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Attenuation of Acute GvHD 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**[*,†,‡]

[*]Lineberger Comprehensive Cancer Centre, University of North Carolina School of Medicine, Chapel Hill, NC

[†]Department of Microbiology and Immunology, University of North Carolina School of Medicine, Chapel Hill, NC

[‡]Department of Medicine, University of North Carolina School of Medicine, Chapel Hill, NC

[§]University of Minnesota Masonic Cancer Center, Minneapolis, MN

[¶]Skirball Institute, New York University School of Medicine, New York NY

Abstract

Graft-versus-host disease (GvHD) remains the most significant complication after allogeneic stem cell transplantation (allo-SCT). Previously, acute GvHD had been considered to be mediated predominantly by Th1 polarized T cells. Recently, investigators have identified a second pro-inflammatory lineage of T cells termed Th17 that is critically dependent on the transcription factor RORγt. Here, 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 aGvHD. This was not found when $CD4^+$ T cells from $RORC^{-/-}$ mice were given to completely mismatched BALB/c mice, and correlated with absolute differences in the generation of TNF in the colon post transplant. Thus, $CD4^+$ T cell expression of RORC is important in the pathogenesis of acute GVHD.

Introduction

Allogeneic 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 antigen presenting cells (APC) 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 tract (8).

Corresponding Author: Jonathan S Serody MD, 450 West Drive, Campus Box #7295, Lineberger Comprehensive Cancer Center, Chapel Hill, North Carolina 27599-7295, Jonathan_Serody@med.unc.edu, Phone: 919 966-6975, Fax: 919 966-8212.

Previous GvHD research has focused on cytokine production in T cell subsets. High levels of interferon γ (IFN-γ) and interleukin-2 (IL-2) found in patients after allo-SCT led investigators to conclude that GvHD was mediated predominantly by proinflammatory Th1 cells (9, 10). However and conversely, inhibition of Th1 cytokines leads to disease exacerbation in GvHD (11, 12). As 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 towards a new subset of $CD4^+$ T cells, Th17 cells. Th17 cell differentiation and expansion requires TGF-β1, IL-6, IL-23, TNF, and IL-1β (14-16). The development of Th17 cells is dependent on the transcription factors retinoid-related orphan receptor (ROR)γt, RORα, IRF-4 and STAT3 (17, 18). Th17 cells produce proinflammatory cytokines such as TNF, IL-21, and IL-22 (19-21) in addition to IL-17A and IL-17F. IL-21 has been found by our group to be critical for blocking the generation of inducible T_{reg} cells (19) while IL-22 has been found to be important for the induction of psoriasis in experimental models (22). IL-17A and IL-17F bind to the IL-17 receptor found on leukocytes, epithelial cells, mesothelial cells, endothelial cells, keratinocytes, and fibroblasts. Binding of IL-17A and IL-17F to the IL-17 receptor enhances production of g-CSF, IL-6, and chemokines that recruit neutrophils such as CXCL1 and CXCL8 (23).

Keppel *et al* using IL-17A knockout (−/−) $CD4^+$ T cells demonstrated that IL-17 contributes to aGVHD (24). In contrast, Yi *et al* has shown that IL-$17A^{-/-}$ T cells exacerbated aGVHD due to augmented release of IFN-γ (25). 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 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 RORγt is important to the pathogenesis of acute GvHD.

Methods

Mice

C57BL/6J ($H2^b$) (termed B6), BALB/cJ ($H2^d$), C.B10-H2b/LiMcdJ (termed BALB.b), B6.129S6-*Tbx21*^{tm1Glm}/J (termed T-bet$^{-/-}$), B6 x DBA/2 F1 (B6D2 F1: $H2^{bxd}$), and B10.BR-$H2^k$ H2-$T18^a$/SjSnJJrep mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6 *RORC*$^{-/-}$ mice were generated as described (27). Donor and recipient mice were age-matched males between 8 and 16 weeks. 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 Cedarlane T recovery column kit or $CD4^+$ T cell recovery kit (Cedarlane, Burlington, NC) respectively, followed by antibody depletion using phycoerythrin (PE) conjugated anti-mouse B220 and anti-mouse CD25 antibodies

(Ebioscience, San Diego, CA) and magnetic bead selection using anti-PE beads (Miltenyi Biotec, Cambridge, MA). Isolated $CD4^+$ T cell were further purified using anti-mouse CD8 PE antibody. T cell depleted bone marrow bone marrow (TCD BM) and conventional T cells were prepared using previously described methods (28). Histopathology specimens were generated as described (29) and analyzed by one of us (APM) blinded to the genotype of donor used. Scoring of tissues was performed per our previous method (30).

Serum and Organ Cytokine Analysis

Transplant recipient animals were anesthetized and perfused with phosphate-buffered saline. Whole organs were removed and homogenized. Cytokine levels were measured using enzyme-linked immunosorbent assay (ELISA) kits against IFN-γ, IL-17A, and TNF (Biolegend, San Diego CA).

Intracellular Cytokine Staining

Single cells suspensions of livers were digested using collagenase A and DNAse I. Liver cells were stimulated with phorbol myristate acetate (PMA), ionomycin and brefeldin A for 4 hours. Cells were harvested and stained for anti-mouse TNF (Ebioscience, San Diego, CA). Flow cytometry analyses were conducted using FlowJo analysis software (Ashland, OR).

Real Time PCR Analysis

RNA was extracted from organs using TRIzol reagent (Invitrogen, Carlsbad CA) according to the manufacturer's recommendations. First strand cDNA synthesis was performed with 1μg RNA as previously described (26). Equal amounts of cDNA were analyzed by real time quantitative PCR, in triplicate, using TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA) and the ABI 7300 Real Time PCR System with primer specific standard curves. The expression level of each gene was normalized to the housekeeping gene, GusB, using the standard curve method before fold activation was determined. TaqMan gene expression assay probes for interferon gamma, tumor necrosis factor, and interleukin 17A/F were purchased from Applied Biosystems.

GvHD Scoring

Mice were observed twice weekly for clinical GvHD signs and symptoms based on a previously established clinical scoring system (31).

GvL Analysis

Recipient mice were infused with 1×10^4 P815 murine mastocytoma cells (ATCC: TIB-64) on the transplantation. Weight loss and survival were monitored bi-weekly. Necropsies were performed on mice to confirm death by tumor infiltration.

Statistical Analysis

Survival differences were evaluated using Mantel-Cox log rank test. Survival curves were generated using the method of Kaplan and Meier (32). Differences in GvHD clinical and pathology scores were determined using Mann-Whitney test. P values ≤ 0.05 were considered statistically significant.

Results

Attenuated GvHD in the Absence of *RORC*

Previous work demonstrated that blocking IFN-γ exacerbated aGvHD suggesting that another T cell lineage may be important in GvHD pathology (12). As 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 non-polarizing conditions. These mice lack both RORγ and RORγt isoforms generated from this locus. CD25-negative ($CD25^{-}$) naïve whole T cells (comprised of $CD4^{+}$ T cells and $CD8^{+}$ T cells; termed Tconv) from WT C57BL/6 (WT) or $RORC^{-/-}$ donors were transferred into lethally irradiated B6D2 F1 recipients. In addition to T cells, mice were injected with T cell depleted bone marrow (TCD BM) cells from WT donors. Recipient mice given $RORC^{-/-}$ Tconv had a substantial improvement in survival with all B6D2 F1 recipient mice surviving until day 60 post transplantation (Fig 1a). Using a semi-quantitative scoring system we evaluated the clinical manifestations of aGvHD (33) in B6D2 F1 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 F1 recipient mice transplanted with $RORC^{-/-}$ Tconv compared to WT Tconv (Fig 1b).

To determine whether the reduced aGVHD lethality observed with the infusion of $RORC^{-/-}$ Tconv vs 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 to WT Tconv. Similarly, the median survival was improved when BALB.B mice were administered $RORC^{-/-}$ Tconv compared to WT Tconv (Supp Fig 1). However, in BALB.B recipients, there was only a transient improvement in GvHD score from days 10-17 post 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 if $RORC^{-/-}$ 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 to WT Tconv recipients. Fifteen days post transplantation the organs of recipient animals were harvested and pathology analyses conducted. Recipients of $RORC^{-/-}$ Tconv displayed significantly less pathology in the liver, colon, lung, and spleen compared to 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.

In Vivo Cytokine Production Using $RORC^{-/-}$ Tconv Cells

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 vs WT Tconv in B6D2 F1 recipients on day 14 post 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 to those receiving WT Tconv (Fig 3a). A substantial decrease in

IL-17 and TNF were seen in the serum of recipients *RORC*$^{-/-}$ Tconv compared to 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 however no differences were seen in cytokine production in other organs (Fig 3b).

To determine if the lack of differences in pro-inflammatory 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 post transplantation. No difference was found in the expression of these cytokines in the colon, liver or spleen of recipients of WT compared to RORC$^{-/-}$ T cells plus TCD B6 bone marrow. Thus, the absence of *RORC* in donor T cells led to a marked decrease in the generation systemically of the pro-inflammatory 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 if the different T cell inoculums mediate the changes in outcome initially, we confirmed the data from Icozlan et al. BALB/c mice receiving *RORC*$^{-/-}$ CD4^{+} T cells did not have improved survival or GvHD scores compared to recipients given WT CD4^{+} T cells (Fig 4a). Next, we determined if 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, while recipients of WT CD4^{+} T cells succumbed to disease by day 35 post 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 due to the ability of a smaller number of donor T cells to mediate GvHD, or GvHD mediated through different pro-inflammatory pathways. To elucidate the differences in outcome using *RORC*$^{-/-}$ CD4^{+} T cells in the B6 into BALB/c transplant model compared to 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 3×10^6 *RORC*$^{-/-}$ or WT CD4^{+} T cells with 3×10^6 WT TCD BM cells while lethally irradiated BALB/c recipients were infused with 5×10^5 *RORC*$^{-/-}$ or WT CD4^{+} T cells supplemented with 5×10^6 WT TCD BM cells. Serum and tissue homogenates from the liver, GI tract, lung and spleen were collected from recipients 14 days post transplantation. We found that B6D2 recipients of *RORC*$^{-/-}$ CD4^{+} T cells had increased TNF production in the serum with decreased IFN-γ production compared to B6D2 recipients of WT CD4^{+} T cell (Fig 4c), however 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 to 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 times higher than IL-17 production in B6D2 recipients (Fig 4d) and not altered by the infusion of

donor T cells lacking *RORC*. To determine if 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 3×10^6 WT or *RORC*$^{-/-}$ CD4$^+$ T cells with 3×10^6 TCD BM. TNF and IFN 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 if 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 2×10^6 *RORC*$^{-/-}$ CD4$^+$ T cells with 2×10^6 WT CD8$^+$ T cells supplemented with 3×10^6 TCD BM cells. A second group received 2×10^6 WT CD4$^+$ T cells with *RORC*$^{-/-}$ CD8$^+$ T cells supplemented with 3×10^6 WT TCD BM cells. A final group received only 3×10^6 TCD BM cells. Interestingly, more than 80 percent of mice that received *RORC*$^{-/-}$ CD4$^+$ T cells with WT CD8$^+$ T cells survived until day 50 post transplantation while those receiving WT CD4$^+$ T cells with *RORC*$^{-/-}$ CD8$^+$ T cells died from GvHD by day 30 post 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 days post 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 if 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 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 to T-bet$^{-/-}$ Tconv (Fig 6a). However, analysis fifteen days post transplantation revealed statistically significant decreased pathology in the ileum of recipients of T-bet$^{-/-}$ compared to wild type Tconv cells ($p < 0.05$, Fig 6b). A trend for decreased pathology was also seen in the colon ($p = 0.08$, Fig 6b). However, we did not find a difference in tissue pathology in other GvHD target organs given WT compared to 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).

GvL Response in the Absence of *RORC*

Next, we addressed whether the loss of *RORC* would impact the anti-tumor activity of SCT. Anti-tumor activity after transplantation was evaluated by adding 1×10^4 P815 cells to the donor bone marrow inoculum on day 0. One group of B6D2 F1 mice received *RORC*$^{-/-}$ Tconv cells in addition to WT TCD BM cells infused with P815 tumor cells. Since recipients of WT Tconv often succumb to GvHD before anti-tumor properties can be

analyzed syngeneic T cells 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*$^{-/-}$ Tconvs and P815 cells indicating that the 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).

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 acute GvHD. Over 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 subset(s) critical for the pathophysiology of acute GvHD is currently unclear and the focus of this manuscript. Here, we find unexpectedly that the loss of the Th17 transcription factor, *RORC*, in donor CD25-depleted T cells led to markedly diminished acute GvHD. In three different models, recipient mice given *RORC*$^{-/-}$ Tconv cells had significantly less GvHD and increased survival compared to recipients given WT Tconv cells. The absence of *RORC* was associated with diminished GvHD in all target organs evaluated and correlated with diminished systemic generation of pro-inflammatory cytokines. The difference in pathology of GvHD target organs was not associated with a difference in frequency of regulatory T cells in these organs post transplant (Fulton and Serody unpublished). 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 mice receiving bone marrow alone, indicating the presence of an anti-tumor 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 due to the diminished generation of TNF.

Previous work has clearly indicated a critical role for Th1/Tc1 T cells in the pathophysiology of acute GvHD particularly involving the GI tract. Thus, it was somewhat unexpected that the absence of T-bet alone, while diminishing GvHD in the small bowel and to a lesser extent 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 acute GvHD.

Quite recently, Yu *et al* evaluated the ability of T cells from mice deficient in *RORC* or *Tbx21* to induce GvHD (37). 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 T_{reg} 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 to B6D2 recipient mice after transfer of B6 T cells and TCD BM. Interestingly, the production of IL-17 was not impacted 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. Currently, 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 to WT T cells. Our previous data has indicated that TNF is critical for the systemic manifestations of GvHD mediated by Th17 cells. Interestingly, here 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 post SCT. We found an increase in the generation of dual positive IL-17A/IFN-γ T cells when WT Tconv cells were infused compared to $RORC^{-/-}$ Tconv cells 12 days post transplantation (Supp Fig 2). The generation of these cells, which may eventually become Th1 cells (Carlson and Serody unpublished), 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 anti-tumor, GvL activity of donor T cells. Here we found that donor T cells lacking *RORC* still mediated an anti-tumor response against the mastocytoma cell line, P815. Killing of P815 cells is dependent on the generation of IFN-γ and TNF (38). This suggests that the decreased generation of TNF in the absence of *RORC* is not sufficient to completely lose the anti-tumor activity of donor T cells.

In summary, we have shown that donor T cells lacking *RORC* do not mediate substantial acute GvHD 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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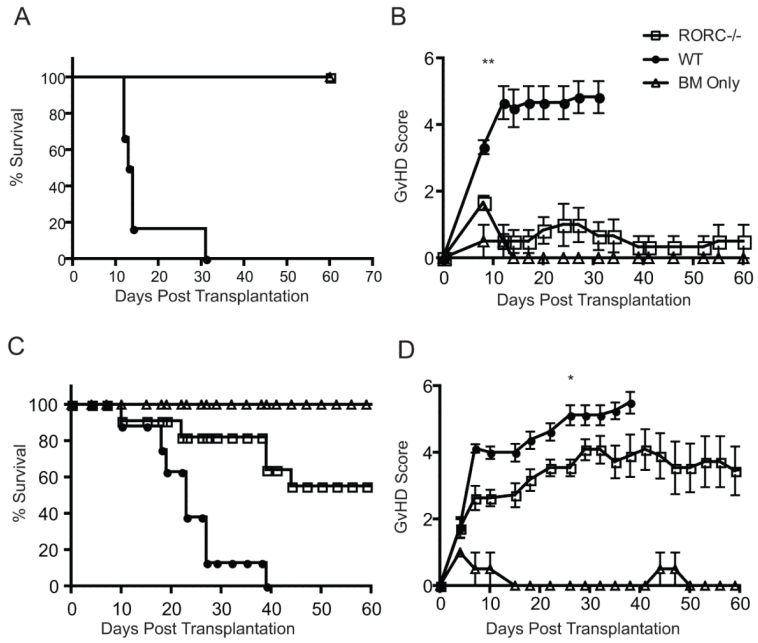

Figure 1. Survival and GvHD scores of B6D2 recipients

(A-B) B6D2 recipients were lethally irradiated (950 cGy) on day -1. One day following irradiation, 4×10^6 WT or *RORC*$^{-/-}$ CD25-Tconv cells supplemented with 3×10^6 TCD BM were injected intravenously into recipient mice. Recipient mice were monitored and scored weekly. Control mice received TCD bone marrow cells alone (C-D) BALB/c recipients were lethally irradiated (800 cGy) on day -1. One day after irradiation, 5×10^5 WT or *RORC*$^{-/-}$ CD25-T cells supplemented with 5×10^6 WT TCD BM cells were injected intravenously into irradiated recipients. Survival was determined using the method of Kaplan-Meier. Statistics determined using log-rank test for survival and Mann-Whitney for scores. *$p<0.05$, **$p<0.001$. A-B n = 13 B6D2 F1 recipients transplanted with *RORC*$^{-/-}$ or WT Tconvs; n = 4 bone marrow controls; C-D n = 11 Balb/c recipients given *RORC*$^{-/-}$ Tconvs and 8 Balb/c recipients given WT Tconvs; n = 3 BM controls. Data are combined from 2 individual experiments.

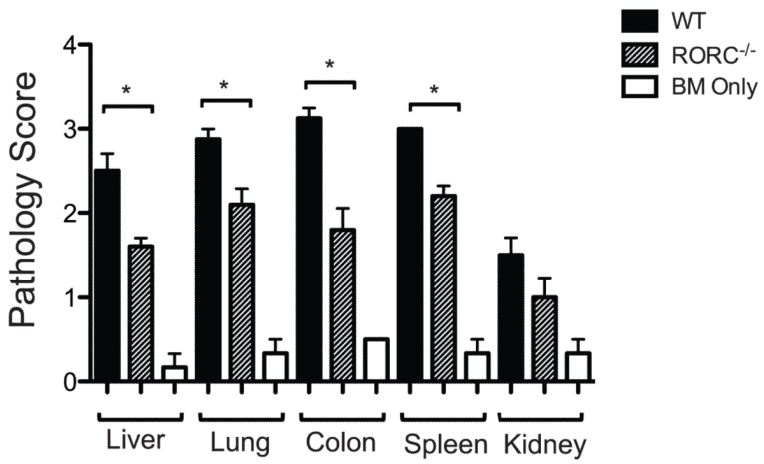

Figure 2. Decreased tissue pathology in recipient mice given *RORC*$^{-/-}$ donor T cells

4×10^6 ($CD25^-$) Tconv cells from *RORC*$^{-/-}$ or WT mice with WT TCD BM were transplanted into lethally irradiated (950 cGy) B6D2 F1 recipients. Organs were harvested on day 15 post-transplantation and processed as described. Tissues were evaluated by one of us (APM) blinded to the treatment group and scored using a semi-quantitative GvHD scoring system. Shown are the mean scores with error bars indicating SEM. Statistical significance was determined using Mann-Whitney test, *$p<0.05$. n = 5 mice analyzed given WT or *RORC*$^{-/-}$ T cells. n=4 for bone marrow controls. Data pooled from an individual transplant using *RORC*$^{-/-}$ or WT Tconv.

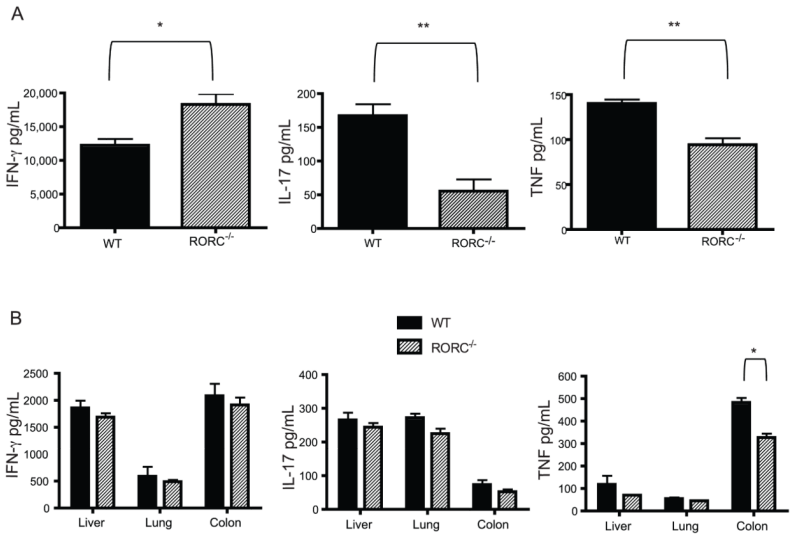

Figure 3. Increased serum IFN-γ and decreased TNF expression *RORC*$^{-/-}$ recipients

WT TCD BM and *RORC*$^{-/-}$ or WT Tconv were transplanted into lethally irradiated B6D2 mice. 14 days post 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 5 individual B6D2 receiving mice *RORC*$^{-/-}$ or WT Tconv. Statistical analyses were conducted using Mann-Whitney test. Data are combined from 2 individual experiments *p<0.05, **p<0.01

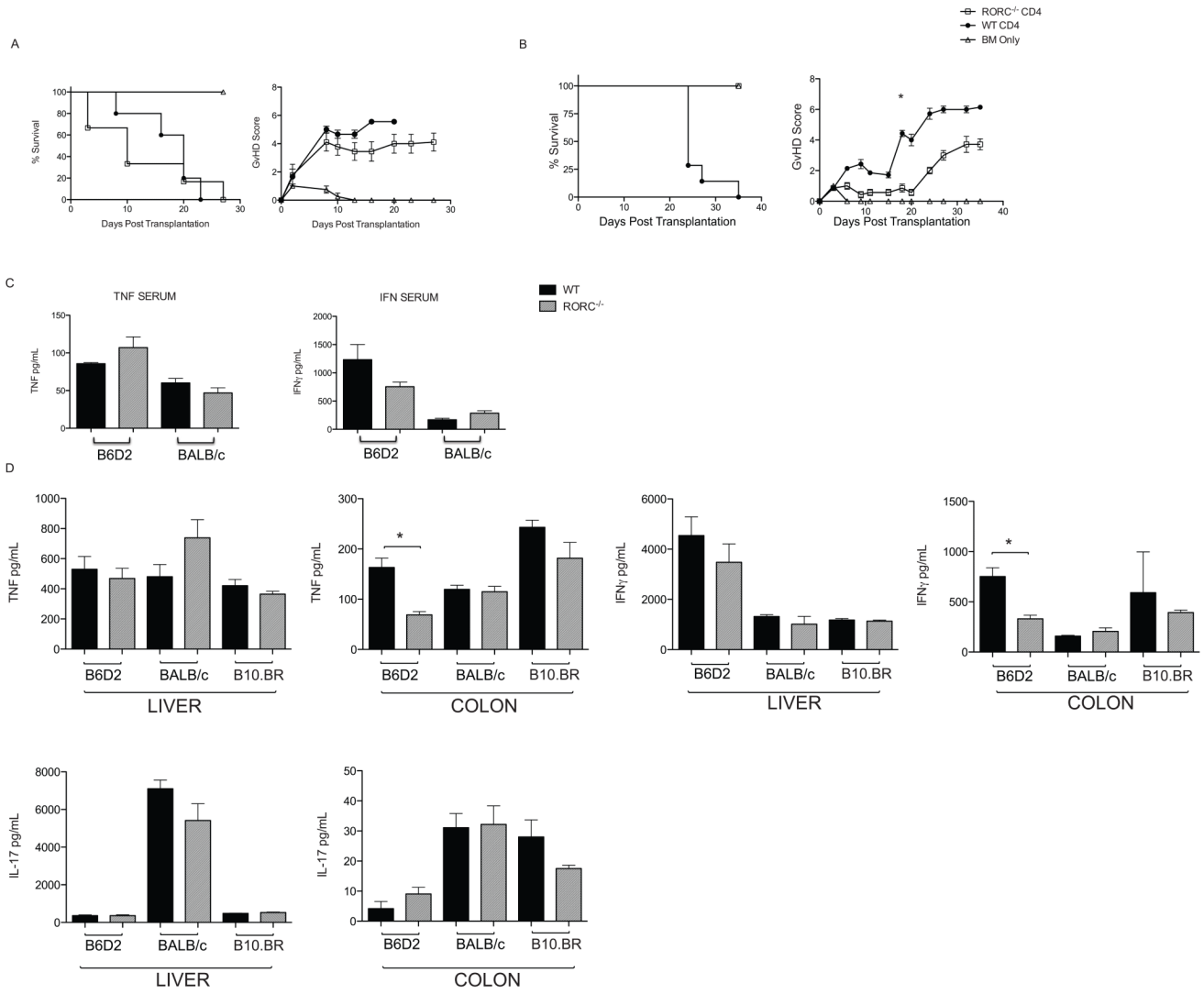

Figure 4. Function of *RORC* in Donor CD4$^+$ T cells is Model Dependent

(A) Lethally irradiated BALB/c recipients were injected with 5 × 10^5 *RORC*$^{-/-}$ CD4$^+$ or WT CD4$^+$ T cells supplemented with 5 × 10^6 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 × 10^6 *RORC*$^{-/-}$ CD4$^+$ T cells or WT CD4$^+$ T cells supplemented with 3 × 10^6 WT TCD BM. n= 7 for *RORC*$^{-/-}$ CD4$^+$ T cells, n=7 for WT CD4$^+$ T cells, n=3 bone marrow only. $p<0.05$ for survival. $p < 0.05$ from day 17 until the completion of the experiment for the difference in GvHD score. Data are combined from 2 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 days post transplantation. WT B10.BR recipients were harvested 10 days post transplantation. TNF, IFN-γ, and IL-17 production were determined by ELISA. Data pooled from 5 *RORC*$^{-/-}$ CD4$^+$ T cell BALB/c recipients and 4 WT CD4$^+$ T cell BALB/c recipients, 6 *RORC*$^{-/-}$ CD4$^+$ T cell B6D2 recipients and 4 WT CD4$^+$ T cell B6D2 recipients, 4 *RORC*$^{-/-}$ CD4$^+$ T cell B10.BR and 3 WT CD4$^+$ T cell B10.BR recipients. Statistical analysis determined by Mann-Whitney test. * $p<0.05$.

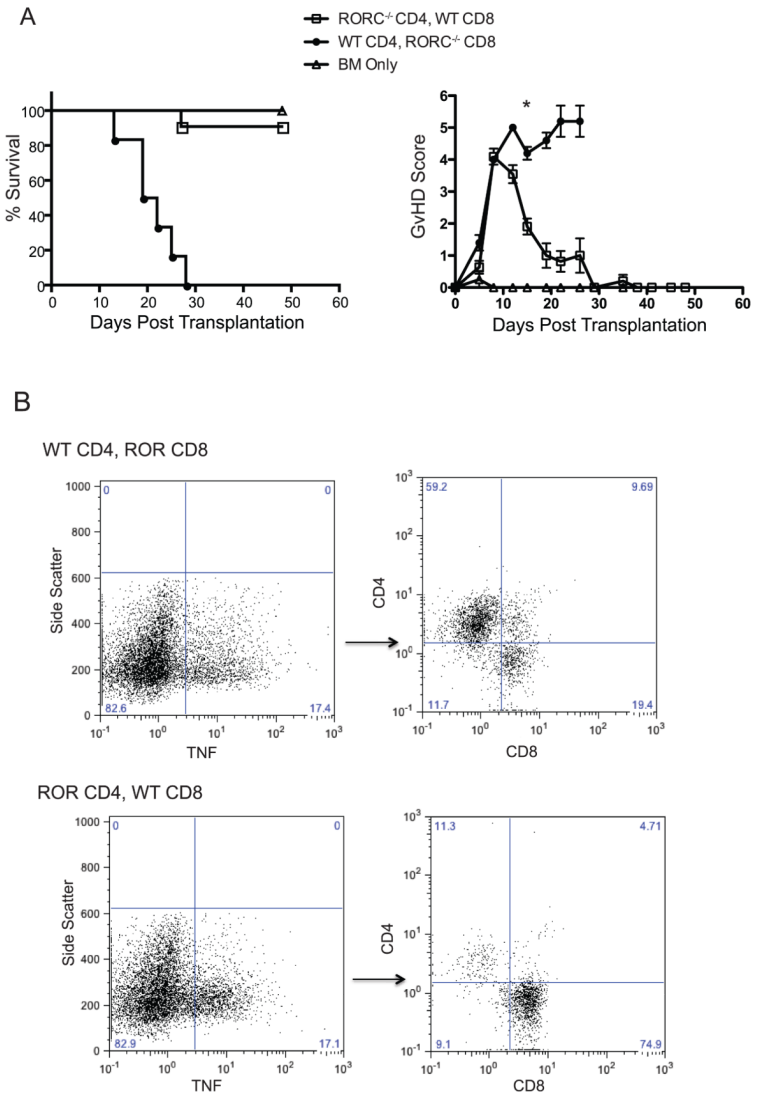

Figure 5. Attenuated GvHD using *RORC*$^{-/-}$ Tconv cells is mediated by CD4^{+} T cells

(A) Lethally irradiated B6D2 F1 mice were injected with 3 × 10^{6} TCD BM. In addition to BM one group received 2 × 10^{6} *RORC*$^{-/-}$ CD4^{+} T cells and 2 × 10^{6} WT CD8^{+} T cells, one group received 2 × 10^{6} WT CD4^{+} T cells and 2 × 10^{6} *RORC*$^{-/-}$ CD8^{+} T cells, and a final group received only BM cells. Recipients of *RORC*$^{-/-}$ CD4^{+} T cells with WT CD8^{+} Tcells showed less GvHD reaching statistical significance by day 15 post transplantation. n= 11 recipient mice receiving *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 only. Data are combined from 2 individual experiments *p<0.05. (B) 10 days post 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 3 WT CD4, *RORC*$^{-/-}$ CD8^{+} recipients and 4 *RORC*$^{-/-}$ CD4^{+}, WT CD8^{+} recipients.

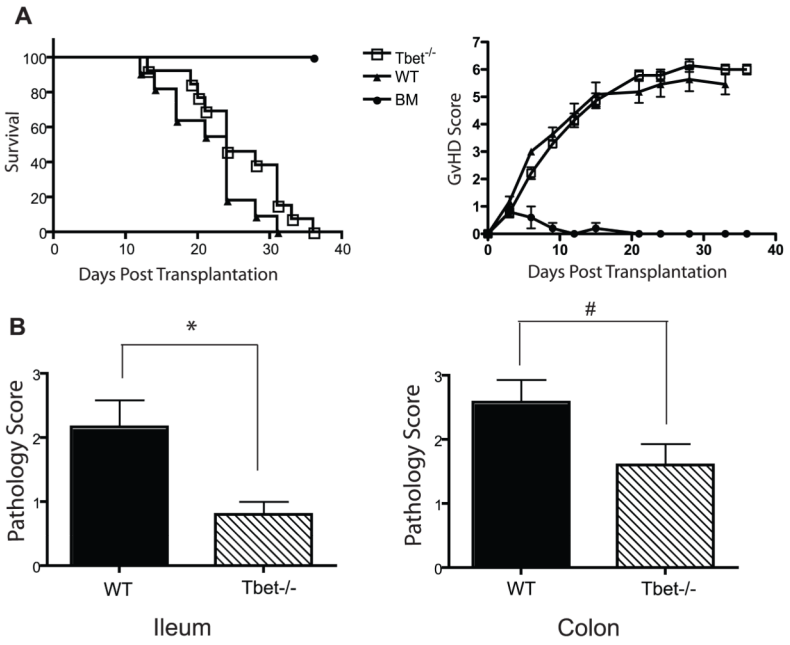

Figure 6. T-bet$^{-/-}$ Tconv cells decrease pathology in the GI tract but do not attenuate GvHD

(A) B6D2 F1 recipient mice were lethally irradiated (950 cGy) on day -1. Following irradiation on day 0 mice were injected intravenously with 4×10^6 WT or T-bet$^{-/-}$ Tconv cells supplemented with 3×10^6 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 post 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 Mann-Whitney test. *$p<0.05$, # $p=0.09$. Data are combined from 2 individual experiments.

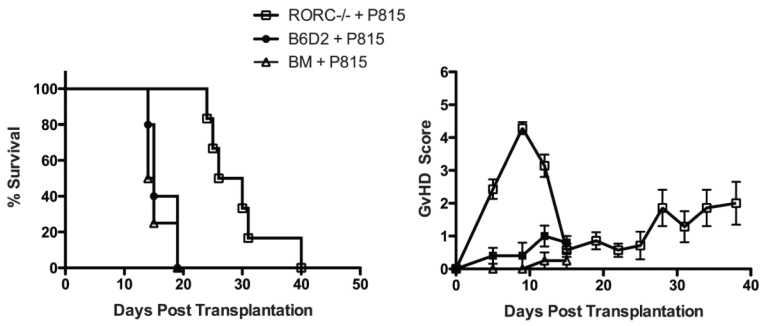

Figure 7. Improved anti-tumor responses in the absence of *RORC*

Lethally irradiated B6D2 F1 mice were injected with 3×10^6 TCD BM with or without 4×10^6 WT B6D2 or $RORC^{-/-}$ Tconv cells. Additionally all recipient mice received 1×10^4 P815 cells with the BM inoculum. Survival was determined by Kaplan and Meier method. An improvement in overall survival was found in B6D2 F1 mice given $RORC^{-/-}$ Tconv cells compared to B6D2 T cells or BM + P815 cells ($p < 0.05$). n=7 recipients receiving RORC null T cells, n=5 recipients receiving B6D2 T cells, n=4 recipients receiving BM. Data combined from 2 individual experiments.