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Effects of Donor Vitamin A Deficiency and Pharmacologic Modulation of Donor T Cell Retinoic Acid Pathway on the Severity of Experimental Graft-versus-Host Disease

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Graft-versus-host disease (GVHD) is the major cause of morbidity and mortality after allogeneic hematopoietic stem cell transplantation (HSCT). A combination of genetic and nongenetic factors dictates the incidence and severity of GVHD. Recent studies have identified the potential role of the retinoic acid (RA)/retinoic acid receptor (RAR) pathway in the pathogenesis of GVHD. RA is the active metabolite of vitamin A. Thus, a clinically relevant question is whether HSCT donor and/or recipient vitamin A status affects the development of GVHD. It has been previously reported that recipient vitamin A deficiency is associated with reduced intestinal GVHD and prolonged overall survival after experimental allogeneic HSCT. However, it is still unknown whether donor vitamin A status influences GVHD development. In the current study, we report that chronic vitamin A deficiency changes the composition of T cell compartment of donor mice with a reduction in the percentage of $CD4^+$ T cells. We showed that although vitamin A deficiency does not affect donor T cell alloreactivity on a per cell basis, a decreased proportion of donor $CD4^+$ T cells in marrow graft inoculums leads to reduced incidence and severity of GVHD. Furthermore, our proof of principle studies using a pan-RAR antagonist demonstrated that transient inhibition of donor T cell RAR signaling can reduce T cell alloreactivity and their ability to cause lethal GVHD. Our studies provide preclinical evidence that donor vitamin A deficiency may be a nongenetic factor that can modulate the severity of GVHD and pharmacologic interfering RA/RAR pathway in donor T cells might be a valuable approach for mitigating GVHD after allogeneic HSCT.

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (HSCT) is a life-saving treatment for a number of nonmalignant and malignant hematologic diseases. However, its wider application is limited by graft-versus-host disease (GVHD), the leading cause of morbidity and mortality after this treatment [1-3]. GVHD occurs because immunocompetent donor T cells recognize genetically disparate host antigens as foreign and attack the transplant recipient's tissues [4,5]. Donor T cells undergo initial activation, differentiation, and expansion in secondary lymphoid tissues and subsequently migrate to a restricted set of target organs (skin, liver, lung, and intestines) to cause profound tissue damage that characterizes acute GVHD [6-10]. Although genetic disparity between donor and recipient is the primary factor predisposing patients to GVHD, various donor and/or host nongenetic factors can also profoundly influence the incidence and severity of GVHD. For example, it has been well documented in clinical and experimental studies that older recipient age is an independent risk factor for increased GVHD severity [11-14]. In fact, it has been shown that enhanced donor T cell activation by aged host antigen-presenting cells is primarily responsible for this phenomenon [14].

Advances in immunology have established that environmental factors, including micronutrients, play significant roles in modulating T cell–mediated immune responses under physiologic and pathologic conditions. In this regard, lipid-soluble vitamin A has received a significant amount of attention [15]. Retinoic acid (RA), the active metabolite of vitamin A, is critically involved in a variety of biologic processes, including immune responses in vivo. Different effects of vitamin A and RA on adaptive immunity in mice have been reported. Earlier studies demonstrated that a vitamin A–deficient (VAD) environment favors Th1 cell differentiation

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and high dietary vitamin A is associated with increased Th2 cytokine production [16-18]. However, there was also evidence that vitamin A deficiency decreases Th1 cell development and is associated with diminished protective mucosal immune responses [19-21]. In humans, vitamin A deficiency increases susceptibility to various pathogens and vitamin A supplementation enhances immunity to infectious diseases [22]. These data provide strong evidence that vitamin A and RA modulate T cell–mediated adaptive immune responses.

The important role of vitamin A and RA as nongenetic factors in regulating GVHD, a T cell–mediated inflammatory disease, is not well understood. We and others identified RA and RA receptor (RAR) signaling as critical factors in the pathogenesis of GVHD [23,24]. Several studies demonstrated that exogenous RA administration exacerbates GVHD [23-25]. Importantly, genetic ablation of RA signaling in donor T cells markedly reduced their ability to cause lethal GVHD [23,24]. Furthermore, Koenceke et al. showed [26] that recipient vitamin A deficiency is associated with reduced intestinal GVHD and prolonged overall survival, although hepatic damage is somewhat increased. These data suggest that vitamin A levels of bone marrow transplantation (BMT) recipients can profoundly affect GVHD development. However, whether donor vitamin A status similarly affects GVHD pathogenesis remains unknown. This is a clinically relevant question because vitamin A deficiency is a major global health concern that may affect many potential HSCT donors [27]. We hypothesized that donor vitamin A deficiency is associated with a decreased ability to cause GVHD. We further hypothesized that pharmacologic interference of the RA/RAR pathway in BMT donors can reduce T cell alloreactivity. Therefore, the purpose of the current study was to test these hypotheses using a well-established murine GVHD model in which RA signaling in donor mice was modified nutritionally and pharmacologically.

METHODS

Mice

C57BL/6 (B6; H-2K^b) and Balb/cJ (H-2K^d) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred in the Biomedical Resource Center at the Medical College of Wisconsin. VAD mice on a B6 background were generated as previously described using a VAD diet [20]. Briefly, pregnant female mice were fed a VAD diet obtained from Harlan Laboratories (Madison, WI) from day 14.5 of gestation and maintained on this special diet until weaning of the litter. After weaning, litters were fed the same VAD food for a minimum of 9 weeks before experimentation. Control vitamin A–normal (VAN) mice were maintained on standard mouse chow. All animals were housed in the Association for Assessment and Accreditation of Laboratory Animal Care–accredited Biomedical Resource Center of the Medical College of Wisconsin. All experiments were carried out under protocols approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Bone Marrow Transplantation

Bone marrow (BM) was flushed from donor femurs and tibias with 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 T cells.

Treating Mice with BMS493

For experiments using BMS493 (Tocris Bioscience, Minneapolis, MN), naive B6 mice were treated with either DMSO or BMS493 (440 μg/mouse), a pan RAR antagonist, by intraperitoneal injection daily for 10 consecutive days. T cells were then purified from these mice and used for mixed lymphocyte reactions or transplants.

Histologic Analysis

Histologic examination of representative samples of liver, lung, and colon was performed as described previously [23].

Mixed Lymphocyte Reaction

Highly purified pan T cells (10^5 cells/well) were isolated from the spleens of VAD or VAN 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 cocultured with 5×10^4 Balb/c dendritic cells (DCs) in 96-well U-bottomed plates at 37°C for 4 days in complete RPMI medium containing 10% FBS. Isolation of CD11c$^+$ DCs using the magnetic cell separation system has been described [23]. T cell proliferation was assessed by dye dilution using an LSRII flow cytometer.

Flow Cytometry and Antibodies

Cells were isolated from spleens or mesenteric lymph nodes of transplant recipients and were labeled with monoclonal antibodies conjugated to FITC, PE, PE-Cy5.5, or allophycocyanin. FITC-anti-CD11c, FITC-anti-H-2K^b, PE-Cy5-anti-CD4, PE-anti-TCRβ, PE-anti-CD4, FITC-anti-CD8, FITC-anti-CD69, PE-anti-CD62L, and FITC-anti-CCR9 were all purchased from BD Biosciences (Franklin Lakes, NJ), and PE-anti-α4β7 was purchased from eBioscience. 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, OR).

Cytokine Analysis

Serum was collected from mice by retro-orbital bleeds. Cytokine levels in serum were analyzed on a Bioplex System (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. All samples were run in duplicate.

Real-Time Quantitative PCR

Real-time quantitative 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). The β_2-microglobulin reference gene as well as IFN-γ and tumor necrosis factor (TNF)-α genes were amplified using a QuantiTect Primer Assay Kit (Qiagen). Specificity for all quantitative 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.

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 $P \leq .05$ was deemed to be significant in all experiments.

RESULTS

Splenocytes from VAD Donor Mice Show Reduced Ability to Cause GVHD

To examine the potential effects of donor vitamin A deficiency on T cell alloreactivity, we generated VAD mice on a B6 background using a diet essentially devoid of vitamin A. We first compared the proliferation of T cells from VAD or VAN mice in mixed lymphocyte reactions. Highly purified T cells were isolated from the spleens of VAD or VAN mice and labeled with CellTrace Violet dye. They were then cocultured with allogeneic CD11c$^+$ DCs from Balb/c mice. We found a significant reduction in the percentage of CellTrace Violet low CD4$^+$ and CD8$^+$ T cells from VAD mice compared with T cells from VAN mice, indicating that chronic vitamin A deprivation reduces the proliferative potential of T cells in response to alloantigen stimulation in vitro (Figure 1A).

To examine the effects of vitamin A deficiency on the ability of donor cells to cause GVHD in vivo, we used a well-established B6 (H2K^b)→Balb/c (H2K^d) MHC-mismatched BMT model. Lethally irradiated Balb/c mice were transplanted with BM and spleen cells from either VAD or VAN donors. Spleen cell inoculums were adjusted so that the dose of TCR-β$^+$ T cells was equivalent in both groups. Recipient mice transplanted with BM and spleen cells from VAN donors showed clinical