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Attenuation of Acute Graft-versus-Host Disease in the Absence of the Transcription Factor RORγt

LeShara M. Fulton,*† Michael J. Carlson,* James M. Coghill,*† Laura E. Ott,* Michelle L. West,* Angela Panoskaltsis-Mortari,§ Dan R. Littman,§ Bruce R. Blazar,§ and Jonathan S. Serody*†‡

Graft-versus-host disease (GVHD) remains the most significant complication after allogeneic stem cell transplantation. Previously, acute GVHD had been considered to be mediated predominantly by Th1-polarized T cells. Recently, investigators have identified a second proinflammatory lineage of T cells termed Th17 that is critically dependent on the transcription factor retinoic acid-related orphan receptor (ROR)γt. In this study, we have evaluated the role of Th17 cells in murine acute GVHD by infusing donor T cells lacking RORC and as a consequence the isoform RORγt. Recipients given donor CD4+ and CD8+ T cells lacking RORC had significantly attenuated acute GVHD and markedly decreased tissue pathology in the colon, liver, and lung. Using a clinically relevant haploidentical murine transplantation model, we showed that RORC−/− CD4+ T cells alone diminished the severity and lethality of acute GVHD. This was not found when CD4+ T cells from RORC−/− mice were given to completely mismatched BALB/c mice, and it was correlated with absolute differences in the generation of TNF in the colon after transplant. Thus, CD4+ T cell expression of RORC is important in the pathogenesis of acute GVHD. The Journal of Immunology, 2012, 189: 000–000.

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llogeneic stem cell transplantation (allo-SCT) is a common treatment for patients with high-risk leukemia, recurrent low-grade lymphomas, aplastic anemia, and congenital bone marrow failure syndromes (1–3). The effectiveness of allo-SCT is limited by the development of acute graft-versus-host disease (aGVHD). aGVHD, a disease characterized by selective epithelial damage to target organs, is mediated by mature T cells present in the stem cell or bone marrow inoculums (4–7). Interactions of donor T cells with predominantly host APCs leads to activation and differentiation of donor T cells, ultimately resulting in inflammation in GVHD target organs, which includes primarily the skin, liver, and gastrointestinal (GI) tract (8).

Previous GVHD research has focused on cytokine production in T cell subsets. High levels of IFN-γ and IL-2 found in patients after allo-SCT led investigators to conclude that GVHD was mediated primarily by proinflammatory Th1 cells (9, 10). However and conversely, inhibition of Th1 cytokines leads to disease exacerbation in GVHD (11, 12). Because both protective and detrimental effects are seen with Th1 cytokines, the exact role of these cytokines in GVHD remains elusive (13). More recent investigations of T cell subsets in GVHD have been directed toward a new subset of CD4+ T cells, Th17 cells. Th17 cell differentiation and expression of cytokines in GVHD remains elusive (13). The development of Th17 cells is dependent on the transcription factors retinoid-related orphan receptor (ROR)γt, RORα, IFN regulatory factor -4, and STAT3 (17, 18). Th17 cells produce proinflammatory cytokines such as TNF, IL-21, and IL-22 (19–21) and as a consequence the isoform RORγt. In the absence of a particular cytokine, in this case IL-17A, the outcome of aGVHD is altered in a manner that suggests a role for Th17 cells in the pathogenesis of this disease (22). IL-17A promotes the activation and recruitment of neutrophils such as CXCL1 and CXCL8 (23).

Kappel et al. (24), using IL-17A knockout (−/−) CD4+ T cells, demonstrated that IL-17−/− T cells exacerbated aGVHD due to augmented release of IFN-γ. Recent studies in our laboratory demonstrated that in vitro-differentiated Th17 cells generated substantial cutaneous and pulmonary pathology in murine models of aGVHD (26), but multiple pathways may have been involved, with IL-17A and TNF being dominant. To better understand the effects of Th17 cells that are differentiated or activated in vivo, we elected not to focus on a particular cytokine effector pathway such as IL-17A itself, which would limit conclusions that can be drawn regarding Th17 cells. Instead, we performed studies using RORC−/− donor T cells that are incapable of producing the array of cytokines generated by Th17 cells, including IL-17A, IL-17F, IL-21, IL-22, and TNF. In the absence of
RORC conventional T cells attenuated GVHD in a haploidentical, minor, and complete mismatched model. The absence of RORC expression by CD4\(^+\) T cells alone was sufficient to attenuate GVHD in the haploidentical model, but it had little impact on GVHD in a complete mismatched model. Interestingly, we found increased generation of IL-17 from lesional tissue in BALB/c recipient mice even when transplanted with donor T cells lacking RORC. These data indicate that T cell generation of ROR\(\gamma\)t is important to the pathogenesis of aGVHD.

Materials and Methods

Mice

C57BL/6J (H2\(^b\)) (termed B6), BALB/cJ (H2\(^k\)), C.B10-H2b/LiMcddJ (termed BALB.b), B6.129S6-Tbx21tm1Glm1/L (termed T-bet\(^-/-\)), B6 × DBA/2 F\(_1\) (B6D2 F\(_1\); H2\(^med\)), and B10.BR-H2\(^d\) H2-T18\(^{Sj}\)SnJjep mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 ROR\(\gamma\)t/− mice were purchased as described (27). Donor and recipient mice were age-matched males between 8 and 16 wk. All experiments were performed in accordance with protocols approved by the University of North Carolina Institutional Animal and Care Use Committee.

Transplantation models

Total T cells or CD4\(^+\) T cells were isolated using Cederlane T cell recovery kit (North Carolina Institutional Animal and Care Use Committee). Recipient mice were infused with 1 \(\times\) 10\(^6\) P815 murine mastocytoma cells (ATCC TIB-64) on the day of the transplantation. Weight loss and survival were monitored biweekly. Necropsies were performed on mice to confirm death by tumor infiltration.

Differences in GVHD clinical and pathology scores were determined using a Mann–Whitney U test. A \(p\) value of \(\leq 0.05\) was considered statistically significant.

Results

Attenuated GVHD in the absence of ROR\(\gamma\)t

Previous work demonstrated that blocking IFN-\(\gamma\) exacerbated aGVHD, suggesting that another T cell lineage may be important in GVHD pathology (12). Because our previous work using in vitro-differentiated Th17 cells demonstrated their ability to induce lethal aGVHD, we used mice in which the RORC locus (RORC\(^{−/−}\)) was altered using homologous recombination to further clarify the contribution of the Th17 subset to GVHD induction under nonpolarizing conditions. These mice lack both ROR\(\gamma\) and ROR\(\gamma\)t isoforms generated from this locus. CD25\(^−\) naive whole T cells (comprised of CD4\(^+\) T cells and CD8\(^+\) T cells (conventional T cells, or Tconv)) from wild-type (WT) C57BL/6 or RORC\(^{−/−}\) donors were transferred into lethally irradiated B6D2 F\(_1\) recipients. In addition to T cells, mice were injected with TCD BM cells from WT donors. Recipient mice given RORC\(^{−/−}\) Tconv had a substantial improvement in survival, with all B6D2 F\(_1\) recipient mice surviving until day 60 after transplantation (Fig. 1A). Using a semiquantitative scoring system we evaluated the clinical manifestations of aGVHD (33) in B6D2 F\(_1\) recipient mice. A significant difference in the aGVHD score starting on day 10 and continuing through the completion of the experiment was found in irradiated B6D2 F\(_1\) recipient mice transplanted with RORC\(^{−/−}\) Tconv compared with WT Tconv (Fig. 1B).

To determine whether the reduced aGVHD lethality observed with the infusion of RORC\(^{−/−}\) Tconv versus WT Tconv was model-dependent, we evaluated two additional transplantation models. Lethally irradiated BALB/c mice given CD25-depleted donor Tconv from either WT or RORC\(^{−/−}\) donors with WT TCD BM had improved median survival (Fig. 1C) with a diminished GVHD score (Fig. 1D) when receiving RORC\(^{−/−}\) compared with WT Tconv. Similarly, the median survival was improved when BALB. B mice were administered RORC\(^{−/−}\) Tconv compared with WT Tconv (Supplemental Fig. 1). However, in BALB.B recipients, there was only a transient improvement in GVHD score from days 10 to 17 after transplant. Thus, in three different GVHD models using CD25-depleted Tconv, the absence of RORC in donor T cells improved survival.

Decrease tissue pathology in GVHD target organs using RORC\(^{−/−}\) donor T cells

Clinically, multiple organs can be affected in aGVHD, including the skin, liver, GI tract, and the lung. To determine whether RORC\(^{−/−}\) Tconv affected aGVHD at a specific site, we evaluated the tissue pathophysiology in the liver, GI tract, lung, and spleen of RORC\(^{−/−}\) Tconv recipients compared with WT Tconv recipients. Fifteen days after transplantation the organs of recipient animals were harvested and pathology analyses conducted. Recipients of RORC\(^{−/−}\) Tconv displayed significantly less pathology in the liver, GI tract, lung, and spleen of RORC\(^{−/−}\) Tconv recipients compared with WT Tconv recipients (\(p < 0.05\), Fig. 2). Decreased pathology in recipient mice transplanted with RORC\(^{−/−}\) donor Tconv was specific to GVHD target organs, as minimal GVHD pathology was detected in the kidney of WT and RORC\(^{−/−}\) Tconv recipients. The aggressive nature of GI tract GVHD precluded the development of significant cutaneous GVHD in this model, and therefore cutaneous tissue was not evaluated. These data demonstrate that the function of RORC in the pathophysiology of aGVHD is not limited to a specific organ site.
BALB/c recipients given RORC one day after irradiation, 5 × 10^6 WT or RORC^-/- CD25^- Tconv supplemented with 3 × 10^6 TCD BM were injected i.v. into recipient mice. Recipient mice were monitored and scored weekly. Control mice received TCD bone marrow cells alone. (C and D) BALB/c recipients were lethally irradiated (800 cGy) on day -1. One day after irradiation, 5 × 10^6 WT or RORC^-/- CD25^- T cells supplemented with 5 × 10^6 WT TCD BM cells were injected i.v. into irradiated recipients. Survival was determined using the method of Kaplan–Meier. Statistics determined using a log-rank test for survival and Mann–Whitney for scores. *p < 0.05, **p < 0.001. For (A) and (B), n = 13 B6D2 F1 recipients transplanted with RORC^-/- or WT Tconv; n = 4 bone marrow controls. For (C) and (D), n = 11 BALB/c recipients given RORC^-/- Tconv and 8 BALB/c recipients given WT Tconv; n = 3 BM controls. Data are combined from two individual experiments.

In vivo cytokine production using RORC^-/- Tconv

Th17 cells generate a number of cytokines that may be important to the pathogenesis of aGVHD such as TNF, IL-17F, IL-21, and/or IL-22. Cytokine analyses were performed on serum and organ samples from RORC^-/- Tconv versus WT Tconv in B6D2 F1 recipients on day 14 after transplantation. Interestingly, the administration of donor T cells unable to express RORC was associated with a modest increase in the production of IFN-γ in the serum of recipient mice compared with those receiving WT Tconv (Fig. 3A). A substantial decrease in IL-17 and TNF was seen in the serum of recipient RORC^-/- Tconv compared with WT Tconv recipients (Fig. 3A). The decrease in TNF production in the serum was associated with statistically significant decreased production of TNF in the colon, but no differences were seen in cytokine production in other organs (Fig. 3B).

To determine whether the lack of differences in proinflammatory cytokines outside of the difference in the generation of TNF in the colon was due to the time point we evaluated, we analyzed mRNA expression of IFN-γ and IL-17A from lesional tissue on days 10 and 18 after transplantation. No difference was found in the expression of these cytokines in the colon, liver, or spleen of recipients of WT compared with RORC^-/- T cells plus TCD B6 bone marrow (data not shown). Thus, the absence of RORC in donor T cells led to a marked decrease in the generation systemically of the proinflammatory cytokines TNF and IL-17A, and of TNF specifically in the colon.

RORC^-/- CD4^+ T cells mediate GVHD in a haploidentical transplantation

Previous investigators have found that the infusion of donor T cells lacking RORC did not affect the incidence or severity of aGVHD when administered to lethally irradiated BALB/c recipients (34). However, the T cell inoculum for these experiments was comprised exclusively of CD4^+ T cells. The difference found by our group in the outcome of BALB/c recipients receiving RORC^-/- T cells occurred when infusing CD4^+ and CD8^+ T cells. To determine whether the different T cell inocula mediate the changes in outcome initially, we confirmed the data from Icozlan et al. (34). BALB/c mice receiving RORC^-/- CD4^+ T cells did not have improved survival or GVHD scores compared with recipients given WT CD4^+ T cells (Fig. 4A). Next, we determined whether the absence of RORC by donor CD4^+ T cells would impact the outcome in the haploidentical B6 into B6D2 model. All B6D2 recipients of RORC^-/- CD4^+ T cells survived until completion of the experiment, with minimal evidence of clinical GVHD, whereas recipients of WT CD4^+ T cells succumbed to disease by day 35 after transplantation (Fig. 4B). This indicated that the difference in the outcome of recipient mice given donor RORC^-/- CD4^+ T cells was model dependent. These data demonstrate a requirement for RORC CD4^+ T cell expression for GVHD pathogenesis in the haploidentical transplant setting.

Cytokine production in RORC^-/- CD4^+ T cell recipients

Differences in outcome using RORC^-/- CD4^+ T cells in the haploidentical versus the complete mismatch model are likely due to increased genetic disparity and potentially increased GVHD owing to the ability of a smaller number of donor T cells to mediate GVHD, or to GVHD mediated through different proin-
flamatory pathways. To elucidate the differences in outcome using RORC−/− CD4+ T cells in the B6 into BALB/c transplant model compared with the B6 into B6D2 transplant model, we evaluated cytokine production in the serum and organs from recipient animals. Lethally irradiated B6D2 recipients were transplanted with $3 \times 10^6$ RORC−/− or WT CD4+ T cells with $3 \times 10^6$ WT TCD BM cells whereas lethally irradiated BALB/c recipients were infused with $5 \times 10^5$ RORC−/− or WT CD4+ T cells supplemented with $5 \times 10^6$ WT TCD BM cells. Serum and tissue homogenates from the liver, GI tract, lung, and spleen were collected from recipients 14 d after transplantation. We found that B6D2 recipients of RORC−/− CD4+ T cells had increased TNF production in the serum with decreased IFN-γ production compared with B6D2 recipients of WT CD4+ T cell (Fig. 4C), but neither of these values reached statistical significance. B6D2 recipients of RORC−/− CD4+ T cells had a significant decrease in the production of TNF and IFN-γ in the colon compared with B6D2 recipients of WT CD4+ T cells (Fig. 4D). This was not found in BALB/c recipients given either RORC−/− or WT donor CD4+ T cells. Interestingly, IL-17 production in the liver of BALB/c recipients was 8-fold higher than IL-17 production in B6D2 recipients (Fig. 4D) and was not altered by the infusion of donor T cells lacking RORC. To determine whether differences in the production of IL-17A was specific to BALB/c recipients, we analyzed a second MHC mismatched model. Lethally irradiated B10.BR mice were injected with $3 \times 10^6$ WT or RORC−/− CD4+ T cells with $3 \times 10^6$ TCD BM. TNF and IFN-γ generation in the liver and colon of B10.BR recipients did not differ in the absence of RORC−/−. Interestingly, similar to BALB/c recipients, increased expression of IL-17 was seen in recipient B10.BR mice given either RORC−/− or WT CD4+ T cells (Fig. 4D). These data suggest that the generation of IL-17A in the completely mismatched MHC transplant models is more dependent on production by cells other than donor T cells. Moreover, we found that the absence of RORC in donor T cells mediated protection against GVHD only in models in which there was a decrease in the production of TNF systemically and in the colon after the infusion of RORC−/− T cells.

**RORC and TNF production**

Our data indicate a role for RORC in the function of CD4+ T cells in the haploidentical transplant model. To determine whether there was a function for RORC in donor CD8+ T cells, we transplanted mice with either RORC−/− or WT CD4+ or CD8+ T cells. Three cohorts of lethally irradiated B6D2 F1 recipients were used for these experiments. One group received $2 \times 10^6$ RORC−/− CD4+ T cells with $2 \times 10^6$ WT CD8+ T cells supplemented with $3 \times 10^6$ TCD BM cells. A second group received $2 \times 10^6$ WT CD4+ T cells with RORC−/− CD8+ T cells supplemented with $3 \times 10^6$ WT TCD BM cells. A final group received only $3 \times 10^6$ TCD BM cells. Interestingly, >80% of mice that received RORC−/− CD4+ T cells with WT CD8+ T cells survived until day 50 after transplantation, whereas those receiving WT CD4+ T cells with RORC−/− CD8+ T cells died of GVHD by day 30 after transplantation (Fig. 5A). Intracellular cytokine analyses of TNF and IFN-γ production were conducted on T cells isolated from liver of WT CD4+ T/RORC−/− CD8+ T cell and RORC−/− CD4+/WT CD8+ T cell recipients 10 d after transplantation. Overall production of both TNF and IFN-γ were equivalent between the two groups. However, in both cohorts independent of whether RORC−/− CD4+ T cells or RORC−/− CD8+ T cells were injected, WT T cells were the primary producers of TNF (Fig. 5B). These data suggest that the production of TNF by CD4+ and not CD8+ T cells is critical to the pathogenesis of GVHD in this model.

**Tissue-specific role for T-bet in aGVHD**

To determine whether the inability to produce proinflammatory cytokines was sufficient to attenuate aGVHD, we investigated the transcription factor that controls the expression of the Th1 cytokine IFN-γ, Tbx21 (T-bet). Donor CD25+ Tconv from T-bet−/− or WT

![FIGURE 3. Increased serum IFN-γ and decreased TNF expression RORC−/− recipients. WT TCD BM and RORC−/− or WT Tconv were transplanted into lethally irradiated B6D2 mice. Fourteen days after transplantation (A) serum and (B) organs were collected from B6D2 F1 recipients and analyzed by ELISA for the expression of IL-17, TNF, and IFN-γ. Shown are the mean values with error bars representing SEM. Data are pooled from five individual experiments. *p < 0.05, **p < 0.01.](http://www.jimmunol.org/DownloadedFrom)
mice supplemented with WT TCD BM were transplanted into lethally irradiated B6D2 F1 recipients. Interestingly, in this model, no difference was found in survival or GVHD score in mice receiving WT compared with T-bet−/− Tconv (Fig. 6A). However, analysis 15 d after transplantation revealed statistically significant decreased pathology in the ileum of recipients of T-bet−/− compared with WT Tconv (p, 0.05, Fig. 6B). A trend for decreased pathology was also seen in the colon (p = 0.09, Fig. 6B). However, we did not find a difference in tissue pathology in other GVHD target organs given WT compared with T-bet−/− T cells (data not shown). These data support the established function for Th1 cells in the pathophysiology of GVHD in the GI tract, but indicate that in this haploidentical transplant model, T cell generation of T-bet was not critical for GVHD lethality (35).

Graft-versus-leukemia response in the absence of RORC

Next, we addressed whether the loss of RORC would impact the antitumor activity of SCT. Antitumor activity after transplantation was evaluated by adding 1 × 10⁶ P815 cells to the donor bone marrow inoculum on day 0. One group of B6D2 F1 mice received RORC−/− Tconv in addition to WT TCD BM cells infus ed with P815 tumor cells. Because recipients of WT Tconv often succumb to GVHD before antitumor properties can be analyzed, syngeneic controls were used as a control. Syngeneic controls were given B6D2 Tconv supplemented with WT TCD BM infused with P815 tumor cells. Control mice received only WT TCD BM infused with P815 tumor cells. All mice receiving only WT TCD BM with P815 tumor cells died by day 20 due to tumor growth. Recipient mice receiving B6D2 Tconv died by day 20 due to tumor infiltration (Fig. 7). Interestingly, survival was extended to day 40 in recipient mice given RORC−/− T conv and P815 cells, indicating that the graft-versus-leukemia (GvL) response remained somewhat intact in mice given T cells lacking RORC. To demonstrate that this difference was not mediated by donor bone marrow cells, we administered RORC−/− TCD BM or WT TCD BM cells plus P815 cells to lethally irradiated B6D2 F1 recipient mice. As expected, all recipient mice succumbed to tumor infiltration by day 30 (data not shown).

FIGURE 4. Function of RORC in donor CD4+ T cells is model dependent. (A) Lethally irradiated BALB/c recipients were injected with 5 × 10⁵ RORC−/− CD4+ or WT CD4+ T cells supplemented with 5 × 10⁶ WT TCD BM. Survival and GVHD scores are shown (n = 9 for RORC−/− recipients, n = 9 for WT recipients, n = 4 for bone marrow only recipients). (B) Lethally irradiated B6D2 F1 recipients were injected with 2 × 10⁶ RORC−/− CD4+ T cells or WT CD4+ T cells supplemented with 3 × 10⁶ WT TCD BM (n = 7 for RORC−/− CD4+ T cells, n = 7 for WT CD4+ T cells, n = 3 bone marrow only). Data indicate p < 0.05 for survival and p < 0.05 from day 17 until the completion of the experiment for the difference in GVHD score. Data are combined from two individual experiments. (C) Serum and (D) organs were harvested from lethally irradiated BALB/c, B6D2 F1, or B10.BR recipients transplanted with RORC−/− or WT CD4+ T cells 14 d after transplantation. WT B10.BR recipients were harvested 10 d after transplantation. TNF, IFN-γ, and IL-17 production were determined by ELISA. Data were pooled from five RORC−/− CD4+ T cell BALB/c recipients and four WT CD4+ T cell BALB/c recipients, six RORC−/− CD4+ T cell B6D2 recipients and four WT CD4+ T cell B6D2 recipients, and four RORC−/− CD4+ T cell B10.BR and three WT CD4+ T cell B10.BR recipients. Statistical analysis was determined by a Mann–Whitney U test. *p < 0.05.
Acute GVHD is mediated by donor T cells that recognize minor or major MHC disparities presented predominantly by host APCs. This process leads to activation, differentiation, and T cell effector responses that are critical for the pathophysiology of aGVHD. During the past decade multiple investigators have identified new T cell subsets characterized by the activity of canonical transcription factors and the generation of specific cytokines. The T cell subsets critical for the pathophysiology of aGVHD are currently unclear and are the focus of this manuscript. In this study, we find unexpectedly that the loss of the Th17 transcription factor, RORC, in donor CD25-depleted T cells led to markedly diminished aGVHD. In three different models, recipient mice given RORC−/− CD4+ T cells and WT CD8+ T cells, n = 5 for recipient mice receiving WT CD4+ T cells and RORC−/− CD8+ T cells, n = 4 for bone marrow alone). Data are combined from two individual experiments. *p < 0.05. (B) Ten days after transplantation the livers of RORC−/− CD4+/WT CD8+ T cell or WT CD4+/RORC−/− CD8+ T cell recipient mice were harvested and T cells isolated. Data are representative from three WT CD4+/ RORC−/− CD8+ recipients and four RORC−/− CD4+/ WT CD8+ recipients.

**Discussion**

Acute GVHD is mediated by donor T cells that recognize minor or major MHC disparities presented predominantly by host APCs. This process leads to activation, differentiation, and T cell effector responses that are critical for the pathophysiology of aGVHD. During the past decade multiple investigators have identified new T cell subsets characterized by the activity of canonical transcription factors and the generation of specific cytokines. The T cell subsets critical for the pathophysiology of aGVHD are currently unclear and are the focus of this manuscript. In this study, we find unexpectedly that the loss of the Th17 transcription factor, RORC, in donor CD25-depleted T cells led to markedly diminished aGVHD. In three different models, recipient mice given RORC−/− Tconv had significantly less GVHD and increased survival compared with recipients given WT Tconv. The absence of RORC was associated with diminished GVHD in all target organs evaluated and correlated with diminished systemic generation of proinflammatory cytokines. The difference in pathology of GVHD target organs was not associated with a difference in frequency of regulatory T cells in these organs after transplant (L.M. Fulton and J.S. Serody, unpublished observations). As was previously found, the absence of RORC on CD4+ T cells had no effect on GVHD outcome in a completely mismatched B6 into BALB/c model. Interestingly, in the B6 into B6D2 model, the absence of T-bet in donor T cells led to diminished pathology in the GI tract but no overall survival benefit. When challenged with P815 tumor cells, recipient mice receiving donor T cells lacking RORC survived longer than did mice receiving bone marrow alone, indicating the presence of an antitumor GvL response. However, in both instances recipient mice succumbed eventually to tumor growth, indicating that the GvL response is modestly compromised using T cells unable to generate RORC, perhaps because of the diminished generation of TNF.

Previous work has clearly indicated a critical role for Th1/Tc1 T cells in the pathophysiology of aGVHD particularly involving the GI tract. Thus, it was somewhat unexpected that the absence of T-bet alone, although diminishing GVHD in the small bowel and to a lesser extent in the colon, was not associated with an improved overall survival. T-bet has been found to be critical for the generation of IFN-γ by CD4+ T cells and NK cells. However, the generation of IFN-γ by CD8+ T cells is not impaired in the absence of T-bet, which may be responsible for the similar survival (36). As we have found that RORC is required in the CD4+ T cell compartment, our data would be consistent with a role for IFN-γ generation by CD8+ T cells and TNF production by CD4+ T cells in the pathogenesis of aGVHD.

Quite recently, Yu et al. (37) evaluated the ability of T cells from mice deficient in RORC or Tbx21 to induce GVHD. They found diminished GVHD using T cells from B6 Tbx21−/− donors, but no...
difference in GVHD using CD4⁺ T cells from RORC⁻/⁻ donors when given to lethally irradiated BALB/c recipients. Interestingly, they did find a modest survival benefit when infusing CD25-depleted T cells lacking RORC, suggesting that the regulatory T cell compartment may not function in RORC mice as it does in WT mice. They found that BALB/c recipient mice given T cells from mice deficient in both RORC and Tbx21 had markedly diminished GVHD. This was associated with diminished generation of Th1 and Th17 cells and impaired expression of chemokine receptors important for the trafficking of donor T cells to GVHD target organs. Our data confirm and extend these findings as they relate to the function of RORC by evaluating the mechanism for the decreased GVHD when CD25-depleted donor T cells lacking RORC are given to lethally irradiated recipients. Additionally, we confirmed their previous data regarding the absence of an effect by infusing CD4⁺ T cells lacking RORC in the B6 into BALB/c model. We found substantially increased IL-17 in the colon and liver of BALB/c compared with B6D2 recipient mice after transfer of B6 T cells and TCD BM. Interestingly, the production of IL-17 was not affected by the infusion of T cells lacking RORC, suggesting that other donor or perhaps host cells generate substantial quantities of IL-17 in BALB/c recipients. At present, we are evaluating which recipient cells generate IL-17 in BALB/c mice. Nonetheless, these data indicate that the model used may be critically important in interpreting the function of IL-17 after bone marrow transplantation.

We found a substantial difference in the generation of TNF and IL-17A in the serum and TNF in the colon of recipient mice given RORC⁻/⁻ compared with WT T cells. Our previous data have indicated that TNF is critical for the systemic manifestations of GVHD mediated by Th17 cells. Interestingly, in this study we found that TNF production by CD4⁺ and/or CD8⁺ T cells was markedly reduced when that subset did not express RORC. However, this was compensated for by production of TNF from the WT T cells when both were given. However, GVHD was decreased only when TNF production was diminished by CD4⁺ T cells and not from CD8⁺ T cells, indicating cell-intrinsic differences in the function of TNF after SCT. We found an increase in the generation of dual-positive IL-17A/IFN-γ T cells when WT Tconv were infused compared with RORC⁻/⁻ Tconv 12 d after transplantation (Supplemental Fig. 2). The generation of these cells, which may eventually become Th1 cells (M.J. Carlson and J.S. Serody, unpublished observations), may be one mechanism for the decreased incidence and severity of aGVHD after the infusion of T cells unable to generate RORC.

For allogeneic transplantation to be successful requires the elimination of GVHD without compromising the antitumor GvL activity of donor T cells. In this study, we found that donor T cells lacking RORC still mediated an antitumor response against the mastocytoma cell line, P815. Killing of P815 cells is dependent on the absence of RORC (38). This suggests that the decreased generation of TNF in the absence of RORC is not

**FIGURE 6.** T-bet⁻/⁻ Tconv decrease pathology in the GI tract but do not attenuate GVHD. **A)** B6D2 F₁ recipient mice were lethally irradiated (950 cGy) on day −1. Following irradiation on day 0 mice were injected i.v. with 4 × 10⁶ WT or T-bet⁻/⁻ Tconv supplemented with 3 × 10⁶ WT TCD BM. Mice were monitored for survival and scored twice weekly for clinical GVHD (n = 14 for T-bet⁻/⁻ recipients, n = 11 for WT recipients, n = 4 bone marrow only). All recipient mice receiving BM only cells survived until the completion of the experiment. **B)** On day 15 after transplantation organs were harvested from WT and T-bet⁻/⁻ recipients and evaluated for pathology as described above. Error bars indicate SEM. Statistical significance was determined using a Mann–Whitney U test. *p < 0.05, **p = 0.09. Data are combined from two individual experiments.

**FIGURE 7.** Improved antitumor responses in the absence of RORC. Lethally irradiated B6D2 F₁ mice were injected with 3 × 10⁶ TCD BM with or without 4 × 10⁶ WT B6D2 or RORC⁻/⁻ Tconv. Additionally, all recipient mice received 1 × 10⁴ P815 cells with the BM inoculum. Survival was determined by the Kaplan–Meier method. An improvement in overall survival was found in B6D2 F₁ mice given RORC⁻/⁻ Tconv compared with B6D2 T cells or BM plus P815 cells (p < 0.05) (n = 7 recipients receiving RORC null T cells, n = 5 recipients receiving B6D2 T cells, n = 4 recipients receiving bone marrow). Data are combined from two individual experiments.
sufficient to completely lose the antitumor activity of donor T cells.

In summary, we have shown that donor T cells lacking RORC do not mediate substantial aGVHD in three different transplant models. This finding is dependent on the absence of RORC in CD4+ T cells, correlated with reduced generation of TNF and IL-17A systemically and TNF in the colon, and was important for the diminished GVHD that occurred in clinically relevant transplant models.

Disclosures

The authors have no financial conflicts of interest.

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