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RADIATION AND HOST RETINOIC ACID SIGNALING PROMOTE THE INDUCTION OF GUT-HOMING DONOR T CELLS AFTER ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Abstract

Intestinal graft-versus-host disease (GVHD) remains a devastating complication after allogeneic hematopoietic stem cell transplantation (HSCT). Although it has been well established that gut-tropic donor T cells expressing integrin $\alpha 4\beta 7$ are required to cause intestinal damage, the factors that control the induction of this pathogenic T cell population remain to be identified. Retinoic acid (RA) plays an important role in inducing $\alpha 4\beta 7$ expression on T cells. In this study, we showed that gene expression of retinaldehyde dehydrogenase, the key enzyme involved in RA biosynthesis, is significantly increased in the spleen and mesenteric lymph nodes (MLNs) of irradiated mice. In a C57BL/6 into B6D2F1 allogeneic HSCT model, irradiation significantly increased the induction of $\alpha 4\beta 7^+$ -donor T cells in MLNs and spleen. Furthermore, we found that the RA pathway modulates the ability of dendritic cells (DCs) to imprint gut-homing specificity on alloreactive T cells. We also showed that host DC RA signaling influences GVHD risk. Our studies identified radiation and recipient RA signaling as two primary factors that dictate the magnitude of gut-homing donor T cell induction after allogeneic HSCT. Attenuating radiation-associated inflammation and modulating host RA signaling represent feasible strategies to mitigate intestinal GVHD by reducing gut-seeking pathogenic donor T cells.

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DISCLOSURE

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INTRODUCTION

Graft-versus-host disease (GVHD) remains the major obstacle for broader clinical application of allogeneic hematopoietic stem cell transplantation (HSCT), an effective treatment for many serious diseases^{1–4}. While donor T cells are the major pathogenic cells responsible for causing GVHD target organ damage, host hematopoietic or non-hematopoietic antigen presenting cells (APCs) are required for initiating GVHD^{5–8}. In particular, host dendritic cells (DCs), the most potent APCs of the immune system, trigger the activation of alloreactive donor T cells early after allogeneic bone marrow transplantation (BMT)^{9–11}. An important aspect of donor T cell activation is the acquisition of tissue homing specificity when they encounter host DCs in the secondary lymphoid organs (SLOs). GVHD mainly targets the skin, liver, and intestines of BMT recipients^{1–4}. However, GVHD with intestinal involvement is associated with particularly poor prognosis and the mortality rate for patients with steroid-refractory intestinal GVHD is very high^{12,13}. Furthermore, it has been well demonstrated that the gut is not only a major target organ of GVHD, but also serves to amplify the systemic inflammatory responses associated with GVHD^{12–14}. Thus, selectively reducing intestinal damage has the potential to mitigate systemic GVHD severity.

In order to gain access to the intestines and cause tissue damage, donor T cells need to express gut-homing molecules including integrin $\alpha 4\beta 7$ ¹⁵. The importance of $\alpha 4\beta 7$ in the pathogenesis of intestinal GVHD has been well demonstrated in both preclinical and clinical studies^{16–20}. It is thus conceivable that the quantity of gut-tropic donor T cells can significantly influence the development and severity of intestinal GVHD. However, it is not completely understood how the induction of gut-seeking donor T cells is regulated after allogeneic BMT. This is a clinically relevant question for devising strategies to mitigate GVHD by reducing the number of this pathogenic donor T cell population.

Retinoic acid (RA) is a metabolite of vitamin A and is required to induce the expression of gut-homing molecules $\alpha 4\beta 7$ and CCR9 on T cells under steady state conditions²¹. Retinaldehyde dehydrogenases (RALDHs) are the key enzymes involved in RA biosynthesis and they are mainly expressed in gut-associated lymphoid tissues (GALTs) such as mesenteric lymph nodes (MLNs)^{22,23}. Recent pre-clinical and clinical studies have demonstrated an important role of vitamin A and RA in the pathophysiology of GVHD^{24–29}. However, the underlying immunological mechanisms are not completely understood. In particular, published animal studies focused largely on RA's effects on donor T cells^{25,26}. It is unclear whether the RA pathway can modulate the alloimmune response by targeting other immune cells critically involved in GVHD pathogenesis.

It has been reported that retinoids can regulate the survival and maturation of human DCs³⁰. In addition, recent studies have demonstrated a proinflammatory function of RA that acts on DCs to enhance the pathogenic intestinal immune response in a celiac disease model³¹. Therefore, we hypothesized that RA signaling targets host APCs, specifically DCs, to modulate GVHD severity. In this paper, we demonstrate that the radiation-induced inflammatory response augments vitamin A metabolism and enhances the generation of gut-homing donor T cells in MLNs after allogeneic HSCT. Interestingly, we found enhanced

RALDH activity in the spleen of irradiated mice, suggesting an alternative SLO distant from the intestine with the ability to imprint gut-homing specificity on donor T cells. In addition, host RA signaling plays an important role in regulating the induction of gut-homing donor T cells. Our results identified a potential mechanism by which radiation intensifies GVHD and indicate that recipient RA signaling is actively involved in GVHD pathogenesis at least partially through modifying host DC function.

MATERIAL AND METHODS

Mice.

C57BL/6 (B6; H-2^b), B6.SJL CD45.1 (H-2^b; CD45.1⁺), Balb/cJ (H-2^d), and B6xDBA/2 F1 (B6D2F1, H-2^{b/d}) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in the Biomedical Resource Center (BRC) at the Medical College of Wisconsin (MCW). Vitamin A-deficient (VAD) mice on a Balb/cJ background were generated as previously described using a vitamin A-deficient diet²⁵. Vitamin A-normal (VAN) control mice were maintained on a mouse chow containing 4 IU/g of vitamin A. Experiments were carried out under protocols approved by the MCW Institutional Animal Care and Use Committee.

Reagents.

All-trans RA was purchased from Sigma-Aldrich (St Louis, MO), dissolved in dimethylsulfoxide (DMSO) at a concentration of 100 mM, and stored as aliquots at -20°C. The RAR- α antagonist (BMS195614), RAR- β antagonist (LE135), RAR- γ antagonist (MM11253), and pan-RAR antagonist (BMS493) were purchased from Tocris Bioscience (Ellisville, MO) and used at the concentrations indicated.

Bone marrow transplantation.

Bone marrow (BM) was flushed from donor femurs and tibias and passed through sterile mesh filters to obtain single-cell suspensions. BMT recipients were conditioned with total body irradiation (900 cGy for Balb/cJ mice; 1100 or 1300 cGy for B6D2F1 mice) using a Shepherd Mark I Cesium Irradiator (J. L. Shepherd and Associates, San Fernando, CA). Lethally irradiated recipients received a single intravenous injection of donor marrow grafts in the lateral tail vein.

Flow cytometry and antibodies.

Cells were labeled with monoclonal antibodies (mAbs) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy5.5, PE-Cy7, or allophycocyanin (APC). Anti-CD11c (HL3), anti-CD45 (30-F11), anti-MHC-II (M5/114.15.2), anti-H-2K^b (AF6-88.5), anti-H-2K^d (34-1-25), anti-TCR β (H57-597), anti-CD4 (GK1.5), anti-CD8 (53-6.7), and anti-interferon (IFN)- γ (XMG1.2) Abs were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-CCR9 (eBio CW-1.2) and anti- α 4 β 7 (DATK-32) Abs were purchased from eBioScience. Intracellular cytokine staining for IFN- γ was performed as described³². Cells were analyzed on a LSRII flow cytometer (Becton-Dickinson, Mountain View, CA). Data were analyzed using FlowJo software (TreeStar, Ashland, Oregon). Cell sorting experiments

were performed on a FACS Aria (Becton Dickinson) and sort purity consistently averaged 98% to 99%.

Mixed lymphocyte reaction (MLR).

Highly purified pan-T cells (10^5 cells/well) were isolated from the spleen of B6 mice using the magnetic cell separation system (Miltenyi Biotech, Auburn, CA) and labeled with CellTrace Violet (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. They were co-cultured with 5×10^4 purified DCs from Balb/cJ mice in 96 well U-bottomed plates at 37°C for four days in complete RPMI medium containing 10% FBS. Isolation of CD11c⁺ DCs using the magnetic cell separation system has been described³². T cell proliferation was assessed by dye dilution using an LSR II flow cytometer.

Generation of BM-derived DCs.

Bone marrow cells were flushed from the femurs and tibias of naïve Balb/cJ mice and single cell suspension was prepared. They were then cultured in complete RPMI medium containing 10% FBS in the presence of 20 ng/ml GM-CSF (R & D Systems) for 6 days. BMS493 (1 μ M) or DMSO was added to the cultures for 24 hours.

Real-time q-PCR.

Real-time q-PCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) and run in a CFX C1000 Real-time Thermal Cycler (Bio-Rad, Hercules, CA). The β 2-microglobulin reference gene as well as *Aldh1a1*, *Aldh1a2*, *Aldh1a3*, *RAR- α* , *RAR- β* , and *RAR- γ* genes were amplified using a QuantiTect Primer Assay Kit (Qiagen). Specificity for all q-PCR reactions was verified by melting curve analysis. To calculate fold-change in gene expression, the average C_q values from triplicate wells were combined from separate experiments.

Aldefluor assay.

RALDH enzyme activity was measured using ALDEFLUOR staining kit (StemCell Technologies, Vancouver, Canada), according to the manufacturer's protocol with some modifications. Briefly, cells were suspended in ALDEFLUOR assay buffer containing the activated ALDEFLUOR substrate and incubated for 45 min at 37 °C. They were then stained with cell surface antibodies and ALDEFLUOR⁺ cells were measured by flow cytometry.

Statistics.

Data analysis was performed using Prism software (GraphPad, La Jolla, CA). Differences between experimental groups were analyzed using a 2-tailed unpaired Student's t test. A p value .05 was deemed to be significant in all experiments.

RESULTS

Irradiation increases RALDH gene expression in the MLNs and spleen.

RA is a key molecule for inducing the expression of gut-homing receptors on T cells²¹. An irreversible step in RA biosynthesis is the conversion of retinaldehyde to RA by RALDH

that is constitutively expressed in cells of mucosal organs, in particular those of GALTs. Lethal irradiation, as a part of allogeneic HSCT procedure, represents a huge stressor to the recipients. Radiation-induced inflammatory responses perturb host homeostasis and can potentially alter the expression of genes involved in various biological processes. We hypothesized that radiation causes aberrant enhancement of RALDH activity in MLNs where imprinting of gut-homing specificity on donor T cells occurs. To test this hypothesis, we examined the effects of radiation on RALDH gene expression by real-time q-PCR analysis. Naïve Balb/cJ mice received total body irradiation (TBI) at 900 cGy to simulate host conditioning for experimental allogeneic HSCT. 72 hours after irradiation, the expression of the *Aldh1a1*, *Aldh1a2*, and *Aldh1a3*, genes encoding for all three RALDH isoforms, increased substantially in the MLNs of irradiated mice compared to that of non-irradiated mice (Fig. 1A). Interestingly, we also observed significantly increased expression of *Aldh1a2* and *Aldh1a3*, but not *Aldh1a1*, in the spleen of irradiated mice (Fig. 1B). The magnitude of *Aldh1a2* gene induction was substantially higher than the other two RALDH isoforms in both MLNs and spleen after irradiation, consistent with the prominent role of RALDH2 in RA synthesis. In contrast, the expression of RALDH was not changed in peripheral tissues such as the liver of irradiated mice (Fig. 1C). Thus, gene expression of key enzyme that is required for RA synthesis is significantly increased in MLNs and spleen after irradiation, with the potential to enhance vitamin A metabolism.

Radiation enhances RALDH enzyme activity and the priming of gut-homing donor T cells early after BMT.

To examine RALDH enzyme activity, we used a well-established ALDEFLUOR assay^{33,34}. Flow cytometry analysis revealed a significant increase in the percentage of ALDEFLUOR-positive 7-AAD⁻CD45⁺CD11c⁺MHC-II⁺ DCs from spleen and MLNs of irradiated mice compared to non-irradiated mice, reflecting an enhanced RALDH enzyme activity (Fig. 2A–2D). It is worth noting that MLN DCs showed substantially higher RALDH activity than splenic DCs. Moreover, we found a significant increase in the percentage, but not absolute cell number, of DCs in the MLNs of irradiated mice versus non-irradiated mice (Supplemental Figs. 1A, 1B). We then assessed whether radiation-induced augmentation of RALDH expression leads to enhanced imprinting of gut-homing molecules on donor T cells after allogeneic BMT. We used a “parent-to-F1” (C57BL/6->B6D2F1) GVHD model that allows us to examine the effect of radiation independent of alloimmunity^{35–37}. While an alloimmune response alone was able to induce the expression of $\alpha 4\beta 7$ on donor T cells isolated from MLNs of B6D2F1 mice, the induction of this molecule was significantly increased in irradiated (1300 cGy) recipients compared to that of non-irradiated mice (Figs. 2E, 2F). Consistent with gene expression data, the expression of $\alpha 4\beta 7$ was significantly increased on donor T cells isolated from the spleen of irradiated mice on day 4 after transplantation (Figs. 2G, 2H). Interestingly, we found persisted upregulation of $\alpha 4\beta 7$ on splenic donor T cells on day 7 after BMT in irradiated mice, suggesting that this is not a transient event (Supplemental Fig. 1B). We also performed transplants using a lower dose of irradiation (1100 cGy) and found similar upregulation of $\alpha 4\beta 7$ on splenic donor T cells (Supplemental Fig. 1C). However, there was no significant difference in the expression other integrins such as $\alpha 4\beta 1$ between the two groups and the magnitude of $\alpha 4\beta 1$ expression was substantially lower than $\alpha 4\beta 7$ (Supplemental Fig. 1D). Compared to allogeneic transplant

recipients, the expression of $\alpha 4\beta 7$ was significantly lower on donor T cells isolated from syngeneic transplant recipients (Supplemental Fig. 2). These results indicate that radiation has the potential to endow spleen with the ability to produce RA and imprint gut-homing specificity on T cells. Thus, irradiation augments RA metabolism in MLNs and spleen, creating a priming environment that promotes the induction of gut-tropic donor T cells early after allogeneic BMT.

Enhancing RA signaling in DCs increases their ability to stimulate the proliferation of alloreactive T cells with heightened gut-homing potential.

T cell activation and acquisition of tissue homing specificity are initiated by their interaction with DCs. Recent studies have demonstrated that RA can act on DCs to modulate their immunological function^{30,31}. The potential effects of RA on host DCs in the context of GVHD have not been studied. We thus tested the hypothesis that RA signaling modulates the ability of DCs to imprint gut-homing specificity on alloreactive T cells. Naïve Balb/cJ mice received TBI and were intraperitoneally injected with either RA or the vehicle control DMSO. After 18 hours, highly purified CD11c⁺ DCs were isolated from the spleens of these mice and co-cultured with CellTrace Violet-labeled naïve T cells from B6 mice in mixed lymphocyte reactions (MLRs). The expression of gut-homing molecules CCR9 and $\alpha 4\beta 7$ was significantly increased on alloreactive CD4⁺ T cells stimulated by RA-exposed DCs (Figs. 3A–3C). Furthermore, CD11c⁺ DCs from RA-treated mice show significantly enhanced capacity to stimulate the proliferation of alloreactive CD4 and CD8 T cells (Fig. 3D). We also performed intracellular cytokine staining and found no significant difference in the percentage of IFN- γ producing CD4 and CD8 alloreactive T cells activated by DMSO- or RA-exposed DCs (Fig. 3E). These data establish that enhancing RA signaling in DCs increases their ability to stimulate the proliferation of alloreactive T cells with heightened gut-homing potential.

Host retinoic acid signaling modulates the induction of gut-homing molecule expression on donor T cells.

To examine how endogenous RA signaling in host DCs modulates gut-homing molecule expression on donor T cells, we generated vitamin A-deficient (VAD) Balb/cJ mice as described previously²⁵. In MLRs, DCs isolated from MLNs of VAD mice show significantly decreased capacity to induce $\alpha 4\beta 7$ expression on alloreactive T cells compared to DCs from vitamin A-normal (VAN) animals (Figs. 4A, 4B). To examine whether acute abrogation of RA signaling on DCs affects their immunostimulatory function, we treated irradiated Balb/cJ mice with a pan-RAR antagonist BMS493. We found that DCs isolated from BMS493-treated mice have significantly diminished ability to induce $\alpha 4\beta 7$ expression on alloreactive T cells (Fig. 4C). To determine how host RA signaling affects the priming of gut-homing donor T cells in vivo, we used VAD and VAN mice as BMT recipients in a B6->Balb/c GVHD model. A large proportion of donor T cells isolated from MLNs of VAN recipients expressed $\alpha 4\beta 7$. In contrast, the expression of this gut-homing receptor was significantly reduced on donor T cells isolated from VAD recipients (Fig. 4D). Remarkably, transient RA supplementation before transplantation completely restored $\alpha 4\beta 7$ expression on donor T cells in these animals (Fig. 4D). To directly tie RA signaling on host DCs to GVHD development, we generated host-type DCs from BM precursors and treated them

with BMS493 to interfere with RA signaling. These host-type DCs were then added to the donor marrow grafts in the B6->Balb/cJ GVHD model. We found a significantly decreased GVHD-associated mortality in BMS493-treated DC recipients compared to control DC recipients, demonstrating the important role of host DC RA signaling in GVHD development (Fig. 4E). Collectively, these data indicate that RA signaling on host DCs can modulate their ability to prime gut-seeking donor T cells that are critically involved in GVHD pathogenesis.

RAR- α signaling is involved in mediating RA's effect on DCs.

We next performed experiments to confirm that DCs express retinoid receptors that can mediate RA signaling. We found that fully differentiated DCs from naïve Balb/cJ mice express RARs. Furthermore, radiation had marginal effects on the expression of RAR- α , RAR- β , and RAR- γ genes in DCs (Fig. 5A). We then examined whether irradiation *per se* can modify the ability of DCs to induce gut-homing molecule expression on alloreactive T cells. We purified CD11c⁺ DCs from naïve Balb/cJ mice and irradiated them *in vitro*. In MLRs, irradiated DCs (at two different DC to T cell ratios) showed significantly increased ability to induce $\alpha 4\beta 7$ expression on CD8⁺, but not CD4⁺ alloreactive T cells (Figs. 5B, 5C). Thus, irradiation *per se* may be sufficient to enhance the ability of DCs to imprint gut-homing molecule expression on alloreactive CD8⁺ T cells *in vitro*. It is plausible that the mucosal barrier dysfunction caused by TBI releases DAMPs and PAMPs that may further activate the RA pathway in DCs *in vivo*.

Since there are three RAR isoforms and all of them can potentially mediate RA's effects on DCs, we sought to determine which RAR isoform is indispensable for the induction of gut-homing molecules using isoform-specific antagonists. Purified Balb/cJ DCs were pre-incubated with BMS195614 (a RAR- α selective antagonist), LE135 (a RAR- β selective antagonist), or MM11253 (a RAR- γ selective antagonist) and washed extensively before coculturing with purified pan-T cells from B6 animals. We found that BMS195614-treated DCs, but not LE-135 or MM11253-treated Balb/c DCs, show significantly decreased capacity to induce the expression of $\alpha 4\beta 7$ on alloreactive T cells (Fig. 5D). Interestingly, a pan-RAR antagonist BMS493 almost completely abrogated the ability of DCs to imprint gut-homing molecule $\alpha 4\beta 7$ on alloreactive T cells (Fig. 5E). These results suggest that RAR- α signaling in DCs plays a major role in modulating the induction of gut-homing molecule expression on alloreactive T cells.

DISCUSSION

Intestinal GVHD is a devastating complication following allogeneic HSCT that causes significant mortality in this patient group. Although it has been well documented that gut-seeking donor T cells expressing $\alpha 4\beta 7$ actively participate in mediating local tissue damage, the major factors that control the induction of this pathogenic cell population remain to be defined. In this study, we found that radiation alters vitamin A metabolism by augmenting the expression of RALDH. Importantly, the enhanced RALDH enzyme activity leads to a corresponding increase in the induction of $\alpha 4\beta 7$ ⁺-donor T cells in the spleen and MLNs early after HSCT. Furthermore, we found that RA signaling through host DCs modulates the

imprinting of gut-homing specificity on alloreactive T cells *in vitro* and influences GVHD lethality *in vivo*.

Total body irradiation is used clinically as an element of pretransplant conditioning regimen, in particular for patients with acute lymphoblastic leukemia. It has been well demonstrated that radiation contributes significantly to the pathogenesis of GVHD. In particular, TBI compromises intestinal barrier function and allows for the leakage of bacterial products into circulation that can amplify inflammatory responses³⁸. On the other hand, lethal irradiation represents a tremendous stressor to the BMT recipient with the potential to disrupt homeostasis and modify biological processes. Indeed, we found irradiation alters vitamin A metabolism by augmenting RALDH expression. Although the expression of all three isoforms of RALDH gene in MLNs is increased, the induction of *Aldh1a2* was substantially higher than the other two. A previous study has demonstrated increased RALDH activity in MLNs and small intestines of allogeneic BMT recipients 14 days after transplantation²⁶. Our current study, however, examined how radiation alone modifies host vitamin A metabolism in the SLOs shortly after irradiation. We reasoned that the activation of donor T cells, including the imprinting of tissue-homing specificity, occurs at the very early time point after HSCT^{39–41}. Thus, it appears that vitamin A metabolism is altered during the early priming phase in SLOs (as shown in this study), as well as during effector phase in GVHD target tissues such as the intestines (as demonstrated by Aoyama and colleagues)²⁶. Although our results suggest that radiation alone is sufficient to increase RALDH activity in host SLOs, it is formally possible that donor T cell mediated alloimmunity can further increase this enzyme activity.

The finding that there is a significant increase in gene expression of *Aldh1a2* and *Aldh1a3* in the spleen of irradiated mice is intriguing. It is generally accepted that the imprinting of gut-homing molecules including $\alpha 4\beta 7$ on T cells is induced in GALTs such as MLNs due to their unique ability to produce RA. However, the involvement of irradiation during allogeneic HSCT may change this default setting. Indeed, we found a significantly higher percentage of $\alpha 4\beta 7$ -expressing donor T cells in the spleen of irradiated mice compared to that of nonirradiated mice in a haploidentical B6->B6D2F1 GVHD model. Thus, our results demonstrate an aberrant enhancement in RALDH enzyme activity in host spleen after irradiation and suggest an alternative site for priming gut-homing donor T cells during GVHD. These observations are consistent with published studies showing a sizable proportion of $\alpha 4\beta 7^+$ -donor T cells in the spleen of recipient mice early after allogeneic BMT^{16,40,42}. These data are also in line with previous findings that the absence of Peyer's patches and/or MLNs is not sufficient to prevent GVHD in BMT involving irradiation^{43,44}. It is likely that gut-homing donor T cells generated in the spleen can compensate for the loss of GALTs and these cells are sufficient to cause intestinal damage and systemic GVHD in those studies. Thus, we propose that radiation-induced aberrant enhancement of RALDH activity in the spleen and MLNs creates an RA-enriched priming environment that promotes the generation of large number of gut-seeking pathogenic donor T cells that contribute significantly to the pathophysiology of GVHD. This may represent a potential mechanism by which radiation intensifies GVHD severity.

We showed that compared to allogeneic transplants, T cells from syngeneic transplant recipients expressed lower levels of gut-homing molecule $\alpha 4\beta 7$, especially in CD8⁺ donor T cells. The data suggest that alloantigen-driven donor T cell activation is required for the optimal expression of gut-homing receptors. In addition, lymphopenia-induced homeostatic T cell proliferation is relatively slow with only 2–3 rounds of cell division per week. This may also explain why we did not observe high levels of $\alpha 4\beta 7$ expression on syngeneic transplant recipients early (day 4) after BMT.

Under homeostatic conditions, a subset of CD11c⁺ DCs residing in GALTs are the major cellular source of RA^{45,46}. In this study, we found significantly increased RALDH enzyme activity in CD11c⁺ DCs in both MLNs and spleen of irradiated mice. These results are consistent with previous findings that MLN CD103⁺ DCs play an important role in inducing gut-homing molecule expression on donor T cells⁴². Importantly, depleting host CCR7⁺ DCs by anti-CD3 preconditioning significantly reduces donor T cell expression of tissue-homing molecules⁴⁷. Thus, host CD103⁺ DCs are likely the major RA producers and are responsible for inducing gut-homing donor T cells in MLNs early after BMT. MLN stromal cells, however, can also produce RA⁴⁸. These radioresistant stromal cells may become activated and contribute to RA production after host CD103⁺ DCs are gradually eliminated by lethal irradiation. We are actively investigating the relative contribution of MLN DCs and stromal cells in RA production after irradiation.

In recent years, there has been increasing recognition of pleiotropic and potent effects of RA on various immune cells under steady state and pathologic conditions⁴⁹. In the context of allogeneic HSCT, several studies have shown that the administration of exogenous RA fuels the inflammatory response and significantly increases GVHD-associated mortality^{25,26,50}. How RA exerts such pro-inflammatory functions to intensify GVHD remains unclear. Given the multifaceted roles of RA in the body, we hypothesized that RA can target host DCs to regulate their immunostimulatory function. Previous studies have indicated that vitamin A metabolism affects the induction of gut-seeking donor T cells after allogeneic BMT^{24–26}. It is tempting to speculate that RA acts on donor T cells directly to regulate the expression of $\alpha 4\beta 7$. However, we show in this study that RA signaling can regulate the expression of gut-homing molecules on alloreactive T cells indirectly through modifying DC function. Remarkably, transient supplementation of RA to the vitamin A-deficient mice prior to BMT almost completely restore $\alpha 4\beta 7$ expression on donor T cells. We also showed that host DC RA signaling influences GVHD-associated mortality. These results indicate that RA signaling can target not only donor T cells, but also host APCs to modulate GVHD. Thus, patient vitamin A levels could influence the severity of GVHD at least partially by modifying APC function. In this study, we also identified RAR- α as the major RAR isoform that mediates RA's effects on DCs. Since previous studies have demonstrated an important role for donor T-cell RAR- α signaling in GVHD pathogenesis^{25,26}, targeting this single RAR isoform has the potential to modulate both donor T cells and host APCs to reduce GVHD risk.

We wanted to point out that there are still several important questions that need to be addressed in future studies. First, we demonstrated in this paper that radiation augments RALDH enzyme activity in host DCs. Whether chemotherapy with significant GI toxicity

has similar effects on activating RA metabolism warrants further study. Secondly, our studies show the important role of host DC RA signaling in regulating GVHD. Whether therapeutic targeting RAR- α pathway will result in reduced GVHD without compromising the graft-versus-leukemia response needs to be investigated. Thirdly, the potential contribution of irradiation-induced lymphodepletion in gut-homing molecule induction should be examined.

In summary, this study defines radiation and host RA signaling as two primary factors that dictate the magnitude of gut-homing donor T cell induction after allogeneic HSCT. Our results point to the modulation of host RA signaling as a feasible strategy to reduce intestinal homing of donor T cells to mitigate intestinal and systemic GVHD. Our study also provides a novel explanation for the detrimental effects of radiation on GVHD. Thus, developing a conditioning regimen devoid of radiation and targeting the RA pathway hold promise for reducing intestinal GVHD risk and improving the outcome of allogeneic HSCT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

APCs	antigen presenting cells
BM	bone marrow
BMT	bone marrow transplantation
DCs	dendritic cells
DMSO	dimethylsulfoxide
GALTs	gut-associated lymphoid tissues
GVHD	graft-versus-host disease
HSCT	hematopoietic stem cell transplantation
MLNs	mesenteric lymph nodes
MLR	mixed lymphocyte reaction
RALDHs	retinaldehyde dehydrogenases
RA	retinoic acid
RAR	retinoic acid receptor

SLOs	secondary lymphoid organs
TBI	total body irradiation
VAD	vitamin A-deficient
VAN	vitamin A-normal

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Figure 1: Irradiation increases RALDH gene expression in the MLNs and spleen.

Naïve Balb/cJ mice received total body irradiation (TBI) at 900 cGy. 24 and 72 hours after irradiation, MLNs (A), spleen (B), and liver (C) were excised from these mice. RNA was extracted from these tissues and gene expression of *Aldh1a1*, *Aldh1a2*, and *Aldh1a3* was analyzed by real-time q-PCR. Data are normalized for β 2-microglobulin RNA and presented as fold increase over gene expression in non-irradiated animals. MLNs are pooled from 3 mice in each group and data are the cumulative results from 3 independent experiments. Data for spleen are the cumulative results from 2 experiments (n = 6 per group). Data for liver are derived from one representative of two experiments (n = 3 per group). Statistics: *P .05, ****P .0001.

Figure 2: Radiation enhances RALDH enzyme activity and the induction of gut-homing donor T cells early after allogeneic BMT.

(A-D) Balb/cJ mice received TBI at 900 cGy and were euthanized on day 2 after irradiation. 0.5×10^6 MLN or spleen cells were suspended in ALDEFLUOR assay buffer containing the activated ALDEFLUOR substrate and incubated for 45 minutes at 37 °C. Cells were then stained with anti-CD45, anti-CD11c and anti-MHC-II antibodies and 7-AAD. Live (7-AAD⁻) CD45⁺CD11c⁺MHC-II⁺ALDEFLUOR⁺ cells were quantified by flow cytometry. Representative contour plots depicting ALDEFLUOR⁺ DCs from spleen (A) and MLN (C) are shown. (B, D) Mean ($\pm$ SEM) percentage of ALDEFLUOR⁺ DCs from spleen (B) and MLN (D). Data are the cumulative results from 5 independent experiments; (E-H) Lethally irradiated (1300 cGy) or non-irradiated B6D2F1 mice were transplanted with 5×10^6 B6 BM together with B6 splenocytes (adjusted to yield a T-cell dose of 4×10^6). Mice in both groups were euthanized on day 4 after transplantation. The expression of integrin $\alpha 4\beta 7$ on donor T cells isolated from MLNs and spleen of recipient mice was examined by gating on H-2^bH-2^d- CD4⁺ or H-2^bH-2^d- CD8⁺ donor T cells. (E, G) Representative contour plots for $\alpha 4\beta 7$ expression on donor T cells isolated from MLNs (E) and spleen (G) are shown. (F, H) Mean ($\pm$ SEM) percentage of $\alpha 4\beta 7$ ⁺ donor T cells isolated from MLNs (F) and spleen (H)

(n = 4 mice per group). Data are derived from one representative of 3 experiments. Statistics:
P .01, *P .001, ****P .0001.

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Figure 3: RA-exposed DCs show increased ability to stimulate the proliferation of alloreactive T cells with heightened gut-homing potential.

Naïve Balb/cJ mice received TBI and were intraperitoneally injected with either DMSO or RA (900 μ g). After 18 hours, highly purified CD11c⁺ DCs were isolated from the spleens of these mice. 5×10^4 DMSO- or RA-exposed DCs were co-cultured with 1×10^5 purified B6 splenic pan-T cells labeled with CellTrace Violet dye. Four days later, cells were harvested for flow cytometric analysis. **(A, B)** Representative flow plots for CCR9 and $\alpha 4\beta 7$ expression on CD4⁺ and CD8⁺ alloreactive T cells. **(C)** Mean ($\pm$ SEM) percentage of alloreactive CD4⁺ and CD8⁺ T cells expressing gut-homing molecules CCR9 and $\alpha 4\beta 7$ from triplicate wells. **(D)** Mean ($\pm$ SEM) percentage of proliferating (CellTrace Violet low) CD4⁺ and CD8⁺ T cells from triplicate wells. **(E)** Mean ($\pm$ SEM) percentage of CD4⁺IFN- γ ⁺ and CD8⁺IFN- γ ⁺ cells from triplicate wells as assessed by intracellular cytokine staining. Data are derived from one representative of at least three experiments. Statistics: *P $\leq$.05, **P $\leq$.01, ***P $\leq$.001, ****P $\leq$.0001.

Figure 4: Abrogating RA signaling in DCs inhibits their ability to induce gut-homing molecule expression on alloreactive T cells.

Purified CD11c⁺ DCs were isolated from the MLNs of vitamin A normal (VAN) or vitamin A deficient (VAD) mice. 5×10^4 of these DCs were co-cultured with 1×10^5 purified spleen pan-T cells labeled with CellTrace Violet dye from naïve B6 mice. Four days later, cells were harvested for flow cytometric analysis. **(A)** Representative flow plots for $\alpha 4 \beta 7$ expression on CD4⁺ and CD8⁺ alloreactive T cells. **(B)** Mean ($\pm$ SEM) percentage of proliferating alloreactive CD4⁺ $\alpha 4 \beta 7$ ⁺ and CD8⁺ $\alpha 4 \beta 7$ ⁺ T cells from triplicate wells. Data are derived from one representative of three experiments. **(C)** Naïve Balb/cJ mice received TBI and were intraperitoneally injected with either DMSO or BMS493 (440 μ g). After 18 hours, purified CD11c⁺ DCs were isolated from the spleens of these mice. 5×10^4 DMSO- or BMS493-exposed DCs were co-cultured with 1×10^5 purified spleen pan-T cells labeled with CellTrace Violet dye from naïve B6 animals. Four days later, cells were harvested for flow cytometric analysis. Mean ($\pm$ SEM) percentage of proliferating alloreactive CD4⁺ and CD8⁺ T cells expressing $\alpha 4 \beta 7$ from 3 wells is shown. Data are derived from one representative of two experiments. **(D)** Naïve VAD mice received 4 doses of either DMSO or RA (450 μ g/mouse) by intraperitoneal injections from day -3 to day 0 of BMT. Lethally

irradiated VAN and DMSO- or RA-treated VAD mice were transplanted with B6 BM (5×10^6) and splenic T cells (adjusted to yield a T-cell dose of 1.5×10^6). Recipient mice were euthanized on day 4 after BMT. Percentage of CCR9 and $\alpha 4\beta 7$ positive donor T cells (H-2^bCD4⁺ or H-2^bCD8⁺) isolated from MLNs of recipient mice is depicted. Data are presented as the mean $\pm$ SEM and are the cumulative results from 2 experiments (n = 4 mice per group). Statistics: **P .01, ***P .001, ****P .0001. (E) Lethally irradiated (900 cGy) Balb/c mice were transplanted with B6 BM (6×10^6) and purified pan-T cells (0.6×10^6) together with DMSO- or BMS493-treated BM-derived DCs (0.6×10^6) from Balb/c mice. Overall survival is depicted. Data are the cumulative results from 3 experiments (n = 12–14 mice per group).

Figure 5: RAR- α signaling is involved in mediating RA's effect on DCs.

(A) Naïve Balb/cJ mice were irradiated at 900 cGy. RNA was extracted from sort-purified CD11c $^{+}$ DCs isolated from spleens of these mice 18 hours after irradiation. Gene expression of RAR- α , RAR- β , and RAR- γ was analyzed by real-time q-PCR. Data are normalized for β 2-microglobulin RNA and presented as fold increase over gene expression in sort-purified CD11c $^{+}$ DCs from naïve animals. Data are the cumulative results from two independent experiments. (B, C) Highly purified CD11c $^{+}$ DCs were isolated from the spleen of naïve Balb/cJ mice. Equal numbers of irradiated (3000 cGy) or non-irradiated DCs at 5×10^4 per well (B) or 1×10^4 per well (C) were co-cultured with 1×10^5 purified spleen pan-T cells labeled with CellTrace Violet dye from naïve B6 animals. Four days later, cells were harvested for flow cytometric analysis. Mean ($\pm$ SEM) percentage of proliferating alloreactive CD4 $^{+}$ and CD8 $^{+}$ T cells expressing α 4 β 7 from 7 wells is shown. Data are the cumulative results of two experiments. (D, E) Purified CD11c $^{+}$ DCs were isolated from naïve Balb/cJ mice and incubated with 1 μ M BMS195614, LE135, MM11253 (D), or BMS493 (E) for 2–3 hours and washed extensively with cell culture media. 5×10^4 DCs from each treated group were co-cultured with 1×10^5 purified spleen pan-T cells labeled with CellTrace Violet dye from naïve B6 animals. Four days later, cells were harvested for flow cytometric analysis. Mean ($\pm$ SEM) percentage of alloreactive CD4 $^{+}$ and CD8 $^{+}$ T cells expressing α 4 β 7 from 5 wells is shown. Data are the cumulative results from 2 experiments. Statistics: *P .05, **P .01, ****P .0001.