Preformed antibody, not primed T cells, is the initial and major barrier to bone marrow engraftment in allosensitized recipients

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Multiply-transfused individuals are at higher risk for BM rejection. We show that whereas allosensitization resulted in the priming of both cellular and humoral immunity, preformed antibody was the major barrier to engraftment. The generation of cross-reactive alloantibody led to rejection of BM of a different MHC-disparate strain. Imaging studies indicated that antibody-mediated rejection was very rapid (< 3 hours) in primed recipients, whereas T-cell–mediated rejection in nonprimed mice took more than 6 days. Antibody-mediated BM rejection was not due to a defect in BM homing as rejection occurred despite direct intra-BM infusion of donor BM. Rejection was dependent upon host FcR+ cells. BM cells incubated with serum from primed mice were eliminated in nonprimed recipients, indicating that persistent exposure to high-titer antibody was not essential for rejection. High donor engraftment was achieved in a proportion of primed mice by mega-BM cell dose, in vivo T-cell depletion, and high-dose immunoglobulin infusion. The addition of splenectomy to this protocol only modestly added to the efficacy of this combination strategy. These data demonstrate both rapid alloantibody-mediated elimination of BM by host FcR+ cells and priming of host antidonor T cells and suggest a practical strategy to overcome engraftment barriers in primed individuals. (Blood. 2007;109:1307-1315)

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Introduction

Allosensitization can be a formidable barrier to bone marrow (BM) engraftment due to priming of the host’s cellular and humoral immune responses.1-7 Despite the well-recognized hazard of antibody-mediated rejection in solid organ transplantation,8-11 donor BM rejection is generally attributed to cytolytic host antidonor T and natural killer (NK) cells that survive the conditioning regimen.16-23 However, antibody-mediated BM failure after allogeneic bone marrow transplantation (BMT) can occur either by antibody-dependent cell-mediated cytotoxicity or complement-mediated cytotoxicity.2-4,6 Preformed antibody present at the time of BM infusion is unaffected by standard transplantation conditioning regimens or T- or B-cell immunosuppressive or modulatory strategies given in the peri-transplantation period. Although plasmapheresis, high-dose intravenous immunoglobulin, splenectomy, and immunoadsorption are commonly used in solid organ transplantation for the abrogation of alloantibody-mediated rejection,8-11,13,14,24-27 these strategies generally are not part of the BMT conditioning regimen.

We show that although allosensitization resulted in the priming of both the cellular and humoral arms of the immune response, preformed antibody was the major barrier to engraftment. A single priming event led to high, long-lived antibody levels that practically precluded waiting for the waning of antibody titer for successful BMT. Imaging studies indicated that antibody-mediated rejection of a moderate BM dose was nearly complete by 3 hours. Antibody-mediated rejection of donor BM in primed mice was dependent on a host FcR+ mechanism. Of significant clinical relevance, priming against one strain could result in the rejection of donor BM of a different strain. Furthermore, incubation of donor BM with serum from primed mice resulted in the destruction of the alloantibody-coated BM cells when transferred into a nonprimed recipient, suggesting that persistent exposure to endogenous high antibody titer was not requisite for antibody-mediated BM rejection. Despite the formidable barrier to engraftment, high levels of donor chimerism could be achieved in a proportion of primed, irradiated mice by a combination of high BM cell dose, in vivo T-cell depletion (TCD), and high-dose murine immunoglobulin (mIg).

Materials and methods

Mice

B10.BR (H2b), C57BL/6 (H2b) (termed B6), and B6 muMT mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 muMT (formerly designated B6.129S2-Igh-6tm1cgn) (H2b) mice are B-cell deficient due to an immunoglobulin heavy chain defect. BALB/c (H2k) mice were purchased from the National Institutes of Health (Bethesda, MD). B6.129P2-FcεRIg−/−N12 (H2b) (termed B6.FceRIg−/−) mice, deficient in the gamma chain subunit of the FcγRI, FcγRIII, FcγRIV, and FcεRI receptors were purchased from Taconic (Hudson, NY). B6 green fluorescent protein (GFP) transgenic (Tg) mice were obtained from the laboratory of Dr Jonathan Serody and bred at the University of Minnesota. Mice were housed in a...
specific pathogen-free facility in microisolator cages and were used at 6 to 12 weeks of age.

Serum antibody detection

A flow cytometric assay published by Valujskikh et al. was used for the detection of serum antibody in primed mice. Mice were primed by the intraperitoneal administration of $2 \times 10^6$ antigen-disparate splenocytes at various intervals from 7 days to 6 months prior to sera collection. Serum (100 μL; diluted at 1:10, 1:40, 1:160) was incubated for 1 hour with thymocytes (or BM cells) (10%) of the priming strain (and in some cases, third-party strain). Cells were washed and incubated with FITC-conjugated goat anti–mouse Ig antibody (Pharmingen, San Diego, CA) for 1 hour, washed, and analyzed by flow cytometry. Controls for determination of serum antibody levels included thymocytes incubated with serum from nonprimed naive mice and thymocytes incubated with no serum.

Bone marrow transplantation (BMT)

For survival studies not involving imaging, B6, B6 muMT, or B6 Fcer1g−/− mice were irradiated with 6.0 Gy (a dose that permits a greater contribution by host T cells and allows for autologous recovery in the event of BM rejection) or 8.0 Gy (a dose that minimizes T-cell–mediated rejection; mice must engraft to avoid lethal aplasia) TBI by x-ray on day −1. BALB/c (or B10.BR) BM cells (range, 20-200 x10^6) were infused on day 0. Cohorts of mice were primed by the intraperitoneal administration of $2 \times 10^6$ BALB/c or B10.BR splenocytes at various times prior to transplantation. Where indicated, anti-CD8 (clone 2.43), anti-CD4 (clone GK1.5), and anti-NK1.1 (clone PK136) mAbs (400 μg each) were administered intraperitoneally on days −2, 1, 4, and 7 to deplete T and NK cells around the time of transplantation. One dose of mAb depleted more than 95% of its target cell (P.A.T., unpublished data, September 2000).

Survival was monitored daily. Packed red blood cell volumes (PCVs) were monitored weekly in some experiments as a measure of BM aplasia. Donor chimerism of long-term survivors was documented by the phenotyping of peripheral blood leukocytes (PBLs) at 4 to 6 weeks and 3 months after transplantation. PBLs were stained with fluorochrome-conjugated antibodies (anti-CD8, -CD4, -MAC-1, -CD19, -H2b, -H2a, and -H2d, and isotype controls; Pharmingen) and analyzed using CellQuest software on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

For experiments designed to image the fate of B6 GFP+ BM under conditions of nonprimed rejection, primed rejection, or engraftment (Figure 4), BALB/c mice were irradiated on day −1 with 3.5 Gy by x-ray and infused with $2 \times 10^6$ T-cell–depleted B6 GFP+ BM cells on day 0 (conditions designed to ensure graft rejection in nonprimed mice). To ensure engraftment in a cohort of mice, anti-CD8, anti-CD4, and anti-NK1.1 (400 μg each) was administered on days −2, 1, 4, and 7. The primed cohort received 20 x10^6 B6 splenocytes intraperitoneally on day −28. PBL phenotyping in survival experiments verified that untreated controls and primed recipients uniformly rejected donor bone marrow. In contrast, all nonprimed mice receiving T- and NK-depleting antibodies engrafted with high levels of donor chimerism.

In vivo imaging

Images were taken with a Retiga Exi color camera and QCapture software (QImaging, Burnaby, BC) mounted on a Leica MZFLIII stereomicroscope using a GFP2 or a GFP/dsRED-bandpass filter and a 1.0× transfer lens (Leica Microsystems, Bannockburn, IL). Zoom factors of 3.5× to 10× were used for imaging on days 1 to 14 (3.5× for ileum and Peyer patch; 7.0× for femoral bone marrow cavity, liver, spleen, and kidney; 10.0× for lung) and 7× to 10× for imaging at 3 hours. Exposure times were optimized for each organ and identical times and settings were used for all mice imaged on any given day. To obtain optimal images, mice were killed and dissected for imaging but no tissue processing is required. Three mice per group were examined at each time point and results were reproduced in a second experiment (Figure 4A-G only). One experiment imaging mice at 3 hours was done (n = 3/group). Mice within a group yielded very similar results at each time point so a representative image is illustrated.

Incubation of donor BM with serum from naive or primed mice prior to BMT

BALB/c or B10.BR T-cell–depleted BM cells ($2 \times 10^6$/mL) were incubated with a 1:20 dilution of serum obtained from either naive B6 mice or BALB/c–primed B6 mice for 1 hour on ice. (B6 serum donors were primed by intraperitoneal administration of $2 \times 10^6$ BALB/c splenocytes 28 days prior to serum collection.) After incubation, BM was washed 3 times prior to infusion of $2 \times 10^6$ donor BM into lethally irradiated (8.0 Gy—a dose chosen to minimize contribution of T-cell–mediated rejection), nonprimed B6 mice. The only alloantibody present is that which bound to the donor BM cells during the brief in vitro incubation prior to infusion.

Splenectomy and BMT

B6 recipients were primed on day −56 with $2 \times 10^6$ BALB/c splenocytes intraperitoneally. Splenectomy (splx) was performed on day −28 under pentobarbital anesthesia. An incision was made over the spleen, vessels were ligated at both ends, and the spleen was removed. The peritoneum was closed with nonabsorbable sutures and the skin closed with small staples. High-dose mouse Ig (20 mg/mouse; Rockland, Gilbertsville, PA) was administered intraperitoneally on day −15 and day −7 where indicated. Some mice were depleted of T cells by the administration of anti-CD4 and anti-CD8 mAbs on days −2, 0, 2, 4, and 7. Mice were irradiated with 6.0 Gy (a dose that permits a greater contribution by host T cells and allows for autologous recovery in the event of BM rejection) by x-ray on day −1 and infused with high-dose BM ($10^6$ cells) on day 0. To determine effect of splx and mIg on antibody levels, serum was taken just prior to splx (day −29) and again just prior to BMT (day −2).

Statistics

Survival data were analyzed by life-table methods and actuarial survival rates are shown. Group comparisons were made by log-rank test statistics. To assess engraftment data, group comparisons of percentage donor chimerism were analyzed by Student t test. Group comparisons of engraftment and survival rates were analyzed by chi-squared test. P values below .05 were considered significant in all tests.

Results

Alloantigen priming is a potent barrier to engraftment

Initial studies focused on the kinetics of priming. Cohorts of B6 mice were primed against BALB/c alloantigen, either 7, 14, or 28 days prior to transplantation by the intraperitoneal administration of BALB/c splenocytes. Mice were lethally irradiated and infused with BALB/c BM. All nonprimed mice survived and engrafted with an average of more than 95% donor chimerism (Figure 1A). In contrast, all primed mice, regardless of whether they were primed 7, 14, or 28 days prior, died of BM aplasia by 2 weeks after transplantation, similar to irradiated controls that did not undergo transplantation (Figure 1A, irradiation controls not shown). Additionally, rigorous host depletion of CD8+, CD4+, and NK+ cells failed to rescue primed mice from lethal BM aplasia, indicating that neither primed T cells nor NK cells surviving lethal irradiation were responsible for BM rejection. Increasing BM cell dose to $160 \times 10^6$ also failed to rescue primed mice from death (data not shown).

To detect the presence of anti-BALB/c antibody in primed B6 mice, an assay published by Valujskikh et al. was used. Serum was collected 7, 14, or 28 days after priming, diluted, and incubated with BALB/c thymocytes (thymocytes have few Fc receptors
thereby minimizing nonspecific binding). A second incubation with FITC-conjugated goat antimouse permitted the flow cytometric evaluation of serum antibody. As early as 7 days after priming, high levels of antibody were present in the serum of primed mice. A 1:10 serum dilution resulted in more than 95% positive binding in 4 of 5 mice at day 7, and all 5 mice at days 14 and 28 (Figure 1B). Even at a 1:160 dilution, serum from 11 of 15 primed mice bound more than 50% of BALB/c thymocytes (data not shown).

To determine how long one single priming event would preclude survival and engraftment, B6 mice were primed against BALB/c alloantigen and, after an interval of 28, 55, 83, or 111 days, were lethally irradiated and infused with BALB/c BM (Figure 1C). All primed mice, whether primed 28 or 111 days before BMT, died by 10 days after transplantation. Serum (1:10 dilution) collected just prior to BMT bound 97% to 99% of BALB/c thymocytes, indicating high levels of antidonor antibody in all mice (Figure 1D). The average mean fluorescent intensity (MFI) of serum binding to thymocytes generally declined over time, suggesting a gradual reduction in anti-BALB/c antibody levels albeit of insufficient magnitude to impact survival. At dilutions of 1:40, serum binding was high in all primed mice (97%, 96%, 81%, and 82% positive whether primed on days −28, −55, −83, or −111, respectively [data not shown]). Dilutions of 1:160 resulted in 80%, 84%, 42%, and 36% positive binding for mice primed on days −28, −55, −83, and −111, respectively (data not shown). In a subsequent experiment, sera collected 6 months after priming showed an average of 47% positive binding at a 1:160 dilution (n = 5, range of 20%-75% positive). These experiments indicated that a single priming event resulted in an early and long-lasting antibody response that would practically preclude waiting for waning antibody titer for successful transplantation.

**To determine the degree of priming specificity, B6 mice were primed against either BALB/c or B10.BR alloantigen on day −28, lethally irradiated on day −1, and infused with either BALB/c or B10.BR T-cell-depleted BM on day 0. Antibody-mediated BM rejection was not entirely alloantigen specific as B6 mice primed...**
against BALB/c also rejected B10.BR BM, albeit more slowly than BALB/c BM (Figure 2A). However, B6 mice primed against B10.BR rejected B10.BR BM but not BALB/c BM. All long-term survivors were more than 90% donor by PBL phenotyping at 3 months.

Examination of serum from primed mice indicated that BALB/c priming resulted in a more vigorous and broadly cross-reactive antibody response than did B10.BR priming, perhaps due to the multiple minor antigen differences as well as MHC disparities that exist between BALB/c and B6 mice but not between B10.BR and B6 mice. Serum from BALB/c-primed mice bound 93% of BALB/c BM, whereas serum from B10.BR-primed mice bound 66% of B10.BR BM, suggesting less robust priming (Figure 2B top panels). With regard to third-party BM, serum from BALB/c-primed mice bound 71% of B10.BR BM, whereas serum from B10.BR-primed mice bound only 37% of BALB/c BM (Figure 2B bottom panels).

Preformed antibody, not T cells, is the major barrier to engraftment in primed mice

To further study the role of humoral immunity in primed rejection, engraftment was compared in primed B6 WT versus B6 muMT (Ig heavy chain deleted, B-cell deficient) mice that are unable to make antibody. Cohorts of B6 WT or B6 muMT mice were primed with BALB/c splenocytes 28 days prior to 6.0 Gy TBI and infusion with BALB/c BM, and survival and chimerism were monitored (Table 1). All nonprimed B6 WT and muMT control mice receiving 40 to 200 $\times 10^6$ BM cells survived and engrafted (all > 95% donor by PBL phenotyping). Primed B6 WT mice receiving 40 $\times 10^6$ BM cells died of BM aplasia. Although increasing the BM dose to 200 $\times 10^6$ cells rescued 3 of 4 B6 WT mice from lethal aplasia, none of the 3 surviving mice had any evidence of donor chimerism. In contrast to the WT mice, all but one of the primed B-cell-deficient muMT mice survived, and all receiving a BM dose of at least 100 $\times 10^6$ cells had donor chimerism levels more than 95% (Table 1). A more modest BM cell dose (20 $\times 10^6$), a role for T-cell–mediated primed rejection was uncovered in muMT mice. Whereas all 8 unprimed muMT mice receiving 20 $\times 10^6$ BM cells engrafted, only 2 of 11 primed muMT mice had evidence of donor chimerism (Table 1). High BM cell dose abrogated primed rejection in muMT mice but not in WT mice, indicating that antibody, rather than primed T cells, was the major barrier to engraftment in primed WT mice.

If preformed antibody, per se, rather than primed B or T cells, was the major barrier to engraftment in the primed recipient, then a brief ex vivo incubation of donor BM with sera from mice primed against the donor alloantigen would effect elimination of the donor BM in a nonprimed recipient. To test this, BALB/c (or specificity control B10.BR) BM was incubated with diluted serum obtained from either naive B6 mice or BALB/c-primed B6 mice. After incubation, BM was washed thoroughly to remove free serum and unbound antibody and infused into lethally irradiated naive (ie, nonprimed) B6 mice.

Prior to infusion, an aliquot of BM was removed and incubated with FITC-conjugated goat anti–mouse Ig to determine the level of serum antibody that had bound to the donor BM. Incubation of BALB/c or B10.BR BM with serum obtained from unprimed, naive mice resulted in low-level binding likely due to the high percentage of FcR$^+$ cells in BM (Figure 3A). As expected, BALB/c BM indicating nonspecificity in the priming response. Figure 2B. Priming to one alloantigen can result in the elimination of third-party BM indicating nonspecificity in the priming response. (A) B6 mice were primed against BALB/c or B10.BR (BR) by the intraperitoneal administration of 20 $\times 10^6$ splenocytes, lethally irradiated on day – 1 (8.0 Gy), and infused with BALB/c or BR BM (20 $\times 10^6$) on day 0. Survival is shown. n = 8/group. (B) Serum was pooled from BALB/c- or B10.BR-primed B6 mice (n = 5) and incubated with BALB/c or B10.BR BM. Histograms illustrate binding of serum antibody to BM of priming strain (top panels) and third-party strain (bottom panels). Percentage positive binding is given. Negative control is shown by thin dashed line.

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<th>Table 1. In contrast with B6 WT, primed B-cell-deficient recipients engraft if given high donor BM cell doses</th>
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<td><strong>Recipient and no. BM cells</strong></td>
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<td><strong>B6 WT, 40 $\times 10^6$</strong></td>
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<td>Unprimed</td>
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<td><strong>B6 WT, 200 $\times 10^6$</strong></td>
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<td><strong>B6 muMT, 200 $\times 10^6$</strong></td>
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<td><strong>B6 muMT, 100 $\times 10^6$</strong></td>
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<td>Unprimed</td>
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<td>Primed</td>
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B6 WT or B6 muMT mice were primed on day – 28 with 20 $\times 10^6$ BALB/c splenocytes intraperitoneally, irradiated on day – 1 (6.0 Gy), and given non-T-cell-depleted BALB/c BM cells at the indicated number on day 0. Survival was monitored. PBLs were phenotyped 6 weeks after transplantation for donor chimerism. All engrafting mice were high-level donor chimeras (> 90%). Shown is percentage of mean group donor chimerism ± 1 SEM. NA indicates not available.

* $P < .01$ versus nonprimed control at same BM cell dose.
The kinetics of BM rejection in primed versus nonprimed mice is unknown. Imaging studies were performed to compare the kinetics of T-cell–mediated BM rejection in nonprimed mice to antibody-mediated BM rejection in primed mice. To image the fate of infused BM, the strain combination was switched to use B6 GFP donor BM and BALB/c recipient mice. Of 2 groups of BALB/c mice that were primed against B6 on day −28, one group received anti-CD4 and anti-CD8 mAbs and one did not. A nonprimed cohort received in vivo anti-CD4– and anti-CD8–depleting mAbs around the time of transplantation to ensure BM engraftment in one group of mice. BALB/c mice were sublethally irradiated (3.5 Gy TBI) to ensure that untreated, nonprimed mice would ultimately reject their BM graft. A moderate donor BM dose of 20 × 10^6 was chosen for imaging studies. Separate long-term chimerism studies substantiated the imaging model by verifying rejection in all primed and untreated nonprimed mice and high engraftment levels in all nonprimed mice receiving anti-CD4 and anti-CD8 mAbs (data not shown).

Initial imaging studies focused on days 1 (approximately 18 hours), 2, 3, 6, and 14 (Figure 4A-G and data not shown). Strikingly, antibody-mediated BM rejection in the primed mice was complete by 18 hours. Furthermore, primed mice, depleted of host T cells by the administration of anti-CD4 and anti-CD8 mAbs, also eliminated donor BM by 18 hours, indicating that acute BM rejection in primed mice was T-cell independent (data not shown). In contrast, T-cell–mediated rejection by nonprimed mice took more than 6 days. Untreated, nonprimed mice that would ultimately reject their BM graft showed equivalent numbers of GFP^+ cells in most organs as engrafting mice on days 1, 2, and 3 (Figure 4 and data not shown). In fact, untreated mice destined to reject their BM graft had increased numbers of GFP^+ cells in the spleen and Peyer patches on day 3 compared with mice that would ultimately accept their BM graft. By day 6, GFP^+ BM was reduced in mesenteric LNs, spleen (see patchy distribution of BM loss in Figure 4C), Peyer patches, and gut-associated lymphoid tissue in the ileum in nonprimed graft-rejecting mice, consistent with BM rejection by host T cells that were likely primed in the lymphoid organs. In contrast to the reduction of donor BM in lymphoid organs by day 6, GFP BM was increased in femoral BM cavity, liver, and lung of rejecting, nonprimed mice. However, by day 14, donor BM was absent in all organs of rejecting, nonprimed mice. These data indicated that T-cell rejection occurred first in lymphoid organs and, only later, in BM and parenchymal organs. That this loss of donor graft was T-cell mediated was evident by the increasing GFP^+ cell number in all organs in nonprimed mice that received anti-CD4 and anti-CD8 mAbs to ensure long-term stable engraftment.

Because antibody-mediated rejection of donor BM was complete by 18 hours, mice were imaged 3 hours after BMT. At this time, donor BM cells could be found in the Peyer patches, mesenteric LN, spleen, and lung of all 3 nonprimed mice (Figure 4H-I). In addition, 1 of 3 nonprimed mice had very few GFP^+ cells in the inguinal LN and femoral BM cavity (data not shown). In contrast, donor BM was found in only the lung of 1 of 3 primed mice and in no other organs, suggesting hyperacute BM elimination prior to initial organ homing events.

We hypothesized that if donor BM cells could be delivered directly to the bone marrow cavity they might bypass early
destruction by the RES system or a BM homing defect. To address this, BALB/c mice were primed against B6 alloantigen, lethally irradiated, and infused with B6 BM by either an intravenous or an intra-BM route of administration. Direct intra-BM infusion of donor BM did not abrogate rejection in primed recipients. Regardless of route of donor BM infusion, all primed mice died by 2 weeks (n = 10/group, data not shown). In contrast, all nonprimed controls, regardless of route of donor BM delivery, survived and were more than 99% donor by PBL phenotyping (data not shown).

**Fc receptor–expressing cells are required for antibody-mediated elimination of donor BM in primed mice**

Although antigen specificity is determined by a small part of the variable region of an antibody, effector functions are determined by the Fc portion of the antibody.29,30 To examine the role of host FcR+ cells in antibody-mediated BM rejection, mice deficient in the gamma chain subunit of the FcγRI, FcγRIII, FcγRV, and FcεRI receptors (B6.Fcγ1γ−/−) were primed against BALB/c, lethally irradiated, and infused with BALB/c BM. Despite having equivalent antibody levels to primed B6 WT mice, primed Fcε1γ−/− mice had a superior survival rate compared with primed WT mice (Figure 5A-B, 63% vs 0% survival, respectively; P < .001). In vivo TCD of primed WT or Fcε1γ−/− mice had no impact on survival in this experiment, indicating that the few deaths occurring in the Fcε1γ−/− mice were not the result of primed T-cell-mediated rejection. The residual activity might be due to complement-mediated lysis and/or phagocytic activity mediated by low-level FcγRI expression.31

**Engraftment can be achieved by the combination of mega-BM cell doses, in vivo TCD, and high-dose murine Ig**

The data indicated that preformed antibody was a formidable barrier to successful transplantation in the primed recipient. The near-complete elimination of a moderate BM cell dose by 3 hours after infusion allowed for no therapeutic window of intervention after transplantation. Waiting for antibody levels to decline might not be clinically feasible. A strategy of high BM cell dose, in vivo TCD, high-dose mIg, and splx was chosen for testing. Administration of depleting anti-CD4 and anti-CD8 mAbs was used to target T cells that would survive TBI conditioning. Our data indicated that a high BM cell dose abrogated primed T-cell rejection (Table 1). We hypothesized that in combination with other strategies, sufficient...
number of stem cells in a high BM cell dose might escape antibody-mediated elimination to permit engraftment. High-dose Ig, widely used in solid organ transplantation in highly sensitized individuals, has multiple immunomodulatory effects, including enhanced clearance of antibody, complement modulation, and inhibition of FcR-mediated clearance of cells. Splx was performed after priming but prior to transplantation in an attempt to reduce alloantibody levels and eliminate splenic RES clearance of antibody-coated BM cells that may occur despite FcR blockade with mIg. To test these strategies, B6 mice were primed on day −56 against BALB/c alloantigen. Splx was performed on cohorts of mice on day −28. High-dose mlg (20 mg/mouse) was administered on day −15 and day −7. Anti-CD4 and anti-CD8 mAbs were administered around the time of transplantation to ensure depletion of host T cells. (A) Overlay T-cell–depleted BM cells on day 0. A cohort received anti-CD4 and anti-CD8 mAbs in a second experiment. Each bold line represents a different mouse.

Discussion

More often used as therapy for hematologic malignancies, allogeneic hematopoietic cell transplantation (HCT) is also potentially curative for aplastic anemia and other hematologic malignancies. The results in mouse models indicate that antibody-mediated BM rejection in the primed recipient is dependent on host FcR+ cells. B6 and B6.Fcr1γ−/− mice were primed against BALB/c on day −28, lethally irradiated (8.0 Gy) on day −1, and infused with 2 × 10^6 BALB/c T-cell–depleted BM cells on day 0. A cohort received anti-CD4 and anti-CD8 mAbs around the time of transplantation to ensure depletion of host T cells.

Figure 5. Antibody-mediated BM rejection in the primed recipient is dependent on host FcR+ cells. B6 and B6.Fcr1γ−/− mice were primed against BALB/c on day −28, lethally irradiated (8.0 Gy) on day −1, and infused with 2 × 10^6 BALB/c T-cell–depleted BM cells on day 0. A cohort received anti-CD4 and anti-CD8 mAbs around the time of transplantation to ensure depletion of host T cells. (A) Overlay T-cell–depleted BM cells on day 0. A cohort received anti-CD4 and anti-CD8 mAbs in a second experiment. Each bold line represents a different mouse.

Table 2. Engraftment can be achieved in donor-primed mice by the combination of high numbers of bone marrow cells, in vivo TCD, and megadose mlg with or without splenectomy

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<tr>
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B6 recipients were primed on day −56 with 2 × 10^6 BALB/c splenocytes intraperitoneally. Splenectomy (Splx) was performed on day −28. Mouse Ig (20 mg) was administered intraperitoneally on day −15 and day −7. In vivo TCD was achieved by intraperitoneal administration of 400 μg anti-CD4 and anti-CD8 mAbs on days −2, −1, 0, 2, 4, and 7. Mice were lethally irradiated (8.0 Gy) on day −1 and infused with 100 × 10^6 BALB/c BM cells on day 0. All deaths occurred within 2 weeks after transplantation. All engrafted mice were more than 95% donor by peripheral blood leukocyte phenotyping 3 months after BMT.

*P < .01 versus primed, no treatment (primed, TCD, Ig vs primed, TCD, Ig, Splx; P = .32).

To determine the effect of mlg and mlg combined with splx on alloantibody levels, serum was obtained from mice 4 weeks after priming and then again just prior to irradiation. As expected, evaluation of serum 4 weeks after priming but prior to intervention strategies verified that all groups had equivalently high levels of antibody (not shown). Serum obtained just prior to transplantation demonstrated the effect of strategies on antibody level (Figure S1, available on the Blood website; see the Supplemental Figure link at the top of the online article). A 1:10 serum dilution resulted in more than 99% positive binding in primed controls, mlg-treated primed mice, and mlg-treated, splenectomized primed mice, indicating high levels of sera antibody in all mice, although there was a significant reduction in MFI from serum in mlg-treated, splenectomized primed mice compared with primed controls. Serum antibody from control, mlg-treated, and mlg-treated, splenectomized mice bound 97%, 93%, and 86% of cells at a 1:40 serum dilution and 79%, 60%, and 41% of cells at a 1:160 serum dilution, respectively (Figure S1). Although these data indicated that mlg (especially in combination with splx) reduced antibody levels, the reduction was unlikely to be of sufficient magnitude to account for the increased survival and engraftment seen in mice when used in conjunction with in vivo TCD. The higher engraftment rate in TCD, mlg-treated mice was more likely due to other known effects of mlg, including inhibition of Fe receptor–mediated clearance of antibody-bound cells and possibly, of the complement cascade.

Table 2. Engraftment can be achieved in donor-primed mice by the combination of high numbers of bone marrow cells, in vivo TCD, and megadose mlg with or without splenectomy
hemoglobinopathies. However, these patient populations often have been sensitized to alloantigens by multiple blood transfusions required for the palliative treatment of their disease that also may render them more resistant to BM engraftment-promotion strategies in the eventuality of allogeneic BMT. Increased rates of BM graft failure in sensitized patients have been attributed to priming of both the cellular and the humoral branches of the immune response. Although antibody-mediated acute rejection is a well-recognized problem in multiply-transfused individuals undergoing solid organ transplantation, it has been less well studied in the field of BMT.

Imaging data presented in this paper show for the first time that a moderate allogeneic BM dose was eliminated in the primed recipient by 3 hours after transplantation compared with more than 6 days in the naive recipient. These data graphically illustrate that preformed antibody is both the initial and most formidable barrier to successful BMT in the heavily allosensitized recipient. The speed of antibody-mediated BM rejection indicates there is no window of opportunity for clinical intervention after BMT and dictates the need for greater patient evaluation and more innovative therapeutic strategies prior to BMT, as standard conditioning and immunosuppressive agents given at the time of BMT are ineffective against preformed antibody.

Data indicating that host FcR+ cells played a major role in the elimination of donor BM in the primed recipient suggest a potential target for future preclinical studies.

ADCC, the cytotoxic destruction of antibody-coated target cells by FcR+ host cells, is triggered when antibody bound to the surface of a cell interacts with Fc receptors on NK cells or macrophages. Although NK cells are the most widely recognized mediators of ADCC, depletion of NK cells did not abrogate lethal aplasia in primed mice, indicating that other FcR+ cells are sufficient for this process (Figure 1A). Furthermore, primed host Ly49A transgenic (NK deficient) mice died of lethal aplasia with similar kinetics as primed WT mice, indicating that NK cells are not essential for antibody-mediated BM rejection (data not shown). In addition to directly targeted cytotoxicity, Fc-FcR engagement can destroy antibody-coated cells by engulfment and phagocytosis. Although the latter process should be absent in Fcer1γ−/− mice, recent work has demonstrated residual expression and function of the FcγR1 in these mice. Low-level activity of the FcγR1 could account for the lethality observed in some of the allogeneic FcγR1−/− mice. We cannot exclude a contribution of complement-mediated lysis in primed FcγR1−/− mice, although a primary role for complement-mediated lysis of antibody-coated BM cells seems unlikely as primed C3−/− mice died with similar kinetics as primed WT mice (data not shown).

Our data also indicate that continual exposure to high-titer circulating antibody is not required for the targeted elimination of antibody-coated donor BM. A brief ex vivo incubation of donor BM with serum from primed mice resulted in sufficient antibody coating of cells to mark them for destruction in nonprimed mice. Additionally, our data do not support approaches that might use intra-BM injection in allosensitized patients as direct intra-BM injection of donor BM did not rescue BM from destruction.

Of significant clinical relevance are those data that indicate that priming to one alloantigen can result in the elimination of donor BM of a different alloantigen (Figure 2A). BALB/c-primed B6 mice rejected third-party B10.BR BM and died of lethal aplasia. In contrast, B10.BR-primed B6 mice accepted BALB/c BM. In addition to being fully MHC disparate, B6 and BALB/c mice, unlike B6 and B10.BR mice, also differ at multiple minor antigens. As a result, BALB/c priming, but not B10.BR priming, of B6 mice resulted in a more vigorous and broadly cross-reactive antibody response that culminated in the rejection of third-party BM. These data imply that not only specific, but also cross-reactive or broadly reactive, alloantibodies found in a multiply-transfused individual might target that individual for BM rejection. Additionally, the highly proinflammatory milieu surrounding conditioning and transplantation may also contribute to the antigen-nonspecific activation of host macrophages and other FcR+ cells potentially increasing the efficiency of elimination of antibody-coated BM cells. Of potential clinical interest, although intraperitoneally and intravenously nonirradiated splenocytes appeared to result in equivalent priming, irradiated splenocytes were less effective at priming than nonirradiated splenocytes (P.A.T., unpublished data, November 2003).

Despite the formidable barrier presented by a single priming event, near-complete donor chimerism was achieved in a significant proportion of the mice by a combination of high BM cell number, high-dose mlg, and in vivo TCD. This triad of intervention perhaps along with pre-BMT plasmapheresis and immunoadsorption to reduce preformed antibody levels as used in allosensitized recipients of solid organ grafts should warrant consideration as strategies to specifically target antibody-mediated acute BM rejection in sensitized patients known to have antidonor reactive alloantibodies before BMT.

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Authorship

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References


Preformed antibody, not primed T cells, is the initial and major barrier to bone marrow engraftment in allosensitized recipients

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