A CRITICAL ROLE FOR THE RETINOIC ACID SIGNALING PATHWAY IN THE PATHOPHYSIOLOGY OF GASTROINTESTINAL GRAFT-VERSUS-HOST DISEASE

Xiao Chen\textsuperscript{1,2}, Joseph Dodge\textsuperscript{1,2}, Richard Komorowski\textsuperscript{3}, William R. Drobyski\textsuperscript{1,2}

From the Division of Hematology/Oncology\textsuperscript{1} and the Departments of Medicine\textsuperscript{2} and Pathology\textsuperscript{3}, Medical College of Wisconsin, Milwaukee, WI 53226, USA

Running title: RA Signaling in gastrointestinal GVHD

Scientific Category: Transplantation

Address correspondence to:
Xiao Chen, MD, PhD
Division of Hematology & Oncology
Medical College of Wisconsin
8701 Watertown Plank Road
Milwaukee, WI 53226
Phone: 414-955-4656, E-mail: xchen@mcw.edu
KEY POINTS

1. Retinoic acid regulates donor T cell trafficking during GVHD.
2. The retinoic acid receptor alpha signaling pathway plays a critical role in the pathophysiology of gastrointestinal graft versus host disease.

ABSTRACT

Damage to the gastrointestinal tract during graft-versus-host disease (GVHD) is one of the major causes of morbidity and mortality in allogeneic hematopoietic stem cell transplant (HSCT) recipients. In the current study, we have identified a critical role for the retinoic acid (RA) signaling pathway in the induction and propagation of gastrointestinal GVHD. The administration of exogenous RA significantly increased expression of the gut-homing molecules, CCR9 and α4β7, on donor T cells in mesenteric lymph nodes, and augmented the accumulation of pro-inflammatory CD4+ and CD8+ T cells within the gut mucosa, leading to a selective exacerbation of colonic GVHD and increased overall mortality. Conversely, depletion of RA in recipient mice by vitamin A deprivation resulted in a dramatic reduction of gut homing molecule expression on donor T cells after HSCT. Significantly, absence of the retinoic acid receptor-α on donor T cells markedly attenuated the ability of these cells to cause lethal GVHD and this observation was attributable to a significant reduction in pathological damage within the colon. These findings identify an organ-specific role for RA in GVHD, and provide evidence that blockade of the RA signaling pathway may represent a novel strategy to mitigate the severity of colonic GVHD.
INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a potentially life-saving therapeutic modality for patients with hematological malignancies and non-malignant disorders. Successful outcomes, however, are compromised by graft-versus-host disease (GVHD) which remains the major complication of this treatment and the leading cause of morbidity and mortality. GVHD is induced by donor T cells recognizing host alloantigens expressed by host antigen presenting cells (APCs). This results in the activation and expansion of donor T cells leading to pro-inflammatory cytokine production and the induction of cytotoxic T cell responses which both lead to tissue damage. Acute GVHD typically develops in a restricted set of organs including the skin, liver and gastrointestinal tract. Of these target organs, the gastrointestinal tract is of particular importance. Compelling data in experimental animal models indicate that the gut is not only a major target organ of GVHD, but plays a crucial role in the amplification of systemic GVHD severity. Clinically, involvement of the gastrointestinal tract in patients with acute GVHD is a major cause of morbidity and mortality.

The gut-associated lymphoid tissue (GALT), which consists of Peyer’s patches (PPs), mesenteric lymph nodes (MLNs), and lymphoid cells in the lamina propria and epithelium, is not only responsible for eliciting, but also regulating, immune responses in the intestinal mucosa. The adaptive immune responses that occur in the gut are modulated by a complex interplay of regulatory mechanisms within these lymphoid tissue sites. Recently, all-trans retinoic acid (RA) has emerged as a critical regulator of gut immunity. RA is an active metabolite of vitamin A that is involved in many important biological processes in vivo. Within the immune system, RA influences many immune cell lineages and regulates an array of immune responses in vivo. RA is produced by a population of CD103+ dendritic cells in the gut and plays a pivotal role in the regulation of inflammation within the colon. RA is also able to enhance the stability of Foxp3 in natural Tregs (nTregs) and facilitate the conversion of CD4+Foxp3− T cells into induced Tregs (iTregs) by up-regulating Foxp3. Recent studies have
demonstrated that RA can influence the lineage decisions of CD4+ T cells. Culture of naïve CD4+ T cells under Th17 polarizing conditions in the presence of RA has been shown to reduce the number of IL-17-secreting cells while resulting in a commensurate increase in the number of iTregs.\textsuperscript{20-22} Thus, RA appears able to alter the balance between effector and regulatory arms of the immune system similar to what has been described for blockade of IL-6 signaling.\textsuperscript{23} Additionally, RA has been shown to augment the expression of gut-homing receptors, such as CCR9 and α4β7 on T cells under steady state conditions,\textsuperscript{24} and mediate the recruitment of Tregs into sites of inflammation.\textsuperscript{25} The ability to drive gut homing along with the capacity to stabilize nTreg function and facilitate the induction of iTregs, even in the presence of inflammation, suggests that administration of RA might be a strategy to reduce inflammatory responses during GVHD, particularly within the colon microenvironment. The purpose of the current study was therefore to define the role of RA in the pathophysiology of GVHD and to determine to what extent endogenous and exogenous RA was able to modulate the balance between inflammation and tolerance during GVH reactivity.

**MATERIAL AND METHODS**

**Mice.** C57BL/6 (B6; H-2\textsuperscript{b}), Balb/cJ (H-2\textsuperscript{d}) mice, C.129S7 Rag-1 (Balb/c Rag), and B6 Foxp\textsuperscript{3EGFP} animals\textsuperscript{26} were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in the Animal Resource Center (ARC) at the Medical College of Wisconsin (MCW). RAR-α-deficient (RAR-α\textsuperscript{−/−}) mice (B6129 background) were kindly provided by Dr. Pierre Chambon (IGBMC, Strasbourg, France).\textsuperscript{27} Vitamin A-deficient (VAD) and vitamin A-sufficient (VAS) mice were generated as previously described.\textsuperscript{28} All animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)–accredited ARC of the MCW. Experiments were all carried out under protocols approved by the MCW Institutional Animal Care and Use Committee.
Reagents. All-trans retinoic acid (RA) was purchased from Sigma-Aldrich (St Louis, MO), dissolved in DMSO at a concentration of 100 mM, and stored as aliquots at -20°C. The RAR-α agonist, AM80, and the RAR-α antagonist, BMS 195614, were purchased from TOCRISbioscience (Ellisville, MO) and used at the concentrations indicated.

Bone marrow transplantation. Bone marrow (BM) was flushed from donor femurs and tibias with Dulbecco’s Modified Eagle Medium (DMEM) (Gibco-BRL, Carlsbad, CA) and passed through sterile mesh filters to obtain single-cell suspensions. Host mice were conditioned with total body irradiation administered as a single exposure at a dose rate of 74 cGy using a Shepherd Mark I Cesium Irradiator (J. L. Shepherd and Associates, San Fernando, CA). Irradiated recipients received a single intravenous injection in the lateral tail vein of BM with or without added spleen cells.

Leukemia model. A20 murine lymphoma cells of Balb/c background (H-2d) were obtained from the American Type Culture Collection and transfected with firefly luciferase as previously described. For in vivo bioluminescence imaging (BLI), mice were given an intra-peritoneal injection of luciferin (150 mg/kg body weight) and then anesthetized with isoflurane gas using a Xenogen XGI Gas Anesthesia System (Perkin Elmer, Waltham, MA) and imaged using the IVIS Imaging system to assess bioluminescence 10 minutes after injection of the substrate. Imaging data were analyzed with Living Image Software (Xenogen).

Cell sorting and flow cytometry. Spleen cells were collected from naive mice or BMT recipients and sorted on a FACSAria (Becton Dickinson, Mountain View, CA). Sort purity for these studies consistently averaged 98% to 99%. Cells were isolated from spleen, lymph nodes, and GVHD target organs (liver, lung, and colon) of transplant recipients as described previously and were labeled with monoclonal antibodies (mAbs) conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-Cy5.5, or allophycocyanin (APC). FITC-anti-CD11c (clone HL3, hamster IgG), PE-anti-CD11b (clone...
MV70, rat IgG2b), FITC-anti-H-2Kb (clone AF6-88.5, mouse IgG2a), PE-Cy5-anti-CD4 (clone RM4-5, rat IgG2a), PE-Cy5-anti-CD8 (clone 543-6.7, rat IgG2a), PE-anti-TCRβ (clone H57-597, hamster IgG), APC-antiCD8a (clone 53-6.7, rat IgG2a), PE-anti-CD4 (clone GK1.5, rat IgG2b), FITC-anti-CD8a (clone 53-6.7, rat IgG2a), FITC-anti-IFN-γ (clone XMG1.2, rat IgG1), and PE-anti-IL-17 (clone TC11-18H10, rat IgG1) were all purchased from BD Biosciences (Franklin Lakes, NJ). FITC-anti-CCR9 (clone CW-1.2, mouse IgG2a), APC-anti-CCR9 (clone CW-1.2, mouse IgG2a), and PE-anti-α4β7 (clone DATK32, rat IgG2a) were obtained from eBioscience (San Diego, CA). Intracellular cytokine staining for IFN-γ and IL-17 was performed as described. In some experiments, cells were labeled with CellTrace Violet (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were analyzed on a FACSCalibur or LSRII flow cytometer with CellQuest or FACSDiva software (Becton-Dickinson). Data were analyzed using FlowJo software (TreeStar, Ashland, Oregon).

Histological analysis. Representative samples of liver, colon, tongue, and lung were obtained from transplant recipients and fixed in 10% neutral-buffered formalin. Samples were then embedded in paraffin, cut into 5-μm-thick sections, and stained with hematoxylin and eosin. One-two sections per organ were evaluated. A semiquantitative scoring system was used to account for histological changes in the colon, liver, and lung as previously described. The scoring system employed to define the severity of GVHD in the tongue denoted 0 as normal, 1 as mononuclear cell infiltration into the dermis and stroma, 2 as inflammatory cells extending into the epithelium, and 3 as blistering or ulceration present in the epidermis. All slides were coded and read in a blinded fashion. Images were visualized using a Nikon Eclipse E400 microscope and a Nikon Plan APO 10X/0.45 objective lens (Nikon, Tokyo, Japan). Image acquisition was performed with a Zeiss Axiom camera and Axiovision 3.0.6 SP2 software (Zeiss, Berlin, Germany).
Mixed lymphocyte culture (MLC). B6 splenocytes (adjusted to yield a T-cell dose of $1 \times 10^5$ cells/well) were co-cultured with $5 \times 10^4$ Balb/c dendritic cells in 96 well U-bottomed plates at 37°C for four days. Isolation of CD11c+ dendritic cells using the magnetic cell separation system (Miltenyi Biotech, Auburn, CA) has been described.32 One microcurie (0.037 MBq) of $^3$H-thymidine was added to triplicate wells for the final 12 to 18 hours prior to harvest. Thymidine incorporation was assessed using a Wallac 1450 Microbeta liquid scintillation counter (Perkin Elmer, Shelton, CT).

Real-time q-PCR. Real-time q-PCR was performed using QuantiTect SYBR Green PCR Kit (Qiagen) and run in a CFX C1000 Real-time Thermal Cycler (Bio-Rad, Hercules, CA). The 18S reference gene was amplified using QuantiTect Primer Assay Kit (Qiagen, Valencia, CA). The primers for RAR-α have been reported33 and were purchased from Integrated DNA Technologies (Coralville, IA). Specificity for all q-PCR reactions was verified by melting curve analysis. To calculate fold-change in gene expression, the average ΔΔCq values from triplicate wells were combined from separate experiments.

Quantification of RA in tissues by mass spectrometry. Excised tissues were weighed and all-trans Retinoic Acid-d₅ (2 ng) was added to each sample as an internal standard. The tissues were homogenized in acetonitrile and prepared as previously described.34 Retinoic acid was determined by liquid chromatography-electrospray ionization-mass spectrometry (Agilent 1100 LC-MSD, SL model). The samples were separated on an ACE 5 C18-PFP column, 250 x 2.1mm 5um (MAC MOD, Chadds Ford, PA) using water and acetonitrile containing 0.1% formic acid as a mobile phase. The concentrations of RA were calculated by comparing the ratio of peak areas to the standard curve.

Statistics. Data analysis was performed using Prism software (GraphPad, La Jolla, CA). Survival comparisons were performed using the Log-Rank test. Other differences between experimental groups were analyzed using a 2-tailed unpaired Student’s t test. A p value $\leq 0.05$ was deemed to be significant in all experiments.
RESULTS

Administration of RA selectively intensifies GVHD of the colon. Initial experiments were performed to determine whether exogenous RA administration had any salutary effect on the severity of acute GVHD. Lethally irradiated Balb/c mice were transplanted with B6 BM alone or together with B6 spleen cells to induce GVHD. Cohorts of mice that received adjunctive spleen cells were then administered either DMSO (vehicle) or RA every other day beginning at the day of transplantation through day 20. The dose, route, and administration schedule were derived from previously published studies that had demonstrated potent immune effects in vivo.\textsuperscript{22,35} Unexpectedly, we observed significantly accelerated GVHD-associated mortality in RA-treated mice compared with DMSO-treated animals (p< 0.0001; Figure 1A). This was accompanied by more weight loss over the first four weeks which corresponded to the period of time when both groups could be comparably assessed given the early mortality in RA-treated mice (Figure 1B). Histological examination of GVHD target organs (colon, liver, and lung) and the tongue which served as a surrogate for skin damage\textsuperscript{36} was performed 5 weeks after transplantation in separate experiments. There was a significant increase in pathologic damage in the colons of RA-treated mice compared with DMSO-treated animals (9.7 ± 0.8 versus 3.3 ± 0.9, p<0.001), whereas there was no significant difference in pathology scores in either the liver, tongue or lung (Figures 1C, 1D). Administration of RA to animals reconstituted with BM only resulted in no mortality and pathological analysis showed no evidence of GVHD in the colon at 90 days post transplantation (data not shown). Thus, the detrimental effect of RA was dependent upon the presence of alloreactive donor T cells. These studies demonstrated that exogenous RA administration exacerbates GVHD lethality and augments GVHD-associated damage within the colon microenvironment.
RA enhances gut-homing molecule expression on donor T cells in mesenteric lymph nodes. The acquisition of tissue-homing molecule expression on T cells after activation in secondary lymphoid tissues is important in determining the subsequent migration pattern of effector T cells into non-lymphoid tissues. Recent studies have demonstrated that an important biological function of RA is to induce the expression of gut-homing molecules, α4β7 and CCR9, on T cells under steady state conditions. Since we observed a significant enhancement of colonic damage in RA-treated mice, we sought to determine whether RA exposure resulted in increased expression of these molecules. We first addressed this question using a mixed lymphocyte culture to mimic the in vivo alloimmune response. Culture in the presence of physiological concentrations of RA (10 nM) resulted in increased T-cell proliferation (Supplementary Figure 1A). There was also decreased expression of CD62L and increased expression of CD25 on CD4+ and CD8+ T cells indicative of an activated T-cell phenotype (Supplementary Figure 1B). More importantly, in the presence of RA, there was a dose-dependent increase in the expression of CCR9 and α4β7 on CD4+ and CD8+ T cells (Supplementary Figure 1C). To determine whether RA affects T cell proliferation in the absence of alloreactivity, we performed syngeneic bone marrow transplantation and examined early T cell division in syngeneic marrow transplant recipients. Recipients of syngeneic marrow grafts that were administered RA had no increase in donor T cell proliferation, indicating that RA did not enhance T cell division under conditions of homeostatic expansion (Supplementary Figure 2).

To further evaluate the acquisition of gut-homing molecule expression by RA exposure in vivo, DMSO or RA-treated animals were euthanized on day 7 after transplantation and examined for the expression of tissue-homing molecules on donor T cells in secondary lymphoid tissues. Expression of CCR9 and α4β7 was significantly higher on donor T cells (both CD4+ and CD8+) isolated from the MLNs of RA-treated mice in comparison with DMSO-treated animals (Figure 2A, 2B). The augmentation of CCR9 and α4β7 expression by RA was also observed to be dose-dependent (Figure 2C). Expression of other chemokine receptors that have been implicated in GVHD pathogenesis, such as CCR5, CXCR3, and CCR10 in other secondary lymphoid tissues, was not affected by RA treatment (Figures 2D-2F). Notably, we
observed that there was also an increase in the percentage of donor T cells that expressed CCR9 and α4β7 in peripheral lymph nodes, suggesting that exogenous RA could effect expression of gut homing molecules at nodal sites distant from the mesentery (Figure 2G). Collectively, these data indicated that RA treatment enhances the priming of gut-homing donor T cells in the MLNs, but does not affect the acquisition of chemokine receptors on T cells designated for other GVHD target organs.

**Effect of RA administration on pro-inflammatory donor T cells and Tregs within the colon microenvironment.** Since we observed a significantly higher number of T cells expressing gut-homing molecules in MLNs after RA administration, we sought to determine if this led to increased recruitment of pro-inflammatory donor T cells into the colon in the later stage of GVHD. To examine the effect of RA more specifically in GVHD target tissues, we isolated mononuclear cells from the spleen, liver, lung, and colon of recipient mice treated with either DMSO or RA three weeks after transplantation. There was a significant eight-fold increase in the absolute numbers of both CD4+IFN-γ (T_H1) (mean 0.08 x 10^4 versus 0.01 x 10^4) and CD4+IL-17 (T_H17) cells (mean 0.42 x 10^3 versus 0.05 x 10^3) in the colon of RA-treated mice versus DMSO-treated animals, whereas there were no statistical differences in T_H1 or T_H17 cells in the spleen, liver and lung (Figures 3A, 3B). The absolute number of CD8+CD103+ cells, which has been shown to represent a specific gut-destructive T-cell population, was also significantly augmented (nine-fold) in the colons of RA-treated mice (mean 1.0 x 10^3 versus 0.11 x 10^3) (Figure 3C). We also examined the effects of RA on Treg reconstitution using Foxp3EGFP reporter mice as donors for transplantation. We observed that there was a significant increase in the absolute number of Tregs in the colon of RA-treated mice as well (Figure 3D). However, the increase in Tregs (three-fold, mean 0.022 x 10^4 versus 0.07 x 10^4) was noted to be more modest than was observed for T_H1, T_H17 and CD8+ T cells. Notably, there was a corresponding increase in the percentage of CD4+ and CD8+ T cells that expressed α4β7 in the colon indicating that these cells are indeed able to traffic to the colon. Since CCR9 has been show to be important in directing T cell trafficking into the small intestines, we examined this tissue site
and observed an increased percentage of CCR9-expressing T cells in RA-treated mice (Figure 3E). Collectively, these data indicated that RA administration resulted in a significant increase in the absolute number of pro-inflammatory donor T cells in the colon without a proportional increase in the Treg compartment, resulting in a more pronounced imbalance between the effector and regulatory arms of the immune system.

**Vitamin-A deficiency in recipient mice impairs the acquisition of gut-homing molecule expression on donor T cells after allogeneic BMT.** Since exogenous RA administration augmented the generation of gut-homing T cells, we examined whether depletion of RA would result in a corresponding reduction in these cells as additional confirmation for the role of this metabolite in GVHD. To address this question, we generated vitamin A-deficient mice and employed them as recipients for these studies. Due to superior breeding on a vitamin A restricted diet, we employed Balb/c Rag as opposed to Balb/c mice. Lethally irradiated vitamin A-sufficient (VAS) or vitamin A-deficient (VAD) Balb/c Rag mice were transplanted with B6 BM and spleen T cells to induce GVHD. Three days after transplantation, a sizable proportion of donor T cells from the MLNs of VAS mice expressed $\alpha_4\beta_7$ and CCR9, whereas expression of these gut-homing receptors was significantly reduced on donor CD4$^+$ and CD8$^+$ T cells from VAD mice (Figures 4A, 4B). Interestingly, expression of the skin homing molecule CCR10 in the PLNs was increased in the VAD group (Figure 4C, 4D), suggesting that vitamin A may have divergent actions on chemokine receptor expression. To determine whether vitamin A deficiency affected alloreactive T cell proliferation, B6 donor T cells were labeled with CellTrace Violet dye and adoptively transferred along with unlabeled BM cells into lethally irradiated VAS or VAD recipients. Splenic donor CD4$^+$ and CD8$^+$ T cells were then examined three days post transplantation when prior studies have shown that robust cell division has occurred in the spleen. There was no significant difference in the percentage of CellTrace Violet low donor CD4$^+$ and CD8$^+$ T cells between the two groups (Figures 4E, 4F), indicating that lack of vitamin A did not appear to affect T-cell proliferation. Consistent with an earlier report by Koenecke and colleagues, we also observed that VAD recipients had more severe GVHD in the liver and increased
splenic cellularity (Supplementary Figure 3). These data indicated that vitamin A deficiency in recipient mice impairs the acquisition of gut-homing molecules on donor T cells in MLNs, and further reinforces a role for RA in imprinting gut-homing donor T cells during GVHD.

**RAR-α is critical for mediating the effects of RA in alloreactive T cells.** Two distinct families of nuclear receptors for RA have been identified; the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), both of which have three isoforms known as α, β and γ.44 Since previous studies indicated a dominant role of RAR-α in mediating the effects of RA on T cells during adaptive immune responses,21,28 we therefore focused our studies on this RAR subfamily. We employed a pharmacologic agonist and antagonist specific for RAR-α to dissect the role of this pathway in mediating the effects of RA. B6 T cells were co-cultured with Balb/c DCs in the presence or absence of the RAR-α agonist, AM80. We found that AM80 significantly enhanced expression of the gut-homing molecules CCR9 and α4β7 on both CD4+ and CD8+ alloreactive T cells (Figures 5A, 5B), indicating that direct targeting of the RAR-α signaling pathway can mimic the effect of RA on alloreactive T cells. To further confirm the involvement of RAR-α, B6 spleen cells were co-cultured with purified Balb/c DCs in the presence of RA alone or together with an RAR-α-specific antagonist, BMS195614. We observed that BMS195614 significantly inhibited the up-regulation of CCR9 and α4β7 on CD4+ and CD8+ T cells that is induced by RA (Figure 5C). Furthermore, addition of BMS195614 resulted in increased expression of CD62L and decreased expression of CD25 indicating that RAR-α blockade led to a reduction in the percentage of T cells with an activated phenotype (Figure 5D). Finally, BMS195614 abrogated the RA-induced increase in T cell proliferation (Figure 5E). These studies demonstrated that an RAR-α agonist could replicate the augmentation of gut-homing molecule expression observed with RA, and that this effect could be blocked by specific antagonism of the RAR-α pathway.
Endogenous RA production and gene expression of RAR-α in donor CD4+ T cells are enhanced after allogeneic BMT. While the above studies established that exogenous RA significantly exacerbated GVHD within the colon microenvironment, it is unclear how endogenous RA production and RAR-α expression on donor T cells contribute to the pathophysiology of GVHD. To address these questions, we measured endogenous RA levels in the colons of mice undergoing either syngeneic or allogeneic BMT. These studies revealed that RA was significantly increased in the colon tissue of allogeneic transplant recipients compared with syngeneic transplant recipients early after BMT (day 7) (Figure 6A). Thereafter, there was a trend towards higher RA levels in recipients of allogeneic versus syngeneic marrow grafts, but this did not reach statistical significance at these later time points. Given prior results supporting a role for the RAR-α signaling pathway in mediating the effects of RA (Figure 5), we examined whether RAR-α gene expression levels were augmented in donor T cells during GVHD. Real time q-PCR analysis revealed a mean 3.5-fold increase in RAR-α expression in sort-purified donor splenic CD4+ T cells 4 days post transplantation when compared to purified naïve CD4+ T cells obtained from original donor animals (Figure 6B). Gene expression levels were also noted to be higher 7 days after BMT, although somewhat less than at the earlier time point. In contrast, mRNA levels of RAR-α in purified CD8+ T cells obtained from the spleens of GVHD animals did not significantly differ from levels observed in naïve CD8+ T cells from original donor mice (Figure 6B). Collectively, these results indicated that endogenous RA levels are increased early during GVHD in the colon and that there is also concomitant increased expression of RAR-α in donor CD4+ T cells.

Recipients of RAR-α-deficient T cells are protected from GVHD and retain the ability to mount a GVL response. To further define the role of the RAR-α signaling pathway in GVHD pathogenesis, we examined the ability of RAR-α-deficient T cells, which lack the capacity to respond to endogenous RA, to cause GVHD. BM and splenocytes from RAR-α-deficient (RAR-α−/−) mice or wild type (WT, RAR-α+/+) littermates were transplanted into lethally irradiated Balb/c mice. Recipients of RAR-α-deficient
marrow grafts had decreased GVHD-associated mortality when compared with the recipients of WT T cells (93% versus 36%, p=0.002) (Figure 7A). Moreover, mice that received RAR-α−/− T cells had significantly less GVHD-associated weight loss (data not shown). Moreover, histological examination of GVHD target organs on days 10-12 after transplantation revealed a significant reduction in pathologic damage in the colon of mice that received RAR-α−/− T cells compared with control mice (2.1 ± 0.7 versus 8.4 ± 1.0, p<0.001; Figure 7B), whereas there was no difference in the liver. We did observe more pathological damage in the lung in recipients of RAR-α−/− grafts which was due exclusively to increased perivascular cuffing without any parenchymal damage. Early post transplantation, there was a significant decrease in the percentage of T H1, but not T H17, cells in the spleens of animals reconstituted with RAR-α−/− marrow grafts (Supplementary Figure 4). Quantification of donor T cells in GVHD target organs revealed a significant and selective reduction in the absolute number of donor CD4+ and CD8+ T cells in the colons of recipients of RAR-α−/− T cells. There was a corresponding increase in the absolute number of donor CD4+ and CD8+ T cells in the lung and spleen (Figures 7C and 7D). The observed differences in GVHD-induced mortality could not be ascribed to any pre-existing differences in the T cell compartment of the donor mice because there was no difference in the percentage of CD4+, CD8+, and CD4+Foxp3+ T cells in the spleen between naïve RAR-α−/− and WT littermate controls (Supplementary Figures 5A, 5B). There was also no difference in proliferation of CD4+ or CD8+ T cells in response to alloantigen between the two donor types (Supplementary Figure 5C). These results demonstrated that abrogation of RA signaling through RAR-α in donor T cells markedly diminished their capacity to cause lethal GVHD, and that this was attributable to selective protection in the colon. Despite the reduction in GVHD, animals transplanted with marrow grafts from RAR-α−/− donors retained their ability to mount a GVL response and had significantly longer survival when compared to leukemia or GVHD control mice (Figure 7E). Moreover, bioluminescence imaging studies confirmed that these animals were free of tumor at the conclusion of these studies.
DISCUSSION

Severe GVHD of the gastrointestinal tract is primarily responsible for much of the morbidity and mortality that is observed in experimental murine models and BMT patients. In this report, we have identified RA as a novel and critical factor in the induction of gastrointestinal GVHD. In gain-of-function studies, we found that exposure of recipient mice to exogenous RA not only failed to ameliorate the severity of GVHD, as we had initially postulated, but selectively exacerbated colonic GVHD, resulting in significantly increased mortality in recipient mice. This observation was associated with enhanced generation of gut-tropic, donor T cells in the MLNs early after BMT and an increased influx of pro-inflammatory donor T cells into the gut mucosa during later stages of disease development. Conversely, vitamin A deprivation, which resulted in RA deficiency in recipient mice, inhibited the acquisition of the gut-homing molecules CCR9 and α4β7 on donor T cells after BMT, confirming a role for RA in this process. Importantly, genetic ablation of RAR-α signaling on donor T cells markedly attenuated the ability of these cells to cause GVHD lethality and, more specifically, colonic GVHD which was associated with a significant reduction in donor CD4+ and CD8+ T-cell accumulation in this tissue. Thus, these results demonstrated that RA facilitates the initiation and progression of gastrointestinal GVHD and thereby has an important pro-inflammatory role in GVHD biology.

Trafficking of donor T cells after in vivo infusion is an important aspect of GVHD pathophysiology since these cells exert their pathogenic effect only after infiltrating into target organs. The acquisition of gut-homing molecules on donor T cells is essential for them to migrate specifically to the gut mucosa. The important role of the integrin α4β7 in the pathogenesis of gastrointestinal GVHD has been well demonstrated in experimental BMT models. Specifically, Petrovic and colleagues showed that recipients of sort-purified α4β7-negative donor T cells had significantly less GVHD-associated morbidity...
and mortality compared with recipients of α4β7-positive donor T cells. This phenomenon was associated with a selective reduction of pathological damage in the intestine and liver but not other target organs, demonstrating an important role of α4β7 in the selective migration of donor T cells to the intestinal mucosa.\textsuperscript{39,45} In addition, similar studies were performed using β7-deficient donor T cells where it was confirmed that this molecule is critically involved in the development of intestinal GVHD.\textsuperscript{46,47} Clinically, up-regulation of α4β7 on peripheral blood T cells has been associated with the subsequent development of intestinal GVHD in allogeneic HSCT recipients.\textsuperscript{48} Although the importance of α4β7 in the pathogenesis of colonic GVHD has been established, it is unclear how the expression of this molecule on donor T cells is regulated during GVHD. Our studies extend these previous findings and now show that the expression of gut-homing molecules that are critically involved in the induction of intestinal GVHD is regulated by RA.

The observation that RA dose-dependently augmented the induction of gut-homing molecule expression on donor T cells indicates that the bioavailability of RA in vivo during donor T-cell activation profoundly influences the generation of donor T cells that are destined to migrate to the gut and initiate colonic GVHD. Notably, RA treatment did not affect the expression of other chemokine receptors mediating T-cell migration to other tissues. For example, the expression of CCR10, which directs T-cell migration into the skin, was not altered on donor T cells isolated from PLNs after RA exposure. The same was true for the expression of CCR5 and CXCR3 on alloreactive T cells primed at other secondary lymphoid tissues such as spleen. Interestingly, RA administration was associated with increased expression of α4β7 and CCR9 on T cells in peripheral lymph nodes, raising the possibility that RA may be able to direct T cells into the GI tract from distal nodal sites as well. Thus, RA appears to play a dominant and selective role in regulating gut immunity during the alloimmune response, although we cannot exclude the possibility that other chemokine receptor expression could also be regulated by RA after prolonged exposure. It was noteworthy that we observed an increased number of donor T cells in the spleen and lung in recipients of RAR-α\textsuperscript{−/−} grafts. While the splenic data, in particular, may have been attributable to
enhanced T-cell reconstitution in mice with reduced GVHD, an alternative possibility is that altered trafficking away from the colon led to higher levels of donor T cells in the circulation and was manifested as augmented perivascular cuffing in the lung and T-cell recovery in the spleen.

RA has been generally accepted to have an immunoregulatory role because of its ability to drive T cell differentiation towards the Treg pathway\textsuperscript{17-19} and has been proposed as a crucial factor in the induction of oral tolerance and the maintenance of intestinal homeostasis.\textsuperscript{11} However, emerging evidence suggest that RA is also essential for the activation and function of effector T cells and may play a pro-inflammatory role under certain pathogenic conditions.\textsuperscript{28,49,50} For example, RA was shown to promote T\textsubscript{H}1 polarization and inhibit Treg induction in the presence of the pro-inflammatory cytokine IL-15 in a murine celiac disease model.\textsuperscript{50} Therefore, RA can function as either an anti-inflammatory or a pro-inflammatory molecule which appears to depend, in part, upon the existing cytokine milieu. It is noteworthy that we did observe an increase in the absolute number of Tregs in the colons of animals that were treated with exogenous RA. However, the magnitude of Treg expansion was substantially less than that observed for the corresponding pro-inflammatory effector T-cell populations, and was insufficient to prevent increased pathological damage within the colon. Thus, we infer from these data that the pro-inflammatory effects of RA outweighed any salutary effects on Treg reconstitution during GVHD.

Despite the well-known effect of RA in imprinting gut-homing molecules on T cells under steady state conditions, our studies demonstrate that this functional property of RA is indispensable even in a systemic inflammatory disease such as GVHD. The finding that imprinting of gut-homing molecules on donor T cells was significantly impaired in vitamin A deficient mice indicates that although irradiation and the alloimmune response induce the secretion of many proinflammatory cytokines, RA plays a non-redundant role in generating gut-homing donor T cells during GVHD. These results are consistent with a recent publication by Koenecke and colleagues\textsuperscript{43} who also showed that vitamin A deficiency resulted in reduced expression of CCR9 and $\alpha4\beta7$ on donor T cells and accumulation of these cells in the intestines during
GVHD. Rodent models of BMT use highly inbred experimental mice that are typically fed with commercially available diets that are well supplemented with vitamin A. This is in contrast to humans who consume highly variable diets and who therefore may have more variability in nutritional status, and hence vitamin A levels. Our results suggest that recipient vitamin A levels could be a heretofore unrecognized variable that influences the severity of GVHD in the GI tract after allogeneic BMT. Future studies will be of interest to determine if there is a correlation between recipient vitamin A levels and the severity of GVHD within this tissue site.

We observed that RAR-α signaling was important in mediating the effects of RA. The concomitant increase in RAR-α gene expression in donor CD4 T cells and endogenous RA production in the colon early after allogeneic BMT suggests that RA acts directly on T cells to modulate their function through RAR-α. However, since RAR is broadly expressed by many cell types, it is formally possible that RA can act on other cells and modulate T-cell phenotype and function indirectly. For example, it has been shown that non-mucosal dendritic cells can take up and deliver RA to T cells to up-regulate gut-homing molecule expression. Therefore, it is possible that RA modulates donor T-cell function indirectly through DCs. In addition, our data do not exclude that other RAR isoforms, namely RAR-β and RAR-γ, may also participate in mediating RA signaling and contribute to the pathogenesis of acute GVHD in the gastrointestinal tract. Nonetheless, these results suggest that blockade of RAR-α signaling with small molecule inhibitors or through other approaches may be a clinically relevant strategy to reduce the priming of gut-homing donor T cells and selectively prevent or mitigate GVHD in the colon. We should note, however, our results differ somewhat from those reported by Nishimori and colleagues who noted that administration of AM80, a synthetic retinoid that binds to both RARα and RARβ, resulted in a reduction in GVHD severity in the skin, and did not exacerbate pathological damage in the colon. Of note, the authors employed a murine model of chronic GVHD in which cutaneous and not visceral manifestations of GVHD are most pronounced as opposed to the acute model utilized in the current
studies. Thus, it is formally possible that the effects of RAR-α signaling blockade may vary to some extent in the setting of acute versus chronic GVHD. Further studies will be necessary to determine if this is indeed the case.

In summary, these studies identify RA as a critical molecule that plays a major role in the regulation of gut-homing molecule expression and, by extension, the pathophysiology of GVHD in the gastrointestinal tract. Manipulation of the RA signaling pathway may therefore represent a novel strategy to mitigate the severity of colonic GVHD and reduce overall mortality. Finally, our results suggest that the vitamin A status of the recipient can significantly affect the ability of T cells to traffic into the colon. Consequently, determination and/or modification of vitamin A levels in patients may be useful for the prognostication of GVHD severity in the colon as well as may represent a less toxic, non-pharmacological approach by which GVHD can be successfully modulated.
ACKNOWLEDGMENTS

This research was supported by the Amy Strelzer Manasevit Research Program which is funded through The Be The Match Foundation and the National Marrow Donor Program and an Advancing a Healthier Wisconsin award from the MCW Cancer Center (to XC). This work was also supported in part by grants from the National Institutes of Health (HL64603, HL081650, and DK083358) and by awards from the Midwest Athletes Against Childhood Cancer Fund (to WD). We thank Dr. Kasem Nithipatikom for assistance with the mass spectrometry analysis.

Author Contributions: X.C. designed and performed research, analyzed and interpreted data, generated the figures, and wrote the manuscript. J.D. performed research. R.K. performed pathological analysis of all tissue samples. W.R.D. designed experiments, interpreted data and wrote the manuscript.

The authors declare no competing financial interest.
REFERENCES


44. Soprano DR, Qin P, Soprano KJ. Retinoic acid receptors and cancers. **Annu Rev Nutr** 2004; 24: 201–221.


FIGURE LEGENDS

Figure 1: Administration of exogenous RA selectively intensifies GVHD of the colon. Lethally irradiated Balb/c mice received a transplant of 10 x 10^6 B6 BM alone (○) or together with B6 spleen cells (adjusted to yield a dose of 0.6 x 10^6 T cells). Cohorts of mice that received adjunctive spleen cells were then treated by intraperitoneal injection with either DMSO (□) or RA (●)(450μg/mouse) every other day beginning on the day of transplantation until day 20. (A) Overall survival is depicted. Data are cumulative results of 3 experiments (n=6 for BM control group, and n=12 for both DMSO and RA-treated groups). (B) Mean percent initial body weight of recipient mice transplanted as in panel A over the first 35 days is depicted. (C) Lethally irradiated Balb/c mice were transplanted with B6 BM and spleen cells as above with the exception that the spleen cell dose was adjusted to yield a dose of 0.3 x 10^6 T cells. Groups were then treated with either DMSO or RA. Mice were euthanized 5 weeks after transplantation. Pathological damage in the tongue, liver, lung, and colon was examined using a semi-quantitative scoring system as described in Material and Methods. Data are presented as the mean (± SEM) and are the cumulative results from 3 experiments (n=12 for both groups). Statistics: **p ≤ 0.01. (D) Histology of tongue, liver, lung, and colon from representative Balb/c recipients transplanted with B6 BM and spleen cells and treated with either DMSO or RA. Tongues in DMSO and RA-treated mice reveal lymphocytic infiltration into the epidermis. Livers in the two groups show mononuclear cell infiltration in the portal triads, while lungs both demonstrate perivascular cuffing attributable to mononuclear cells along with associated interstitial inflammation. Colon in DMSO-treated animals demonstrates loss of mucin and lamina propria inflammation but preservation of crypt cell architecture. Conversely, RA-treated mice show extensive loss of crypts with goblet cell depletion and inflammation extending into the muscle. Original magnification is 200X for liver, colon and tongue, and 100X for lung images.
Figure 2: RA enhances the expression of α4β7 and CCR9 on donor T cells in mesenteric lymph nodes. Lethally irradiated Balb/c mice were transplanted with B6 BM and spleen cells and treated with either DMSO or RA. Mice from both groups were euthanized at day 7 after BMT. The expression of integrin and chemokine receptors on donor T cells isolated from different secondary lymphoid tissues of recipient mice was examined by gating on donor-derived H-2Kb+ CD4+ or H-2Kb+ CD8+ cells. (A,D) Representative contour plots for α4β7 and CCR9 expression on donor T cells isolated from MLNs (A), and CXCR3 and CCR5 expression on donor T cells isolated from spleen (D) is shown. (B, E, F, G) Mean percent positive (± SEM) donor T cells (n=6-8 per group) isolated from MLNs (B), spleen (E), or peripheral lymph nodes (F,G) for the specified integrin and chemokine receptors is depicted. Data are combined results from two independent experiments. Statistics: *p ≤ 0.05, **p < 0.01, ***p < 0.001. (C) Lethally irradiated Balb/c mice were transplanted with B6 BM cells and spleen cells (adjusted to yield a T cell dose of 0.6 x 10⁶) and then treated with escalating doses of RA. The expression of the gut-homing molecules α4β7 and CCR9 on donor H-2Kb+ CD4+ and CD8+ cells from the MLNs of recipient mice 7 days after BMT is depicted (n=3/group). Data are from one representative experiment of two with similar results.

Figure 3: Effect of exogenous RA administration on effector and regulatory T cell infiltration into the colon. Lethally irradiated Balb/c mice were transplanted with 10 x 10⁶ B6 BM together with B6 spleen cells (adjusted to yield a T-cell dose of 0.6 x 10⁶). Cohorts of mice were then treated with either DMSO (n=6-8/group, black bars) or RA (n=6-8/group, white bars). (A, B) Absolute cell number of CD4+IFN-γ+ or CD4+IL-17+ T cells in the spleen, liver, lung and colon of animals treated with either DMSO or RA and then euthanized 19-21 days after transplantation. (C) Absolute cell number of CD8+CD103+ cells in the colon of recipient mice. Data are presented as the mean (± SEM) and are the cumulative results from 2 independent experiments. (D) Lethally irradiated Balb/c mice were transplanted with 10 x 10⁶ Foxp3EGFP BM and spleen cells (adjusted to yield a T-cell dose of 0.6 x 10⁶). Cohorts of mice were then treated with either DMSO (n=8) or RA (n=8). Mice in both groups were
euthanized 19-21 days after transplantation. The absolute cell number of Foxp3 + (GFP+) Tregs in the spleen, liver, and colon of mice is depicted. Data are presented as the mean (± SEM) and are the cumulative results from two independent experiments.  (E) Percentage of CD4+ and CD8+ T cells that expressed either α4β7 in the colon or CCR9 in the small intestines of transplant recipients that were treated with either DMSO or RA. Animals were euthanized 19-21 days post transplantation. Data are presented as the mean (± SEM) and are the cumulative results from 2 independent experiments. Statistics: *p ≤ 0.05, **p < 0.01, ***p < 0.001.

Figure 4: **Vitamin-A deprivation in recipient mice impairs the acquisition of gut-homing molecule expression on donor T cells after BMT.** Lethally irradiated Balb/c Rag mice fed with either vitamin A-sufficient (VAS) or vitamin A-deficient (VAD) diets were transplanted with B6 BM and CellTrace Violet labeled-splenic T cells (adjusted to yield a T-cell dose of 1 x 10^6). Mice from both groups were euthanized on day 3 after BMT. (A, C) Representative contour plots of CCR9 and α4β7 expression on donor T cells isolated from the MLNs (A) and CCR10 expression on donor T cells isolated from the PLNs (C) of recipient mice after gating on H-2K b+ CD4+ or H-2K b+ CD8+ cells are shown. (B, D) The mean percentage positive donor T cells (± SEM) expressing each of the specified integrin or chemokine receptors from 4 mice per group. Data are from one of two representative experiments with similar results. Statistics: *p ≤ 0.05, **p < 0.01, ***p ≤ 0.001. (E) Representative example of CellTrace Violet labeled-donor H-2K b+ CD4+ or CD8+ T-cell proliferation in the spleen of VAS and VAD recipients 3 days after transplantation. (F) Mean (± SEM) percentage of proliferating (CellTrace Violet low) splenic donor T cells (n= 4 mice per group). One of two representative experiments is shown.

Figure 5: **RAR-α signaling is important in mediating the effects of RA on T cells.** B6 spleen cells (adjusted to 1 x 10^5 T cells/well) were co-cultured with 5 x 10^4 Balb/c dendritic cells in the presence or absence of 1μM AM80, an RAR-α agonist. Four days later, cells were harvested for flow cytometric
analysis. (A) Representative contour plots of α4β7 and CCR9 expression on CD4+ and CD8+ T cells. (B) Mean (± SEM) percentage of CD4+ and CD8+ T cells that expressed α4β7 and CCR9. Data are cumulative results from 4 independent experiments. Statistics: *p ≤ 0.05, **p < 0.001 (C, D) B6 spleen cells (adjusted to 1 × 10^5 T cells/well) were co-cultured with 5 × 10^4 Balb/c dendritic cells in the presence of 10 nM exogenous RA. An RAR-α-antagonist, BMS195614 (1μM), was added to some cultures one hour prior to the addition of RA. Cells were analyzed after four days in culture. Representative contour plots of CCR9, α4β7, CD62L, and CD25 expression on CD4+ and CD8+ T cells is depicted. Data are from one of four representative experiments with similar results. (E) Cells were cultured as in panels C and D with the exception that ^3H-thymidine was added for the last 18 hours to assess proliferation. Data are presented as mean cpm ± SEM and are representative of one of two experiments with similar results. Statistics: *p ≤ 0.05

Figure 6: Endogenous RA production and RAR signaling on donor T cells are enhanced after allogeneic BMT. (A) Lethally irradiated Balb/c mice were transplanted with Balb/c BM (10 × 10^6) and spleen cells (0.4-0.5×10^6, syngeneic) or B6 BM and an equivalent number of B6 spleen cells (allogeneic). Cohorts of animals were euthanized weekly and colon tissue was analyzed for RA levels by mass spectrometric analysis as described in “Quantification of RA in tissues by mass spectrometry”. Data are derived from 9-10 mice per group and are cumulative results from two independent experiments. Statistics: *p ≤ 0.05. (B) Lethally irradiated Balb/c mice were transplanted with B6 BM and spleen cells to induce GVHD. RNA was extracted from sort-purified CD4+ and CD8+ T cells isolated from spleens of allogeneic transplant recipients at the indicated time points after BMT, and gene expression of RAR-α was analyzed by real-time q-PCR as described in “Real-time q-PCR.” Data are normalized for 18S ribosomal RNA and presented as fold increase over RAR-α expression in sort-purified original naïve CD4+ or CD8+ donor T cells used for transplantation. Data are derived from two independent experiments and are presented as the mean ± SEM.
Figure 7: Recipients of RAR-α-deficient T cells are protected from lethal GVHD yet are able to mount a GVL response. Lethally irradiated Balb/c mice were transplanted with BM cells (5 x 10⁶) from either RAR-α⁻/⁻ (□) or wild type (WT) littermate controls (○) or BM and spleen cells (adjusted to yield a T cell dose of 0.7–1.0 x 10⁶ T cells) from either RAR-α⁻/⁻ (■) or WT littermate controls (●). (A) Overall survival is depicted. Data are cumulative results from three independent experiments (n=3 for BM control groups, and n=14 for each BM + T-cell group). (B) Lethally irradiated Balb/c mice were transplanted with BM (5 x 10⁶) and spleen cells (adjusted to yield a T-cell dose of 2.1 x 10⁶) from either RAR-α⁻/⁻ (n=9) or WT littermate controls (n=5). Mice were euthanized at day 10-12 after BMT and pathological damage in the liver, lung, and colon was examined. Data are derived from 2 independent experiments and are presented as the mean (± SEM). Representative photo micrographs of the colon from mice transplanted with WT versus RAR-α⁻/⁻ marrow grafts are shown. Colon from recipients of WT grafts show loss of mucin and destruction of crypt architecture, while animals reconstituted with grafts from RAR-α⁻/⁻ mice have normal-appearing crypts. Statistics: *p ≤ 0.05, ***p ≤ 0.001. (C,D) The absolute number of donor-derived CD4⁺ (C) and CD8⁺ cells (D) in the liver, spleen, lung, and colon is shown for each group. Data are derived from 2 independent experiments and are presented as the mean (± SEM). (E) Lethally irradiated Balb/c mice were administered 0.6 x 10⁶ A20 cells transfected with firefly luciferase (A20-luc) and then transplanted with either WT BM alone (n=5), WT BM plus spleen cells (adjusted to yield a dose of 1.2 x 10⁶ T cells)(n= 5), or RAR-α⁻/⁻ BM plus spleen cells (adjusted to yield an equivalent T cell dose)(n= 5). Overall survival and whole body distribution of A20-luc cells using in vivo BLI is depicted.
Figure 1

Graphs and images showing survival rates and pathology scores after transplantation.
Figure 3
Figure 4
Figure 6

A

B

CD4⁺

CD8⁺
Figure 7

A

For personal use only.

B

RAR-α^{+/+}

RAR-α^{-/-}

C

D

E

D27

D41

D58

38
A critical role for the retinoic acid signaling pathway in the pathophysiology of gastrointestinal graft-versus-host disease

Xiao Chen, Joseph Dodge, Richard Komorowski and William R. Drobyski