R treated non-leukoreduced blood followed two weeks later by a second transfusion with untreated non-leukoreduced blood, or 4) the group 3 transfusions given in reverse. Two weeks after their final transfusions, blood and spleens were collected.

Circulating antibodies were measured in each recipient by incubation of serum with splenocytes collected from C57Bl/6 (donor type) mice, followed by stains with anti-Igκ antibody to detect bound antibodies, and anti-TCRβ and anti-B220 to detect T, and B cells respectively. MFI was measured in the $TCR\beta^{+}B220^{-}$ gate to measure levels of antibodies. This was done to exclude B cells (most of which express the Igκ chain), and to focus on a uniform population. This limited our detection to antibodies against antigens expressed on T cells, predominantly class I MHC which is the dominant antigen in platelet alloimmunization, and excluded detection of antibodies against class II MHC and platelet-specific antigens, though similar results were obtained using the $TCR\beta^{-}B220^{-}$ gate as targets, which should contain some class II expressing cells (data not shown). This also restricted our detection to antibodies using the Igκ light chain, but these make up ~95% of the antibodies in mice.[57,58]

High levels of antibody were detected in the group receiving a single untreated transfusion (Figure 2A). In contrast, while they still had a detectible alloantibody response, those receiving a single UV+R treated transfusion had significantly lower levels of antibody than the mice given untreated blood. Furthermore, when mice given UV+R treated transfusions were subsequently transfused with untreated blood from the same donor type, these antibody responses were lower than those in mice given only an untreated transfusion, suggesting the generation of weak humoral tolerance. This protection was order specific as transfusion of UV+R treated blood after transfusion of untreated blood from the same donor type did not significantly alter antibody responses relative to mice given a single untreated transfusion.

To evaluate the impact of PRT treatment with UV+R on leukoreduced whole blood, mice were given either no transfusion, or a single transfusion with either untreated leukoreduced C57Bl/6 whole blood or UV+R treated leukoreduced C57Bl/6 whole blood, and antibodies

were measured in the serum 2 weeks after transfusion. Levels of antibody were very low in recipients of leukoreduced whole blood, as expected, though still detectibly higher than non-transfused controls in the absence of UV+R treatment (Figure 2B). Levels of antibody in the mice given UV+R treated leukoreduced whole blood fell between the non-transfused and untreated leukoreduced group, and were not significantly different from the untreated leukoreduced group.

To confirm that WBCs were the primary source of MHC alloantigen, whole blood from C57Bl/6J and BALB/cJ mice was stained with surface markers to identify WBC, platelet, and red blood cell (RBC) subsets (Figure 3A), along with either anti-H2Db (Figure 3B), or anti-H2Dd (Figure 3C). H2Db is the class I MHC expressed by C57Bl/6J but not BALB/cJ mice, and H2Dd is a class I MHC expressed by BALB/cJ but not C57Bl/6J mice. Matched cell populations from the strain that does not express each MHC were used as negative controls. As expected, high levels of class I MHC were seen on WBCs, low levels were seen on platelets, and no expression was seen on RBCs. We next evaluated the effect of UV+R treatment on the size of each cell compartment and expression of class I MHC within each cell type (Figure 3D). The concentration of each cell component was not affected by treatment (Figure 3D, left panel), but there was a slight reduction in class I MHC associated with treatment among the WBC compartment (Figure 3D, right panel). There were slightly higher MFIs seen in the platelet and RBC compartments following treatment (Figure 3D, right panel), but this is most likely due low level expression in combination with weak fluorescence of the riboflavin, as UV+R treated unstained controls were also brighter in this channel than the untreated unstained controls (data not shown).

In addition to antibody responses, we evaluated *ex vivo* cytokine responses in splenocytes collected from the mice to look for signs of cell priming from *in vivo* alloantigen exposure. Freshly collected splenocytes were cultured either alone or with mitomycin C treated splenocytes from C57Bl/6 (donor type) mice for 48 hours and levels of cytokines were measured using a multiplexing technique (Figure 4). Cells from the non-transfused naïve mice produced a number of cytokines in response to stimulation with the allogeneic C57Bl/6 cells, but cytokine responses were significantly enhanced by prior exposure to C57Bl/6 antigens in the untreated transfused control group, demonstrating cell priming (Figure 4A). Mice given UV+R treated transfusions behaved more like the naïve mice with significantly reduced responses compared to the untreated transfusion controls. Furthermore, when mice given UV+R treated transfusions were subsequently given untreated transfusions their responses for a number of cytokines were significantly lower than those given the untreated transfusion only. The one exception was IL-4, which was elevated in this group, and only this group, compared with the recipients of untreated transfusion only (Figure 4B). As was seen with the antibody responses, this protection from priming was order dependent, requiring exposure to the UV+R treated product prior to the untreated product.

T_{reg} cells have been shown to play a role in ECP induced tolerance,[31,32] as well as in tolerance induced by UV treated allogeneic WBCs in a different model system.[59] As we saw signs of both humoral and cellular tolerance induction following transfusion with UV+R treated allogeneic whole blood, and we next assessed if there was any evidence of elevated T_{reg} cells in these mice. Cryopreserved splenocytes from these experiments were thawed and

stained with our T_{reg} flow cytometry panel. Live $CD4^+$ T cells were gated (Figure 5A), and the frequency of $FoxP3^+$ T_{reg} cells was assessed in this population (Figure 5B). These frequencies were plotted by group, and no differences were seen between groups (Figure 5C).

DISCUSSION

In this study we have demonstrated that pathogen reduction treatment of whole blood with UV+R significantly reduces, but does not eliminate the alloantibody response to foreign MHC. This treatment also blocks priming of cytokine responses *ex vivo*, suggesting that other cellular responses may also be prevented. In addition, transfusion of UV+R treated allogeneic whole blood induces some tolerance to subsequent exposures to the same alloantigens, with reduced alloantibody and cytokine responses following subsequent transfusion with untreated whole blood from the same donor. This tolerance does not appear to be mediated by T_{reg} cell induction, as the frequency of these cells is not elevated following UV+R treated transfusion.

These findings differ somewhat from what we have observed following transfusion of UV+R treated PRP.[24] Compared with treated PRP, UV+R treatment of whole blood provides less protection against the primary antibody response, with levels of antibody roughly half of what is seen with untreated whole blood transfusion, but still significantly higher than the non-transfused controls. In contrast, protection against secondary exposure may be improved with whole blood treatment, with reduced antibody levels following transfusion of untreated whole blood in mice given UV+R treated blood from the same donor type first. This modulation of the humoral response has not been seen with UV+R treated PRP transfusion. These differences in protection could be the result of the different cell composition of these products, or of the high dose of UV light utilized in the whole blood treatment.

Cytokine responses appear similar with the two different treated blood products, with high levels of protection seen following primary exposure and indications of tolerance following secondary exposure to the same donor type. IL-4 was the only cytokine that followed a different pattern in the current study, with slightly elevated levels seen in the mice transfused with treated allogeneic whole blood prior to untreated. IL-4 is a classic T_H2 cytokine, but no differences in the levels of other T_H2 cytokines such as IL-5 or IL-13 were seen for this group (data not shown). Interestingly, IL-4 has been shown to be important in indirect allogeneic stimulation. Production of IL-4 by T cells during allogeneic stimulation *in vitro* stimulates expression of B7.1 and B7.2 on the antigen presenting cells, and skin allograft rejection is reduced when IL-4 is blocked.[60] The higher levels seen in cells from mice receiving treated followed by untreated allogeneic transfusion may be the result of increased production or decreased utilization by tolerogenic antigen presenting cells.

In contrast with what has been observed with ECP and some other UV-based treatments,[31,32,59] we did not see evidence of T_{reg} expansion following transfusion of UV+R treated allogeneic whole blood. This failure to detect a difference in T_{reg} levels could mean that non-T_{reg} mechanisms are responsible for the tolerance we have observed, or that the

role they play does not result in a detectible increase in frequency at the time-point evaluated here (two weeks after final transfusion).

Leukoreduction alone resulted in a significant decrease in alloantibody responses that exceeded the protection seen with UV+R treatment alone. This is consistent with the expression of MHC alloantigen seen in Figure 3, as the majority of MHC alloantigen is derived from WBCs, and even the low MHC expressing platelets are reduced with these leukoreduction filters.[61] When UV+R was combined with leukoreduction, the mean levels of detected antibody were approximately halfway between the non-transfused and the leukoreduction only groups, but were not significantly different from the leukoreduction only group. This suggests that if leukoreduction is already in place, adding UV+R treatment may not add meaningful additional protection against alloimmunization following a single whole blood transfusion. An additional benefit of combining these protective measures may be seen in recipients of repeated transfusions, as we have observed with repeated PRP transfusions.[25]

UV+R treatment did not itself alter the levels of WBCs in the product, but did result in a small reduction in their expression of class I MHC, consistent with what we have seen with human WBCs in UV+R treated plasma.[23] While the numbers of WBCs, platelets, and RBCs in the transfused product was not altered by UV+R treatment, this treatment can decrease donor platelet survival in the recipient as has been seen with both treated murine and human platelets, though more severe in mouse model.[25,62] Furthermore, UV+R treatment of whole blood has been shown to effectively kill WBCs *in vitro*.[33,56] Clearance of dying WBCs or damaged platelets can result in indirect presentation of MHC antigens and may explain the partial tolerance observed with transfusion of treated blood.

The addition of a viable whole blood pathogen reduction treatment would help to close the gap in protection that is achievable with currently available treatments. While UV+R pathogen reduction treatment does not appear to prevent red cell alloimmunization in mice,[55] it does appear to provide some protection against both primary and secondary WBC alloimmunization. This, in combination with protection from transfusion-transmission of infection and graft-versus-host disease suggests that UV+R treatment may provide significant protection to transfusion recipients from these complications. As appropriate clinical data become available, additional work will be needed to determine if this protection translates to human transfusion recipients.

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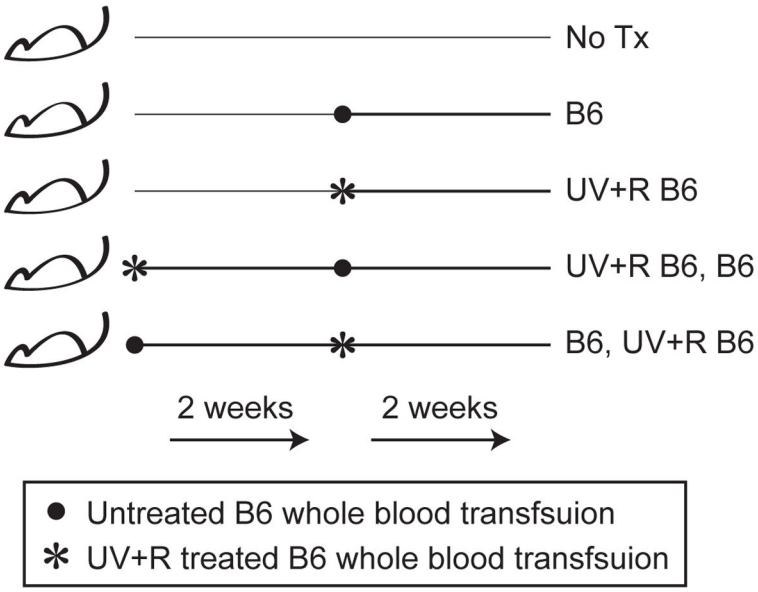

Figure 1. Experimental groups

Balb/c mice were given either no transfusion (No Tx), a single untreated B6 whole blood transfusion (B6), a single UV+R treated B6 whole blood transfusion (UV+R B6), a UV+R treated B6 whole blood transfusion followed 2 weeks later with an untreated B6 whole blood transfusion (UV+R B6, B6) or an untreated B6 whole blood transfusion followed 2 weeks later with a UV+R treated B6 whole blood transfusion (B6, UV+R B6). Mice were sacrificed 2 weeks after their final transfusion, with serum and splenocytes harvested for further analysis.

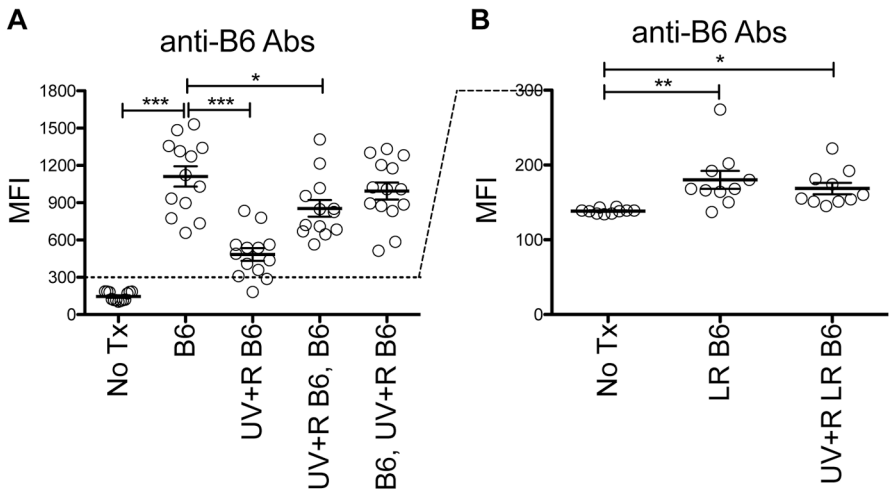

Figure 2. Decreased alloantibody production and induction of partial humoral tolerance following transfusion of UV+R treated whole blood

Serum levels of anti-B6 antibodies were measured by flow using B6 splenic T cells as target cells. MFI for each mouse is plotted by group. Dashed lines indicate change in scale between plots. Error bars display mean and standard error. (A) Balb/c mice were treated as described in Figure 1. Data from 2 experiments were combined. Groups were compared by ANOVA using Dunnett's post-test to compare each group to the B6 control group, *$p<0.05$, ***$p<0.001$. (B) Balb/c mice were given either no transfusion (No Tx), a single untreated leukoreduced B6 whole blood transfusion (LR B6), or a single leukoreduced UV+R treated B6 whole blood transfusion (UV+R LR B6). Mice were sacrificed 2 weeks after their transfusion and serum levels of anti-B6 antibodies were measured by flow using B6 splenic T cells as target cells. MFI for each mouse is plotted by group. Groups were compared by ANOVA using Tukey's post-test to compare all groups pairwise, **$p<0.01$, *$p<0.05$.

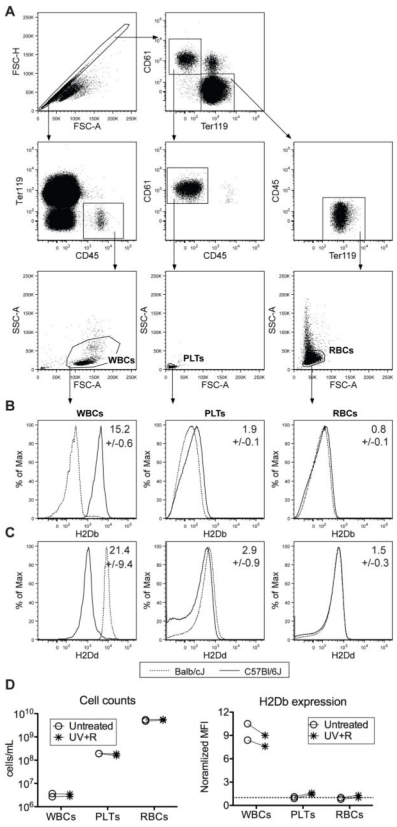

Figure 3. MHC expression in the blood

Whole blood from C57Bl/6J and BALB/cJ mice was stained with antibodies against CD45, CD61, and Ter119 along with either anti-H2Db (class I MHC expressed by C57Bl/6J but not BALB/cJ mice) or anti-H2Dd (class I MHC expressed by BALB/cJ, but not C57Bl/6J mice). (A) Gating strategy used to identify WBCs, platelets (PLTs), and RBCs. Cells were gated first on single cell events, then by appropriate surface marker, and finally by light scatter properties. (B) Expression of H2Db on C57Bl/6J WBCs, PLTs, and RBCs with staining on BALB/cJ WBCs, PLTs, and RBCs used as matched negative controls. (C) Expression of H2Dd on BALB/cJ WBCs, PLTs, and RBCs with staining on C57Bl/6J WBCs, PLTs, and RBCs used as matched negative controls. This experiment was repeated 3 times, with representative flow data shown. The fold change was calculated by dividing the MFI of the stained cells by the MFI of the negative control (cells from opposite strain). The fold change values from each of the 3 experiments were averaged, with the mean +/− standard error for the three experiements displayed for each plot. (D) Cell counts and H2Db expression were compared in untreated versus UV+R treated C57Bl/6J blood. C57Bl/6J blood samples were collected and a portion of each sample was treated with UV+R. Samples were stained as described above with the addition of known concentrations of beads to allow for determination of the concentration of each component. Cells were gated using the same strategy shown in A. The concentration of WBCs, PLTs, and RBCs was calculated using reference beads (left panel). MFIs for H2Db stains were determined and normalized against control BALB/cJ cells as described above, and these normalized values are plotted by cell

type (right panel). Dashed line at 1 (no difference from background stain). Paired pre/post-UV+R treatment samples connected by a line.

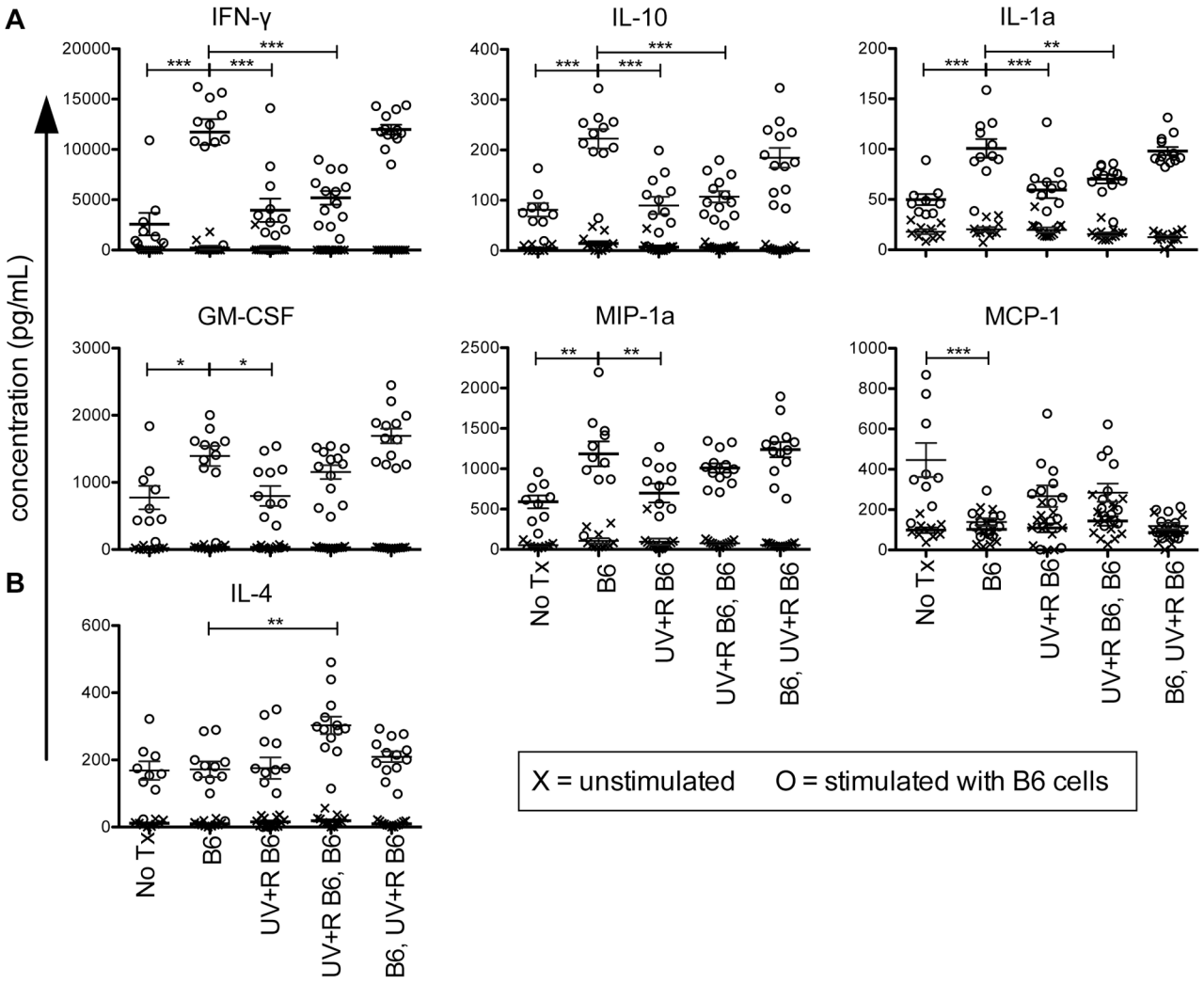

Figure 4. Altered ex vivo cytokine response following transfusion of UV+R treated whole blood

Balb/c mice were treated as described in Figure 1. Splenocytes were isolated and cultured either alone (X) or with mitomycin C treated B6 splenocytes (O) for 48 hours. Culture supernatants were screened for cytokines using multiplexing methods. Data from 2 experiments were combined. Groups were compared by ANOVA using Dunnett's post-test to compare each group to the B6 control group, *p<0.05, **p<0.01, ***p<0.001. Error bars display mean and standard error. (A) Most cytokines followed a similar pattern with UV+R transfusion either alone or before untreated transfusion resulting in responses closer to the naïve No Tx group. (B) IL-4 followed a different pattern with elevated IL-4 seen with a UV +R treated transfusion followed by an untreated transfusion.

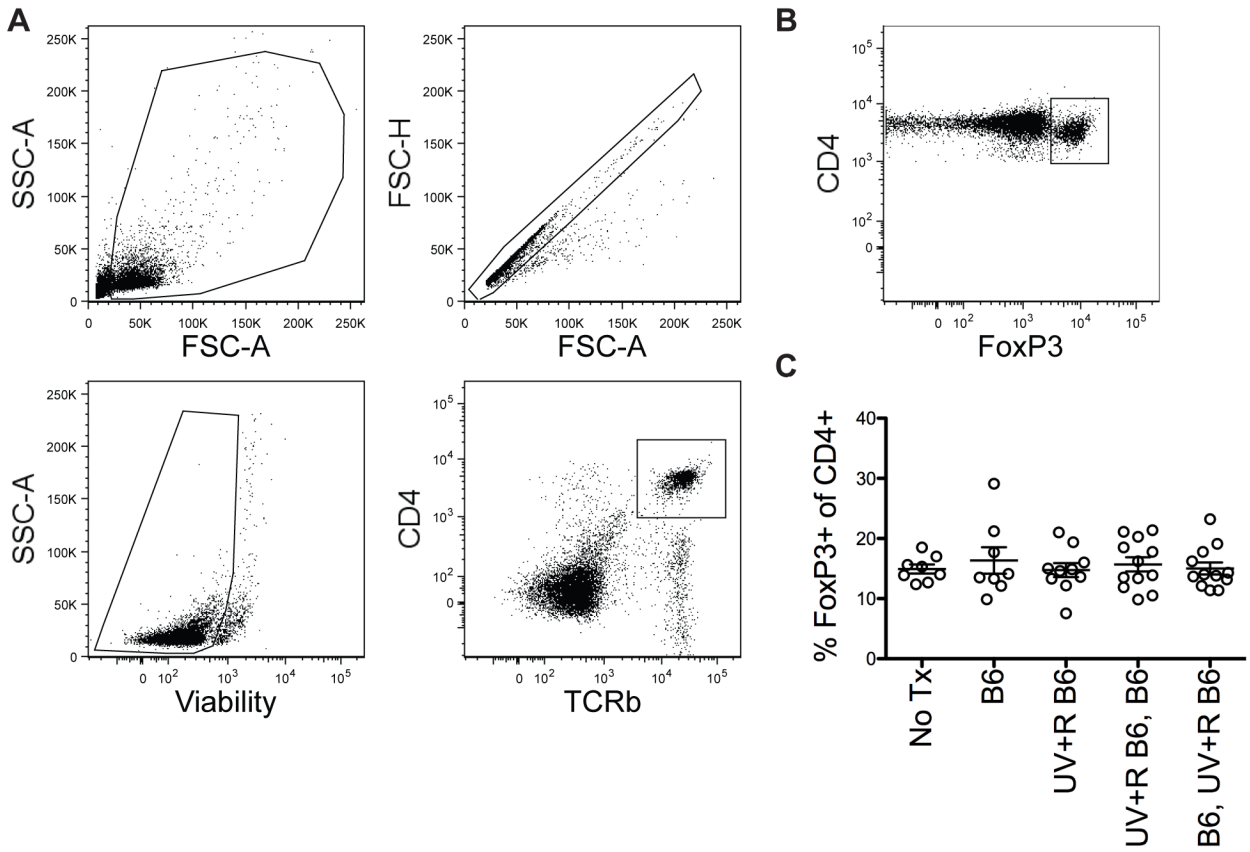

Figure 5. No increase in Treg cells detected following transfusion of UV+R treated whole blood

Balb/c mice were treated as described in figure 1. Splenocytes were stained with a viability marker, surface TCRβ and CD4, and intracellular FoxP3. (A) Gating strategy for Treg stain. Cells were gated 1) on total lymphocytes, 2) single cells, 3) live cells, and then 4) on TCRβ +CD4+ cells. (B) CD4+ T cells were evaluated for intracellular FoxP3. (C) Plots the percent of CD4+ T cells that are FoxP3+. Groups were compared by ANOVA. Error bars display mean and standard error.