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Bortezomib decreases the magnitude of a primary humoral immune response to transfused RBCs in a murine model

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Abstract

Background—Few therapeutic options currently exist to prevent or to mitigate transfusion associated RBC alloimmunization. We hypothesized that bortezomib, a proteasome inhibitor currently being utilized for HLA alloantibody and ADAMTS13 autoantibody reduction, may be beneficial in a transfusion setting. Herein, we utilized a reductionist murine model to test our hypothesis that bortezomib would decrease RBC alloimmune responses.

Study designs and methods—Wild type mice were treated with bortezomib or saline and transfused with murine RBCs expressing the human KEL glycoprotein. Levels of anti-KEL immunoglobulins in transfusion recipients were measured by flow cytometry. The impact of bortezomib treatment on recipient plasma cells and other immune cells was also assessed by flow cytometry and immunofluorescence.

Results—Following bortezomib treatment, mice had a 50% reduction in splenic leukocytes and a targeted reduction in bone marrow plasma cells. Mice treated with bortezomib prior to the transfusion of KEL RBCs became allommunized in 3/3 experiments, although their serum anti-KEL IgG levels were 2.6-fold lower than those in untreated mice. Once a primary antibody response was established, bortezomib treatment did not prevent an anamnestic response from occurring.

Conclusion—To the extent that these findings are generalizable to other RBC antigens and to humans, bortezomib monotherapy is unlikely to be of significant clinical benefit in a transfusion setting where complete prevention of alloimmunization is desirable. Given the impact on plasma cells, however, it remains plausible that bortezomib therapy may be beneficial for RBC alloimmunization prevention or mitigation if used in combination with other immunomodulatory therapies.

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Keywords

red blood cell; alloimmunization; proteasome inhibitor

INTRODUCTION

Exposure to foreign antigens on RBCs through transfusion or pregnancy may lead to the generation of alloantibodies[1]. Approximately 3–10% of transfusion recipients become alloimmunized to RBC antigens[2–4], and approximately 1 in every 600 pregnancies are affected by maternal RBC alloantibodies[5,6]. In a transfusion setting, RBC alloantibodies may lead to premature clearance of transfused RBCs, hemolytic transfusion reactions, or even death[7]. In a pregnancy setting, these alloantibodies may lead to hemolytic disease of the fetus and newborn (HDFN)[5,8].

To date, few therapies exist to prevent the formation of RBC alloantibodies, or to mitigate the dangers of existing alloantibodies induced through transfusion. Avoidance of RBC or antigens altogether is desirable but not often feasible, and little knowledge exists about steps that initiate a humoral immune response to these antigens[9]. In theory, immunomodulatory treatments that impact plasma cells (PCs) may decrease alloimmunization, though targeting these cells poses a significant therapeutic challenge in alloimmune and autoimmune conditions alike. Given the high rate of antibody synthesis, however, PCs are particularly sensitive to proteasome inhibition, which blocks NF-κB activation and results in the accumulation of misfolded proteins in the endoplasmic reticulum. This triggers the terminal unfolded protein response, leading to the apoptosis of PCs [10,11].

Bortezomib, a drug that binds reversibly to the 26S proteasome, is FDA approved for use in multiple myeloma and mantle cell lymphoma, and has been used in an off-label manner for diseases involving pathologic allo or autoantibodies. It has increasingly been used as an adjunct therapy to mitigate HLA antibody mediated rejection in solid organ transplantation[12–18]. It has also been recently used for refractory autoimmune hemolytic anemia[19,20], for refractory thrombotic thrombocytopenic purpura [21–23], for Factor VIII inhibitor eradication (in a single case involving an acquired inhibitor)[24], and for mitigation of antibody responses to enzyme replacement therapy in patients with Pompe's disease[25]. Bortezomib treatment in animal models of lupus leads to mitigation of the clinical symptoms, and significantly decreases double-stranded DNA specific autoantibody production[26,27]. In a chronic graft versus host disease setting in mice, bortezomib treatment ameliorated cutaneous lesions and decreased total germinal center B cells and B-cell activation factor gene expression[28]. However, the benefit of bortezomib, as compared to that of bortezomib in combination with therapeutic plasma exchange, rituximab, and other immunomodulatory therapies, has been difficult to assess in some instances.

Given the plasma cell focused target of bortezomib, we hypothesized that the drug would mitigate the formation of alloantibodies in a transfusion setting. We tested this hypothesis in a reductionist murine model, in which donor RBCs express the human KEL glycoprotein and in which serum anti-KEL alloantibodies can be readily measured in wild type recipients after transfusion[7].

STUDY DESIGN AND METHODS

Mice

C57BL/6 mice were purchased from NCI- the National Cancer Institute (Frederick, MD) or Taconic (Hudson, NY). Transgenic mice expressing the human KEL glycoprotein on the red blood cells, previously published as KEL2b mice, were generated previously [29]. All procedures and protocols were approved by Yale University's Institutional Care and Use Committee.

Bortezomib treatment and transfusion

Mice were given bortezomib (Millennium Pharmaceuticals, Cambridge, MA or Santa Cruz Biotechnology, Dallas, TX) or PBS injection via the intravenous (IV) route (unless mentioned otherwise) 0.75mg/kg body weight[30] twice with a 36h interval. Mice were then either sacrificed 36h to 48h post second injection for analysis or were transfused (IV injection) with the equivalent of 1 human unit of transgenic KEL RBCs (75 microliters of packed RBCs) that had been collected in the anticoagulant preservative solution CPDA-1 and filter leukoreduced over a Pall (East Hills, NY) syringe filter. Throughout the course of the experiment, mice were given intraperitoneal (IP) injection of bortezomib or PBS twice a week unless mentioned otherwise. To avoid dehydration as a side effect of bortezomib, treated mice were also given 200μL PBS IP injection as needed.

Flow cytometry and analysis

Flow cytometric crossmatch—Anti-KEL alloantibodies in transfusion recipients were measured by flow cytometric crossmatch as previously described[31] either using total Igs, IgG (BD Biosciences, San Jose, CA) or specific subtype of IgG secondary antibody (Bethyl Laboratories, Montgomery, TX). An adjusted mean fluorescence intensity was calculated by subtracting the signal obtained with sera crossmatched with antigen negative (C57BL/6) RBCs from that obtained with sera crossmatched with antigen positive (KEL) RBCs. For the flow cytometric crossmatch assay, samples were analyzed on a 4-color BD FACS Calibur, with analysis completed using Flo Jo software.

Immune cell analysis—Single-cell suspensions from bone marrow and spleen tissues at specified time points were collected. In brief, spleens were harvested and homogenized into a single cell suspension in hanks balanced salt solution (HBSS) using the plunger of a 5mL syringe. Single cells from bone marrow tissues were obtained by pipetting in HBSS. For flow cytometric analysis of immune cells, RBCs were lysed using ammonium chloride and potassium bicarbonate salt solution. Cells were stained with antibodies in buffer containing 0.1% EDTA and 0.01% bovine serum albumin. Different immune cell subsets in splenocytes and bone marrow cells were analyzed via flow cytometry using fluorochrome-conjugated monoclonal antibodies to mouse surface markers CD19, CD45R (B220), CD5, CD1d, CD21/35, CD23, CD25, GL7, CD95, CD138, CD3, CD4, CD44 (BD Biosciences, San Jose, CA or eBiosciences San Diego, CA). To detect regulatory T cells (Tregs), intracellular Foxp3 staining with Fix & Perm Cell Permeabilization Kit was performed (eBiosciences, San Diego, CA) according to the manufacturer's instructions. Live cells were first gated

using livedead stain (BioLegend, San Diego, CA). Samples were analyzed on a LSR II flow cytometer (BD Biosciences, San Jose, CA) or on Miltenyi's Macsquants analyzer.

Immunofluorescence

Spleens were cryopreserved in optimal cutting temperature medium, sectioned on a cryostat and stained as described previously[32]. In brief, frozen sections of 8μm thickness were fixed in acetone, washed in ice cold PBS, and stained at room temperature for 60 minutes with anti-B220 (AF488) and biotinylated PNA in 5% FCS (fetal calf serum)/PBS solution. For consistency, near middle sections of the spleens were used for staining and were then analyzed using an automated wide-field microscope (Nikon Eclipse Ti) and CCD camera (Qimaging Retiga 2000R) with NIS elements software. The follicles present in the entire field of view of each section were analyzed.

Statistics

All statistical analyses were performed using Graph Pad Prism software (San Diego, CA). The data were analyzed using the Mann Whitney U test (to determine significant differences between two groups), the Kruskal-Wallis test with Dunn's post-test (to determine significant differences between multiple groups), and the Wilcoxan matched pairs signed-rank test (to evaluate changes in paired samples). Error bars represent one standard deviation, and significance was determined by a p-value of <0.05.

Results

Bortezomib pretreatment of recipients decreases the magnitude of but does not prevent a primary KEL IgG alloantibody response

To investigate whether the proteasome inhibitor bortezomib inhibits RBC alloantibody generation during a primary immune response, mice were treated with bortezomib before transfusion (2 injections at 36 hour interval with the second injection 48 hours before transfusion) and after transfusion (one injection every 3–4 days) of transgenic KEL RBCs (Figure 1A). The formation of KEL specific RBC alloantibodies in sera was assessed at multiple time points post-transfusion, with no statistically significant differences in KEL specific IgM noted between bortezomib treated or untreated mice 5 days after transfusion in 3/3 experiments with 3–5 mice/group/experiment (Figure 1B shows a representative experiment). However, mice treated with bortezomib had statistically significantly lower levels of anti-KEL IgG compared to untreated mice 28 days after transfusion in 2 of these experiments ($p=0.004$ between groups in one experiment and $p=0.005$ between groups in a repeat experiment), with a 3rd experiment showing a similar trend but a non-statistically significant p-value ($p=0.09$) between groups; Figure 1B shows a representative experiment. Taking all 3 experiments together, mice treated with bortezomib had a mean 2.6-fold decrease in anti-KEL IgG levels compared to untreated mice. The generation of anti-KEL IgG subtypes was then investigated by flow cytometry, using secondary reagents specific for IgG1, IgG2b, IgG2c, and IgG3. Mice treated with bortezomib had lower levels of all IgG subtypes compared to those transfused without bortezomib treatment, though no targeted depletion of any one particular IgG subtype was observed in bortezomib treated mice (Figure 1C).

Bortezomib given IV or IP decreases the absolute number of all lymphocytes and preferentially decreases plasma cells

To characterize the impact of bortezomib on plasma cells and other immune cells, mice were injected twice with bortezomib intravenously (IV) or intra-peritoneally (IP), 36 hours apart. Recipients were sacrificed 36 hours after the second injection, and different tissues were isolated for analysis (Figure 2A). There was a significant reduction in the total number of lymphocytes in spleens of mice treated with bortezomib IV ($p<0.01$) as compared to mice that were not injected with the drug (Figure 2B). We observed a similar reduction in lymphocyte counts in the bone marrow tissues as well (data not shown), with both routes of bortezomib injection having similar effects. When we analyzed immune cell sub-populations in the spleen, namely total $CD3^+$ T cells and $CD19^+$ $B220^+$ B cells (Figure 2C), $CD21/35^{hi}$ $CD23^-$ marginal zone B cells (Figure 2D), $CD4^+$ $Foxp3^+$ regulatory T cells and $CD5^+$ $CD1d^{hi}$ regulatory B cells (Figures 2E and 2F), we did not observe a specific reduction in their relative frequencies upon treatment with the drug. However, the frequency of $CD138^{hi}$ $B220^{lo}$ plasma cells in bone marrow was significantly lower in mice treated with bortezomib as compared to mice that were not given the drug regardless of route, $p<0.05$ (Figure 2G), as was the frequency of plasma cells in the spleen (Figure 2H, $p<0.05$ IV compared to control group). Thus, consistent with previously published data, bortezomib treatment led to preferential depletion of plasma cells.

Bortezomib treatment decreases recipient plasma cell responses to transfused KEL RBCs

Having observed a significant reduction in the magnitude of KEL specific alloantibodies in bortezomib treated transfusion recipients, we next investigated the activation status of T cells, germinal center (GC) B cells, and antibody producing plasma cells in the bone marrow[33,34] in mice transfused in the presence or absence of bortezomib. No differences in CD4+ T cell CD44 (a marker for T cell activation) expression was noted on splenocytes on D7 or D28 after KEL RBC transfusion, in the presence or absence of bortezomib (Figures 3A and 3B). In contrast, we observed a trend of a decreased frequency of germinal center B cells on day 7 post-transfusion in the spleens of mice transfused in the presence of bortezomib compared to those transfused in the absence of bortezomib (Figures 3C and 3D, $p<0.01$ between mice transfused in the absence of bortezomib and naïve animals). Immunofluorescence showed that mice treated with bortezomib formed fewer GCs (30% of sections from 6 spleens had GC clusters using PNA staining) as compared to mice that were not treated with bortezomib (100% of sections from 6 spleens had GC clusters using PNA staining), Figure 3E shows a representative image. Additionally, the frequency of plasma cells in bone marrow at day 28 post-transfusion was significantly increased in mice transfused in the absence of bortezomib compared to naïve animals ($p<0.01$), with no significant difference noted between mice transfused in the presence of bortezomib compared to naïve animals (Figures 3F and 3G).

Bortezomib treatment prior to primary KEL RBC exposure does not prevent an anamnestic response to secondary KEL RBC exposure

Since bortezomib treatment led to significant suppression of KEL-specific antibody production, we wondered whether treatment prior to an initial KEL RBC exposure may

mitigate alloantibody responses to subsequent KEL RBC exposures. To this end, 9 weeks following the initial transfusion, mice that were or were not treated with bortezomib during primary KEL RBC transfusion were challenged with a secondary KEL RBC transfusion without bortezomib treatment, and assessed for secondary KEL specific alloantibody responses (Figure 4A). Bortezomib treatment did not prevent an anamnestic response from occurring, though the absolute signal of the secondary antibody response showed a lower trend in mice treated with bortezomib prior to the primary RBC exposure compared to those re-transfused in the absence of bortezomib. Figure 4B shows a representative experiment, with no statistically significant differences observed between primary and secondary responses in either group using the Wilcoxan matched pairs signed rank test (but with $p<0.05$ by Mann Whitney U when comparing the secondary responses solely to each other). A repeat experiment showed similar results, with $p=0.25$ between primary and secondary responses in bortezomib treated or untreated mice by a Wilcoxan test and with $p<0.05$ by Mann Whitney U when comparing the secondary responses solely to each other.

Bortezomib treatment (for the first time) prior to secondary antigen exposure does not prevent an anamnestic response

A clinically significant question revolves around whether bortezomib may be utilized to lower alloantibody titers prior to emergent incompatible RBC transfusion. To investigate this question, we transfused mice with KEL RBCs and tested anti-KEL responses at the peak of the antibody response. The alloimmunized mice were then either treated with bortezomib or received no treatment during a secondary KEL RBC transfusion. KEL specific alloantibody responses were assessed 14 days after the secondary transfusion (Figure 5A). There were no statistically significant differences noted between primary and secondary anti-KEL IgG responses in mice treated or not treated with bortezomib prior to secondary antigen exposure using the Wilcoxan matched pairs signed rank test (Figure 5B, $p=0.125$ between primary and secondary responses in both groups). Similar results were observed in a total of 3 experiments, with 3–5 mice/group/experiment.

DISCUSSION

To date, there are no pharmacologic therapies known to decrease primary RBC alloimmune responses in a transfusion setting, and few therapies exist to mitigate secondary alloimmune responses. This current study, which investigated the impact of the proteasome inhibitor bortezomib on alloimmunization to the human KEL glycoprotein expressed on murine RBCs, was undertaken with the hypothesis that targeted plasma cell reduction may prevent this complication which has implications for transfusion and pregnancy. A series of experiments, completed in a reductionist murine model, showed that although bortezomib monotherapy decreased the magnitude of a primary IgG KEL specific RBC alloantibody response following transfusion, it did not prevent an immune response altogether. Further, bortezomib therapy was unable to prevent anamnestic anti-KEL responses altogether.

The fact that bortezomib was unable to completely prevent primary or secondary IgG KEL RBC alloantibody responses is disappointing, but not entirely surprising[35]. In accordance with prior studies, bortezomib decreased but did not eliminate plasma cells in the bone

marrow. Further, although splenic GC B-cells were decreased quantitatively in bortezomib treated mice transfused with transgenic KEL RBCs, this decrease was not statistically significant. The decreased anti-KEL response was likely due principally to the effect of bortezomib on plasma cells, but it cannot be ruled out that the response was also due in part to bortezomib non-selectively decreasing other cell types. For example, bortezomib decreased splenic marginal zone B-cells by approximately 50%, and these cells are known to play a role in responses to other blood borne antigens[36–38]. However, the decrease in marginal zone B-cells was equal to the decrease in other cells types such as CD4+ T-cells, regulatory T-cells, and regulatory B-cells. Of note, the role that these cells play in alloimmune responses to transfused KEL RBCs is not yet fully understood.

One advantage of studying the KEL reductionist RBC alloimmunization model is that the timing of initial and subsequent RBC exposure can be controlled, and the impact of bortezomib as a monotherapy can readily be studied. In contrast, many of the case reports of the use of bortezomib for the treatment of pathologic allo or antibodies involve patients with refractory antibody mediated diseases, previously treated with multiple immunomodulatory therapies. As such, the effect of bortezomib itself on changes in antibody titers, the complement fixing capabilities of antibodies[39], or documented clinical improvements has been difficult to assess. It is plausible that bortezomib in combination with B-cell depletion or other immunomodulatory therapy may more efficiently decrease RBC alloimmunization in our murine model than treatment with bortezomib alone[13,40], as has been shown to lead to clinical improvement in refractory TTP patients[22] and in a single patient with an acquired Factor VIII inhibitor[24]. It is also plausible that combined targeting of the proteosome and the glutamate antiporter might circumvent bortezomib resistance, at least in multiple myeloma cells[41]. Notably, B-cell depletion as a monotherapy has recently been used in an attempt to prevent delayed hemolytic transfusion reactions in highly alloimmunized patients with sickle cell disease, though some patients have had breakthrough hemolysis and some have formed additional RBC alloantibodies despite rituximab therapy[42–45].

The care of alloimmunized patients remains a clinical challenge, regardless of whether the sensitizing antigen is on an RBC, WBC, or tissue, and regardless of whether the sensitizing event occurs through transfusion, pregnancy, or transplantation. Some patients have so many RBC alloantibodies that crossmatch compatible RBCs cannot be located for transfusion[42]; others have so many HLA antibodies that compatible organs cannot be located for transplantation. We initiated these murine studies with the hope that bortezomib treatment of alloimmunized mice might eliminate anti-KEL antibodies altogether, or decrease anti-KEL titers to the point that the animals could be safely transfused with antigen positive RBCs. However, the anti-KEL IgG levels in animals treated with bortezomib were high enough to be functionally significant and capable of leading to rapid clearance of transfused incompatible KEL RBCs. In other situations, potentially including HLA antibody mediated solid organ transplant rejection or thrombotic thrombocytopenic purpura, reduction of an allo or autoantibody below a certain threshold might be more clinically meaningful.

Limitations to this study must be taken into consideration. We studied just one RBC antigen, selecting the KEL glycoprotein given the clinical significance of antibodies against antigens in this family in mice and in humans. However, it cannot be ruled out that different results

may be observed in studies using other RBC antigens, given known structural (and functional) differences. Further, the route by which the antigen is introduced may be impactful, in considering differences between immune responses to antigens on transfused RBCs and solid organs, for example. It should also be considered that humans may respond subtly differently to bortezomib compared to mice, though similar toxicities have been noted. Finally, the primary measure of immunity described in our murine alloimmunization model is KEL specific Igs. It is possible that useful information could have been obtained had KEL specific antibody secreting cells or antigen specific T or B-cells been able to be evaluated, but these studies are difficult with the tools currently available.

In sum, bortezomib decreased but did not eliminate primary alloantibody responses to transfused murine RBCs expressing the KEL glycoprotein. To the extent that these findings are generalizable to other RBC antigens and to humans, bortezomib monotherapy is unlikely to be of significant clinical benefit in a transfusion setting, where complete prevention of alloimmunization is desirable. A better understanding of transfusion associated alloimmunization to RBC antigens, as well as the development of more optimal strategies to prevent or mitigate the formation of such alloantibodies, would benefit patients in transfusion and pregnancy settings alike.

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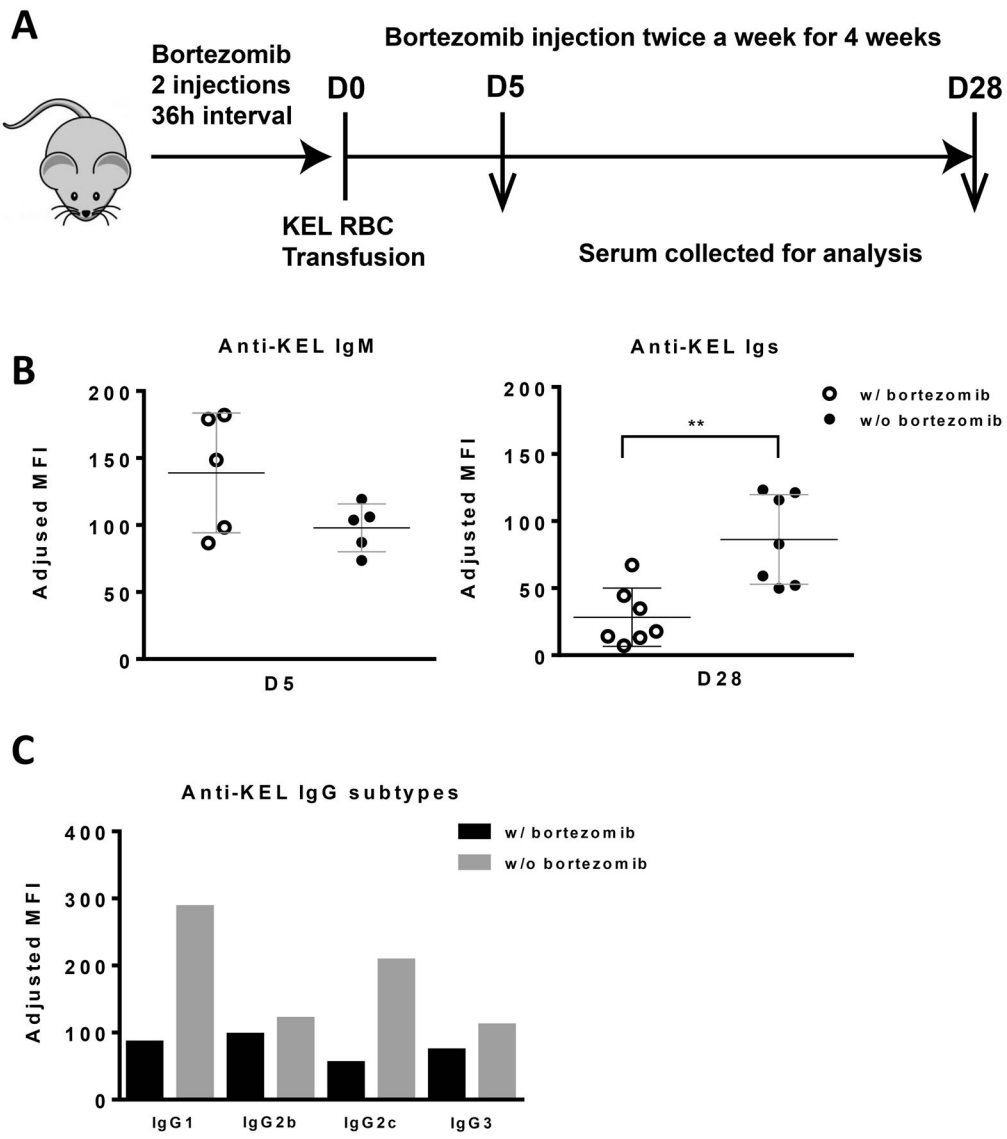
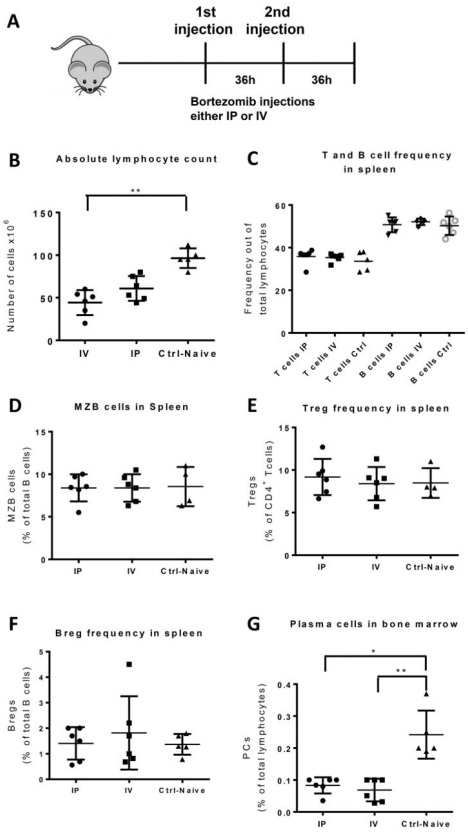


Figure 1. Bortezomib decreases primary alloantibody response to transfused KEL RBC exposure

(A) Schematic of experimental design for treating mice with bortezomib during primary transfusion. (B) Serum anti-KEL IgM at day 5 post-transfusion (left panel) or anti-KEL IgG (right panel) at day 28 post transfusion, represented as adjusted MFI, in mice treated with bortezomib as compared to mice not treated with bortezomib. (C) IgG subtypes in serum of bortezomib treated versus untreated mice represented as adjusted MFI, **p<0.005. Data in B and C are representative of 3 independent experiments with 3–5 mice/group/experiment. Data in C was collected using pooled serum samples from all mice in a particular group, and are representative of 3 experiments.



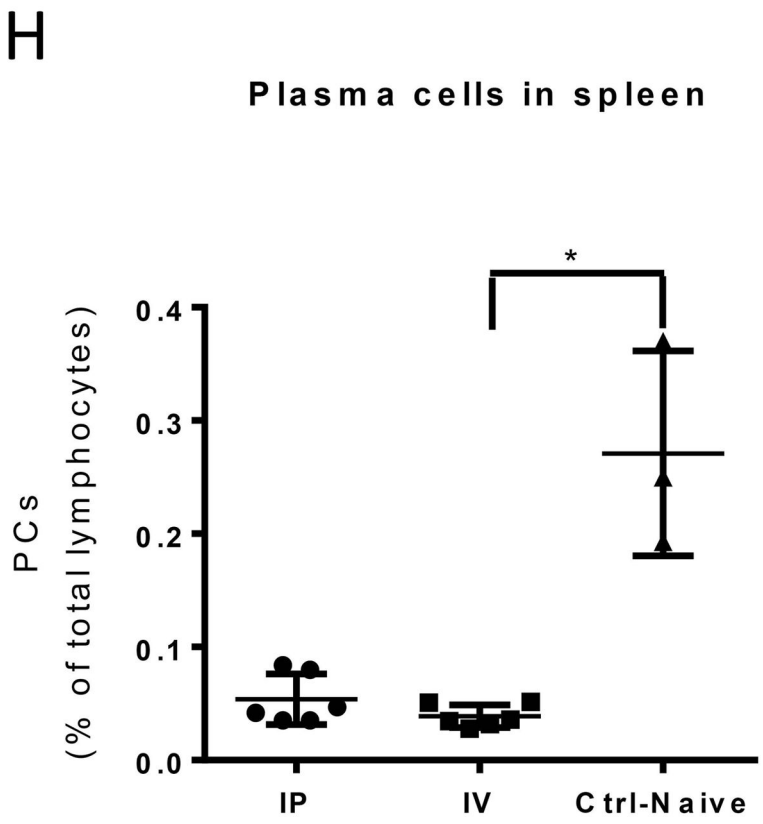
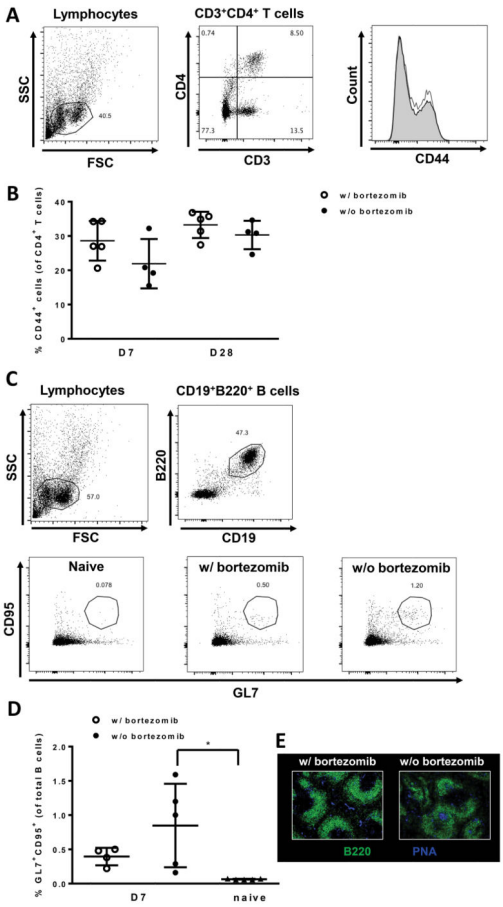


Figure 2. Bortezomib given IV or IP selectively decreases plasma cells

(A) Schematic of experimental design. (B) Absolute number of lymphocytes in the spleens of mice that were injected with PBS:Ctrl-Naive (solid triangle) or bortezomib via IV (solid circle) or IP (solid square) routes. (C) Percentage of $CD3^{+}$T cells and $CD19^{+}B220^{+}$B cells within lymphocyte gate in spleens of mice from different groups. (D) Frequency of marginal zone B cells (MZB cells) gated as $CD21/35^{hi}CD23^{-}$ out of total B cells in the spleen. (E) and (F) comparing frequencies of Tregs ($Foxp3^{+}$ cells out of total $CD4^{+}$ T cells) and Bregs ($CD5^{+}$ $CD1d^{hi}$ out of total B cells) in spleens of mice from the indicated groups. (G) Comparing frequency of plasma cells from the bone marrow or (H) spleen, identified as $B220^{lo}CD138^{hi}$ cells, out of total lymphocytes. * $p<0.05$, ** $p\leq0.01$. Data from 2/2 experiments are shown (n=6 mice total per group).



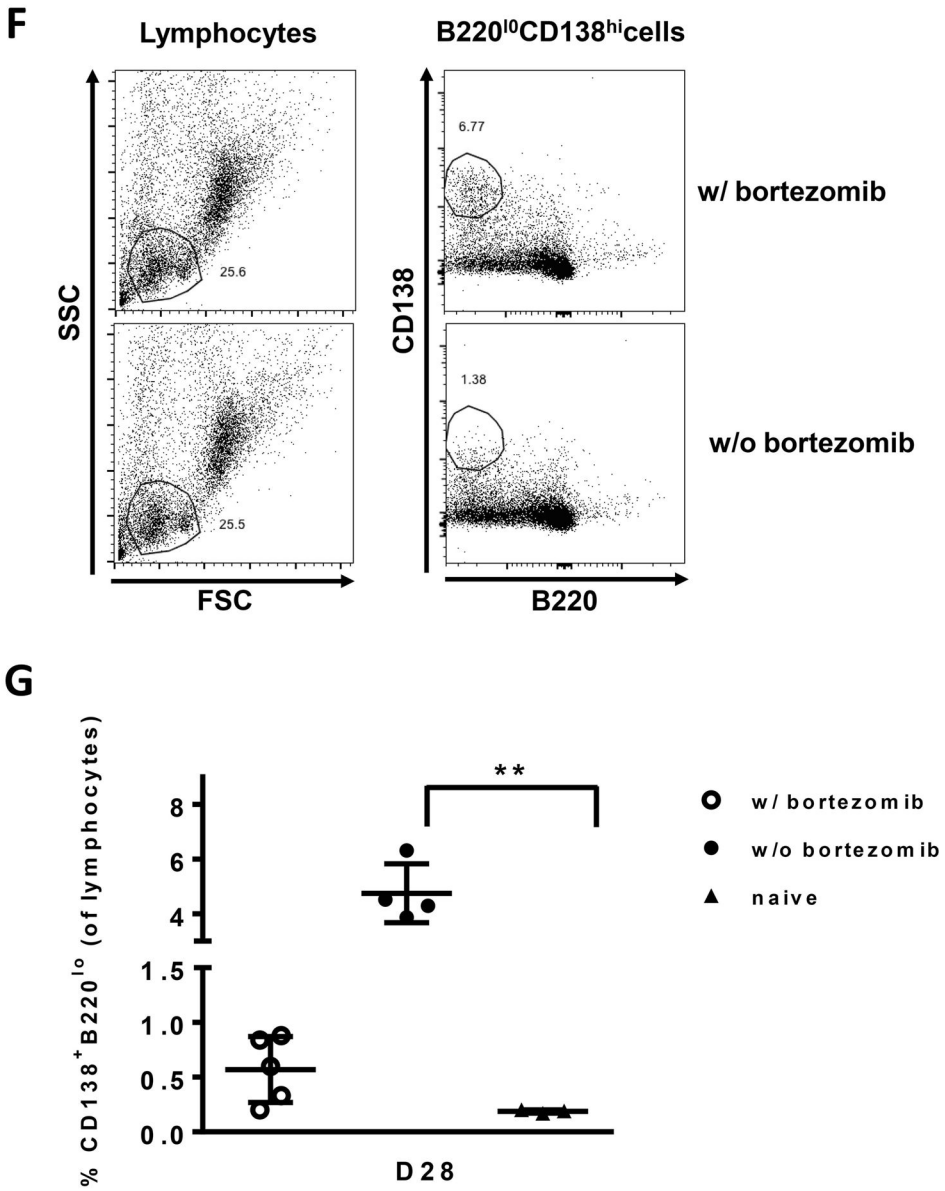


Figure 3. Effect of bortezomib on immune cells after continuous treatment for 4 weeks after transfusion

(A) Representative dot plots showing lymphocytes (pre-gated on live cells), $CD3^+CD4^+$ T cells and histogram comparing CD44 expression on $CD3^+CD4^+$ T cells in bortezomib treated (shaded) versus control mice (solid line). Data shown are collected from spleen at day 7 post transfusion. (B) Gated percentage of $CD44^+$ T cells of total CD4 T cells. (C) Representative dot plots showing the gating strategy for germinal center (GC) B cells. GC B cells are gated as $GL7^+CD95^+$ cells of total $CD19^+B220^+$ B cells. Data shown are collected from spleen at day 7 post transfusion. (D) Gated percentage of GC B cells in mice treated with bortezomib (clear circle), mice not treated with bortezomib (solid circle) and naïve (solid triangle) mice. (E) Representative 10× images of the spleen isolated at D7 post transfusion from mice treated with bortezomib (left panel) or without bortezomib (right

panel). (F) Representative dot plots showing the gating strategy for plasma cells gated as $B220^{lo}CD138^{hi}$ cells of total lymphocytes. Data shown are collected from bone marrow at day 28 post transfusion. (G) Gated percentage of plasma cells in mice treated with bortezomib (clear circle) compared to mice not treated with bortezomib (solid circle) at day 28 (D28) post transfusion. * $p<0.05$, ** $p\leq0.01$. Data in B, D and G are representative of at least 2 experiments (n=3–5 mice/group/experiment). Data in E are representative images from 2 experiments with n= 3 to 5 mice per group.

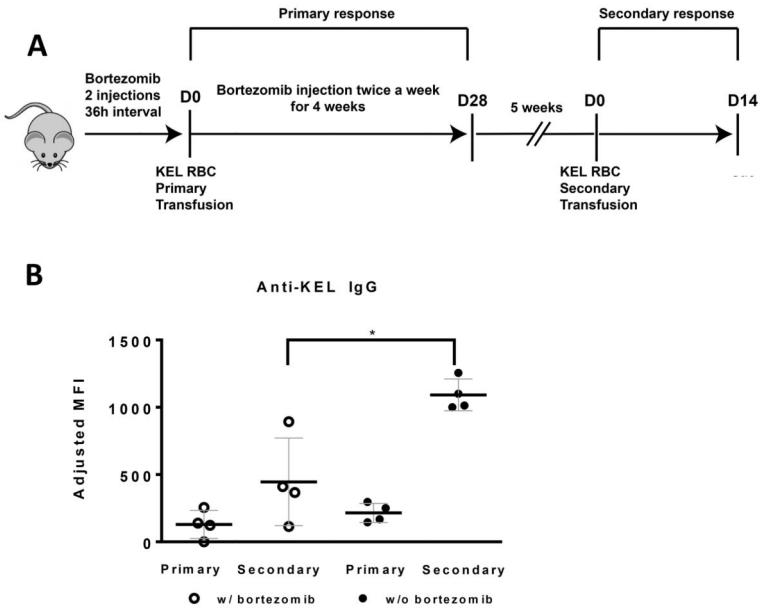


Figure 4. Mice treated with bortezomib during primary but not secondary RBC exposure have anamnestic anti-KEL responses

(A) Schematic of experimental design. (B) Serum anti-KEL IgG antibodies represented as adjusted MFI at day 14 (D14) following primary or secondary transfusion in mice treated (open circle) or untreated (solid circle) with bortezomib during primary challenge. Data in B are representative of 2 experiments (3–5 mice/group/experiment). * $p<0.05$ between these 2 groups by the Mann Whitney U test, with no significant differences between primary and secondary responses by the Wilcoxan matched pairs signed rank test.

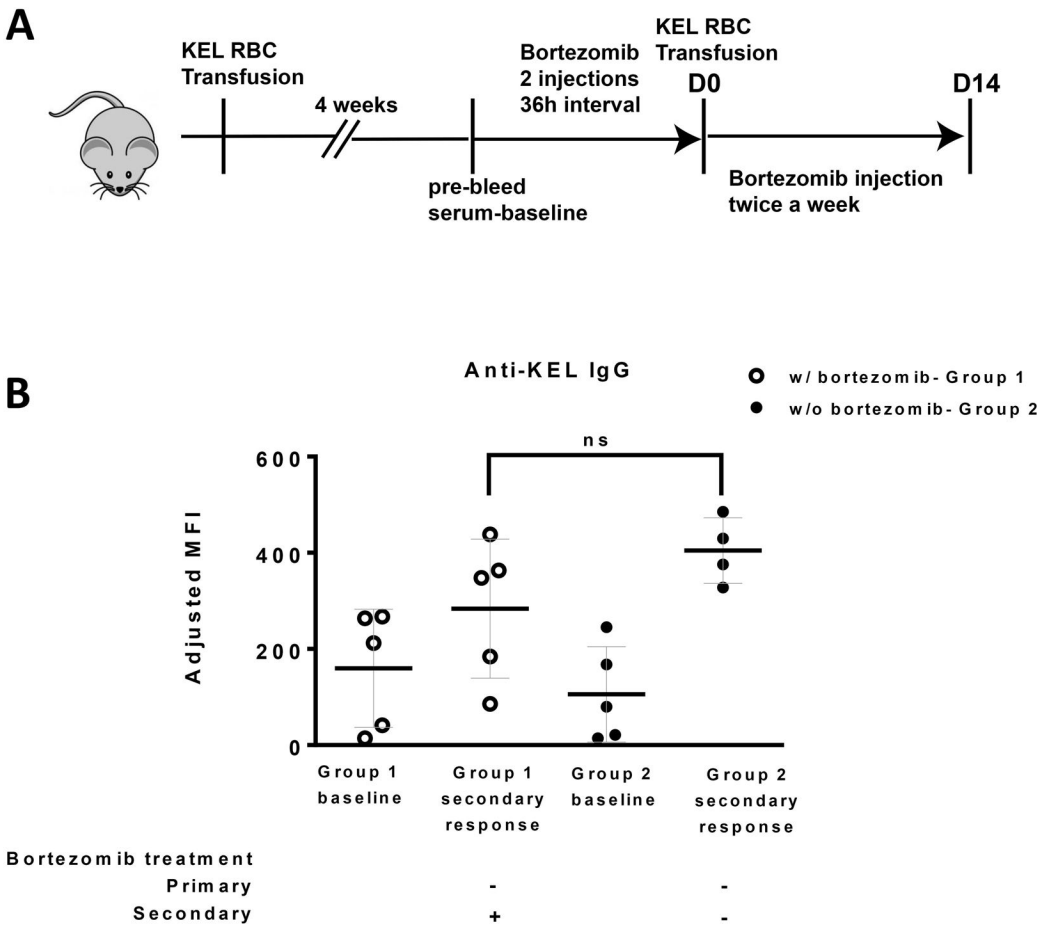


Figure 5. Bortezomib treatment prior to secondary KEL RBC exposure does not impact anamnestic responses

(A) Schematic of experimental design. (B) Serum anti-KEL IgG antibodies represented as adjusted MFI either before secondary transfusion (baseline) or at day 14 (D14) post-secondary transfusion, shown in mice treated with bortezomib (clear circles) as compared to mice that were not treated with bortezomib (solid circles). Data in B are representative of 3 experiments (n=3–5 mice/group/experiment), with significance of secondary responses evaluated by the Mann Whitney U test (ns), and with significance between baseline and secondary responses evaluated by the Wilcoxan matched pairs signed rank test (ns).