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Minor Antigen H60-Mediated Aplastic Anemia Is Ameliorated by Immunosuppression and the Infusion of Regulatory T Cells

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Minor Antigen H60-Mediated Aplastic Anemia Is Ameliorated by Immunosuppression and the Infusion of Regulatory T Cells

Jichun Chen,^{1*} Felicia M. Ellison,* Michael A. Eckhaus,[†] Aleah L. Smith,* Keyvan Keyvanfar,* Rodrigo T. Calado,* and Neal S. Young*

Human bone marrow (BM) failure mediated by the immune system can be modeled in mice. In the present study, infusion of lymph node (LN) cells from C57BL/6 mice into C.B10-*H2^b/LilMcd* (C.B10) recipients that are mismatched at multiple minor histocompatibility Ags, including the immunodominant Ag H60, produced fatal aplastic anemia. Declining blood counts correlated with marked expansion and activation of CD8 T cells specific for the immunodominant minor histocompatibility Ag H60. Infusion of LN cells from H60-matched donors did not produce BM failure in C.B10 mice, whereas isolated H60-specific CTL were cytotoxic for normal C.B10 BM cells *in vitro*. Treatment with the immunosuppressive drug cyclosporine abolished H60-specific T cell expansion and rescued animals from fatal pancytopenia. The development of BM failure was associated with a significant increase in activated CD4⁺CD25⁺ T cells that did not express intracellular FoxP3, whereas inclusion of normal CD4⁺CD25⁺ regulatory T cells in combination with C57BL/6 LN cells aborted H60-specific T cell expansion and prevented BM destruction. Thus, a single minor histocompatibility Ag H60 mismatch can trigger an immune response leading to massive BM destruction. Immunosuppressive drug treatment or enhancement of regulatory T cell function abrogated this pathophysiology and protected animals from the development of BM failure. *The Journal of Immunology*, 2007, 178: 4159–4168.

Aplastic anemia (AA),² paroxysmal nocturnal hemoglobinuria, and myelodysplastic syndrome are typical bone marrow (BM) failure syndromes featuring severe destruction of hemopoietic stem cells (HSCs) and progenitor cells. Patients often have empty BM cavity and suffer the consequences of severe anemia, leukopenia, and thrombocytopenia (1, 2). Activation of T cells is believed to be the major proximal event responsible for the destruction of HSCs and progenitors, and immunosuppressive therapies now routinely used are effective in the majority of patients who have AA and AA with paroxysmal nocturnal hemoglobinuria and in a subset of cases of myelodysplastic syndrome (1, 3). In published work from our laboratory and other published reports, only a limited number of T cell clones, as defined by usage of the CDR3 of the TCR β -chain, appear to be active in BM failure patients (4–7); however, the exact etiology of BM failure, especially the specific Ag or Ags that initiate the immune response, is unknown.

One specific type of AA is the transfusion-related BM failure that shares the pathophysiological mechanism with other diseases: allogeneic lymphocytes contained in the infused cell population attack and destroy various host cells in the form of graft-vs-host (GVH) responses (8–18). Transplanting cells from donors to recipients that differ at MHC generates MHC-mediated GVH re-

sponses in which reaction to thousands of novel MHC-presented peptides results in the activation and expansion of a large number of clonally diverse CD8 T cells, causing fatal injury to a broad range of tissues and organs (12, 14, 19). Previously we produced a transfusion-associated BM failure model by the infusion of lymph node (LN) cells from C57BL/6 mice (B6) into B6D2F₁ and CByB6F₁ recipients using donor-recipient MHC disparity (20, 21).

In human BM transplantation, patients receiving MHC-matched BM from related or unrelated donors are likely to have multiple minor histocompatibility (minor H) Ag differences, which provide epitopes capable of triggering expansion of peptide-restricted T cell clones. Mismatch at minor H Ags can provoke severe immune responses against host cells upon transplantation in clinical settings as well as in experimental models (10, 11, 15–17, 22–25). In one minor H Ag-mismatched cell transplantation model, the infusion of B6 lymphocytes to C.B10-*H2^b/LilMcd* (C.B10) recipients caused restricted T cell expansion of specific V β subfamilies (24, 26) with immunodominant effects against H60 (10, 11, 22), a minor H Ag that interacts with both H2K^b and NKG2D receptors (27, 28). Experimental data indicate, though, that an immunodominant epitope for CD8 T cells, such as H60, cannot elicit a response in the absence of CD4 T cells responding to another helper cell epitope (10, 11, 22).

In the current study, we specifically investigated the role of the minor H Ag H60 in the induction of BM failure by infusing B6 LN cells into H60-mismatched C.B10 recipients. Indirect and direct experimental data indicate that H60-specific CTLs contribute to the severe marrow destruction leading to fatal pancytopenia. Treatment with immunosuppression or with administration of CD4⁺CD25⁺ regulatory T cells (Tregs) abrogated expansion of T cell clones specific for the H60 Ag and effectively prevented the development of BM failure.

Materials and Methods

Mice and cell infusion

Inbred C57BL/6 (B6, *H2^{b/b}*), congenic B6^{CD45.1/CD45.1}, and congenic C.B10-*H2^b/LilMcd* (C.B10, *H2^{b/b}*) mice were purchased from The Jackson Laboratory, whereas congenic B6-H60 mice were provided by Dr. D.

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² Abbreviations used in this paper: AA, aplastic anemia; BM, bone marrow; GVH, graft-vs-host; HSC, hemopoietic stem cell; LN, lymph node; minor H, minor histocompatibility; TBI, total body irradiation; Treg, regulatory T cell.

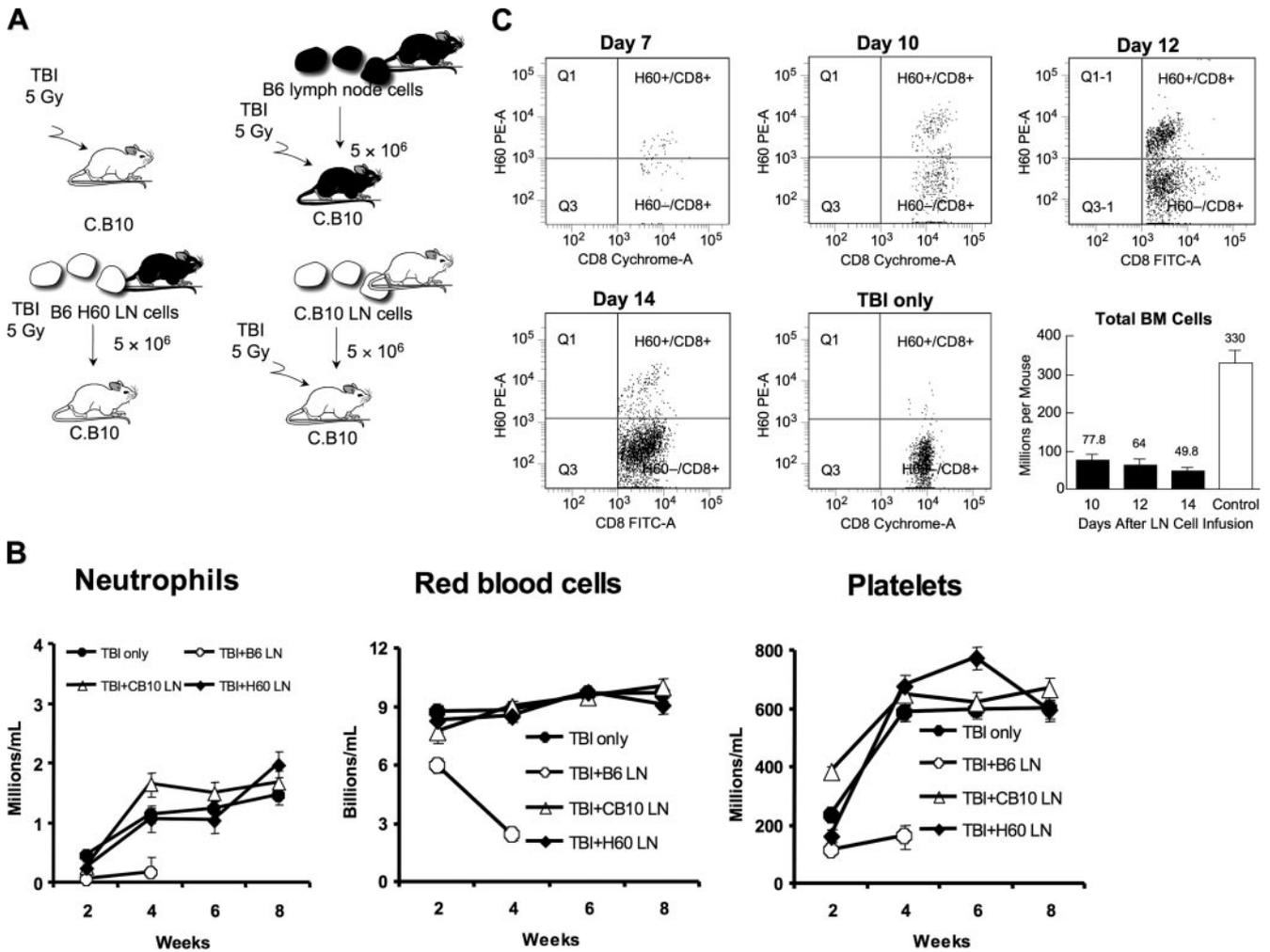


FIGURE 1. Minor H Ag H60-mediated fatal pancytopenia. C.B10 congenic mice were sublethally irradiated with 5 Gy TBI ($n = 7$) (TBI only) and were then injected in the tail vein with 5×10^6 LN cells from B6 ($n = 9$) (TBI+B6 LN), C.B10 ($n = 3$) (TBI+CB10 LN), or B6-H60 congenic ($n = 3$) (TBI+H60 LN) donors. **A**, Animals that received TBI only, TBI+CB10 LN, and TBI+H60 LN treatments survived normally, whereas mice that received TBI+B6 LN treatment were moribund within 2–5 wk. **B**, Complete blood counts revealed pancytopenia in mice from the mice treated with TBI plus B6 LN cells, which all died within 5 wk, whereas mice that received TBI only, TBI plus CB10 LN, and TBI plus H60 LN treatments had blood counts that returned close to normal by 6 wk. BM cells obtained at days 0, 7, 10, 12 and 14 ($n = 2$) for each time point) from mice ($n = 2$) that received TBI plus B6 LN treatment showed an increasing presence of CD8⁺ T cells specific for minor H Ag H60 based on a PE-labeled H60 tetramer staining and flow cytometer analysis, concurrent with loss of total BM cells (**C**).

Roopenian (The Jackson Laboratory, Bar Harbor, ME). All mice were bred and maintained at National Institutes of Health animal facilities under standard care and nutrition. Male and female mice were used at 6–16 wk of age. All animal study protocols were approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee.

Inguinal, brachial, and axillary LNs were obtained from B6, C.B10, and B6-H60 mice and were homogenized, counted, and infused into sublethally irradiated (5 Gy total body irradiation (TBI)) C.B10 mice at 5×10^6 cells per mouse. Untreated control mice, mice that received TBI only, and mice that received TBI plus 5×10^6 LN cells from B6 (TBI+B6 LN), C.B10 (TBI+CB10 LN), or B6-H60 (TBI+H60 LN) donors were analyzed at different time points as specified in each experiment.

Cell analyses and flow cytometry

Complete blood counts were performed using a Hemavet 1700 analyzer (Drew Scientific). BM cells were extracted from tibiae and femurs of each mouse, filtered through a 90- μ m nylon mesh, and counted by a ViaCell counter (Beckman Coulter). PBL and BM cells were analyzed by flow cytometry as previously described (21). mAbs for murine CD3 (clone 145-2C11), CD4 (clone GK 1.5), CD8 (clone 53-6.72), CD11a (clone 2D7), CD11b (clone M1/70), CD19 (clone ID3), CD25 (clone 3C7), CD34 (clone RAM34), CD45R (B220, clone RA3-6B2), CD95 (Fas, clone Jo2), CD117 (c-Kit, clone 2B8), erythroid cells (clone Ter119), granulocytes (Gr1/Ly6-G, clone RB6-8C5), and stem cell Ag 1 (*Sca1*, clone E13-161) were all

from BD Biosciences and were conjugated to FITC, PE, CyChrome, PE-Cyanin 5, biotin, or allophycocyanin. Streptavidin-conjugated Quantum red was from Sigma-Aldrich. An H60-specific peptide LTFNYRLN was synthesized, folded to MHC class I to form the H60 tetramer, and then conjugated to PE (Baylor College of Medicine). Stained cells were analyzed using a LSR II flow cytometer (BD Biosciences).

Pathology

Two untreated, two TBI only-treated, and six TBI plus B6-treated C.B10 mice underwent euthanasia at day 14 after LN cell infusion. Mice were examined for gross pathological changes in various organs/tissues. Sternebrae were fixed in 10% neutral buffered formalin and embodied, sectioned, and H&E stained. Slides were viewed using an Olympus IX50 microscope (Optical Elements), and photographic images of BM morphology were captured at $\times 400$ magnification using a SPOT INSIGHT camera with the SPOT version 4.0.8 software. Two TBI only-treated mice were studied at 4 mo after the date they received sublethal TBI as comparisons.

Fluorogenic cytotoxicity assay

BM cells from three C.B10 mice that had received treatment with TBI plus LN cell infusion from B6 mice 14–21 days earlier were extracted and counted. One aliquot of each BM sample was directly used as effector cells in the cytotoxicity assay. Another aliquot of each BM sample was stained with CD8-FITC and H60 tetramer-PE, and was sorted for H60-specific

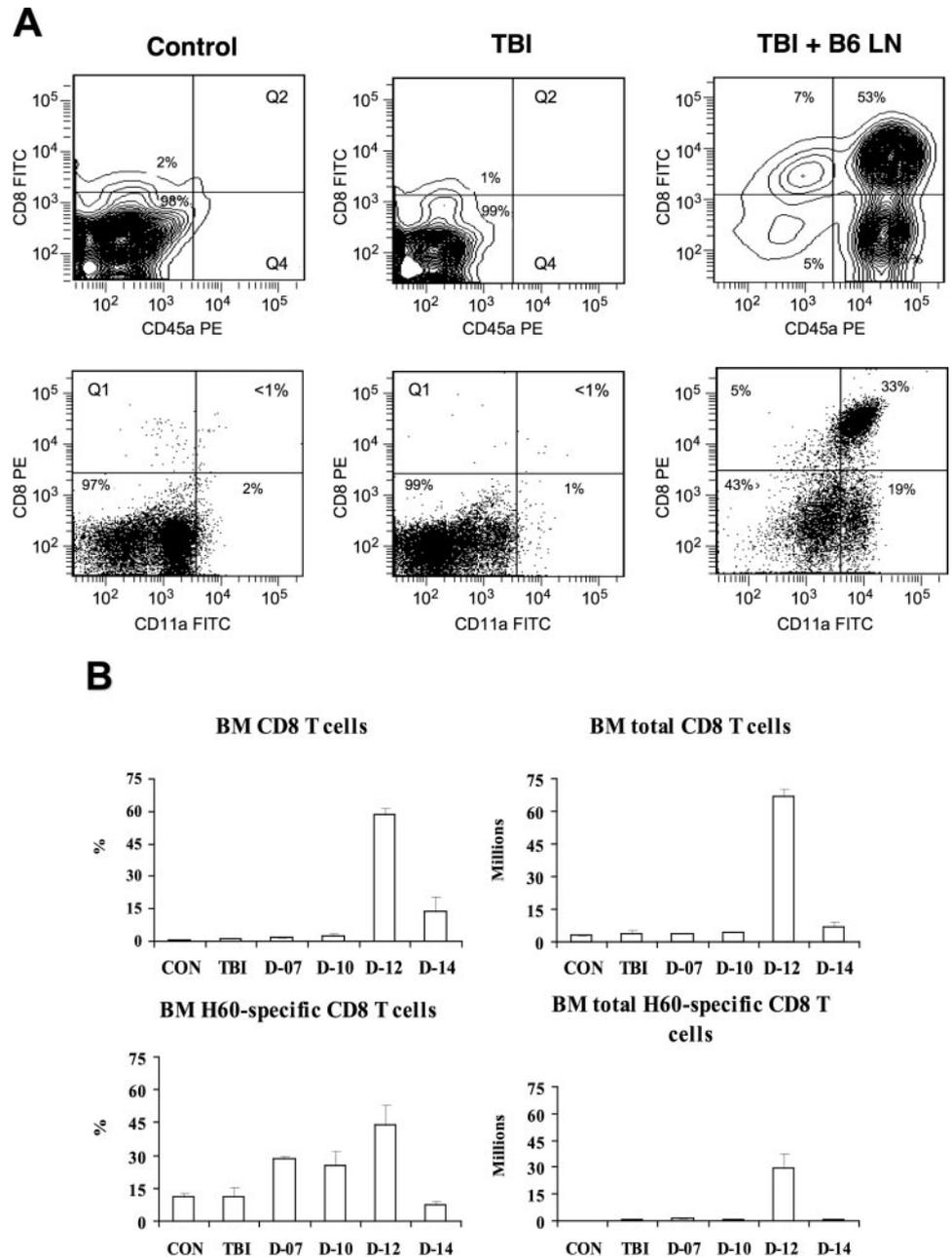


FIGURE 2. Infiltration, expansion, and activation of CD8 T cells, especially H60-specific CD8 T cells, in the BM of affected C.B10 mice. Untreated C.B10 controls and mice that received TBI only or TBI plus B6 LN treatments were euthanized at 2 wk after treatment. *A*, BM cells from mice that received TBI plus B6 LN treatment showed drastic infiltration and expansion of CD8⁺ T cells in the BM that were of the CD45a donor genotype in comparison to BM from control and TBI only mice. The infiltrated/expanded donor T cells were all activated showing high level CD11a expression. Data shown were representative of mice ($n = 3-8$) analyzed for each group. *B*, BM cells obtained at days 0, 7, 10, 12, and 14 ($n = 2$ for each time point) from mice that received TBI plus B6 LN treatment showed an increased proportion and total number of CD8⁺ T cells and H60-specific CD8⁺ T cells in comparison to untreated control ($n = 4$) and TBI only ($n = 4$) mice.

CD8 T cells to be used as effector cells. BM cells from an untreated normal C.B10 mouse were used as targets. Cytotoxicity was assayed using the CyToxiLux PLUS kit (OncoImmune) according to the manufacturer's instructions. In brief, normal C.B10 BM targets were first labeled with the red fluorescence dye at 37°C for 30 min and then dispensed into 96-well culture plates; each well contained 5×10^4 labeled target cells mixed with 10×10^5 TBI plus B6-treated BM cells as effectors (E:T ratio is 20:1), or 10^3 labeled target cells mixed with 10×10^3 H60-specific CD8 T cells as effectors (E:T = 10:1). The effector to target cell mixtures were incubated at 37°C for 60 min and then incubated with caspase substrate for the detection of cell apoptosis by LSR-II flow cytometry. Wells containing only target or effector cells were used as controls.

Immunosuppressive therapy

Nine C.B10 mice all received 5 Gy TBI and were divided into three groups: 1) infused with 5×10^6 B6 LN cells plus treatment with cyclosporine (Sandoz Pharmaceuticals) at $50 \mu\text{g}/\text{g}/\text{day}$ for 5 days injected i.p. beginning 1 h after LN cell infusion; 2) infused with 5×10^6 B6 LN cells with no cyclosporine treatment; and 3) no LN cell infusion and no cyclosporine treatment as controls. Animals were monitored two to three times per week, and surviving animals were bled at 2, 4, and 9 wk after LN cell

infusion for blood cell analyses. In another experiment, six C.B10 mice that received 5 Gy TBI plus a 5×10^6 B6 LN cell infusion were divided into two groups of three animals each, with one group receiving $50 \mu\text{g}/\text{g}/\text{day}$ cyclosporine treatment for 5 days to observe for animal survival. Results from these two experiments were combined.

Effects of Tregs

We first examined the presence of Tregs in the BM of C.B10 mice that received treatment with TBI only, TBI plus B6, or TBI plus LN cells from B6-H60 mice using flow cytometry analysis. Rat anti-mouse FoxP3 Ab (clone FJK-16s) was obtained from eBioscience, in combination with CD4 and CD25 Abs from BD Biosciences as described earlier. We isolated CD4⁺CD25⁺ Tregs from spleen and thymus of normal C.B10 mice or from the spleen of normal B6 donors using the FACS Vantage flow cytometer. In the first experiment, two C.B10 mice received TBI plus LN cells from B6 mice, two C.B10 mice received TBI plus LN cells from B6 mice with 2×10^3 CD4⁺CD25⁺ Tregs from C.B10 spleen, and three C.B10 mice received TBI plus LN cells from B6 mice with 15×10^3 CD4⁺CD25⁺ Tregs from C.B10 thymus. Mice were euthanized at 3 wk after treatment and BM cells were counted to evaluate marrow failure. In a second experiment, six C.B10 mice that received 5 Gy TBI and infusion of

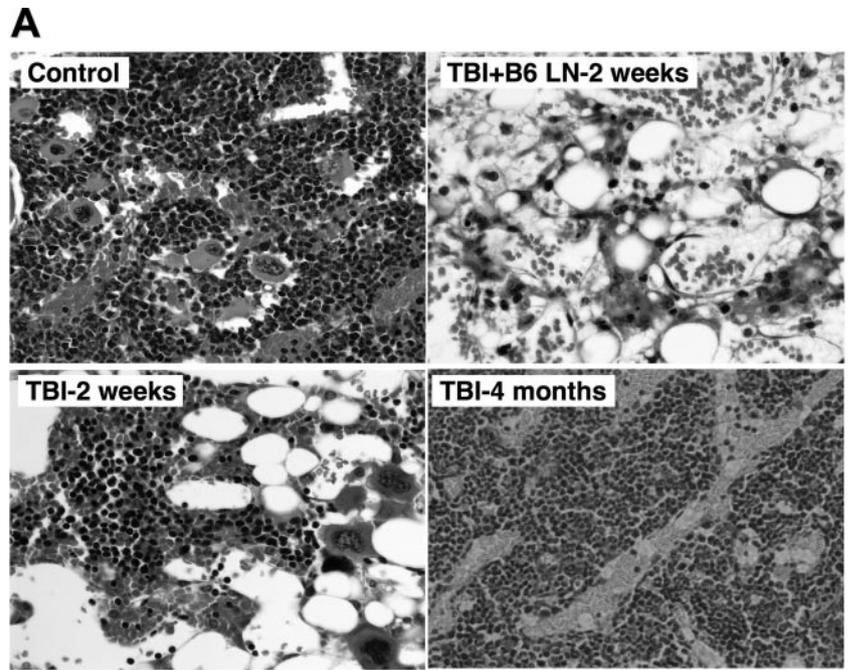
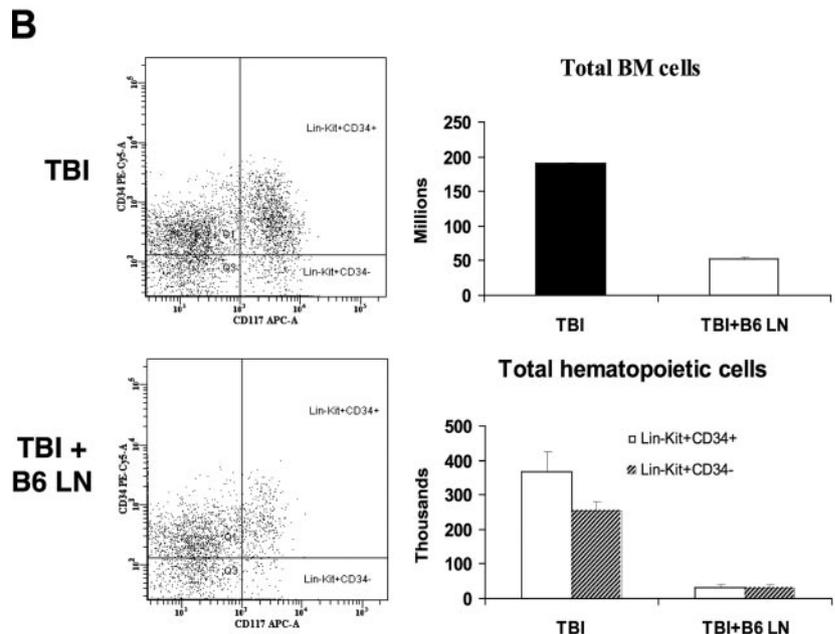


FIGURE 3. BM failure caused by infusion of allogeneic B6 LN cells. **A**, Sternebrae from untreated control mice ($n = 2$), mice that received TBI only ($n = 2$), or mice that received TBI plus B6 LN ($n = 6$) treatment were sectioned, H&E stained, and photographed at day 14. Sternebrae from TBI only-treated mice ($n = 2$) were also analyzed at 4 mo after irradiation. C.B10 mice that received TBI only ($n = 3$) or TBI plus B6 LN ($n = 5$) treatment were euthanized to analyze total BM cells per mouse, assuming that two tibiae and two femurs contain 25% of total BM cells. **B**, The proportion and total number of Lin⁻Kit⁺CD34⁻ and Lin⁻Kit⁺CD34⁺ HSCs and progenitor cells were analyzed by flow cytometry and were drastically reduced in mice that received TBI plus B6 LN treatment. Data shown are from three to five mice used in each group.



5×10^6 B6 LN cells were divided into two groups: two of the six mice received 5×10^3 sorted CD4⁺CD25⁺ Tregs while the other four mice did not. All cells were i.v. injected through the tail vein. Recipient blood cell counts were measured at 2 and 3 wk. All recipients were euthanized at 3 wk. Residual BM cells from each mouse were counted and were analyzed by flow cytometry to detect T cell expansion and the presence of H60-specific T cells.

Data analysis

Blood and BM cell composition data were analyzed by JMP Statistical Discovery software using different ANOVA models (SAS Institute) (29). Data were the mean with SE bars. Statistical significances were shown at values for $p < 0.05$ and $p < 0.01$ levels.

Results

Minor H Ag H60-mediated fatal pancytopenia

Injection of 5×10^6 B6 LN cells to sublethally irradiated C.B10 mice (TBI+B6 LN) caused fatal pancytopenia in which animals

became moribund within 2–5 wk (Fig. 1A) with significant declines ($p < 0.01$) in peripheral blood neutrophils and white blood cells, RBCs, and platelets (Fig. 1B) in comparison to mice that received TBI only or TBI plus 5×10^6 autologous C.B10 LN cells (TBI+CB10 LN). Because B6 and C.B10 mice are matched at the MHC but differ in multiple minor H Ags, we specifically tested the role of minor H Ag H60; mice that received TBI plus 5×10^6 B6-H60 LN cells (TBI+H60 LN) also survived, and their blood cells recovered to levels similar to levels found in TBI only control mice, indicating that H60 disparity was essential in the induction of fatal pancytopenia (Fig. 1, A and B).

To further characterize the role of H60 in immune-mediated pancytopenia, we examined the presence of H60-specific T cells in recipient mice using an H60 MHC-peptide tetramer (H60 tetramer) in flow cytometry. H60-specific CD8 T cells were detectable in the blood, spleen, and BM of B6 LN cell-infused C.B10 recipients. In

the BM, the proportion of H60-specific CD8 T cells was $29 \pm 1.4\%$ at day 7, $25 \pm 6.8\%$ at day 10, $44 \pm 9.5\%$ at day 12, and $7.2 \pm 1.8\%$ at day 14 (Fig. 1C). In the same period of time, affected mice showed progressive loss of BM cells from days 7 to 14 (Fig. 1C). The expansion of H60-specific CTLs and the elimination of recipient BM cells was concurrent with significant increases in the proportion of CD8 T cells in the recipient animals' marrow (Fig. 2A). Expanded cells were of donor origin, as evident by their CD45a genotype when CD45a congenic B6 mice were used as LN cells donors. The expanded lymphocytes were also activated, showing expression of the T cell activation mark CD11a (Fig. 2A). The significant increase in the proportions of CD8 T cells and H60-specific CD8 T cells resulted in a calculated 18- to 21-fold increase in BM CD8 T cells and a 50- to 80-fold increase in BM H60-specific CD8 T cells in comparison to untreated control or TBI only-treated mice (Fig. 2B). Specifically, total H60-specific CD8 T cells increased from 1.14 ± 0.14 million per mouse at day 7 to 29.7 ± 8.1 million per mouse at day 12 (Fig. 2B).

Destruction of BM HSCs and progenitor cells

The severity of pancytopenia and the lethal nature of the TBI plus B6 cell treatment implicated an immune response that attacked and destroyed BM HSC and progenitor cells. Examination of sternbrae sections from untreated control C.B10 mice showed normal cellularity with marrow cavity full of nucleated cells including typical hemopoietic precursor cells such as megakaryocytes (Fig. 3A). In mice treated with TBI only, sternbrae showed moderate loss of BM cellularity at 14 days but BM cellularity recovered spontaneously and appeared normal in marrow 4 mo later (Fig. 3A). In contrast, mice that received treatment with TBI plus LN cells from B6 mice developed severe BM atrophy at 14 days, with marrow cavity empty of hemopoietic precursor cells (Fig. 3A). When residual BM cells were stained and analyzed by flow cytometry, mice that received TBI only treatment had ~ 200 million total BM cells per mouse at 14 days following treatment of which there were high proportions and a total number of $\text{Lin}^- \text{CD117}^+ \text{CD34}^+$ and $\text{Lin}^- \text{CD117}^+ \text{CD34}^-$ HSCs and progenitor cells (Fig. 3B). In contrast, mice that received TBI plus LN cells from B6 mice treatment had significant loss of total BM cells of which the proportions and total number of $\text{Lin}^- \text{CD117}^+ \text{CD34}^+$ and $\text{Lin}^- \text{CD117}^+ \text{CD34}^-$ hemopoietic progenitor cells and HSCs reduced even further (Fig. 3B). The massive destruction of BM cells, especially BM HSCs and hemopoietic progenitor cells, is the key to the development of fatal pancytopenia.

H60-specific CD8 T cell-mediated cytotoxicity

The concurrence of H60-specific CD8 T cell expansion and massive BM loss implicated activated CD8 T cells, especially those specific for the known dominant minor H Ag H60, as the effectors responsible for the development of BM failure. To directly test the effectiveness of activated T cells, we obtained residual BM cells from TBI plus B6 LN cell-treated mice, which contain a large proportion of activated H60-specific CD8 T cells, and used them as potential effectors in an in vitro fluorescence cytotoxicity assay, with normal C.B10 BM cells as targets. We also separated by cell sorting H60-specific CD8 T cells for testing as effectors in the same assay. At an E:T ratio of 20:1, residual BM cells from affected mice caused normal C.B10 BM cell targets to undergo apoptosis, as evidenced by activation of the caspase pathway. Isolated H60-specific CD8 T cells also functioned as effectors and induced apoptosis in C.B10 BM target cells (Fig. 4).

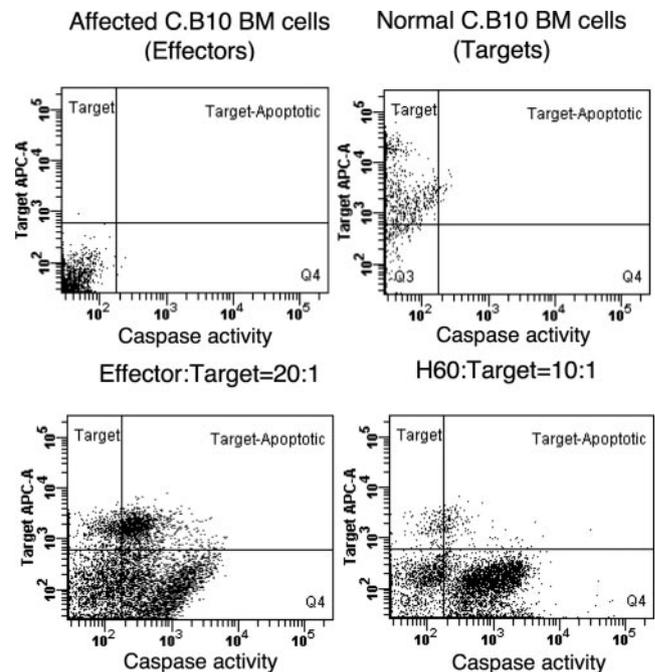


FIGURE 4. H60-specific T cell cytotoxicity in vitro. BM cells from CB10 mice that developed BM failure after TBI plus B6 LN treatment were obtained 14–21 days later and were used as effectors and mixed with normal C.B10 BM cell targets at an E:T ratio of 20:1, or were stained and sorted for H60-specific CD8 T cells as effectors and mixed with C.B10 BM cell targets at 10:1 ratio in a CytoxiLux PLUS cytotoxicity assay. Mixed effector and target cells were incubated at 37°C for 60 min, and cell apoptosis was measured by caspase substrate cleavage and was detected by flow cytometry. BM cells from TBI plus B6 LN cell-infused mice showed obvious cytotoxicity by inducing cell apoptosis in normal C.B10 BM cells targets. Isolated H60-specific CD8 T cells also showed cytotoxicity against normal C.B10 BM cell targets in the same assay. Data shown are representative of three independent studies.

Effectiveness of immunosuppressive therapy with cyclosporine

Because a large portion of human BM failure patients respond to immunosuppressive therapy, we next tested the effectiveness of immunosuppression in the prevention of H60-mediated BM failure. Sublethally irradiated C.B10 mice were injected with 5×10^6 B6 LN cells with or without the treatment of cyclosporine, a drug widely used in the treatment of human acquired AA, at a dose of 50 $\mu\text{g}/\text{g}/\text{day}$ i.v. injected once per day for 5 days starting from the same day of LN cell infusion. Mice that received TBI plus B6 LN cell infusion without cyclosporine had significantly ($p < 0.01$) lower levels of white blood cells, RBCs, and platelets at 2 and 4 wk in comparison to control mice and mice that received TBI plus B6 LN cell infusion with cyclosporine treatment (Fig. 5A). At day 21, mice that received TBI plus B6 LN infusion without cyclosporine treatment had $\sim 7\%$ H60-specific CD8 T cells in the blood, whereas mice that received TBI plus B6 LN infusion with cyclosporine had essentially no H60-specific CD8 T cells (Fig. 6B). All six mice that received TBI plus B6 LN infusion with cyclosporine treatment survived to 10 wk and beyond, whereas the six mice that received TBI plus B6 LN cell infusion without cyclosporine died off gradually starting from week 2 and were all dead by week 5 with a mean survival length of 23 days (Fig. 5B). Cyclosporine prevented H60-specific T cell expansion and effectively rescued animals from fatal pancytopenia.

The role of $\text{CD4}^+ \text{CD25}^+$ Tregs in BM failure

In recent human observations, patients with AA have a profound deficit in Tregs. To assess the role of Tregs in the development of BM failure in the mouse model, we first measured the proportion

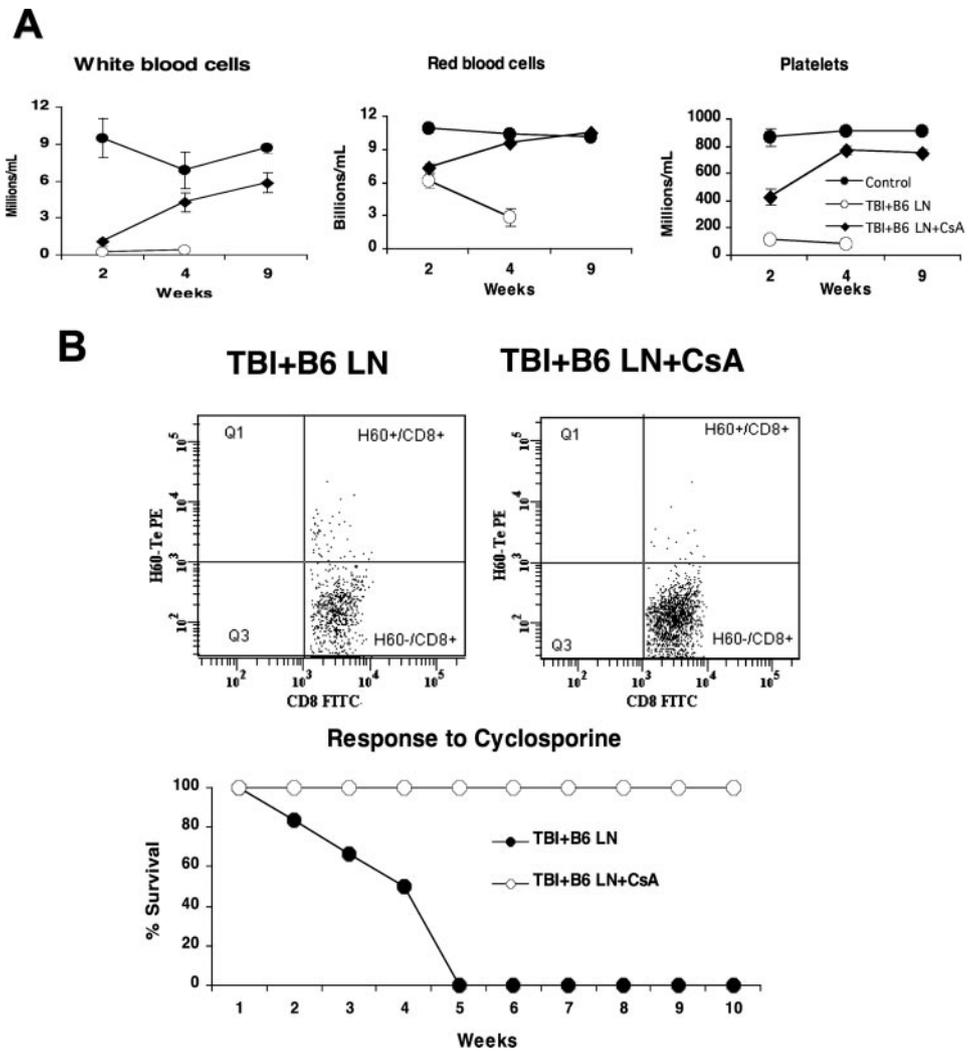


FIGURE 5. Therapeutic effects of the immunosuppressive drug cyclosporine. **A**, Peripheral white blood cells, RBCs, and platelets were drastically reduced in mice that received TBI plus B6 LN (TBI+B6 LN) treatment ($n = 6$) when they were alive. All these three blood cell types recovered between 2 and 9 wk in mice that received TBI plus B6 LN infusion with cyclosporine treatment ($n = 6$) at $50 \mu\text{g/g/day}$ for 5 days injected i.p. beginning 1 h after LN cell infusion. **B**, At 21 days after TBI plus B6 LN treatment, C.B10 mice had 5–7% H60^+ CD8 T cells in peripheral blood, whereas mice that received TBI plus B6 LN infusion with cyclosporine had $>1\%$ H60^+ CD8 T cells in peripheral blood. C.B10 mice that received TBI plus B6 LN treatment died off between 2 and 5 wk with an average survival of 23 days, whereas mice that received TBI plus B6 LN infusion with cyclosporine treatment all survived to 10 wk and beyond.

of $\text{CD4}^+\text{CD25}^+$ T cells and their expression of intracellular transcription factor FoxP3. Mice that received TBI plus B6 LN treatment showed a significant ($p < 0.01$) increase in the proportion of $\text{CD4}^+\text{CD25}^+$ T cells in their residual BM in comparison to mice that received TBI only or mice treated with TBI plus H60 LN cells (Fig. 6, *A* and *B*). However, when we examined intracellular FoxP3 expression, $\text{CD4}^+\text{CD25}^+$ T cells from TBI plus B6 LN-treated mice had a very large proportion of FoxP3⁻ cells (Fig. 6*A*), resulting in a significantly ($p < 0.01$) decreased FoxP3⁺ to FoxP3⁻ ratio (Fig. 6*B*). Because only $\text{CD4}^+\text{CD25}^+$ FoxP3⁺ T cells are considered Tregs, we then calculated Treg to CD4 and Treg to CD8 T cell ratios and found that both ratios were significantly reduced ($p < 0.05$ and $p < 0.01$, respectively) in TBI plus B6 LN-treated mice in comparison to TBI only or TBI plus H60 LN-treated mice (Fig. 6*C*). Because the expanded CD4 and CD8 T cells were mostly activated in TBI plus B6 LN-treated mice, as described (Fig. 2*A*), our data indicate that a drastically reduced Treg to activated T cell ratio led to imbalance between T cell activation and T cell suppression and was possibly contributory in producing BM failure.

To further test the functional role of Tregs, we sorted $\text{CD4}^+\text{CD25}^+$ T cells from the spleen and thymus of the autologous C.B10 mice or from the spleen of allogeneic B6 mice and then transfused them into TBI-treated C.B10 mice in combination with LN cell infusion. TBI plus B6 LN-treated mice that received 2×10^3 splenic or 15×10^3 thymic autologous Tregs had significantly

more residual BM cells than did TBI plus B6 LN-treated mice that did not receive Tregs (Fig. 7*A*). Thus, small a number of autologous Tregs protected against immune-mediated BM failure. In a second experiment, we specifically tested the effects of allogeneic Tregs. In comparison to TBI plus B6 LN cell-infused mice without Treg treatment, which became moribund after 2–3 wk, mice that received TBI plus B6 LN cell infusion with 5×10^3 Tregs from B6 spleen appeared normal (Fig. 7*B*). When residual BM was analyzed at 3 wk after LN cell infusion, mice without Treg injection had a significantly higher CD8^+ T cell proportion (47%) with the majority (70%) being H60-specific T cells (Fig. 7*B*), whereas coinjection of 5×10^3 Tregs with B6 LN cells significantly inhibited donor lymphocyte infiltration/expansion in the recipient BM, evident by a very low proportion of CD8 T cells in the BM (2%), of which only 8% stained positively with H60 tetramer (Fig. 7*B*). In comparison to TBI plus B6 LN-treated mice without Tregs, mice that received the injection of Tregs had significantly ($p < 0.05$) higher levels of residual BM cells and higher concentrations of white blood cells, RBCs, and platelets (Fig. 7*C*). Thus, as few as 5×10^3 allogeneic Tregs produced significant protective effects against immune-mediated BM failure.

Discussion

Infusion of lymphocytes into MHC-mismatched or minor H Ag-mismatched recipients has been used to induce GVH responses in various murine models under which allogeneic BM cells,

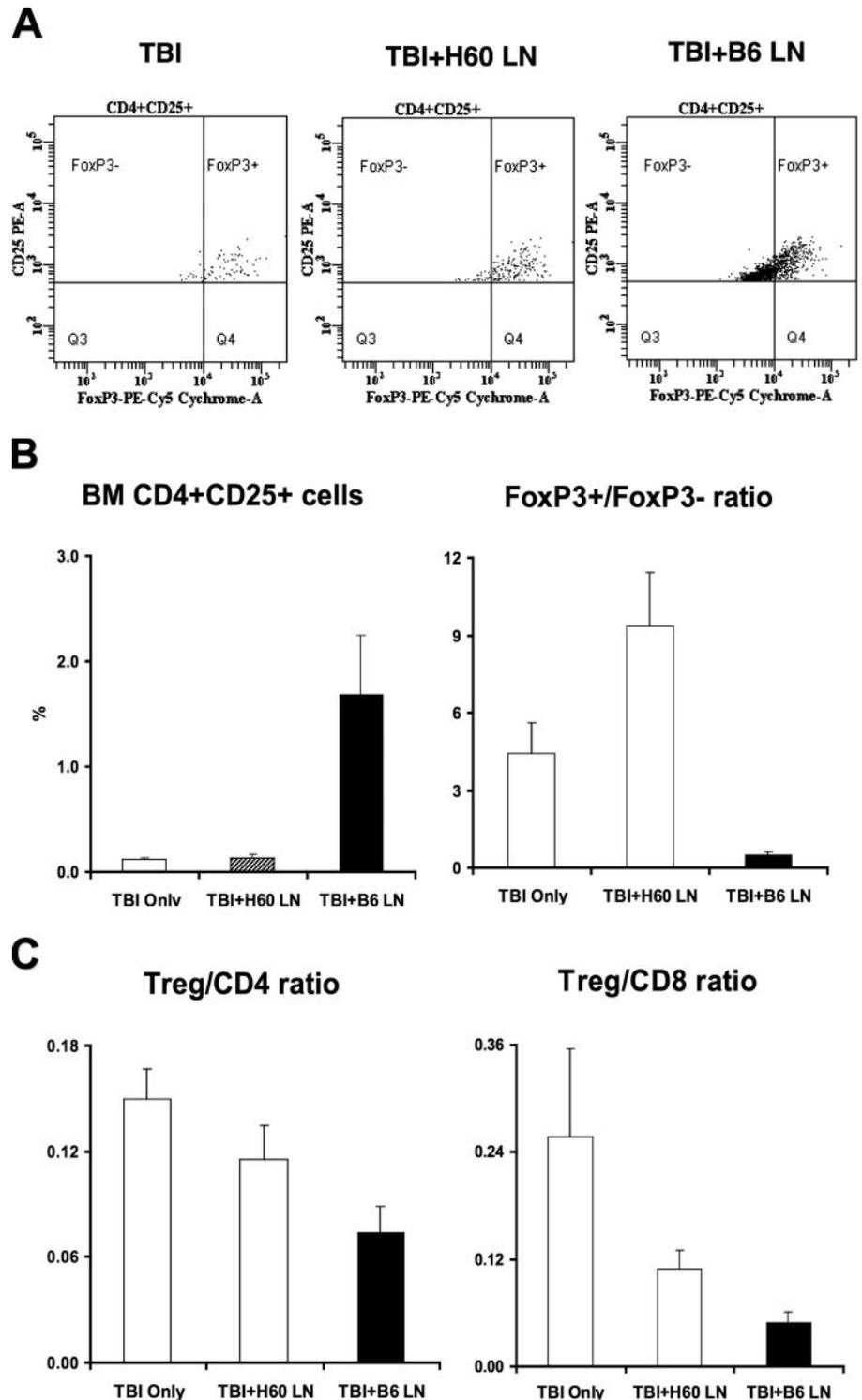


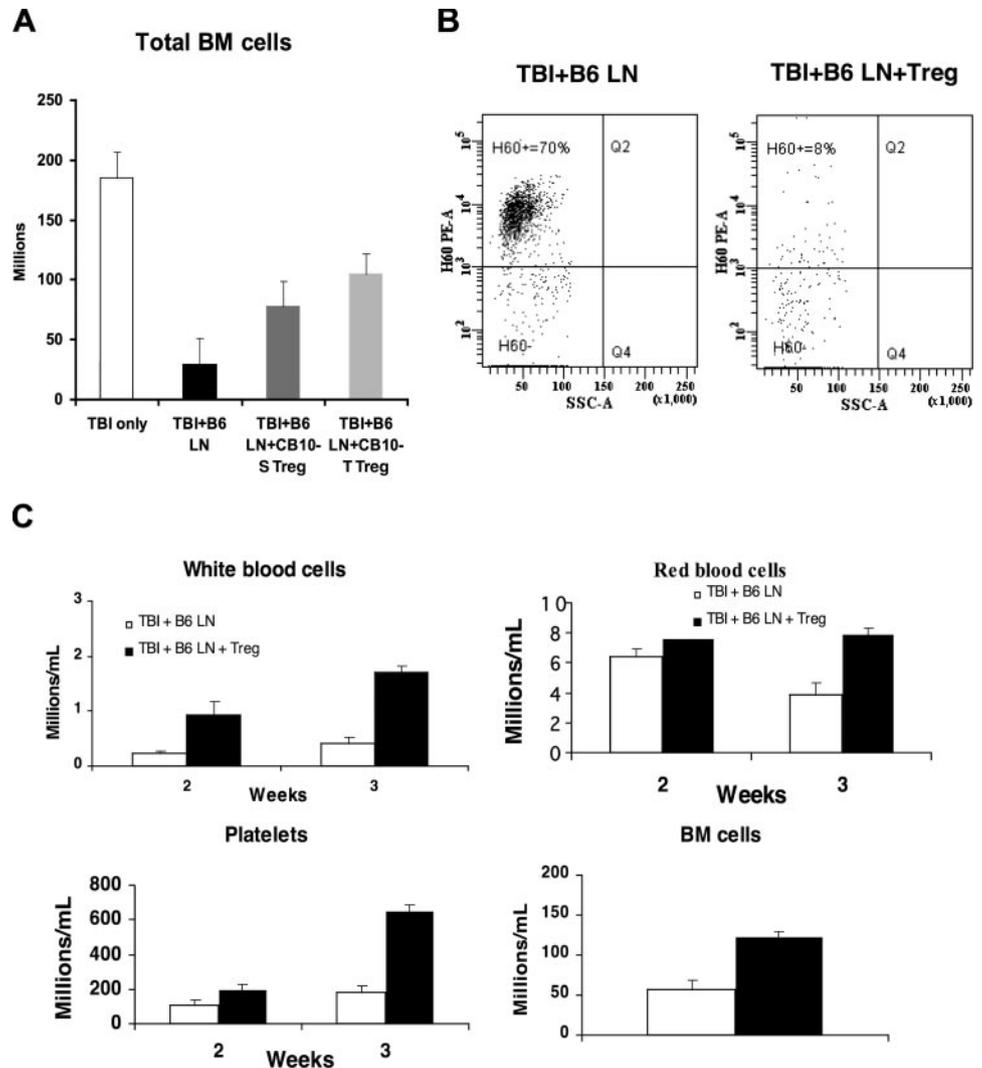
FIGURE 6. Reduced Treg to activated T cell ratios in immune-mediated BM failure. **A**, BM cells from C.B10 mice that received TBI ($n = 5$), TBI plus B6-H60 LN cell infusion (TBI+B6-H60 LN) ($n = 3$), or mice receiving TBI plus B6 LN (TBI+B6 LN) ($n = 7$) treatment were analyzed for the proportion of CD4⁺CD25⁺ T cells. Gated CD4⁺CD25⁺ T cells were further analyzed for the intracellular expression of FoxP3. Mice that received TBI plus B6 LN treatment developed BM failure but had large proportion of CD4⁺CD25⁺ T cells in the BM that do not express the intracellular transcription factor FoxP3. **B**, The proportion of CD4⁺CD25⁺ T cells increased significantly ($p < 0.01$) in the BM of mice that received TBI plus B6 LN treatment. These mice have a significantly reduced FoxP3⁺ to FoxP3⁻ cell ratio ($p < 0.01$) in the CD4⁺CD25⁺ T cell population. **C**, Treg (CD4⁺CD25⁺FoxP3⁺) to CD4 and Treg to CD8 T cell ratios were also significantly reduced in mice that received TBI plus B6 LN treatment and developed BM failure, in comparison to mice that received TBI only or TBI plus B6-H60 LN treatment and did not develop BM failure. Note that CD4 and CD8 T cells in the BM of mice treated with TBI plus B6 LN were all activated as shown earlier in Fig. 2.

combined LN and spleen cells, or combined BM and spleen cells were the usual sources of donor lymphocytes that produce severe inflammatory responses in multiple organs and tissues (12, 23, 24, 26). In the current study, we adopted a similar approach but used only LN cells as the source of lymphocytes. This modification afforded us to develop a murine model specific for BM failure (Fig. 3). Using 5 Gy TBI as a preconditioning regimen enabled us to achieve massive marrow destruction with the infusion of only a minimal 5×10^6 LN cells, whereas the sublethal irradiation itself is not necessary for the induction of marrow failure (20). The fact that we did not observe severe inflammatory response in organs

other than the BM might be related to the rapid development of fatal pancytopenia that causes animal death before immune responses can spread to other organs. Different effector cell types may lead to GVH-associated pathology in different organs, and various cellular and molecular mechanisms might be involved in the development of GVH pathology (8, 9, 23, 25, 30). Based on data previously published and data obtained from current study, we speculate that BM is the tissue most vulnerable to damage caused by GVH responses (12, 23, 26).

For the current model, C.B10 mice had been produced by back-cross of BALB/c \times C57BL/10 (B10) F₁ mice to BALB/c for 13

FIGURE 7. Alleviation of BM failure by infusion of host- and donor-type Tregs. **A**, C.B10 mice that received TBI plus B6 LN infusion (TBI+B6 LN) ($n = 2$) show significant marrow loss, whereas mice that received the same treatment plus 2×10^3 CD4⁺CD25⁺ Tregs from the spleen ($n = 2$) or 15×10^3 Tregs from the thymus ($n = 3$) of normal C.B10 mice had significantly more BM cells. **B**, In a separate study, C.B10 mice receiving TBI plus B6 LN treatment ($n = 4$) became moribund at 2–3 wk with significant expansion of CD8 T cells, especially H60-specific T cells. Mice received the same TBI plus B6 LN treatment plus 5×10^3 Tregs (TBI+B6 LN+Treg) ($n = 2$) stayed healthy and showed no H60-specific T cell expansion in the BM. **C**, Treatment with TBI plus B6 LN infusion caused fatal pancytopenia in the BM as well as in the blood, whereas infusion of 5×10^3 Tregs improved cell counts in the BM and blood. Total BM cells and white blood cells, RBCs, and platelets were all higher in the treatment group receiving an infusion of Tregs.



generations while selecting for the *H2^b* allele that originated from the B10 donor. As a result, C.B10 mice share the same *H2^b* allele with B6 mice at the MHC loci but differ from B6 at multiple minor H Ag loci. It has been well documented that minor H Ag disparity causes GVH responses in many murine models (10, 11, 15–17, 22, 26, 31). In human patients, mismatch of the adhesion molecule CD31 and of H-Y Ags may cause GVH responses, and the effect could even be advantageous when directed against host leukemic cells (15–17). Among the many minor H Ags that differ between B6 and C.B10 mice, H60 is of unique importance because B6 mice carry a null allele and do not express the protein from which the H60 peptide derives (27, 28). The H60 glycoprotein is a member of the retinoic acid early inducible gene family and serves as a ligand for the stimulatory NK receptor NKG2D. In mismatched transplantation settings, H60 displays an immunodominant effect over other minor H Ags due to an abnormally large pool of T cell precursors directed against its unique peptide (10, 11, 22).

Minor H Ag H60 mismatch in the induction of BM failure may model the immune mechanisms underlying human AA in the role of a few minor Ags in mediating massive marrow cell loss due to T lymphocyte activity. We observed a time-dependent H60-specific CTL expansion and retraction, which coincided with BM loss in the current mouse model (Figs. 1C and 2B). That infusion of H60-matched LN cells failed to induce fatal pancytopenia (Fig. 1) provided strong evidence indicating that, under our experimental conditions, H60 disparity was key for the development of immune-

mediated BM failure syndrome. In the absence of H60 allelic difference, the number of donor LN cells feasibly administered to recipients was unable to mount an immune response sufficient to cause marrow injury leading to fatal pancytopenia.

The role of H60-specific T cells in the initiation of immune attack against C.B10 hemopoietic cells was directly tested in a cytotoxicity assay in vitro using H60-specific T cells as effectors (Fig. 4). Induction of apoptosis in targets was more prominent when unfractionated residual BM cells from affected mice, rather than isolated H60-specific CD8 T cells, were used as effectors, likely due to the difference in E:T ratio that could be achieved with the two effector cell populations (E:T ratio of 20:1 vs 10:1). Additionally, other activated T cells present in the whole marrow and absent in the highly purified H60-specific population may contribute to the cytotoxic effect. The role of H60 in the induction of BM failure was further confirmed in the immunosuppressive therapy study in vivo, in which cyclosporine prevented fatal pancytopenia and rescued animals. Cyclosporine treatment abolished H60-specific T cell proliferation in recipient mice and effectively rescued animals (Fig. 5B).

Informed by the suspected role of CD4⁺CD25⁺ Tregs in the pathogenesis, prevention and treatment of autoimmune diseases in other animal models (32–38) and provoked by recent observations that patients with AA and chronic GVH disease have abnormally reduced Treg numbers and function (39, 40), we determined the Treg presence in animals that developed BM failure and we tested

the capability of Tregs to prevent BM failure in our model. Our finding that the proportion of CD4⁺CD25⁺ T cells was increased (Fig. 6A) rather than decreased in mice with BM failure, although initially surprising, was consistent with a previous report of increased CD4⁺CD25⁺ T cell presence in patients with chronic GVH disease (41). However, a distinguishing feature of CD4⁺CD25⁺ T cells in BM failure mice was that the vast majority of these cells did not express the intracellular FoxP3 Ag (Fig. 6A) and were therefore likely incapable of suppression of the immune response. The much reduced Treg to activated T cell ratio in mice with BM failure (Fig. 6C) also was evidence of imbalance of T cell activation and T cell suppression that may figure in the aberrant immune response pathophysiology of massive BM damage.

The role of Tregs was further supported by the effectiveness of their administration in abrogating expansion of H60-specific T cell, alleviating peripheral pancytopenia, preserving marrow cellularity, and rescuing animals from immune-mediated BM failure (Fig. 7). These data are consistent with recent findings of high donor FoxP3-positive Treg content in association with a low risk of GVH disease following HLA-matched allogeneic stem cell transplantation (42). Both autologous and allogeneic Tregs were functionally suppressive, but allogeneic cells appeared more active. Our results are consistent with publications of Treg prevention of autoimmune myocarditis, diabetes mellitus, and hepatitis in various animal models (32–38).

Although H60-specific T cells are important in initiating BM damage, they likely are not the only T cells responsible for the massive marrow destruction. Infusion of 5×10^6 B6 LN cells into 5 Gy TBI-treated B6-H60 congenic mice resulted in only slight reduction in blood counts (data not shown), indicating that other minor H Ags in addition to the dominant H60 Ag also were active in the induction of BM failure in the B6→C.B10 LN cell infusion model. This observation is consistent with previously published data showing that additional CD4⁺ T cells responding to at least one other helper epitope, such as an epitope from the H-Y Ags, are needed to stimulate a response to H60 in a transplantation setting like B6→B6-H60 congenic in which H60 is the only minor H Ag different between donors and recipients (10, 11, 22).

Previously we have observed that activated T cells could destroy autologous BM cells as bystanders (21). In other autoimmune models, such as in the NOD mouse model, animals develop an initially nondestructive peri-insular infiltration of dendritic cells, accessory macrophages, T cells, and B cells that persist for weeks, and only later progress to a destructive insulinitis. The autoimmune response is specific to islet cells, but in some cases thyroid cells and Schwann cells are also casualties (43–45). In experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis, epitope spreading is highly associated with disease progression (46, 47). Although epitope spreading is a potential mechanism for marrow destruction mediated by an “innocent bystander” effect, we do not have specific evidence for such a pathophysiology in the current experimental model. H60-specific T cell numbers increased steadily from days 7 to 12, coincident with massive BM cell loss. H60-specific T cells declined after day 12, as did other T cells due to a general reduction in CTLs in the BM of affected animals from days 12 to 14. Thus, it is not evident that epitope spreading/switching played a major role in the disease progression, although we did not specifically test reactivity to other known minor H Ag epitopes.

We have successfully produced a B6→C.B10 LN cell infusion model that destroys BM progenitor cells and HSCs. CTLs specific for the minor H Ag H60 are responsible for the initiation of BM destruction. Removal of H60-specific T cells or inhibition of H60-specific T cell expansion by immunosuppression or administration

of Tregs aborted the induction of marrow failure and rescued animals from pancytopenia. This model mimics many features of human BM failure and can provide a platform to study the pathophysiological role of Ag-specific T cells and to test new treatments.

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Disclosures

The authors have no financial conflict of interest.

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