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J Immunol 2007; 178:4159-4168; doi: 10.4049/jimmunol.178.7.4159
http://www.jimmunol.org/content/178/7/4159

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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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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-H2b/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.


Received for publication August 25, 2006. Accepted for publication January 19, 2007.

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

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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, TBI plus 5 × 10^6 LN cells from B6 (TBI + B6 LN), C.B10 (TBI + CB10 LN), or B6-H60 congenic (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-μm 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 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 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).
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 (OncoImmunin) 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 \times 10^5 labeled target cells mixed with 10^5 TBI plus B6-treated BM cells as effectors (E:T ratio is 20:1), or 10^3 labeled target cells mixed with 10^3 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 \times 10^6 B6 LN cells plus treatment with cyclosporine (Sandoz Pharmaceuticals) at 50 \mu g/g/day for 5 days injected i.p. beginning 1 h after LN cell infusion; 2) infused with 5 \times 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 \times 10^6 B6 LN cell infusion were divided into two groups of three animals each, with one group receiving 50 \mu g/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 FACSVantage 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 \times 10^3 CD4^+CD25^+ Tregs from C.B10 spleen, and three C.B10 mice received TBI plus LN cells from B6 mice with 15 \times 10^5 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
5 × 10^6 B6 LN cells were divided into two groups: two of the six mice received 5 × 10^5 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
in BM H60-specific CD8 T cells in comparison to untreated control groups (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 sternum sections from untreated control C.B10 mice showed normal cellularity with narrow cavity full of nucleated cells including typical hematopoietic precursor cells such as megakaryocytes (Fig. 3A). In mice treated with TBI only, sternum sections 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 narrow cavity empty of hematopoietic 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 Lin− CD117+ CD34+ and Lin− CD117+ 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 Lin− CD117+ CD34+ and Lin− CD117+ CD34− hematopoietic progenitor cells and HSCs reduced even further (Fig. 3B). The massive destruction of BM cells, especially BM HSCs and hematopoietic 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).

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 cyclosorine, a drug widely used in the treatment of human acquired AA, at a dose of 50 μg/g/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 (~7%) H60-specific CD8 T cells in the blood, whereas mice that received TBI plus B6 LN infusion with cyclosporine treatment (Fig. 5A). Day 21, mice that received TBI plus B6 LN infusion without cyclosporine treatment had ~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 CD4+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...
of CD4⁺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 CD4⁺CD25⁺ T cells in peripheral blood, whereas mice that received TBI plus H60 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.

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,
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 $5 \times 10^6$ 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 backcross of BALB/c × C57BL/10 (B10) F₁ mice to BALB/c for 13
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 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 hematopoietic 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 pathophysiologic 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, like B6/H11001, were therefore likely incapable of suppression of the immune response to mouse non-MHC transplantation antigens in vivo: the H60 histocompatibility antigen dominates over all others. J. Immunol. 166: 4370–4379.

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 insulitis. 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, epoxide spreading is highly associated with disease progression (46, 47). Although epoxide spreading is a potential mechanism for marrow destruction mediated by an “innocent bystander” effect, we do not have specific evidence for such a pathophysiologic 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 epoxide 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.

Acknowledgment We thank David Caden from the Laboratory of Animal Medicine and Surgery, National Heart, Lung, and Blood Institute, for technical assistance in complete blood counts.

Disclosures The authors have no financial conflict of interest.

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