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Persistence of donor-derived protein in host myeloid cells after induced rejection of engrafted allogeneic bone marrow cells

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Abstract

Objective—In recipients of allogeneic hematopoietic stem cell transplantation to treat hematologic malignancies, we have unexpectedly observed anti-tumor effects in association with donor cell rejection in both mice and humans. Host-type CD8 T cells were shown to be required for these anti-tumor effects in the murine model. Since sustained host CD8 T cell activation was observed in the murine bone marrow following the disappearance of donor chimerism in the peripheral blood, we hypothesized that donor antigen presentation in the bone marrow might be prolonged.

Materials and Methods—To assess this hypothesis, we established mixed chimerism with green fluorescence protein (GFP)-positive allogeneic bone marrow cells, induced rejection of the donor cells by giving recipient leukocyte infusions (RLI), and utilized *in vivo* microscopy to follow GFP-positive cells.

Results—After peripheral donor leukocytes disappeared, GFP persisted within host myeloid cells surrounding the blood vessels in the bone marrow, suggesting that the host myeloid cells captured donor-derived GFP protein.

Conclusions—Since the host-versus-graft reaction promotes the induction of anti-tumor responses in this model, this retention of donor-derived protein may play a role in the efficacy of RLI as an anti-tumor therapy.

Keywords

hematopoietic cell transplantation; mouse model; immunotherapy; rejection; *in vivo* microscopy

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Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

Introduction

Anti-tumor effects of allogeneic hematopoietic cell transplantation have been attributed to engrafted donor lymphocytes and long-term donor engraftment is thought to be essential to cure hematological malignancies. However, we have observed that following non-myeloablative hematopoietic cell transplantation (HCT), some patients are cured of advanced hematologic malignancies despite rejection of their donor hematopoietic cell grafts [1]. Other investigators have also reported cases of tumor remission in association with donor hematopoietic cell rejection [2] and a clinical trial of infusion of allogeneic lymphocytes, which were promptly rejected, was associated with significant anti-tumor effects [3]. These phenomena led us to hypothesize that host-versus-graft (HVG) reactions may induce host-derived anti-tumor effects.

In a murine model, we established that HVG responses can indeed promote anti-tumor responses that are dependent on host-type CD8 T cells, IFN γ , and initial donor engraftment [4–6]. The intensity of the HVG reaction correlates with the potency of anti-tumor effects [4]. Host CD8 T cells in the bone marrow secrete high levels of IFN γ even after peripheral donor chimerism has totally disappeared (Saito et al. manuscript in preparation). Thus, we hypothesized that alloresponses may persist in the bone marrow, driven by the presence of alloantigens, either due to sustained engraftment of the donor cells (direct alloantigen presentation) or to presentation of alloantigens by host antigen-presenting cells (APC) (indirect alloantigen presentation) in the bone marrow.

To assess this hypothesis, we used dynamic *in vivo* microscopy to directly visualize events in living animals. Two-photon and confocal microscopy allowed visualization of the bone marrow environment 150 μ m below the skull bone surface of anesthetized mice. With these tools, we were able to track fluorescently labeled donor protein together with bone and blood vessels in real time.

Our studies revealed the persistence of host-derived myeloid cells containing donor protein and residing around the bone marrow blood vessels after peripheral blood chimerism was lost. The results are compatible with the hypothesis that persistent presentation of donor-derived antigens on host APC maintain alloreactive host CD8 T cell activation in the bone marrow, promoting the anti-tumor effect induced by HVG reactions.

Materials and methods

Animals

GFP⁺ C57BL/6 transgenic mice express green fluorescent protein (GFP) ubiquitously under the control of the human ubiquitin C promoter (Jackson Laboratories, stock number: 4353). Female BALB/c mice were purchased from the Frederick Cancer Research Facility, National Cancer Institute. Mice were used in experiments at 8–12 weeks of age and housed in autoclaved microisolator environments. All procedures were performed in a laminar flow hood following approval by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Bone marrow transplantation (BMT) and recipient leukocyte infusions (RLI)

Mixed chimerism was induced in female BALB/c mice using a nonmyeloablative regimen as described previously [7]. The regimen consists of *in vivo* T cell depletion using anti-CD4 (GK1.5) (1.76 mg/mouse) and anti-CD8 (2.43) (1.44 mg/mouse) monoclonal antibodies (mAbs) administered intraperitoneally on Day -5, cyclophosphamide (Cytosan, Mead Johnson, Evansville, IN) at 200 mg/kg intraperitoneally on Day -1, and 7 Gy thymic

irradiation from a ^{60}Co source on Day 0. Donor bone marrow cells were harvested from GFP⁺ C57BL/6 mice, and single cell suspensions were prepared as described previously [7]. Four to six hours after thymic irradiation, 25×10^6 donor bone marrow cells were injected intravenously through the tail vein. Recipient BALB/c spleen cell suspensions were prepared and administered intravenously as RLI at a dose of 30×10^6 splenocytes per recipient 7 to 10 weeks after BMT, as described [4].

FACS analysis

Chimerism in various lineages was analyzed at various times post-BMT by flow cytometry as described previously [4,7]. Non-specific Fc γ R binding was blocked by anti-mouse Fc γ -III/II receptor mAb 2.4G2 [8]. Chimerism was analyzed in various cell lineages with the following antibodies: anti-CD4-PE, anti-CD8 β -PE, anti-B220-PE, anti-Mac-1-PE, anti-H-2Dd-FITC, anti-H-2Dd-Biotin, anti-H-2Db-Biotin and streptavidin-APC (BD Biosciences, San Diego, CA). Relative percentages of donor cells were calculated using the following formula: $100\% \times [1 - \{(\text{recipient phenotype percentage positive} - \text{isotype control}) / (\text{recipient phenotype positive} - \text{isotype control}) + (\text{recipient phenotype percent negative} - \text{isotype control})\}]$.

In vivo microscopy

In vivo imaging of the bone marrow of live mice was carried out as described below [9,10]. We used a video-rate laser scanning hybrid confocal/two-photon microscope that is specifically designed and optimized for live animal imaging. A polygon-based scanning engine allows simultaneous multi-channel image acquisition at video rate (30 frames per second), a feature that is particularly useful for imaging live, moving objects. Fast scanning speed, together with a precision computer-controlled xyz stage, also facilitate surveying large tissue volumes in 3D and searching for rare cells in the bone marrow (BM).

The mice were anesthetized with an intra-peritoneal injection of ketamine/xylazine, and a small incision was made in the scalp to expose the underlying skull. The skull bone was kept intact. Second harmonic microscopy was used to visualize the bone and to identify the major anatomical landmarks such as the central vein and the coronal suture. Bone is rich in type-1 collagen, which is visualized by second harmonic generation using a 840 nm excitation laser and detection at 420 nm. Using the crossing of the central vein and coronal sutures as landmarks, we imaged identical areas of the skull ($\sim 1500 \mu\text{m} \times 1800 \mu\text{m}$) encompassing most of the parasagittal BM cavities [9,10]. The maximum imaging depth was about $150 \mu\text{m}$ below the outer bone surface, allowing us to penetrate between 40 and 60% of the bone marrow cavity space in over 75% of our measurements [10]. To visualize bone marrow vasculature, Evans Blue vascular dye, (2%, 4ml/kg) was administered via tail vein 15 minutes prior to the imaging. Evans Blue signal was excited with a 638 nm helium-neon laser (Radius, Coherent Inc., Santa Clara, CA) and detected with a photomultiplier tube through a 695 ± 27.5 nm bandpass filter (Omega Optical, Brattleboro, VT). GFP signal was excited with a 491 nm solid state laser (Dual Calypso, Cobolt AB, Stockholm, Sweden) and detected with a photomultiplier tube through a 528 ± 19 nm bandpass filter (Semrock, Rochester, NY). Second harmonic generation signals from the bone were excited with an 840 nm Ti:sapphire laser (Spectra Physics, Tucson, AZ) and detected with a photomultiplier tube through a 435 ± 20 nm bandpass filter (Semrock, Rochester, NY). Imaging duration per mouse was approximately 2 hours.

Results

GFP⁺ cells in the donor bone marrow following RLI

We hypothesize that indirect alloreactivity induced by donor graft rejection is essential for the anti-tumor effect of RLI. Based on the observation that high levels of IFN γ were secreted by host CD8⁺ T cells in the bone marrow following the disappearance of measurable donor chimerism in peripheral blood in RLI recipients (Saito et al. manuscript in preparation), we hypothesized that either donor cells or donor-derived protein may persist in the bone marrow after donor chimerism is no longer detectable in the peripheral blood.

To address this possibility, we made use of GFP⁺ C57BL/6 mice as the donor strain. This system allows tracking of GFP as a model donor antigen and discrimination of donor cells from host cells engulfing donor proteins by examining both GFP staining and expression of specific major histocompatibility complex (MHC) alleles.

We established stable mixed allogeneic bone marrow chimeras using GFP⁺ C57BL/6 mice as donors and BALB/c mice as recipients using non-myeloablative conditioning [7]. RLI was infused into some of the mixed chimeras 7 to 10 weeks after initial allogeneic BMT in order to induce rejection of engrafted donor cells. GFP expression in the bone marrow was examined in stable mixed chimeras and in recipients of RLI.

Eleven days post-RLI, when donor chimerism in peripheral blood had become undetectable (Figure 1a), no GFP^{high} cells were detected in any lineage examined in the bone marrow by FACS analysis (Figure 1b), and only small numbers of GFP^{dim} Mac1⁺ cells were found in chimeric recipients of RLI (Figure 1b, left panels). Chimeras not receiving RLI also contained small numbers of these GFP^{dim} Mac1⁺ cells in addition to the GFP^{high} population.

We performed *in vivo* microscopy on the same animals examined in Figure 1. In stable mixed chimeras, clusters of GFP^{high} donor cells were detected as green areas that were evenly distributed and located mainly around the blood vessels in the bone marrow (Figure 2A, arrowheads). Fast-moving and rolling GFP^{high} cells were observed inside the vessels (Figure 2A, arrows and Supplemental video 1). GFP^{high} cells outside the vessels were static, even when we observed the same site for 10 minutes (Supplemental video 1). In RLI recipients, there was an absence of GFP^{high} cells and only a small number of GFP^{dim} cells were observed (Figure 2B, arrowheads) around the vessels and small, GFP^{dim} cells were also detected moving inside the vessels (Figure 2B, arrows and Supplemental video 2). These small circulating cells were also detected in chimeras not receiving RLI (Figure 2A and Supplemental video 1). Thus, donor cells were evenly distributed in clusters throughout the bone along the blood vessels in mixed chimeras and GFP^{dim} cells persisted in this location after rejection of leukocytes was complete in the periphery of RLI recipients. Circulating GFP^{dim} cells in the vessels were smaller than 8 μ m, suggesting they may be erythrocytes, while those around the vessels were larger than 21 μ m.

Recipient Mac1⁺ cells retain donor-derived protein long after donor cell rejection

Even 22 days after RLI administration, we still observed static GFP^{dim} cells surrounding the vessels (Figure 3, arrowheads and Supplemental video 3) and abundant GFP^{dim} small cells inside the vessels (Supplemental video 3). Consistent with the hypothesis that these were donor-derived red blood cells (RBC), when we stained bone marrow cells without RBC lysis, the GFP^{dim} cells were found to be mostly smaller than lymphocytes (Figure 4A) and were confirmed to be RBC by Ter119 staining (Figure 4B). We also confirmed that RBC from GFP⁺ C57BL/6 mice were GFP^{dim} (Figure 4C).

When we gated out most of the RBCs on the basis of forward scatter, a significant number of GFP^{dim} cells in the bone marrow at 22 days post-RLI were Mac1⁺, while a smaller proportion were of the B cell lineage (Figure 5A). When we examined the origin of the Mac1⁺ GFP^{dim} cells using donor and recipient MHC-specific antibodies, they were found to be recipient-derived. (Figure 5B).

These results suggest that not only donor-derived RBC, but also Mac1⁺ host cells that retain donor-derived proteins remain in the bone marrow after rejection of donor marrow, and these cells are located just outside blood vessels.

Discussion

While homing patterns of purified hematopoietic stem cells (HSC) and tumor cells have been described [9], to our knowledge, this is the first detailed large area live imaging study of mixed bone marrow chimeras. The results show that donor cells are not focally localized but, rather, are distributed in clusters throughout the marrow cavity with greatest density around the blood vessels.

Historically, it has not been easy to examine cell localization inside the bone marrow. Immunohistochemistry of bone requires decalcification, and the niche within the bone marrow that has recently been shown to be very important for hematopoietic cells and HSC is actually attached to bone and in between bones [11] and blood vessels [10,12]. Thus, decalcification may disturb observation of cells in the niche attached to the bones, such as HSC [11]. To avoid these problems, we made use of real-time *in vivo* microscopy. This approach is advantageous because of the fast scanning speed and ability to acquire information on cell movement and 3D localization, while maintaining high resolution in intact, live bone marrow.

Donor-derived RBCs persisted long after the rejection of other cell lineages in mixed chimeras given RLI. As is apparent from the lack of requirement for HLA matching for RBC infusions to patients, RBC are not rejected due to MHC mismatching because of their low level of MHC expression. The life span of murine RBC is around 40 days [13] thus explaining the persistent erythrocyte chimerism in RLI recipients in this study.

There are several possible mechanisms by which recipient Mac1⁺ cells might retain or express donor-derived GFP protein. Transfer of genetic information can occur by microvesicles [14] and tumor-derived microvesicles have been shown to transfer surface determinants and mRNA to monocytes [15]. It is also possible that killed donor cell may be captured by granulocytes through neutrophil extracellular traps (NETs). While bacteria are well-known targets of the NETs system [16], neither microvesicle mediated transfer nor NETs has been described in the context of allo-antigen pickup [14,16]. On the other hand, phagocytosis of allogeneic cells [17] and cross-presentation of allogeneic antigens have been well-described [18]. Given that the majority of donor cells are killed when rejection occurs, we believe that conventional phagocytosis is the most likely mechanism of capture of donor-derived protein by recipient Mac1⁺ cells.

The persistence of donor-derived protein in host APC in the bone marrow is compatible with the hypothesis that indirect presentation of donor-derived antigens in the bone marrow promote durable alloresponses, resulting in prolonged IFN γ production by host CD8⁺ T cells in the bone marrow (Saito et al. manuscript in preparation). It is also compatible with our hypothesis that host APC presenting both alloantigens and tumor antigens induce the anti-tumor immunity of RLI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Staining with donor class I MHC-specific mAb (anti-H-2D^d-FITC, clone 34-2-12) is shown for gated Mac1⁺, B220⁺, CD4⁺, and CD8⁺ peripheral leukocytes in chimeras (60 days post BMT, filled gray area) and chimeric recipients of RLI (60 days post BMT, 11 days post-RLI, solid line). (B) Bone marrow chimerism is shown for Mac1⁺, B220⁺, CD4⁺, and CD8⁺ bone marrow cells in chimeras, chimeric recipients of RLI (11 days post-RLI) and naïve BALB/c mice. Data from a representative mouse of four chimeric recipients of RLI are shown.

Figure 2.

Engraftment of donor bone marrow cells and remaining cells after rejection. GFP⁺ cells are shown in green, second harmonic generation of the bone in blue, and blood vessels in red. GFP⁺ cells are visualized *in vivo* inside the skull bone of chimeras (A) and of chimeras that received RLI 11 days earlier (B). Arrowheads point to GFP⁺ cells around the blood vessels and arrows point to GFP⁺ cells in the blood vessels. Data from one representative mouse of two mice are shown. Scale bars indicate 200 μm.

Figure 3.

GFP-positive cells are still detected 22 days after RLI. GFP-positive cells are visualized in vivo in chimeras that received RLI 22 days earlier. Arrowheads point to GFP^{dim} cells around the blood vessels. Data from one representative mouse of two mice are shown. GFP⁺ cells are shown in green, second harmonic generation of the bone in blue, and blood vessels in red. Scale bars indicate 200 μm.

Figure 4.

Donor-derived GFP is preserved in recipient Mac1⁺ cells in the bone marrow 22 days after RLI. (A) Size (FSC) and complexity (SSC) of GFP⁺ cells are shown as black dots (left panel). The control panel is shown for whole bone marrow cells (right panel). (B) Whole bone marrow cell suspension analyzed for expression of GFP and Ter119. (C) GFP-fluorescence intensity of granulocytes (filled black area), lymphocytes (open area with thick line) and red blood cells (open area with thin line) from GFP⁺ C57BL/6 mouse and blood cells from wildtype C57BL/6 mouse (filled gray area).

Figure 5. (A) GFP expression among Mac1⁺, B220⁺, CD4⁺, and CD8⁺ bone marrow cells in RLI recipient. (B) GFP positive leukocytes in the bone marrow are stained for H-2Db and H-2Dd. Data from one representative mouse of two mice are shown.