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A Radio-Resistant Perforin-Expressing Lymphoid Population Controls Allogeneic T Cell Engraftment, Activation, and Onset of Graft-versus-Host Disease in Mice

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Immunosuppressive pretransplantation conditioning is essential for donor cell engraftment in allogeneic bone marrow transplantation (BMT). The role of residual postconditioning recipient immunity in determining engraftment is poorly understood. We examined the role of recipient perforin in the kinetics of donor cell engraftment. MHC-mismatched BMT mouse models demonstrated that both the rate and proportion of donor lymphoid cell engraftment and expansion of effector memory donor T cells in both spleen and BM were significantly increased within 5 to 7 days post-BMT in perforin-deficient ($pfn^{-/-}$) recipients, compared with wild-type. In wild-type recipients, depletion of natural killer (NK) cells before BMT enhanced donor lymphoid cell engraftment to that seen in $pfn^{-/-}$ recipients. This demonstrated that a perforin-dependent, NK-mediated, host-versus-graft (HVG) effect limits the rate of donor engraftment and T cell activation. Radiation-resistant natural killer T (NKT) cells survived in the BM of lethally irradiated mice and may drive NK cell activation, resulting in the HVG effect. Furthermore, reduced pretransplant irradiation doses in $pfn^{-/-}$ recipients permitted long-term donor lymphoid cell engraftment. These findings suggest that suppression of perforin activity or selective depletion of recipient NK cells before BMT could be used to improve donor stem cell engraftment, in turn allowing for the reduction of pretransplant conditioning.

INTRODUCTION

Hematopoietic stem cell (HSC) transplantation is an effective therapy for a number of blood cancers, including leukemia and lymphoma, by reconstituting a patient's immune system with donor immune cells that mediate a graft-versus-tumor effect. Clinical outcome is dependent on rapid donor cell engraftment, immune reconstitution, and potent control of tumor without the development of graft-versus-host disease (GVHD). Pretransplant immunosuppressive conditioning is required for complete donor engraftment of the bone marrow (BM) and to prevent graft rejection. However, preconditioning using chemotherapy with or without total body irradiation (TBI) results in significant morbidity and contributes to the onset of acute GVHD.

Mouse models of BM transplantation (BMT) and GVHD have contributed greatly to our understanding of the early immune events post-transplant leading to the activation of donor cells, mimicking human disease (reviewed in [1]). Damage of the intestinal mucosa after pretransplant conditioning permits the release of pathogen-/danger-associated molecular patterns that triggers activation of residual

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recipient antigen presenting cells (APC), resulting in the rapid secretion of pro-inflammatory cytokines [2,3]. The initial priming phase of allogeneic $CD4^+$ donor T cells largely occurs via the recognition of minor hematopoietic antigens presented on MHC class II–positive non-hematopoietic recipient APC in the gut [4,5] and by hematopoietic APC in the lymphoid tissue for $CD8^+$ T cells [6,7]. Hyperactivation of donor lymphoid cells leads to a "cytokine storm" characterized by elevated IL-6, IFN-γ, tumor necrosis factor-α, IL-21, and IL-17 levels [8]. This provides a pro-inflammatory milieu that subsequently drives the differentiation and expansion of donor regulatory T cells, natural killer (NK) cells, and allogeneic T cells, contributing to acute GVHD.

The cytotoxic granule proteins, perforin and granzymes, have well-characterized donor allogeneic NK and T cell–mediated graft-versus-tumor and GVHD effects [9-11]. In addition to their ability to induce apoptosis of allogeneic target cells, these cytotoxic proteins have increasingly diverse functions, including restricting antigen presentation and T cell activation during virus infection [12], exacerbating sepsis [13], and, counterintuitively, impeding antitumor responses by directly killing $CD8^+$ T cells [14]. Although donor perforin-dependent and -independent, context-dependent effects on GVHD have been previously described [9,10,15-22], the role of perforin in recipient mice remains uncertain. Bennett et al. [23] identified that recipient perforin modulates donor engraftment in an MHC class I–deficient mouse model, consistent with NK mediated rejection of donor stem cells with "missing-self." In contrast, perforin was dispensable in preprimed HLA-matched, $CD8^+$ T cell–dependent mouse models of short-term myeloid engraftment [24,25]. Furthermore, others have described that NK depletion in recipient mice, using anti-Asialo GM1 treatment, increased the kinetics of hematopoietic cell reconstitution and the rate of donor cell engraftment in mouse BMT models [26,27]. Recently, the generation of the *Mcl-1* knockout mouse [28] confirmed the importance of recipient NK cells in regulating BM engraftment. Additionally, recipient NKT natural killer T (NKT) cells can regulate BM engraftment and GVHD by activating donor regulatory T cells via an IL-4–dependent, NK cell–dependent mechanism [29,30]. Therefore, it is becoming increasingly apparent that cytotoxic proteins, such as perforin, and the cells that express them play an important role in post-transplant immune function.

We hypothesized that perforin expression in BMT recipients is important for inhibiting donor lymphocyte engraftment and allogeneic T cell activation. In 2 MHC-mismatched mouse models of BMT, we found that donor lymphoid cell engraftment, expansion of effector memory allogeneic T cells, and release of pro-inflammatory cytokines were accelerated in perforin-deficient recipients compared with wild-type (WT) recipients. Enhanced donor cell engraftment in the absence of perforin in recipient immune cells was attributed to a decreased host-versus-graft effect via radiation-resistant, NK cell–mediated killing of allogeneic donor cells. Long-term donor lymphoid cell engraftment in perforin-deficient recipients persisted when the pretransplant TBI dose was reduced. Therefore, inhibition of recipient perforin function at the time of BMT in combination with reduced-intensity conditioning may improve donor cell engraftment and minimize conditioning-related side effects.

METHODS

Mice and BMT Models

All animal work was conducted in an specific-pathogen-free (SPF) facility at the Peter MacCallum Cancer Centre, under institutional animal ethics committee approved protocols. Female 6- to 14-week-old BALB/c WT and BALB/c.perforin knockout (KO) mice (bred and housed at Peter MacCallum Cancer Centre Animal Facility) ($H\text{-}2K^d$) and C57BL/6 WT and C57BL/6.perforin KO mice ($H\text{-}2K^b$) were used as BMT recipients. MHC-disparate models of BMT were established, transplanting BALB/c donor BM into C57BL/6 recipients ($H\text{-}2K^{d/b}$) or C57BL/6 BM into BALB/c recipients ($H\text{-}2K^{b/d}$).

On day 0, recipient mice were administered a split dose of lethal radiation using a cesium source (2 × 6 gray for C57BL/6 and 2 × 5.5 gray for BALB/c) and injected i.v. with 5^{e6} T cell–depleted BM (TCD-BM) cells from MHC-mismatched or syngeneic donors. On day 2, recipient mice were injected i.v. with 2^{e5} C57BL/6 or 1^{e6} BALB/c purified splenic T cells at a 2:1 $CD4^+/CD8^+$ T cell ratio. Mice were monitored daily and killed at selected time points post-transplant (typically days 5 to 7 for short-term engraftment or day 20 for long-term engraftment) to examine donor lymphoid cell engraftment.

Serum was collected for cytokine analysis and the spleen, gut, and BM harvested for phenotypic and histological analysis. NK cells were depleted by injecting 100 μL rabbit anti-Asialo GM1 antibody (Wako, Chuo-Ku, Osaka, Japan) i.p. into mice on days −1 and 0. Control mice were injected with PBS.

MACS Bead Purification

T cells were depleted from donor BM using a magnetic activated cell sorting (MACS) mouse CD3e microbead kit, following manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Splenic T cells were purified using MACS $CD4^+$ T cell–negative selection or $CD8^+$ T cell–negative selection beads and mixed at a 2:1 ratio before injecting into mice.

Flow Cytometry

Lymphocytes isolated from BM and spleen were washed and resuspended in FACS buffer (PBS + 2% FCS). Nonspecific binding was blocked using the 2.4G2 hybridoma (ATCC HB-197, USA) before staining with anti-mouse mAbs for 30 minutes on ice and then washing and fixing the cells in 2% paraformaldehyde. The antibody panel included $H\text{-}2K^b$-PE, $H\text{-}2K^d$-FITC, CD8a-PeCy7, CD44-FITC, CD62L-APC, CD19-PerCpCy5.5, NK1.1-PeCy7 (BD Biosciences, San Jose, CA), CD3e-eFluor450, CD4-APC AF780 (eBioscience San Diego, CA, Carlsbad, CA), and live/dead fixable AmCyan (Invitrogen, Life Technologies). Mouse CD1d-αGalCer (PBS44) tetramers were provided as a kind gift from Dale Godfrey, University of Melbourne. Cells were acquired on an LSRFortessa (BD Biosciences, San Jose, CA) and results analyzed using FlowJo (TreeStar, Ashland, OR) software.

Cytokine Detection

Serum cytokine levels were measured using Luminex multiplex assays. Serum was stored at −20°C until tested by Luminex assay, as per the manufacturer's instructions (Merck Millipore, Darmstadt, Germany).

Statistical Analysis

Results from pooled experiments were analyzed using GraphPad Prism software (La Jolla, CA), using the unpaired *t*-test. Mouse survival curves were analyzed using the log-rank (Mantel-Cox) test. Significant results were plotted as $P < .05$, $P < .01$, $P < .001$, and $P < .0001$.

RESULTS

Absence of Recipient Perforin Contributes to Donor Lymphoid Cell Engraftment

To investigate the role of recipient perforin in regulating donor lymphoid cell engraftment, we used 2 MHC-mismatched models, transplanting WT donor cells into WT or $pfn^{-/-}$ recipients. The percent of total body weight was determined for spleens harvested on day 5 or 7 post-BMT in C57BL/6 WT or $pfn^{-/-}$ recipient mice (Figure 1A). At both early time points, the spleen size was significantly increased in $pfn^{-/-}$ recipients compared with the WT counterparts ($P < .0001$). Enlarged spleens were only observed in $pfn^{-/-}$ recipients after allogeneic BMT, as after syngeneic transplants the spleen sizes were similar for both WT and $pfn^{-/-}$ mice (Supplementary Figure 1), indicating no underlying difference in spleen size solely because of perforin deficiency.