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Dendritic Cell Expression of Retinal Aldehyde Dehydrogenase-2 Controls Graft-Versus-Host Disease Lethality

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Abstract

Recent studies have underscored the critical role of retinoic acid (RA) in the development of lineage committed CD4 and CD8 T cells *in vivo*. We have shown that under acute graft-versus-host disease (GVHD) inflammatory conditions, RA is upregulated in the intestine and is pro-inflammatory as GVHD lethality was attenuated when donor allogeneic T cells selectively expressed a dominant negative RA receptor α that blunted RA signaling. RA can function in an autocrine and paracrine fashion and as such, the host cell lineage responsible for the production of RA metabolism and the specific RA metabolizing enzymes that potentiate GVHD severity are unknown. Here, we demonstrate that enhancing RA degradation in the host and to a lesser extent donor hematopoietic cells by over-expressing the RA catabolizing enzyme, CYP26A1, reduced GVHD. RA production is facilitated by retinaldehyde isoform-2 (RALDH2) preferentially expressed in dendritic cells (DCs). Conditionally deleted RA synthesizing enzyme, RALDH2, in host or to a lesser extent donor DCs, reduced GVHD lethality. Improved survival in recipients with

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RALDH2 deleted DCs was associated with increased T cell death, impaired T effector function, increased regulatory T cell frequency and augmented co-inhibitory molecule expression on donor CD4+ T cells. In contrast, retinaldehydrogenase isoform-1 (RALDH1) is dominantly expressed in intestinal epithelial cells (IECs). Unexpectedly, conditional host IECs RALDH1 deletion failed to reduce GVHD. These data demonstrate the critical role of both donor and especially host RALDH2+ DCs in driving murine GVHD and suggest RALDH2 inhibition or CYP26A1 induction as novel therapeutic strategies to prevent GVHD.

Introduction

Recent studies have highlighted the importance of retinoic acid (RA), a metabolite of vitamin A, in influencing the outcome of adaptive immune responses by balancing tolerance and immunity (1–3). The immunomodulatory functions of RA are diverse and are involved in: lymph node formation, Tregulatory cells (Tregs) induction, imprinting of gut homing receptors on lymphocytes, innate lymphoid cells type 3 (ILC3) development and inhibition of Th17 differentiation (1, 4–7) as well as a mediator critical in stabilizing CD4+ and CD8+ T cell effector functions.

Broadly described, synthesis of RA from vitamin A (VA) involves two steps: firstly, retinol dehydrogenase converts retinol to retinal and secondly, retinaldehyde dehydrogenase (RALDH) converts retinal to RA (3, 8). RA concentrations and signaling in immune cells is modulated by RALDH enzymes that generate RA and catabolizing enzymes such as cytochrome P450 (CYP), mainly from the CYP26 family, that degrade RA (3) by oxidative metabolism (9). RALDH is expressed in many cell types including dendritic cells (DCs) and intestinal epithelial cells (IECs) (3). Intestinal stromal cells and IECs predominantly express RALDH1 (8, 10), while DCs express RALDH2 (11), Lee YC et al (manuscript in preparation). The local effects of RALDH1 and RALDH2 are to produce RA from retinol, which is then available to bind to the RA receptor (RAR) isoforms, α, β, and γ. It is believed that RA produced by DCs signals T cells differentiation in a paracrine fashion. Counterbalancing the RA synthesis capacity of RALDH enzymes are CYP26 catabolic enzymes that are widely expressed by immune cells and exist in three isoforms namely, CYP26A1, CYP26B1 and CYP26C1.

Although RA has an immune-regulatory role in maintaining immune homeostasis, it also has the capacity to promote pro-inflammatory responses (12–14). For instance, in the context of substantial inflammation as seen in acute graft versus-host-disease (GVHD), donor alloreactive T cells that express an RA response element driving luciferase could sense heightened RA levels in GVHD target organs, an intensity that is magnified as GVHD worsened (12). Exogenous RA increased expression of the gut-homing molecules on donor T cells in mesenteric lymph nodes, and augmented proinflammatory gut mucosal CD4+ and CD8+ T cells, leading to exacerbation of colonic GVHD and increased mortality (13). Impairing normal RA signaling in donor allogeneic T cells by expressing a dominant negative RA receptor alpha chain (dnRARα), deleting RARα or treatment of the recipient with a pan-RAR antagonist attenuated GVHD with reduced Th1 differentiation and GVHD pathology (12, 13, 15).

These data indicated that high levels of RA synthesis during experimental GVHD substantially contributed to pathogenic donor T effector responses and subsequent GVHD lethality. However, the lineage of cells involved in RA metabolism and the RALDH isoform(s) primarily involved in RA synthesis during GVHD are unknown. Following identification of the specific RA metabolic enzymes involved in GVHD, novel and effective targeted therapies for GVHD could be designed. Hence, the goal of the current study was to assess the effects of ablating RA production and signaling in defined cell lineages, in GVHD mice, using novel genetically engineered mice in which synthesis and signaling can be controlled.

Materials and Methods

Mice

All procedures and protocols carried out during this study were approved under The Institutional Animal Care and Use Committee (IACUC) at the University of Minnesota. Female BALB/c (H-2^d), C57BL/6 (B6; H-2^b) mice were purchased from Charles River Laboratories. Female B10.BR (H-2^k), B6.Cg-Tg (Vil1-cre) (B6 villin^{Cre}) and CD11c^{Cre} mice were purchased from The Jackson Laboratory. In villin^{Cre} mice, the expression of Cre recombinase is restricted to villus and crypt epithelial cells of the small and large intestines. B6 CYP26A1 flox/flox (loxP-STOP-loxP-CyP26A1) mice were generated by two of the authors (YCL and RNJ, Dartmouth College, USA). B6 RALDH1 flox/flox frozen embryos and RALDH2 flox/flox mice were kindly provided by Drs. Pierre Chambon and Norbert Ghyselinck (Institute for Genetics and Cellular and Molecular Biology, France). RALDH1 flox/flox embryos were implanted into pseudopregnant mothers to generate RALDH1 flox/flox offspring. Both female and male B6 CYP26A1$^{stop/stop}$, B6 RALDH1 flox/flox, RALDH2 flox/flox and conditional strains of each were used in experiments. Mice were housed in a specific-pathogen free facility in micro-isolator cages maintained by the University of Minnesota.

Bone marrow transplantation (BMT) and GVHD induction

On day −1, recipient mice were lethally irradiated using 11Gy, 8.3Gy, or 7Gy dose, delivered by x-ray, for B6, B10.BR and BALB/c, respectively. On day 0, recipients were infused with a total of 10^7 non-T cell depleted (NTCD) allogeneic bone marrow cells (BM) with or without purified T cells from MHC-disparate donors. Donor T cells were purified from splenocytes and lymph nodes using biotin-labeled anti-CD19 (1D3), CD45R (RA3–6B2), CD11b (M1/70), CD11c (N418), CD49b (DX5), NK1.1 (PK136), TCR γδ (GL3) and TER-119 (TER-119), followed by streptavidin RapidSpheres depletion with EasySep magnet (StemCell Technologies, Vancouver, Canada). For CD25- T cell purification, biotinylated anti- CD25 Ab (PC61.5) was added to the above purification cocktail. Donor T cell purity was >96%. Survival was monitored daily and weights and clinical scores were recorded 2–3 times per week (12). In a survival study, all-trans-retinoic acid (ATRA; 200 μg/mouse; Sigma-Aldrich) was administered (i.p) daily from the day of BMT until day 28.

Intestinal lamina propria lymphocyte (LPL) isolation

Small and large intestine LPLs were isolated using a modified protocol from one previously described (16). Intestines were cut into pieces, followed by washing at 10 minutes twice (37ºc) in 10% FBS PBS with 5mM EDTA (GBiosciences, St. Louis, MO) and 10mM HEPES (Sigma-Aldrich, St. Louis, MO). After washing step, tissues were digested thrice for 20 minutes each at 37ºc in a digestion mix of 10% FBS PBS with 1 mg/ml collagenase D (Roche, Indianapolis, IN), 0.15 IU/mL Dispase (Sigma-Aldrich, St. Louis, MO) and 0.5mg/mL DNAseI (Sigma-Aldrich, St. Louis, MO). LPLs were purified on a Percoll gradient (40%/80%).

Flow cytometry

BD LSRII Fortessa flow cytometer was used to acquire cells. Cells were stained with the following monoclonal antibodies anti- H-2K^{d} (SF1–1.1.1), CD4 (RM4–5/GK1.5), CD8α (53–6.7), CD8β (H35–17.2), CD25 (PC61.5), Lag3 (C9B7W), PD-1 (J43), Tim3 (RMT3–23), Foxp3 (FJK-16s), CCR9 (CW-1.2), α4β7 (DATK-32), IFN-γ (XMG1.2), IL-17 (eBio17B7) and TNF-α (MP6-XT22). Antibodies were purchased either from Thermo Fischer Scientific (Waltham, MA) and Biolegend (San Diego, CA). Cells were stimulated with cell stimulation cocktail and protein transport inhibitor cocktail (Thermo Fischer Scientific, Waltham, MA) for 5 hours to measure intra-cellular cytokines. For intracellular staining, cells were fixed using the Foxp3/Transcription factor staining buffer set or IC fixation kit (Thermo Fischer Scientific, Waltham, MA). Active caspase-3 was measured by CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (Thermo Fischer Scientific; Waltham, MA). Cells were stained with Fixable Viability Dye ef780 (Thermo Fischer Scientific, Waltham, MA) to exclude dead cells for all experiments. Data were analyzed with FlowJo (Tree Star Inc., Ashland, OR).

Detection of RALDH activity

Splenocytes were cultured with 50 μg/mL DNase I (Roche, Indianapolis, IN) and 250 μg/mL Liberase DL (Roche, Indianapolis, IN) at 37°C for 30 minutes, homogenized, and isolated with CD11c microbeads (Miltenyi Biotec, Auburn, CA) according to manufacturer's directions. Resulting cells were cultured with 10 ng/mL each of GM-CSF (Peprotech, Rocky Hill, NJ) and IL-4 (Peprotech, Rocky Hill, NJ) for 24 hours. RALDH activity of CD11c+ cells was estimated using AldeRed ALDH Detection Assay (EMD Millipore, Billerica, MA), according to the manufacturer's protocol. Cells were additionally stained with CD11c (Biolegend, San Diego, CA), and resuspended in SYTOX Blue Dead Cell Stain (Thermo Fisher Scientific, Waltham, MA). In GVHD experiments, RALDH activity was determined in DCs by using the ALDEFLUOR staining kit (STEMCELL Technologies Inc.) according to the manufacturer's instructions.

Induction of gut homing receptors *in vitro*

OT-II T cells (Stem Cell naïve CD4 T cell isolation kit) and MLN DCs (Stem Cell CD11c positive selection kit) were purified. The cell purity for both populations was at least 90%. MLN DCs were co-cultured with OT-II T cells with 2 ng/ml TGF-β and 5 ug/ml OVA 323–339 peptide. The expression of α4β7 on OT-II T cells was analyzed on day 5.

qPCR

RNA was extracted using RNeasy kit (Qiagen) and followed by cDNA synthesis using SuperScript® III First-Strand Synthesis System (Invitrogen). qPCR experiments were performed using SYBR Green (BioRad Laboratories). Primers to measure *RALDH2* expression: *Aldh1a2* Forward: 5' TGGGTGAGTTTGGCTTACGG 3' and *Aldh1a2* Reverse: 5' AGAAACGTGGCAGTCTTGGC.

Statistical Analyses

Log-rank test was used to analyze differences in survival curve. Data were reported as mean values ± SEM. Comparison of groups was made by Student's *t*-tests. Statistical comparisons with p-values < 0.05 were deemed significant.

Results

Overexpression of the RA catabolizing enzyme, CYP26A1, in host hematopoietic cells ameliorated GVHD

Although RALDH1 and RALDH2 metabolizing enzymes are upregulated during GVHD, the potential contribution of RA catabolizing enzymes, which also regulate RA levels in the local environment, is unknown (12). To determine whether heightened RA catabolism in host hematopoietic cells would reduce GVHD, we exploited the biological properties of CYP26A1, a highly specific RA catabolizing enzyme, to reduce available RA (17). To limit overexpression to hematopoietic cells, B6 CYP26A1$^{stop/stop}$ flox/flox mice were crossed to B6 mice expressing VAV^{Cre} that is expressed in all hematopoietic cells. CYP26A1 overexpression, as measured by quantifying GFP positive cells, was detected only in hematopoietic cells and not in non-hematopoietic cells such as IECs of B6 CYP26A1$^{stop/stop}$-VAV$^{Cre\ positive}$ (Cre pos) mice (Supplemental Fig. 1A). Whereas, hematopoietic cells of B6 CYP26A1$^{stop/stop}$-VAV$^{Cre\ negative}$ controls (Cre neg) were GFP negative. RALDH enzymatic activity can be determined by flow cytometry of Aldefluor stained cells (17). Overexpression of CYP26A1 significantly reduced the RA producing capacity of DCs (Supplemental Fig. 1B-C). Lethally irradiated Cre pos or Cre neg controls were given MHC mismatched BALB/c BM ± purified T cells. As shown in Fig. 1A, overexpression of CYP26A1 only in recipient hematopoietic cells present at the time of BMT ameliorated GVHD as evidenced by significantly improved overall survival and clinical scores. To exclude the possibility that reduced GVHD was specific to one strain combination, we used a second MHC mismatched model (B10.BR→B6 CYP26A1$^{stop/stop}$-VAV^{Cre}). Confirming and extending results from the first model, overexpression of CYP26A1 in recipients also led to improved survival and clinical scores compared to Cre neg controls (Fig. 1B).

Host antigen presenting cells (APCs) initiate GVHD by direct MHC or MHC/allopeptide recognition by donor T cells (18), whereas donor APCs typically amplify GVHD by indirect allorecognition by presenting host allopeptides on donor MHC molecules (19). Because the majority (>90%) of hematopoietic cells in the mesenteric lymph nodes and intestinal LP, sites essential for donor T cell priming for gastrointestinal acute GVHD, were donor-derived

on day 14 and GVHD mice exhibited higher RALDH activity than those without GVHD (12), studies were performed to determine whether CYP26A1 expressing donor hematopoietic cells present later post-BMT could ameliorate GVHD. Lethally irradiated B10.BR mice were given donor BM from B6 CYP26A1$^{stop/stop}$-VAV^{Cre} mice and donor T cells from wild type (WT) B6 mice. Increased RA catabolic enzyme expression in donor BM also reduced the incidence of WT donor T cell mediated GVHD (Fig. 1C). However, the magnitude of GVHD protection appeared to be more modest than seen when CYP26A1 was overexpressed in host hematopoietic cells with no recipients in the former surviving beyond 2 months post-BMT. Thus, increased RA catabolic enzyme expression in host and to a significant but lesser extent in donor hematopoietic cells attenuated GVHD.

RALDH2 expression in DCs controls acute GVHD lethality

Having demonstrated that increasing RA catabolism associated with reduced RALDH activity lowered the rate of GVHD lethality, we next sought to determine the cellular lineage and RALDH isoform responsible for increased RA production that drives GVHD. Among APCs, DCs alone are sufficient for inducing GVHD-related mortality (20). Hence, we tested whether DCs were a key source of RA synthesis following BMT. Since DCs predominantly employ the RALDH2 isoform to produce RA (11), we used B6 RALDH2 flox/flox mice mated with B6 CD11c^{Cre} mice to generate conditional DC RALDH2 knockout (RALDH2$^{-/-}$ DC) mice. qPCR analysis confirmed the deletion of RALDH2 in CD11c cells (Supplemental Fig. 2A). As expected, RALDH2$^{-/-}$ DCs showed reduced Aldefluor activity in response to GM-CSF and IL-4, indicative of reduced RA production (Lee YC et al., manuscript in preparation). We next analyzed RALDH activity, by Aldefluor staining, in GVHD settings. On day 3 post-transplant, Aldefluor activity was significantly reduced in the splenic host DCs of recipients with RALDH2 $^{-/-}$ DCs than those of controls (Supplemental Fig. 2B). However, at the later time point (day 16) there was no significant difference observed in Aldefluor activity of LPL DCs between the two groups (Supplemental Fig. 2C, D), as the majority of recipient DCs would have been replaced by donor DCs. RA is required for the upregulation of gut homing molecules on T cells (21). RALDH2$^{-/-}$ DCs were less potent in inducing the upregulation of the gut homing receptor ($\alpha 4\beta 7$) on OT-II T cells (Supplemental Fig. 2E) and WT T cells (Lee YC et al., manuscript in preparation). We further investigated the biological effects of RALDH2$^{-/-}$ DC in regulating GVHD pathogenesis. Lethally irradiated B6 RALDH2$^{-/-}$ DC recipients were given BALB/c BM cells with or without T cells. Compared with controls, B6 RALDH2$^{-/-}$ DC recipients had significantly improved survival, weight, and reduced clinical scores (Fig. 2A). To confirm and extend these results, we used a different MHC mismatch mouse model (B10.BR→B6 RALDH2$^{-/-}$ DC) and observed similar findings (Fig. 2B), indicating that host DCs depend on the production of RALDH2 to drive allogeneic donor T cell responses.

As described above, RA metabolism plays a role in donor APCs and the control of GVHD. Therefore, we further tested whether ablating RALDH2 in donor DCs could affect GVHD. BALB/c mice were lethally irradiated and transplanted with BM from B6 RALDH2$^{-/-}$ DC donors and T cells from B6 WT mice. GVHD was significantly, albeit modestly, ameliorated in mice given donor RALDH2$^{-/-}$ DC BM and WT T cells, suggesting that donor BM-derived DCs are source of RA that can aid in disease progression (Fig. 2C). Collectively,

these data indicate that reducing RA production by selectively deleting RALDH2 in host or donor DCs can ameliorate GVHD.

Selective RALHD1 deficiency in intestinal epithelial cells failed to reduce GVHD

Gastrointestinal (GI) tract injury caused by conditioning regimens creates a pro-inflammatory environment, which initiates and amplifies GVHD (22–25). RALDH1 was upregulated and more dominantly expressed than RALDH2 in IECs of GVHD versus no GVHD control mice (12). Thus, we focused on RALDH1 expression in villin expressing cells in the intestine. To determine whether RALDH1 expression in IECs is essential for GVHD, we generated conditionally deleted RALDH1 in IECs by crossing B6 RALDH1 floxed/floxed with B6 villin^{Cre} mice (RALDH1$^{-/-}$ villin). Lethally irradiated B6 RALDH1$^{-/-}$ villin and their WT Cre negative littermate controls were used as recipients of BALB/c as donor BM plus T cells. Contrary to our hypothesis that GVHD would be reduced by eliminating IEC RALDH1 expression, no beneficial effect of conditional RALDH1 deletion in recipient villin expressing cells was seen (Fig. 3A).

Although RALDH1 is a predominant isoform in IECs, other isoforms expressed in IECs could potentially compensate in generating RA. Alternatively, IECs may fail to regulate RA production during GVHD. Therefore, we generated B6 CYP26A1-villin mice to reduce RA production, as might occur with combined RALDH1 and RALDH2 conditional deletion, by crossing B6 CYP26A1$^{stop/stop}$ and B6-villin^{Cre} mice. In an MHC mismatched model (BALB/c→B6 CYP26A1-villin), CYP26A1 overexpession in IECs also failed to ameliorate GVHD (Fig. 3B). Taken together, these results suggest that IECs do not sufficiently alter RA levels during the GVHD process since that reducing IEC RA production by RALDH1 deficiency or enhancing RA degradation does not improve GVHD lethality.

Reduced donor Th1 differentiation and increased co-inhibitory molecule expression in host DCs with RALDH2 deficiency

The above studies pointed to the critical role of RA metabolism in DCs rather than IECs. To uncover contributing mechanisms responsible for increased survival in RALDH2$^{-/-}$ DCs recipients, donor T cells in lymphoid and non-lymphoid tissues of recipients post- BMT were assessed for IFN-γ and TNF-α production. The percentage and absolute numbers of IFN-γ expressing donor CD4+ and CD8+ T cells and absolute numbers of donor CD8+ T cells expressing TNF-α were significantly lower in spleen, intestine, particularly in the small intestines in RALDH2$^{-/-}$ DC recipients (Fig. 4A-D). Furthermore, donor splenic CD4+ but not CD8+ T cells (data not shown), upregulated PD-1 and Lag3 co-inhibitory molecules (Fig. 4E). Consistent with these results, we also observed upregulation of PD-1, Lag3 and Tim-3 co-inhibitory molecules in donor CD4+ T cells isolated from B6 CYP26A1$^{stop/stop}$-VAV^{Cre} recipients (Supplemental Fig. 3A). Upregulation of PD-1 on donor CD4+ T cells was not associated with exhaustion as CD4+ PD-1^{hi} T cells were either significantly or equally proliferative as CD4+ PD-1^{low} T cells (data not shown). Together, these data suggest that impaired donor T effector cytokine and augmented co-inhibitory molecule expression contributed to protection from GVHD in RALDH2$^{-/-}$ DC recipients.

RALDH2 deficiency in recipient DCs increased donor T cell death and reduced gut homing receptor expression on donor T cells, whereas exogenous RA accelerated GVHD lethality in RALDH2$^{-/-}$ DCs recipients

Reduced number of Th1 cells in RALDH2$^{-/-}$ DCs recipients may be associated with increased apoptosis of donor T cells. Hence, we investigated the survival of donor T cells in RALDH2$^{-/-}$ DCs recipients by measuring active caspase-3. Compared with controls, RALDH2$^{-/-}$ DCs recipient donor T cells had higher active caspase-3 staining suggesting that those cells are undergoing apoptosis (Fig. 5A). Previous studies reported that the downregulation of gut homing molecules ($\alpha 4\beta 7$, CCR9) on donor T cells was associated with the attenuation of GVHD lethality. Therefore, we tested donor T cell gut homing molecule expression in RALDH2$^{-/-}$ DCs recipients. Compared with controls, RALDH2$^{-/-}$ DCs recipient donor T cells had lower gut homing molecule expression in mesenteric lymph nodes (MLN) (Fig. 5B) and small intestine LPL (Supplemental Fig. 2F). However there was no significant difference observed between the groups in donor T cell gut homing molecules on splenic (Fig. 5C) or large intestine LPLs (Supplemental Fig. 2G). To further support the notion that reduced RA levels was associated with reduced GVHD lethality in RALDH2$^{-/-}$ DCs recipients, we administered ATRA to determine whether increased RA signaling by ATRA would override host RALDH2 DC deficiency. Consistent with our previous report in WT mice (12), exogenous RA supplementation significantly accelerated GVHD lethality in RALDH2$^{-/-}$ DCs recipients than those of vehicle controls (RALDH2$^{-/-}$ DCs + vehicle controls) (Fig. 5D). Survival was not significantly different between the two exogenous RA groups (RALDH2$^{-/-}$ DCs + RA vs. littermate controls +RA). However, recipients with RALDH2$^{-/-}$ DCs + vehicle had significantly lower GVHD lethality than littermate controls + vehicle (Fig. 5D).

GVHD amelioration in recipients that have conditional RALDH2 deletion in host DCs is associated with increased Treg frequencies in secondary lymphoid organs and the intestine

On day 7 post-BMT, the percentage of CD4+FoxP3+ Tregs was significantly increased in RALDH2$^{-/-}$ DCs recipient spleens, MLN and intestines compared to their WT Cre negative littermate controls (Fig. 6A-D). However, there was no significant difference in the absolute numbers of Tregs in spleens, MLN (data not shown) and LPLs (Supplemental Fig. 3B) between the two groups. The increase in Tregs frequency observed in B6 RALDH2$^{-/-}$ DC recipients could be due to the expansion of Tregs from BALB/c donor T cell inoculum or generation of donor-derived peripheral Tregs (pTregs), as we previously have shown in settings in which GVHD is controlled (26). To investigate the source of the Tregs, lethally irradiated B6 RALDH2$^{-/-}$ DC recipients were infused with BALB/c BM and T cells or BALB/c BM and CD25- T cells. B6 RALDH2$^{-/-}$ DC recipients of BALB/c BM plus intact T cells or BM plus CD25- T cells survived significantly longer than those of their respective littermate controls (Fig. 6E-F). Together, these results suggest that the GVHD protective effect is associated with increased Treg frequencies likely from *in vivo* pTreg induction rather than the expansion of the few remaining thymic-derived Tregs contained in the donor T cell inoculum within this 7 day time period.

Discussion

In this study, we conditionally ablated RALDH isoforms responsible for RA synthesis *in vivo* in defined cellular lineages and observed that RALDH2 expression in DCs is critical to control GVHD lethality. RALDH exists in multiple isoforms (27) and while selective deletion of RALDH isoforms in immune cells should reduce RA levels, it is possible that sufficient RA would remain to support GVHD initiation and amplification by deleting one or another isoform. By averting any isoform effects from a single RALDH enzyme deletion, overexpression of the RA catabolizing enzyme, CYP26A1, only in hematopoietic cells was found to reduce GVHD lethality, providing evidence for the role of hematopoietically-derived RA in aggravating GVHD lethality. The attenuation of disease in B6 CYP26 transgenic recipients could be shown to be the consequence of increased RA degradation in recipient APCs. Following host APC depletion due to irradiation, donor APCs become the predominant cell population in maximizing alloresponses and reduced donor APC RA production was shown to diminish GVHD lethality. Together, these data suggesting that RA signaling also may play a role in the later post-BMT amplification phase of GVHD at a time when donor APCs predominate over host APCs.

In the current study, we ascertained that RA synthesis in DCs is critical in driving GVHD lethality, as ablation of RA signaling in host DCs alone protected mice from GVHD. Whereas, exogenous RA supplementation accelerated GVHD lethality in those mice. Our data also showed that silencing RA production only in donor DCs attenuated GVHD. Various cell types including host DCs, macrophages, and B cells, can serve as APCs to support GVHD-causing T effector cells (28, 29). For example, the addition of DCs to GVHD-resistant MHC class-II $^{-/-}$ recipients initiated allostimulation and GVHD (20, 30). A few studies have also demonstrated the direct role of donor DCs in regulating GVHD pathophysiology (31, 32), but given the dramatic effect of disease amelioration, it is an important aspect of the disease and of clinical interest. On the other hand, macrophages and B cells are not required for the initiation of GVHD (33, 34). Notably, RALDH2 is a key metabolizing enzyme required specifically in DCs to produce RA and represents an attractive therapeutic target.

Non-hematopoietic hosts IECs are capable of functioning as APCs and are sufficient to induce lethal GVHD (32). IECs form a single layer of cells and possess regulatory functions in maintaining a non-inflammatory milieu of the intestine by secreting RA and cytokines such as TGF-β and thymic stromal lymphopoietin (35). During steady state, RA release by IECs supported DC facilitated generation of suppressive Tregs and maintenance of immune homeostasis (36). Under inflammatory conditions, the RA pathway is a key determinant for the induction of intestinal GVHD by promoting a pro-inflammatory milieu (12, 13). During GVHD, RALDH1, the predominant isoform in IECs, was upregulated in non-hematopoietic cells of intestines (12). However, we did not observe any beneficial effects of RA ablation in the intestines on curbing GVHD lethality either by conditional RALDH1 deletion or CYP26A1 overexpression in IECs. There are at least two potential explanations for these findings. Firstly, other cell types, namely villin negative non-hematopoietic cells and intestinal DCs, may act as a source of RA synthesis in intestines during GVHD to maintain the inflammatory milieu (37). Secondly, RA synthesis by IECs may not be required to

promote GVHD lethality. Regardless of the correct explanation, our findings indicate that RA signaling in DCs played a dominant role in controlling GVHD lethality. While there are a myriad of non-hematopoietic and hematopoietic cells that produce RA, ablation of RA synthesis by DCs and amelioration of disease suggests that RA may be delivered to T cells in close proximity in a cognate paracrine fashion.

Pro-inflammatory cytokines, mainly Th1 cell -derived IFN-γ and TNF-α, have been associated with the development and severity of GVHD (38). Our finding of reduced IFN-γ in RALDH2$^{-/-}$ DC recipients corroborates our previous observations of reduced donor Th1 differentiation in the absence of RA synthesis during GVHD (12) and the absolute need for RA in stabilizing the Th1 phenotype *in vivo* (5). Although a considerable percentage of donor T cells produced pro-inflammatory cytokines in RALDH2$^{-/-}$ DC recipients, the absolute number of those cells may be below the threshold number to cause GVHD lethality. Moreover, lower RA levels in a GVHD setting was associated with upregulated expression of multiple co-inhibitory molecules including PD-1, Lag-3, and Tim-3 on donor CD4+ T cells. While co-inhibitory molecules can be upregulated on activated T cells, our data in aggregate are most consistent with their inhibitory function to control GVHD by dampening alloimmune responses (39–41). In the current study, the upregulation of PD-1 on donor CD4+ T cells was not related to exhaustion, as these cells were proliferative and continued to produce IFN-γ and TNF-α even at the later time point of transplantation. Moreover, upregulation of PD-1 and higher active caspase-3 expressions on donor T cells indicate that the apoptosis may be mediated through activation-induced cell death. Based on these data, we cannot exclude the possibility that the highest IFN-γ producing donor T cells in RALDH2$^{-/-}$ DC recipients may be due to IFN-γ-induced activation-induced cell death, as we have reported, thereby mitigating GVHD lethality (41). Furthermore, reduced gut homing receptor expressions on donor T cells of RALDH2$^{-/-}$ DC recipients may also contribute to reduced GVHD mortality due to their impaired homing to intestines. However as evident by the cytokine expression data lower homing receptor expression is but one mechanism involved in this biological effect.

We observed an increase in the frequency of Tregs in RALDH2$^{-/-}$ DC recipients, which may have actively contributed to GVHD lethality amelioration. On the other hand, there was no significant difference observed in the absolute number of Tregs between the two groups. Because higher Tregs: Teffector (Teff) ratios may blunt Teff responses, the higher frequencies of Tregs in RALDH2$^{-/-}$ DC recipients vs. littermate controls may contribute to GVHD control. Mechanistically, the increased Treg frequencies in RALDH2$^{-/-}$ DC recipients may be due to the reduced number of donor T cells that elaborate pro-inflammatory cytokines such as IL-21, resulting in a permissive environment for pTregs (26). Studies have reported that RA can promote the generation of *in vitro* induced Tregs and *in vivo* pTregs as well as expansion of thymic-derived Tregs (1, 42, 43). On the other hand, in an inflammatory setting of EAE, RA failed to increase the frequency of Tregs *in vivo* (44). In the current study, paradoxically we observed increased frequency of donor Tregs in RALDH2$^{-/-}$ DC recipients despite lower RA levels that would occur in the absence of RALDH2 synthesis in host DCs. These data support the notion that the effect on RA on immune cells is highly context-dependent and varies dependent upon the inflammatory microenvironment and disease model. Further, our data are most consistent with lower

GVHD lethality in RALDH2$^{-/-}$ DC recipients due to reduced Th1 differentiation, IL-21 and pro-inflammatory cytokines that allowed increased generation of pTregs (12, 45). We would speculate based on prior studies (5), the absence of RA signaling to the alloreactive T cell compartment undermines the stable expression of T-bet critical for sustained Th1 differentiation. Although CD103+ DCs of gut associated lymphoid tissues are able to generate Tregs, due to their higher RALDH activity (46) and RA can maintain Treg stability inhibiting Th17 differentiation (47), Treg-depleted donor grafts failed to abrogate the reduced GVHD benefits seen in RALDH2$^{-/-}$ DC recipients. This finding suggests that pTreg generation might have contributed to the increased frequency of Tregs and supports the notion that reduced pro-inflammatory cytokine milieu favors pTreg generation.

Inhibition of full RA signaling in allogeneic donor T cells expressing the dnRARα transgene, as would occur in recipients with RALDH2$^{-/-}$ DCs, did not impair their graft-versus-leukemia (GVL) responses (12). We did not investigate GVL responses in RALDH2$^{-/-}$ DC recipients, as the current study focused on GVHD pathogenesis and biology rather than GVHD prevention or treatment. Therefore, we cannot exclude the possibility that GVL would be inhibited even though this seems unlikely since the dnRARα transgene effect should be more pervasive and robust than selective deficiency of RALDH2 in host DCs. Future studies are warranted to explore the GVL response in RALDH2$^{-/-}$ DC recipients.

In summary, our findings provide novel insights on RA synthesizing cell lineages and RALDH isoforms in regulating GVHD lethality. Selective targeting of RALDH2 or inducing CYP26A1 using new classes of drugs may serve as a novel approaches for preventing GVHD and potentially for inflammatory conditions that affect the GI tract.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article:

APCs	antigen presenting cells
DC	dendritic cell
GVHD	graft-versus-host-disease
RALDH	retinal aldehyde dehydrogenase
RA	retinoic acid

Th	T helper cells
Tregs	Tregulatory cells
WT	wild-type

References:

1. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, and Cheroutre H. 2007 Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 317: 256–260. [PubMed: 17569825]
2. Pino-Lagos K, Guo Y, Brown C, Alexander MP, Elgueta R, Bennett KA, De Vries V, Nowak E, Blomhoff R, Sockanathan S, Chandraratna RA, Dmitrovsky E, and Noelle RJ. 2011 A retinoic acid-dependent checkpoint in the development of CD4+ T cell-mediated immunity. J Exp Med 208: 1767–1775. [PubMed: 21859847]
3. Guo Y, Brown C, Ortiz C, and Noelle RJ. 2015 Leukocyte homing, fate, and function are controlled by retinoic acid. Physiol Rev 95: 125–148. [PubMed: 25540140]
4. van de Pavert SA, Ferreira M, Domingues RG, Ribeiro H, Molenaar R, Moreira-Santos L, Almeida FF, Ibiza S, Barbosa I, Goverse G, Labão-Almeida C, Godinho-Silva C, Konijn T, Schooneman D, O'Toole T, Mizee MR, Habani Y, Haak E, Santori FR, Littman DR, Schulte-Merker S, Dzierzak E, Simas JP, Mebius RE, and Veiga-Fernandes H. 2014 Maternal retinoids control type 3 innate lymphoid cells and set the offspring immunity. Nature 508: 123–127. [PubMed: 24670648]
5. Brown CC, Esterhazy D, Sarde A, London M, Pullabhatla V, Osma-Garcia I, Al-Bader R, Ortiz C, Elgueta R, Arno M, de Rinaldis E, Mucida D, Lord GM, and Noelle RJ. 2015 Retinoic acid is essential for Th1 cell lineage stability and prevents transition to a Th17 cell program. Immunity 42: 499–511. [PubMed: 25769610]
6. Kim MH, Taparowsky EJ, and Kim CH. 2015 Retinoic Acid Differentially Regulates the Migration of Innate Lymphoid Cell Subsets to the Gut. Immunity 43: 107–119. [PubMed: 26141583]
7. Zhang Z, Li J, Zheng W, Zhao G, Zhang H, Wang X, Guo Y, Qin C, and Shi Y. 2016 Peripheral Lymphoid Volume Expansion and Maintenance Are Controlled by Gut Microbiota via RALDH+ Dendritic Cells. Immunity 44: 330–342. [PubMed: 26885858]
8. Larange A, and Cheroutre H. 2016 Retinoic Acid and Retinoic Acid Receptors as Pleiotropic Modulators of the Immune System. Annu Rev Immunol 34: 369–394. [PubMed: 27168242]
9. Stevison F, Jing J, Tripathy S, and Isoherranen N. 2015 Role of Retinoic Acid-Metabolizing Cytochrome P450s, CYP26, in Inflammation and Cancer. Adv Pharmacol 74: 373–412. [PubMed: 26233912]
10. Vicente-Suarez I, Larange A, Reardon C, Matho M, Feau S, Chodaczek G, Park Y, Obata Y, Gold R, Wang-Zhu Y, Lena C, Zajonc DM, Schoenberger SP, Kronenberg M, and Cheroutre H. 2015 Unique lamina propria stromal cells imprint the functional phenotype of mucosal dendritic cells. Mucosal Immunol 8: 141–151. [PubMed: 24938743]
11. Yokota A, Takeuchi H, Maeda N, Ohoka Y, Kato C, Song SY, and Iwata M. 2009 GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. Int Immunol 21: 361–377. [PubMed: 19190084]
12. Aoyama K, Saha A, Tolar J, Riddle MJ, Veenstra RG, Taylor PA, Blomhoff R, Panoskaltsis-Mortari A, Klebanoff CA, Socie G, Munn DH, Murphy WJ, Serody JS, Fulton LM, Teshima T, Chandraratna RA, Dmitrovsky E, Guo YX, Noelle RJ, and Blazar BR. 2013 Inhibiting retinoic acid signaling ameliorates graft-versus-host disease by modifying T-cell differentiation and intestinal migration. Blood 122: 2125–2134. [PubMed: 23814022]
13. Chen X, Dodge J, Komorowski R, and Drobyski WR. 2013 A critical role for the retinoic acid signaling pathway in the pathophysiology of gastrointestinal graft-versus-host disease. Blood 121: 3970–3980. [PubMed: 23529927]
14. DePaolo RW, Abadie V, Tang F, Fehlner-Peach H, Hall JA, Wang W, Marietta EV, Kasarda DD, Waldmann TA, Murray JA, Semrad C, Kupfer SS, Belkaid Y, Guandalini S, and Jabri B. 2011 Co-

adjuvant effects of retinoic acid and IL-15 induce inflammatory immunity to dietary antigens. Nature 471: 220–224. [PubMed: 21307853]

15. Dodge J, Stephans A, Lai J, Drobyski WR, and Chen X. 2016 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. Biol Blood Marrow Transplant 22: 2141–2148. [PubMed: 27596131]
16. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, and Littman DR. 2006 The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126: 1121–1133. [PubMed: 16990136]
17. Kedishvili NY 2013 Enzymology of retinoic acid biosynthesis and degradation. J Lipid Res 54: 1744–1760. [PubMed: 23630397]
18. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, Liu J, Shlomchik MJ, and Emerson SG. 1999 Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science 285: 412–415. [PubMed: 10411505]
19. Matte CC, Liu J, Cormier J, Anderson BE, Athanasiadis I, Jain D, McNiff J, and Shlomchik WD. 2004 Donor APCs are required for maximal GVHD but not for GVL. Nat Med 10: 987–992. [PubMed: 15286785]
20. Duffner UA, Maeda Y, Cooke KR, Reddy P, Ordemann R, Liu C, Ferrara JL, and Teshima T. 2004 Host dendritic cells alone are sufficient to initiate acute graft-versus-host disease. J Immunol 172: 7393–7398. [PubMed: 15187116]
21. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, and Song SY. 2004 Retinoic acid imprints gut-homing specificity on T cells. Immunity 21: 527–538. [PubMed: 15485630]
22. Ferrara JL, Levine JE, Reddy P, and Holler E. 2009 Graft-versus-host disease. Lancet 373: 1550–1561. [PubMed: 19282026]
23. Hill GR, and Ferrara JL. 2000 The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. Blood 95: 2754–2759. [PubMed: 10779417]
24. Shlomchik WD 2007 Graft-versus-host disease. Nat Rev Immunol 7: 340–352. [PubMed: 17438575]
25. Welniak LA, Blazar BR, and Murphy WJ. 2007 Immunobiology of allogeneic hematopoietic stem cell transplantation. Annu Rev Immunol 25: 139–170. [PubMed: 17129175]
26. Bucher C, Koch L, Vogtenhuber C, Goren E, Munger M, Panoskaltsis-Mortari A, Sivakumar P, and Blazar BR. 2009 IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. Blood 114: 5375–5384. [PubMed: 19843883]
27. Erkelens MN, and Mebius RE. 2017 Retinoic Acid and Immune Homeostasis: A Balancing Act. Trends Immunol 38: 168–180. [PubMed: 28094101]
28. Markey KA, Banovic T, Kuns RD, Olver SD, Don AL, Raffelt NC, Wilson YA, Raggatt LJ, Pettit AR, Bromberg JS, Hill GR, and MacDonald KP. 2009 Conventional dendritic cells are the critical donor APC presenting alloantigen after experimental bone marrow transplantation. Blood 113: 5644–5649. [PubMed: 19336758]
29. Zhang Y, Louboutin JP, Zhu J, Rivera AJ, and Emerson SG. 2002 Preterminal host dendritic cells in irradiated mice prime CD8+ T cell-mediated acute graft-versus-host disease. J Clin Invest 109: 1335–1344. [PubMed: 12021249]
30. Koyama M, Hashimoto D, Aoyama K, Matsuoka K, Karube K, Niiro H, Harada M, Tanimoto M, Akashi K, and Teshima T. 2009 Plasmacytoid dendritic cells prime alloreactive T cells to mediate graft-versus-host disease as antigen-presenting cells. Blood 113: 2088–2095. [PubMed: 19144988]
31. Das R, Chen X, Komorowski R, Hessner MJ, and Drobyski WR. 2009 Interleukin-23 secretion by donor antigen-presenting cells is critical for organ-specific pathology in graft-versus-host disease. Blood 113: 2352–2362. [PubMed: 19059877]
32. Koyama M, Cheong M, Markey KA, Gartlan KH, Kuns RD, Locke KR, Lineburg KE, Teal BE, Leveque-El Mouttie L, Bunting MD, Vuckovic S, Zhang P, Teng MW, Varelias A, Tey SK, Wockner LF, Engwerda CR, Smyth MJ, Belz GT, McColl SR, MacDonald KP, and Hill GR. 2015 Donor colonic CD103+ dendritic cells determine the severity of acute graft-versus-host disease. J Exp Med 212: 1303–1321. [PubMed: 26169940]

33. MacDonald KP, Palmer JS, Cronau S, Seppanen E, Olver S, Raffelt NC, Kuns R, Pettit AR, Clouston A, Wainwright B, Branstetter D, Smith J, Paxton RJ, Cerretti DP, Bonham L, Hill GR, and Hume DA. 2010 An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue- and tumor-associated macrophages but does not inhibit inflammation. Blood 116: 3955–3963. [PubMed: 20682855]
34. Matte-Martone C, Wang X, Anderson B, Jain D, Demetris AJ, McNiff J, Shlomchik MJ, and Shlomchik WD. 2010 Recipient B cells are not required for graft-versus-host disease induction. Biol Blood Marrow Transplant 16: 1222–1230. [PubMed: 20338255]
35. Rimoldi M, Chieppa M, Salucci V, Avogadri F, Sonzogni A, Sampietro GM, Nespoli A, Viale G, Allavena P, and Rescigno M. 2005 Intestinal immune homeostasis is regulated by the crosstalk between epithelial cells and dendritic cells. Nat Immunol 6: 507–514. [PubMed: 15821737]
36. Iliev ID, Mileti E, Matteoli G, Chieppa M, and Rescigno M. 2009 Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. Mucosal Immunol 2: 340–350. [PubMed: 19387433]
37. Molenaar R, Knippenberg M, Goverse G, Olivier BJ, de Vos AF, O'Toole T, and Mebius RE. 2011 Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. J Immunol 186: 1934–1942. [PubMed: 21220692]
38. Blazar BR, Murphy WJ, and Abedi M. 2012 Advances in graft-versus-host disease biology and therapy. Nat Rev Immunol 12: 443–458. [PubMed: 22576252]
39. Blazar BR, Carreno BM, Panoskaltsis-Mortari A, Carter L, Iwai Y, Yagita H, Nishimura H, and Taylor PA. 2003 Blockade of programmed death-1 engagement accelerates graft-versus-host disease lethality by an IFN-gamma-dependent mechanism. J Immunol 171: 1272–1277. [PubMed: 12874215]
40. Sega EI, Leveson-Gower DB, Florek M, Schneidawind D, Luong RH, and Negrin RS. 2014 Role of lymphocyte activation gene-3 (Lag-3) in conventional and regulatory T cell function in allogeneic transplantation. PLoS One 9: e86551. [PubMed: 24475140]
41. Veenstra RG, Taylor PA, Zhou Q, Panoskaltsis-Mortari A, Hirashima M, Flynn R, Liu D, Anderson AC, Strom TB, Kuchroo VK, and Blazar BR. 2012 Contrasting acute graft-versus-host disease effects of Tim-3/galectin-9 pathway blockade dependent upon the presence of donor regulatory T cells. Blood 120: 682–690. [PubMed: 22677125]
42. Benson MJ, Pino-Lagos K, Rosemblatt M, and Noelle RJ. 2007 All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. J Exp Med 204: 1765–1774. [PubMed: 17620363]
43. Golovina TN, Mikheeva T, Brusko TM, Blazar BR, Bluestone JA, and Riley JL. 2011 Retinoic acid and rapamycin differentially affect and synergistically promote the ex vivo expansion of natural human T regulatory cells. PLoS One 6: e15868. [PubMed: 21253593]
44. Xiao S, Jin H, Korn T, Liu SM, Oukka M, Lim B, and Kuchroo VK. 2008 Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. J Immunol 181: 2277–2284. [PubMed: 18684916]
45. Chen X, Das R, Komorowski R, Beres A, Hessner MJ, Mihara M, and Drobyski WR. 2009 Blockade of interleukin-6 signaling augments regulatory T-cell reconstitution and attenuates the severity of graft-versus-host disease. Blood 114: 891–900. [PubMed: 19491393]
46. Coombes JL, Siddiqui KR, Arancibia-Cárcamo CV, Hall J, Sun CM, Belkaid Y, and Powrie F. 2007 A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med 204: 1757–1764. [PubMed: 17620361]
47. Zhou X, Kong N, Wang J, Fan H, Zou H, Horwitz D, Brand D, Liu Z, and Zheng SG. 2010 Cutting edge: all-trans retinoic acid sustains the stability and function of natural regulatory T cells in an inflammatory milieu. J Immunol 185: 2675–2679. [PubMed: 20679534]

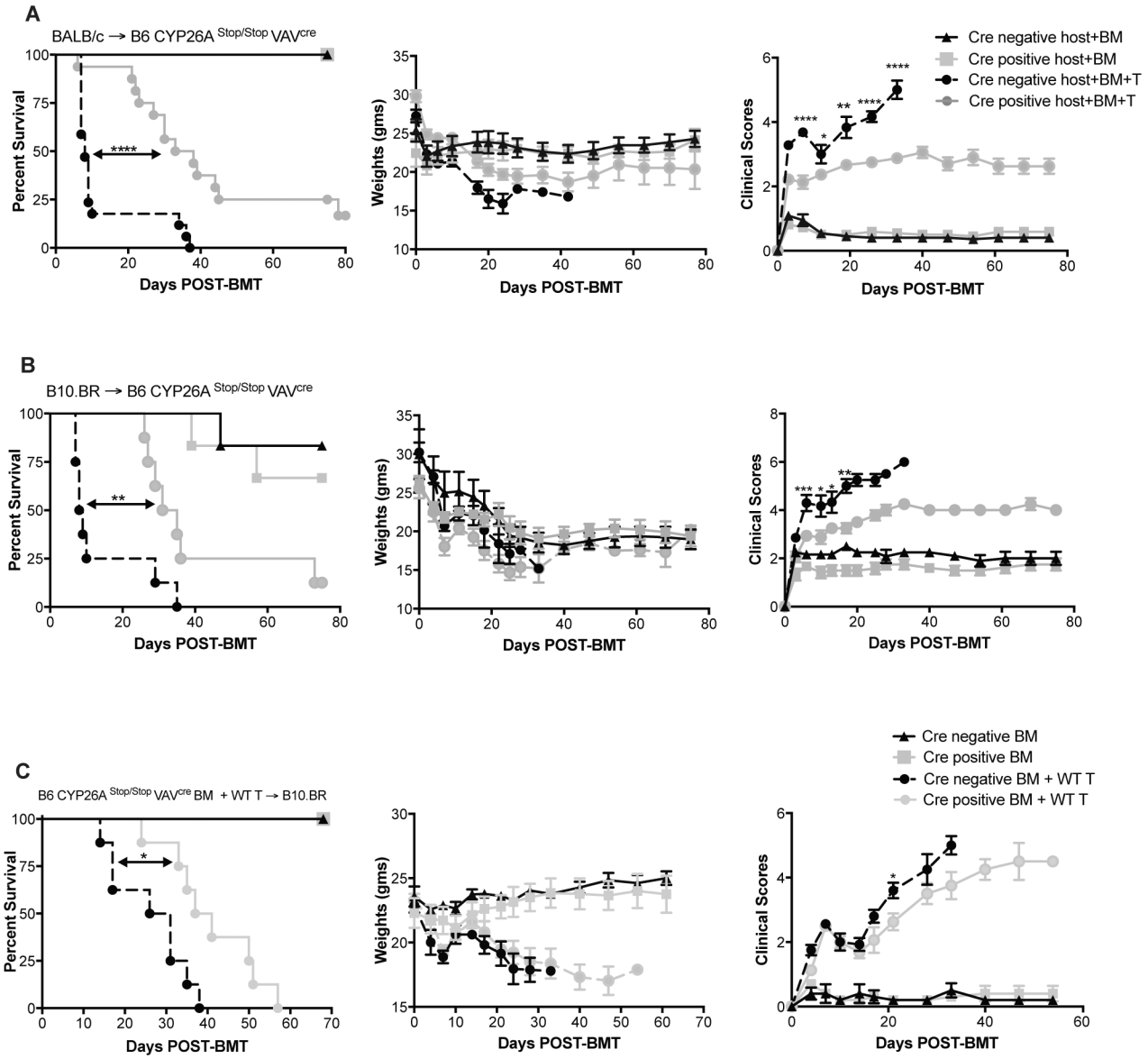

Figure 1: Increased RA catabolism in recipient hematopoietic cells reduces GVHD.

A) C57BL/6 (B6) CYP26A1$^{stop/stop}$ Vav^{Cre} positive (pos) or negative (neg) mice were lethally irradiated on day −1 followed by infusion with BALB/c NTCD bone marrow (BM) with or without 3×10^6 BALB/c purified T cells on day 0. Survival plot, weight curves and clinical scores are shown. Data are pooled from two independent experiments with similar results; n=11 mice/BM group; 16-17 mice/BM+T group; B) Lethally irradiated B6 CYP26A1$^{stop/stop}$ Vav^{Cre} pos or neg recipients were given B10.BR BM with or without 3×10^6 B10.BR purified T cells. n=6 mice/BM group; 8 mice/BM+T group; C) Lethally irradiated B10.BR recipients were given allogeneic B6 CYP26A1$^{stop/stop}$ Vav^{Cre} (pos or neg BM with or without 3×10^6 B6 wild type (WT) purified T cells. Survival plot, weight curves and clinical scores are shown. n=5 mice/BM group; 8 mice/BM+T group. *$P < .05$; **$P < .01$; ***$P < .001$; and ****$P < .0001$.

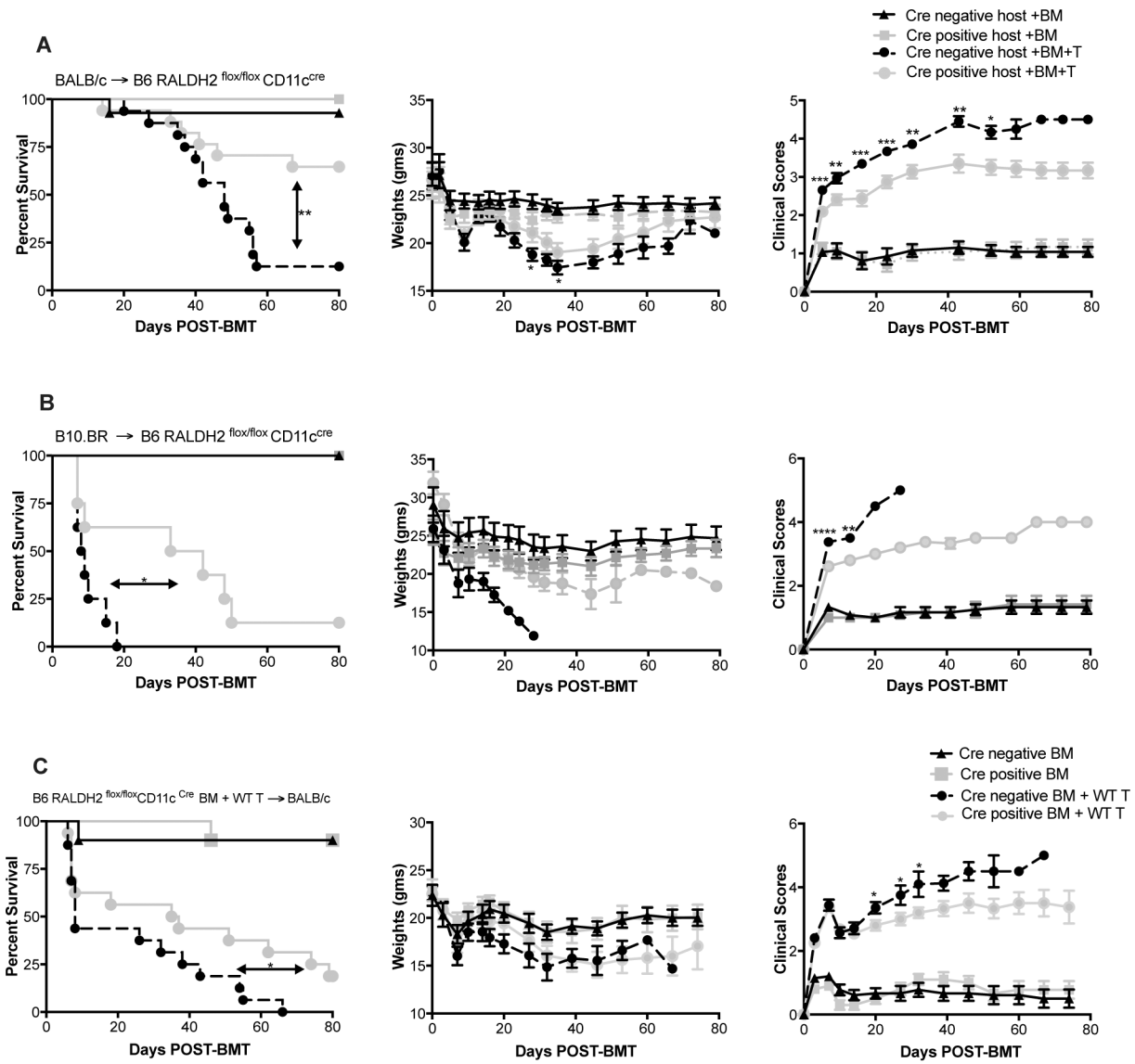

Figure 2: RALDH2 expression is required in recipient DCs and to a lesser extent in donor DCs to accelerate GVHD.

Lethally irradiated B6 RALDH2$^{fl/fl}$ CD11c^{Cre} pos or neg recipients were given BALB/c BM with or without 3×10^6 BALB/c purified T cells. A) Survival plot, weight curves and clinical scores are shown. Pooled data are from two independent experiments; n=14 mice/BM group; 16-17 mice/BM+T group. B) Lethally irradiated B6 RALDH2$^{fl/fl}$ CD11c^{Cre} pos or neg recipients that were transplanted with B10.BR BM with or without 2×10^6 B10.BR purified T cells. Survival plot, weight curves and clinical scores are shown. n=6 mice/BM group; 8 mice/BM+T group; C) BALB/c recipients were lethally irradiated and subsequently transplanted with 10^7 B6 RALDH2$^{fl/fl}$ CD11c^{Cre} pos or neg BM ±1×10^6 B6 WT purified T cells. Survival plot, weight curves and clinical scores are shown. n=10 mice/BM group; 16 mice/BM+T group. *P < .05; **P < .01; ***P < .001; and ****P < .0001.

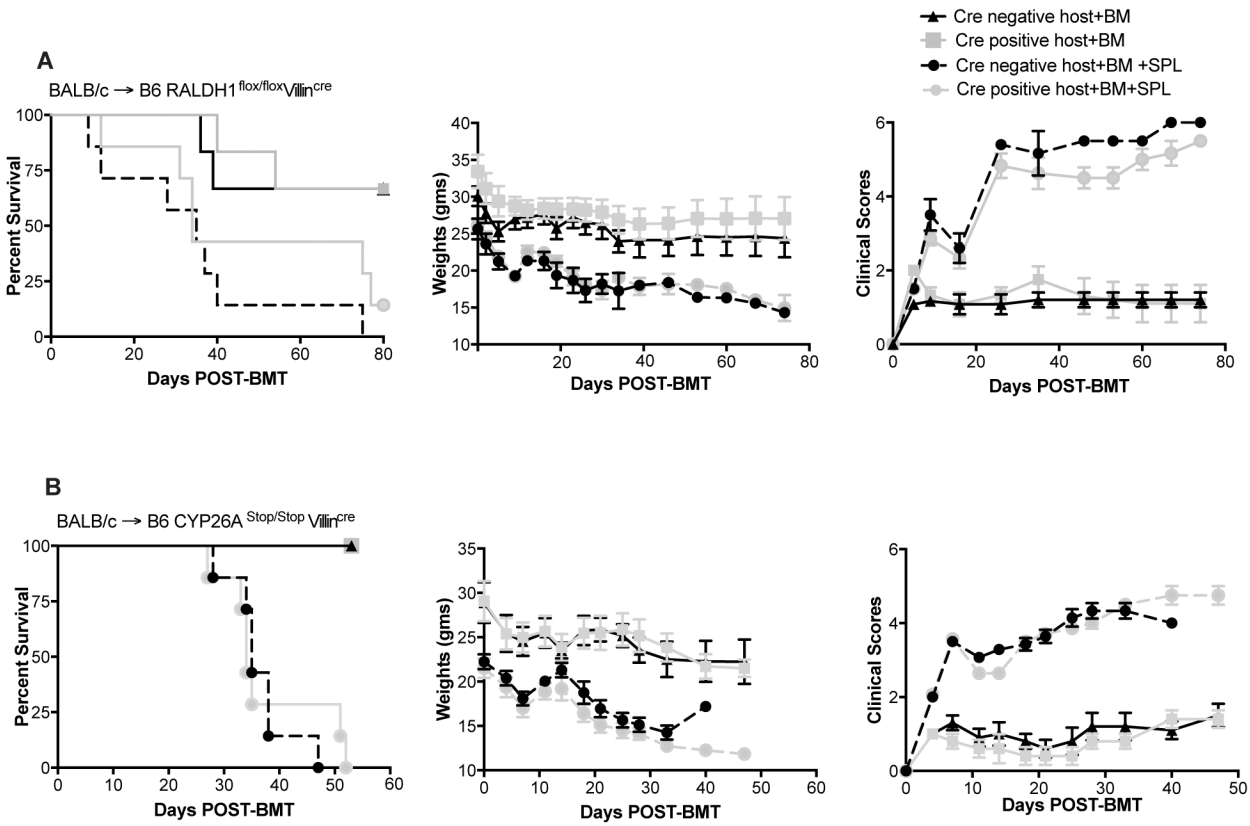

Figure 3: Enhancing RA degradation in intestinal epithelial cells had no effect in controlling GVHD.

A) Lethally irradiated B6 RALDH1$^{fl/fl}$ Villin^{Cre} pos or neg recipients were transplanted with BALB/c BM alone or with 5×10^6 BALB/c splenocytes (SPL). Survival plot, weight curves and clinical scores are shown. n=6 mice/BM group; 7 mice/BM+T group. B) B6 Cyp26A$^{stop/stop}$ Villin^{Cre} pos or neg mice were lethally irradiated and infused with BALB/c BM alone or with 5×10^6 BALB/c SPL. Survival plot, weight curves and clinical scores are shown. n=5 mice/BM group; 7 mice/BM+T group.

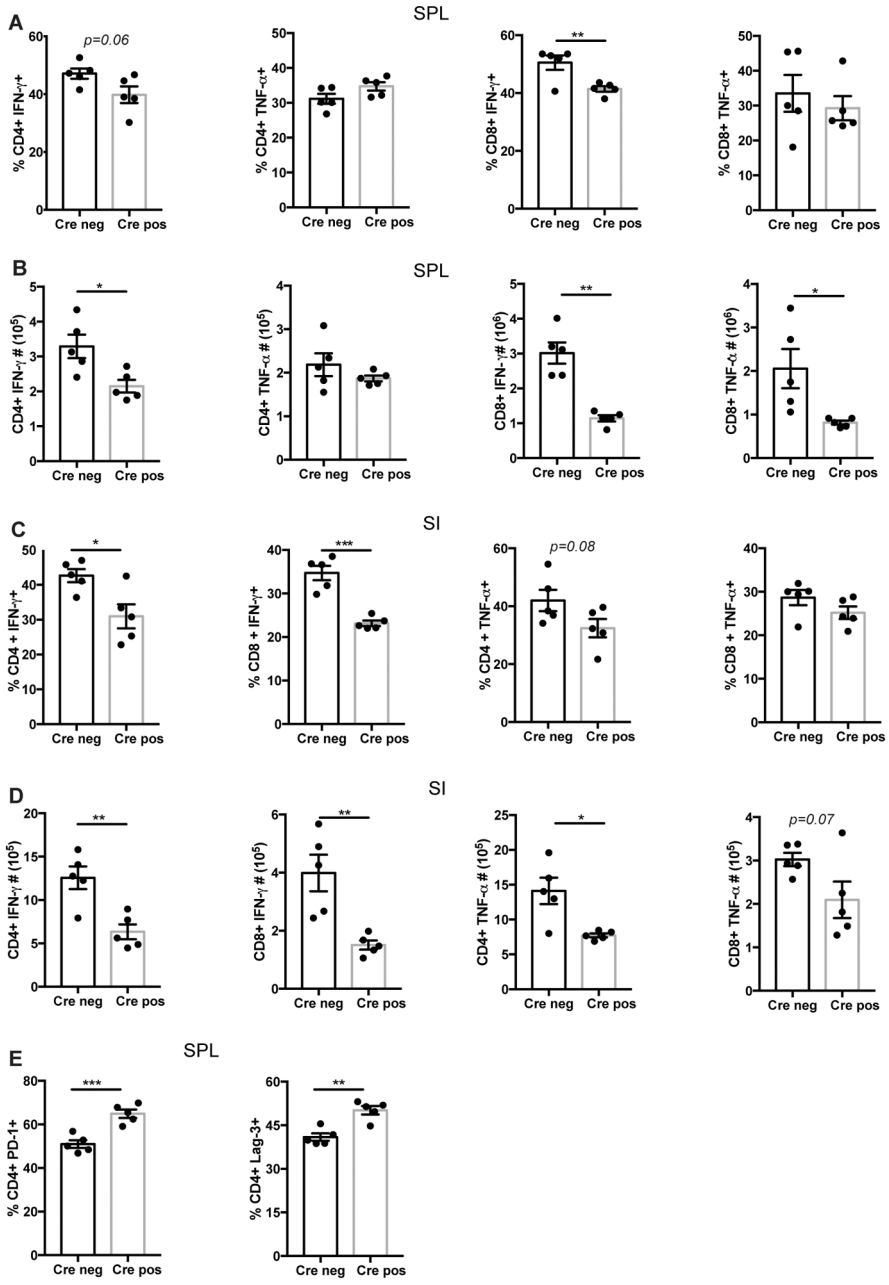

Figure 4: Absence of RALDH2 in recipient DCs reduced Th1 differentiation and increased donor T cell co-inhibitory molecule expression in a GVHD setting.

Lethally irradiated B6 RALDH2$^{fl/fl}$ CD11c^{Cre} pos or neg recipients were given BALB/c BM with 3×10^6 BALB/c purified T cells. Frequency and absolute numbers of donor CD4+ IFN-γ+, CD4+ TNF-α, CD8+ IFN-γ+, and CD8+ TNF-α cells in spleens (SPL; A and B) and small intestines (SI; C and D) of the recipients day 7 and 21 post-transplantation respectively. Data are representative of 1-2 experiments. n=5/group. E) Frequency of donor CD4+ PD-1+, CD4+Lag-3+ cells in recipient spleens on day 7 post transplantation. n=5/group. *P < .05; **P < .01; ***P < .001; and ****P < .0001.

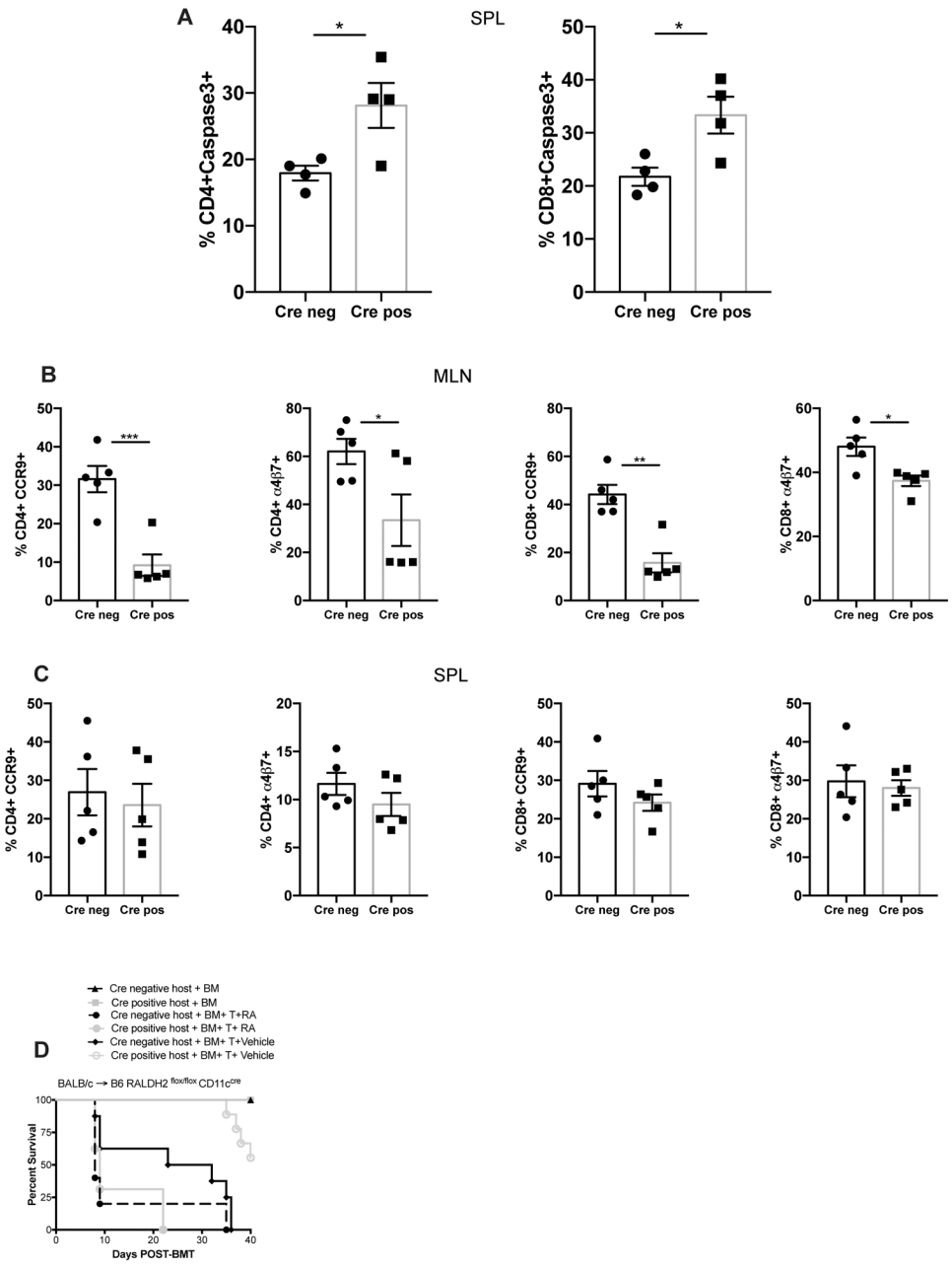

Figure 5: RALDH2 deficiency in recipient DCs increased donor T cell death and reduced gut homing receptor expression on donor T cells, whereas exogenous RA accelerated GVHD lethality in RALDH2$^{-/-}$ DCs recipients

Lethally irradiated B6 RALDH2$^{fl/fl}$ CD11c^{Cre} pos or neg recipients were given BALB/c BM with 3×10^6 BALB/c purified T cells. (A) Frequencies of active caspase-3 expression on splenic donor T cells day 6 post-transplantation. (B-C) Frequencies of gut homing receptor (α4β7, CCR9) expression on donor T cells from (B) mesenteric lymph nodes (MLN) and (C) spleens (SPL). n=4-5/group. Data are representative of two experiments (A) and one experiment. D) Survival of lethally irradiated B6 RALDH2$^{fl/fl}$ CD11c^{Cre} pos or neg recipients that were given BALB/c BM with or without 3×10^6 BALB/c purified T cells. Cohorts, as indicated, were treated with either vehicle or ATRA (RA). n=7 mice/BM group;

8–9 mice/BM+T group. RALDH2$^{fl/fl}$ CD11c^{Cre} pos + vehicle recipients (n=9) were significantly survived longer ($P < .0001$) than either RALDH2$^{fl/fl}$ CD11c^{Cre} pos + RA or RALDH2$^{fl/fl}$ CD11c^{Cre}neg + vehicle (n=8/group). No significant differences were noted between RALDH2$^{fl/fl}$ CD11c^{Cre} pos + RA and RALDH2$^{fl/fl}$ CD11c^{Cre}neg + vehicle (n=8/group). *$P < .05$; **$P < .01$; ***$P < .001$

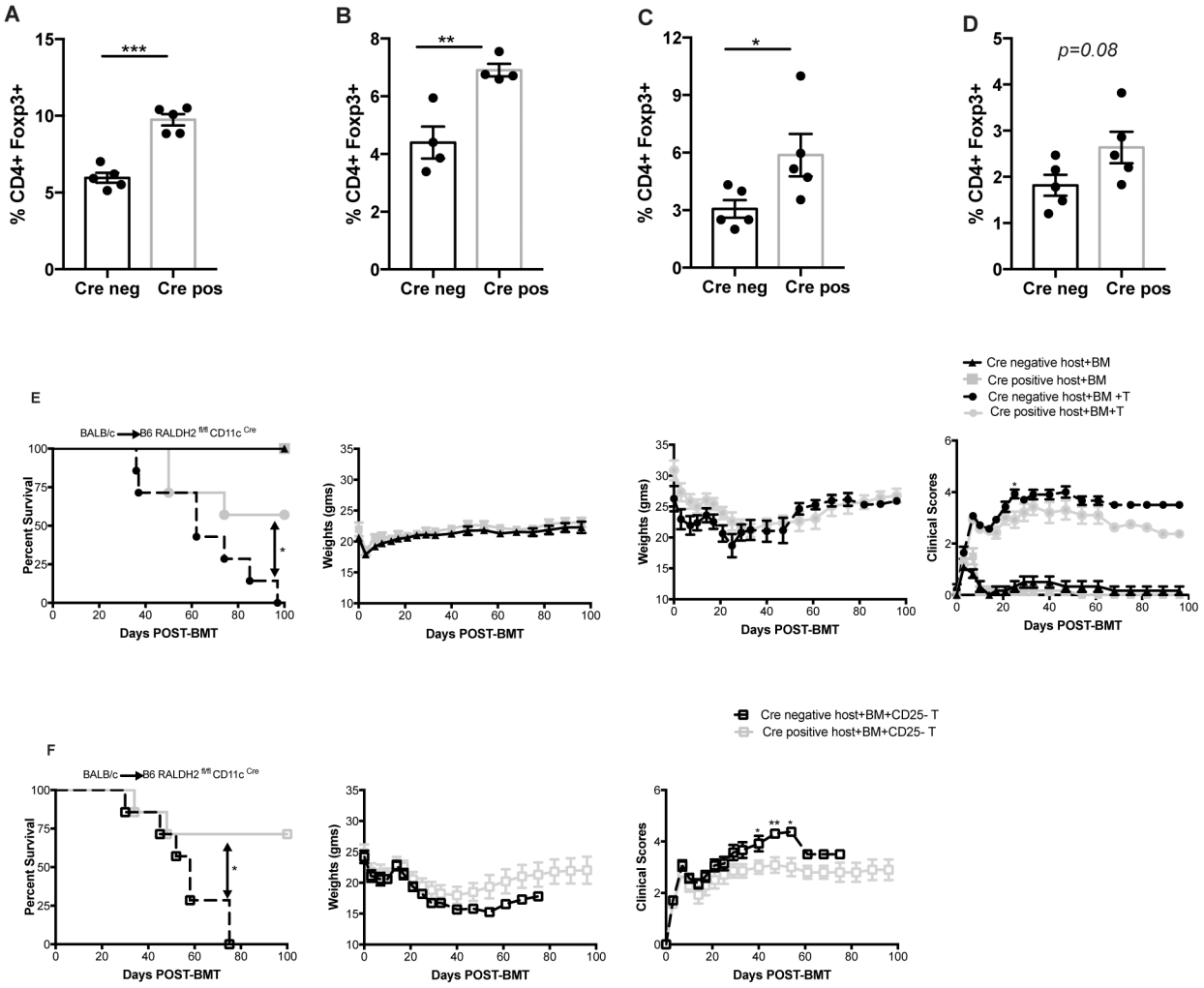

Figure 6: GVHD amelioration in RALDH2$^{-/-}$ DCs recipients is associated with increased Treg frequencies but is not dependent upon Tregs present in the donor graft at the time of BMT.

Lethally irradiated B6 RALDH2$^{fl/fl}$ CD11c^{Cre} pos or neg recipients were given BALB/c BM with 3×10^6 BALB/c purified T cells. Frequency of donor CD4+ Foxp3+ T cells on day 7 post- transplantation in A) spleen B) MLN and day 21 in C) small intestine lamina propria lymphocytes (LPL) and D) large intestine LPL. Data are representative of 1-2 experiments. n=5/group; Lethally irradiated B6 RALDH2$^{fl/fl}$ CD11c^{Cre} pos or neg recipients that were given E) BALB/c BM with or without 3×10^6 BALB/c purified intact T cells; F) BALB/c BM with or without 3×10^6 BALB/c CD25$^-$T cells. Survival plot, weight curves and clinical scores are shown. n=6 mice/BM group; 7 mice/BM+T group. *P < .05; **P < .01; ***P < .001.