Biol Blood Marrow Transplant 19 (2013) 378–386

Homing Characteristics of Donor T Cells after Experimental Allogeneic Bone Marrow Transplantation and Posttransplantation Therapy for Multiple Myeloma

Robbert van der Voort [1,*], Thomas J.H. Volman [1], Viviènne Verweij [1], Peter C.M. Linssen [1,†], Frans Maas [1], Konnie M. Hebeda [2], Harry Dolstra [1]

[1] *Department of Laboratory Medicine, Laboratory of Hematology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands*
[2] *Department of Pathology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands*

Article history:
Received 10 July 2012
Accepted 14 December 2012

Key Words:
Allogeneic transplantation
DLI
GVHD
GVM
Mouse
T cell

A B S T R A C T

Relapse and graft-versus-host disease remain major problems associated with allogeneic bone marrow (BM) transplantation (allo-BMT) and posttransplantation therapy in patients with multiple myeloma (MM) and other hematologic malignancies. A possible strategy for selectively enhancing the graft-versus-myeloma response and possibly reducing graft-versus-host disease is to increase the migration of alloreactive T cells toward the MM-containing BM. In the present study, we characterized the BM-homing behavior of donor-derived effector T cells in a novel allo-BMT model for the treatment of MM. We observed that post-transplantation immunotherapy consisting of donor lymphocyte infusion (DLI) and vaccination with minor histocompatibility antigen-loaded dendritic cells (DCs) was associated with prolonged survival compared with allo-BMT with no further treatment. Moreover, $CD8^+$ effector T cells expressing inflammatory homing receptors, including high levels of CD44, LFA-1, and inflammatory chemokine receptors, were recruited to MM-bearing BM. This was paralleled by strongly increased expression of IFN-γ and IFN-γ–inducible chemokines, including CXCL9, CXCL10, and CXCL16, especially in mice treated with DLI plus minor histocompatibility antigen-loaded DC vaccination. Remarkably, expression of the homeostatic chemokine CXCL12 was reduced. Furthermore, IFN-γ and TNF-α induced BM endothelial cells to express high levels of the inflammatory chemokines and reduced or unaltered levels of CXCL12. Finally, presentation of CXCL9 by multiple BM endothelial cell-expressed heparan sulfate proteoglycans triggered transendothelial migration of effector T cells. Taken together, our data demonstrate that both post-transplantation DLI plus miHA-loaded DC vaccination and MM growth result in an increased expression of inflammatory homing receptors on donor T cells, decreased levels of the homeostatic BM-homing chemokine CXCL12, and strong induction of inflammatory chemokines in the BM. Thus, along with increasing the population of alloreactive T cells, post-transplantation immunotherapy also might contribute to a more effective graft-versus-tumor response by switching homeostatic T cell migration to inflammation-driven migration.

INTRODUCTION

Multiple myeloma (MM) is a severe B cell malignancy characterized by the accumulation of malignant plasma cells in the bone marrow (BM). Allogeneic BM transplantation (allo-BMT) or allogeneic stem cell transplantation (allo-SCT) followed by donor lymphocyte infusion (DLI) can result in long-term remission [1]. This therapeutic effectiveness is attributed to the graft-versus-myeloma (GVM) response, during which donor $CD8^+$ T cells become activated by recipient minor histocompatibility antigens (miHAs) presented on dendritic cells (DCs). Consequently, these alloreactive T cells migrate to the BM and eradicate $miHA^+$ myeloma cells. Unfortunately, subsets of alloreactive T cells migrate to $miHA^+$ healthy tissues, such as the gut, skin, and liver, resulting in graft-versus-host disease (GVHD), causing high morbidity and even mortality. Thus, a major challenge is to design a specific therapy that induces a potent GVM response without severe GVHD. For instance, adoptive transfer of T cells recognizing miHA specifically expressed by malignant cells, including MM cells, or vaccination with DCs presenting these miHA, has recently garnered attention [2,3]. Alternatively, or additionally, strategies that increase the recruitment of alloreactive T cells to the BM could result in a more effective GVM response.

Recruitment of activated T cells from the blood into tissues requires a tightly regulated sequence of adhesion events between the T cells and endothelial cells of the vessel wall [4]. Several families of homing receptors are involved in each of these adhesion events. For instance, selectins are predominantly involved in rolling on the endothelium, and chemokine receptors and integrins are involved mainly in enhanced adhesion and transmigration. Although many of the homing receptors involved in, for instance, skin homing and gut homing have been characterized, those participating in homing to BM, especially inflamed BM, remain incompletely defined. Early work with transgenic mice and blocking antibodies indicated that the T cell-expressed chemokine receptor CXCR4 and the adhesion molecule VCAM-1, which is constitutively expressed on BM endothelial cells (BMECs), play major roles in the homing of T cells to healthy BM [5-7]. These results were confirmed by Mazo et al. [8], who demonstrated that in $CD8^+$ central memory T cells, a major

Financial disclosure: See Acknowledgment on page 385.

* Correspondence and reprint requests: Dr Robert van der Voort, Department of Laboratory Medicine, Laboratory of Hematology, Radboud University Nijmegen Medical Centre, Geert Grooteplein 8, 6525 GA Nijmegen, The Netherlands.
E-mail address: r.vandervoort@labgk.umcn.nl (R. van der Voort).
† Deceased.

1083-8791/$ – see front matter © 2013 American Society for Blood and Marrow Transplantation.
http://dx.doi.org/10.1016/j.bbmt.2012.12.014

subset of BM T cells, activation of chemokine receptor CXCR4 by BM-expressed CXCL12 results in activation of the integrin α4β1 and subsequent firm adhesion via VCAM-1. Those authors also reported that central memory T cells use carbohydrate ligands of E-selectin and P-selectin to roll on BMECs, and L-selectin to mediate tethering to already-adhering leukocytes. Finally, most $CD4^+$ memory T cells in the BM were shown to express α2 integrins, and α2-blocking antibodies inhibited their homing to the BM of healthy or immunized mice [9].

In the present study, we characterized the trafficking of activated donor T cells to the MM-containing BM of mice that had undergone allo-BMT with and without post-transplantation immunotherapy. Our data suggest that the inflammatory environment created by MM growth and immunotherapy specifically recruits activated $CXCR6^+CXCR3^+$ donor T cells by inducing the expression of inflammatory chemokines that are subsequently presented on BMEC heparan sulfate proteoglycans (HSPG).

MATERIALS AND METHODS

Mice

C3.SW-$H2^b$/SnJ (H-2^b, $Ly9.1^+$) mice and OT-1 mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). C57Bl/KaLwRij.hsd mice were purchased from Harlan Laboratories (Indianapolis, IN). All mice were maintained on standard lab chow and sterile water and housed under specific pathogen-free conditions at the Central Animal Laboratory. All animal experiments were approved by the Animal Experimental Committee of the Radboud University Nijmegen Medical Centre and were performed in accordance with institutional and national guidelines. All efforts were made to minimize animal suffering.

Allo-BMT, DC Vaccination, DLI, and MM Challenge

C57Bl/KaLwRij.hsd mice aged 8-12 weeks were irradiated with a linear particle accelerator in 2 treatments of 6 Gy at days -2 and -1 before allo-BMT. Irradiated mice received 5×10^6 T cell-depleted C3.SW-$H2^b$/SnJ BM cells i.v. T cells were depleted using anti-CD4 and anti-CD8 IMag magnetic beads (BD Biosciences, San Jose, CA). Between day -7 and day +7 after allo-BMT, mice received ciprofloxacine in their drinking water. After a 2-month reconstitution period, mice were vaccinated with 5×10^5 $CD11c^+MHCII^+$ DCs i.v. Using anti-CD11c magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), DCs were isolated directly from the BM of C57Bl/KaLwRij.hsd mice that had been injected s.c. with B16-Flt3L cells 14 days earlier, as reported previously [10]. The percentage of $CD11c^+MHCII^+$ DCs was determined by flow cytometry, and DCs were loaded with miHA H7a peptide (KAPDNRETL; Leiden University Medical Centre, Leiden, The Netherlands). On the next day, the mice were injected with 10^6 5T33-GFP MM cells [11], along with DLI in the form of 2×10^7 mononuclear cells from C3.SW-$H2^b$/SnJ spleens and peripheral lymph nodes. In some experiments, nontransplanted C57Bl/KaLwRij.hsd mice with and without 10^6 5T33-GFP MM cells were analyzed.

Genotype Analysis

Genomic DNA was isolated from cell lines using the QIAamp DNA Blood Mini Kit (Qiagen, XXX). 100 ng DNA was amplified in a 50-μL reaction mixture containing 1.25 U AmpliTaq Gold (Life Technologies, Carlsbad, CA), 300 nmol/L MiHA-specific forward and reverse primers (Biolegio, Nijmegen, The Netherlands), 250 μmol/L of each dNTP (Life Technologies), 5 mmol/L $MgCl_2$, and 1× Taqman PCR buffer (Life Technologies, Carlsbad, CA). PCR amplification was performed using a PerkinElmer Cetus DNA Thermal Cycler (Waltham, MA) under the following PCR conditions: enzyme activation for 10 minutes at 95°C, followed by 35 cycles of 15 seconds at 95°C and 1 minute at 66°C. PCR products were analyzed on a 2% agarose Tris-borate-EDTA gel.

RNA Isolation and Quantitative RT-PCR

RNA was isolated and amplified by quantitative RT-PCR (qPCR) as described previously [10]. Primers and probes are listed in Table S1. Expression was normalized against the reference gene porphobilinogen deaminase (*PBGD*) using the $2^{-\Delta Ct}$ method, where ΔCt represents the threshold cycle (Ct) of the sample gene minus the Ct of *PBGD*.

Flow Cytometry, Cell Sorting, and Antibodies

BM was isolated by flushing femurs and tibia with buffer. Staining of cell surface proteins was performed as described previously [10]. Ex vivo 5T33-GFP MM cells were sorted on an EPICS ELITE Cell Sorter (Beckman Coulter, Brea, CA). Then MM cells (93%-98% pure) were lysed for RNA isolation.

Except when noted otherwise, antibodies were purchased from BD Biosciences. Along with the isotype controls FITC-conjugated mouse IgG2b and rat IgG2a (eBioscience); hamster IgG, PE-conjugated rat IgG1, IgG2a, and IgG2b (all eBioscience); and hamster IgG, PE-Cy5-conjugated rat IgG2a (eBioscience), the following rat antibodies were used: syndecans-4; biotinylated CD3, CD11b (eBioscience); B220/CD45R, FITC-conjugated CD4, CD62L (ImmunoTools, Friesoyrth, Germany); H2-D^b, Ly9.1/CD229.1, PE-conjugated CD4, α4/CD49d, α4β7, αL/CD11a (eBioscience); CD86, CCR5, CXCR3 (R&D Systems, Minneapolis, MN); CXCR4 (BD Biosciences and R&D Systems), CXCR6 (R&D Systems); PSGL-1/CD162, I-A/I-E; PE-Cy5-conjugated CD44; APC-conjugated syndecan-1; and PE-Cy7-conjugated CD8. In addition, we used mouse anti-heparan sulfate (10E4) [12]; mouse anti-VSV (Sigma-Aldrich, St Louis, MO); goat anti-glypican-1 (Santa Cruz Biotechnology, Santa Cruz, CA); biotinylated goat anti-mouse CXCL9 and CXCL10 (both R&D Systems); FITC-conjugated mouse anti-H-2Db and hamster anti-CD11c; Alexa Fluor 488-conjugated goat anti-mouse IgG (Life Technologies), Alexa Fluor 488-conjugated goat anti-mouse IgM (Life Technologies); Alexa Fluor 647-conjugated hamster anti-CD11c (AbD Serotec, Kidlington, UK); PE-conjugated hamster anti-CD80; and control (MPB49) and anti-chondroitin sulfate single chains (IO3H10) [13].

ELISA on Soluble Material from Tissues

Soluble material from tissues was isolated essentially as described previously [14]. In brief, BM (from 2 femurs and 2 tibias) single-cell suspensions were prepared in PBS with proteinase cocktail inhibitor (P8340; Sigma-Aldrich). Next, insoluble material was separated from soluble material by 3 consecutive rounds of centrifugation. A sandwich ELISA for mouse CXCL9 was performed in accordance with the manufacturer's instructions (R&D Systems). We performed ELISAs for mouse IFN-γ and CXCL16 as described previously [10]. The detection limit of these ELISAs was ~16 pg/mL for IFN-γ, ~60 pg/mL for CXCL9, and ~24 pg/mL for CXCL16.

BMEC Stimulation and Chemokine Binding

The BMEC lines STR-4, STR-10, and STR-12 [15] were cultured in RPMI 1640 medium supplemented with 1 mM sodium pyruvate, nonessential amino acids (all Life Technologies), 1% penicillin and streptomycin, and 10% FCS (Integro, Zaandam, The Netherlands). For analysis of chemokine expression, BMECs were stimulated with IFN-γ and/or TNF-α (ImmunoTools) for 2 days. For analysis of chemokine binding to heparan sulfate, BMECs were incubated in Iscove's modified Eagle's medium (IMDM) with 0.5% BSA with or without 500 mU/mL heparinase I, II, and II or 1 U/mL chondroitinase ABC (all Sigma-Aldrich) for 1 hour at 37°C. Next, cells were extensively washed and incubated with 10 μg/mL CXCL9 or CXCL10 (ImmunoTools) in PBS for 30 minutes at 4°C. After washing, cells were analyzed by flow cytometry for expression of heparan sulfate and immobilized chemokines.

Adhesion and Migration Assays

$CD8^+$ effector T cells were generated by culturing OT-1 splenocytes in the presence of OVA peptide (SIINFEKL; Leiden University Medical Center) in T cell medium (IMDM with L-glutamine supplemented with 25 mM Hepes, 10 mM sodium pyruvate, 1× nonessential amino acids, 1% penicillin and streptomycin, 50 μM β-mercaptoethanol (all Life Technologies), and 10% FCS (Integro) in a humidified incubator at 37°C for 7-9 days. Migration assays without shear flow were performed with Transwell Costar (Corning Life Sciences, Corning, NY) as described previously [10].

Adhesion and migration assays under flow conditions were performed essentially as described previously [16]. In short, BMECs were cultured to confluence on gelatin-coated coverslips and assembled in a Focht FCS-2 laminar flow chamber (Bioptechs, Butler, PA). The chamber was then rinsed and filled with HBSS (Lonza, Basel, Switzerland) supplemented with 10 mM Hepes (Life Technologies), 0.2% BSA, 1 mM $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$ (all Sigma-Aldrich) at pH 7.4 (TEM medium), with or without 1 μg/mL CXCL9 (ImmunoTools) for 5 minutes, and then washed extensively before T cell perfusion. Subsequently, $CD8^+$ effector T cells ($1\text{-}4 \times 10^6$/mL) were perfused in TEM medium into the chamber and, at a shear stress of 0.18 dyne/cm^2 for 5 minutes, allowed to accumulate and settle on the BMEC monolayer. Shear stress was then adjusted to 2 dynes/cm^2, a physiological level known to support transendothelial migration [17], for 15 minutes. All experiments were performed at 37°C. Video recordings of 1-4 microscopic fields (0.418 mm^2) were read (1 image every 10 seconds) and then analyzed with an in-house software program. The sizes of 4 types of T cells were determined: (1) detaching or (2) stationary/arrested during the migration phase and locomotive from the site of an initial stationary attachment (3) without or (4) with subsequent transendothelial migration, as described