Immune Mechanisms Important for the Pathogenesis of Acute Graft-versus-Host Disease

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ABSTRACT

LESHARA M FULTON: Immune Mechanisms Important for the Pathogenesis of Acute Graft-versus-Host Disease
(Under the direction of Jonathan Serody)

Allogeneic stem cell transplantation is a standard treatment for patients with high-risk relapsed leukemia, aplastic anemia, congenital bone marrow failure syndromes, and relapsed or recurrent lymphoid malignancies. Over 20,000 allogeneic transplants are conducted annually worldwide, confirming its effectiveness as a treatment for patients with otherwise lethal malignancies. Cure rates range from 15-80% depending on preexisting conditions and diagnoses. Patients receive high dose chemotherapy to eliminate malignant cells and allow engraftment of donor cells. However, this intense treatment regimen leaves patients vulnerable to infections, relapse, and acute graft-versus-host disease (aGvHD).

aGvHD is a disease characterized by selective epithelial damage to target organs. Complications from aGvHD result in increased morbidity and mortality in transplant recipients. aGvHD is initiated by mature $CD4^+$ and $CD8^+$ T cells present in the stem cell inoculum. These donor T cells replenish host T cell immunity and promote engraftment. Conversely, damage to target tissue, predominantly the skin, liver, and gastrointestinal tract, in aGvHD is caused by immunologically functional donor T lymphocytes that respond to genetic disparities in host antigens.

Our laboratory has focused on the migration of T cells in aGvHD pathogenesis. Coronins are a family of seven-actin binding proteins found in all eukaryotic organisms. Functional data in non-mammalian systems have shown a role for Coronins in cell migration, motility, and cytokinesis. The most well studied of the proteins, Coronin 1A (Coro 1A) is expressed primarily in hematopoietic cells and the focus of our studies.

Here, we demonstrate a requirement for Coro 1A in the pathogenesis of acute GvHD. Delayed entry and impaired egress from secondary lymphoid tissues were observed in T cells deficient in Coro 1A. Decreased expression of the C-C chemokine receptor type 7 (CCR7) and the signaling lipid receptor, sphingosine 1 phosphate receptor 1 (S1Pr1) were detected in Coro 1A deficient T cells. Egress to target aGvHD was limited by Coro 1A deficient cells due to accumulation in gastrointestinal lymph nodes. These data suggest that therapeutic approaches that prevent entry and egress from secondary lymphoid organs may effective treatment options for acute GvHD.

Dedication

To my family my mother, father, and brother and cousins who kept me uplifted throughout it all

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List of Abbreviations and Symbols

aGvHD	Acute graft-versus-host disease
Allo-SCT	Allogeneic stem cell transplantation
APC	Antigen presenting cells
CCR	C-C chemokine receptor
CD	Cluster of differentiation
Coro 1A	Coronin 1A
DC	Dendritic cell
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
GvL	Graft-versus-leukemia
IL	Interleukin
IFN-γ	Interferon gamma
I.V.	Intravenous
MHC	Major histocompatibility complex
miHA	Minor histocompatibility antigens
MLN	Mesenteric lymph node
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NK	Natural killer cells
PE	Phycoerythrin
PP	Peyer's patch
RORC	RAR related orphan receptor C
SLT	Secondary lymphoid tissue

S1P	Sphingosine 1 phosphate
S1Pr1	Sphingosine 1 phosphate receptor 1
TCD BM	T cell depleted bone marrow
Tconv	T conventional cell
TCR	T cell receptor
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper 17 cell
TNF	Tumor necrosis factor
T_{reg}	Regulatory T cell
WT	Wild type

CHAPTER ONE

INTRODUCTION TO ACUTE GRAFT VERSUS HOST DISEASE AND CORONINS

1.1 Introduction to Transplantation

Overview of Graft versus Host Disease

In the 1950s a syndrome referred to as "runt disease" or "wasting disease" was described in mice following ablative radiation treatment and bone marrow or splenic transfer. Introduction of immunologically functional cells after radiation treatment induced the development of "runt disease", characterized by weight loss, hunched posture, and diarrhea[1]. The development of this *graft-versus-host* response was independent of age or gender however scientists noted that immunological disparity also caused disease progression[1].

In the mid 1960s Billingham developed three requirements for the development of graft-versus-host disease (GvHD): immunologically functional cells must be present in the graft, the recipient must contain tissue antigens that are not found in the donor, and the recipient must be incapable of mounting an immune response[2]. Genetic disparity between the donor and an immune deficient recipient initiates an immune response by donor cells. Cells of the adaptive immune system, specifically T cells, are the major contributor for this immune response during GvHD. These donor T cells are activated after interacting with host innate

immune cells that act as antigen presenting cells. Host antigen presenting cells are often dendritic cells but can also include macrophages, B cells, and some epithelial cell subsets[3].

Genetic differences between host and recipient are recognized by T cells via presentation of peptides by major histocompatibility complex (MHC) antigens. These differences can exist at the level of the MHC protein (major mismatch) or in the type of peptides presented by the MHC protein (miHA) [4]. MHC presentation occurs through the MHC class I or MHC class II pathways of antigen presentation. MHC class I proteins present primarily self-proteins that are cytosolic and transported to the surface for presentation. Conversely extracelluar antigens require endocytosis or phagocytosis presentation by MHC class II proteins. MHC class II proteins are presented by professional antigen presenting cells that include dendritic cells, macrophages, and B cells[3].

The involvement of T cells in the pathogenesis of GvHD is well known. Suppression of helper T cell expansion by regulatory T cells has been shown to inhibit GvHD development[5,6]. However, which helper T cell populations are essential for the development of GvHD remains unclear. With the constant identification of new T cell subsets, the complexity of a seemingly simple question continues.

Allogeneic Stem Cell Transplantation

Allogeneic stem cell transplantation (allo-SCT) is a common treatment for malignant and non-malignant diseases including relapsed or high-risk acute leukemia, recurrent low-grade lymphoma, aplastic anemia, and genetic bone marrow failure syndromes[7-9]. Over 20,000 allogeneic transplants are conducted annually worldwide, confirming its effectiveness as a treatment for patients with otherwise lethal malignancies[10,11]. Cure rates range from 15-

80% depending on preexisting conditions and diagnoses[10]. Patients receive high dose chemotherapy to eliminate malignant cells and allow engraftment of donor cells. Anti-tumor properties that decrease the probability of relapse are mediated by immune cells infused with donor bone marrow or stem cells[12]. However, this intense treatment regimen leaves patients vulnerable to infections, relapse, and acute graft-versus-host disease (aGvHD)[13].

Acute Graft-versus-Host Disease: Phases and Treatments

aGvHD, a disease characterized by selective epithelial damage to target organs, occurs in 30-100% of allogeneic transplant patients[14]. Complications from aGvHD result in increased morbidity and mortality in transplant recipients. aGvHD is initiated by mature $CD4^+$ and $CD8^+$ T cells present in the stem cell inoculum. These donor T cells replenish host T cell immunity and promote engraftment[15]. Conversely, damage to target tissue, predominantly the skin, liver, and gastrointestinal tract, in aGvHD is caused by immunologically functional donor T lymphocytes that respond to genetic disparities in host antigens[16]. T lymphocytes are activated following interactions with host antigen presenting cells (APCs) in lymphoid tissue[17,18]. Following T cell activation there is migration of these cells to GvHD target organs where they mediate tissue destruction (Fig.1.1) [19,20].

Attempts to control aGvHD have been directed at blocking T cell proliferation and/or effector cytokine production. Current drugs used to prevent or treat aGvHD include the calcineurin inhibitor cyclosporine, which mitigates aGvHD by inhibiting expression of NFAT-specific proteins including IL-2 and IL-2R during the activation phase and prednisone which is cytolytic to T cells and blocks cytokine production[21]. Although effective for

some patients, the variability in disease symptoms and the substantial side effects associated with these treatments, especially glucocorticoids, emphasizes the need for more effective treatments.

1.2 T Cell Function

Originating from stem cells in the bone marrow, T cells are an important aspect of cell-mediated immunity. There are multiple types of T cells which can be categorized into three subgroups: cytotoxic T cells, helper T cells, and regulatory (or suppressive) T cells. Cytotoxic T cells, also known killer T cells or $CD8^+$ T cells, are responsible for the elimination of intracellular pathogens. Damage and destruction of pathogens is carried out through the elaboration of cytotoxins and cytokines including granzymes, perforin and interferon gamma. Helper T cells also recognize foreign antigens, however they do not typically possess cytolytic ability. The main function of helper T cells is to assist other immune cell during an adaptive immune response. They are involved in the generation of IL-2 which induces the proliferation of cytotoxic T cells and in B cell antibody class switching.

Regulatory T cells (T_{regs}) are essential for maintaining homeostasis amongst immune cells. This has been shown both clinically in patients with the disease immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) who have mutations in the critical transcription factor FoxP3 that is generated by T_{regs} and in animal models using genetic approaches to ablate the function of FoxP3 in which widespread autoimmune disease occurs[22-24]. Based on these data the primary function of T_{regs} was originally defined as maintenance of self-tolerance to prevent autoimmunity[25]. However, continued research has extended the list of T_{reg} functions to include allergy suppression[26], immune response regulation[27], and induction of oral tolerance[28].

1.3 T Cell Subsets

T Helper Type 1 And T Helper Type 2 Cells

Prior to the 18th century little was known about the blood and lymph. This ignorance sparked the interests of a young scientist by the name of William Hewson. During the late 1700s Hewson observed 'colorless cells' in the thymus that were rare in comparison to the population of flat, red cells[29]. Hewson believed that these 'colorless cells' were the precursors to the more populous red cells. However, with the discovery of stains, a distinction between red cells and white cells was confirmed. Furthermore the white cell population was found to be heterogeneous, containing many varieties of immune cells, amongst them were thymic derived cells involved in cellular immunity. These cells were distinguished from B cells by the absence of antibody secretion[30]. T cells were further divided into cytotoxic and helper T cells by surface marker expression[31].

Worked published in 1986 by Mosmann et al. divided this T cell population into two distinct groups[32]. T helper type 1 (Th1) cells produced interferon gamma (IFN-γ), interleukin 2 (IL-2), IL-3, and granulocyte macrophage colony-stimulating factor (GM-CSF). While T helper type 2 (Th2) cells also secrete IL-3 they were distinguished from Th1 cells by the production of B cell stimulating factor 1, mast cell growth factor 2, and T cell growth factor 2[32]. Later RNA hybridization and monoclonal antibodies were used to show that Th2 cells also generated IL-4 and IL-5[33]. Initially the differentiation of Th1 and Th2 cells from naïve $CD4^+$ T cells was thought to be largely dependent on IL-12 and IL-4 respectively[34]. Further examination revealed an IFN-γ positive feedback looped that introduced T box transcription factor (TBX21) as the transcription factor required for the

generation of Th1 cells[35]. Th2 differentiation however, was dependent on the T cell specific transcription factor GATA-3[36] (Fig 1.2).

Until relatively recently it was thought that Th1 and Th2 cells were the only $CD4^+$ T helper cell lineages. However, in 2005 a novel T cell population distinct from both Th1 and Th2 cells was identified[37]. These cells term Th17 cells are distinct in cytokine secretion and requirements for differentiation.

T Helper 17 Cells

The idea that other T cell lineages aside from Th1 existed was questioned when mice unable to produce IFN-γ or lacking other molecules involved in Th1 differentiation such as IL-12p35, IL-12 receptor β, or IL-18 developed more severe disease than their WT counterparts[38-40]. In 2000 researchers investigating IL-6 homologs identified a new cytokine chain p19, which would later be named IL-23, that formed heterodimers with the p40 subunit of IL-12[41]. Using an experimental autoimmune encephalomyelitis model researchers demonstrated that IL-23 not IL-12 was important for disease manifestations[42]. More importantly IL-23 was shown to generate and expand IL-17 producing cells that could induce disease when adoptively transferred[43]. This research and others lead to the proposal of Th17 cells as a novel T helper subset distinct from Th1 and Th2[37,44]. The differentiation of Th17 cells is dependent on multiple factors. In mice differentiation to Th17 cells require transforming growth factor β (TGF-β), a potent inhibitor of Th1 and Th2 cells[45,46], along with IL-6, IL-21, and in some cases IL-1β[47]. In addition to these cytokines, expression of Th17 cells is dependent on retinoid-related orphan receptor γt (RORγt) [48,49]. The requirement for RORγt in Th17 differentiation was determined as

mice reconstituted with RORγt deficient bone marrow cells had impaired Th17 differentiation[50]. Although RORγt is important for Th17 cell differentiation a close family member, RORα, has similar functions to RORγt. RORα is selectively expressed on T cells and can also induce the transcription of IL-17A and IL-17F[49]. The transcription factor signal transducer and activator of transcription 3 (STAT-3) regulates the expression of RORα and RORγt[51]. Thus both the cooperation of the ROR transcription factors and STAT3 are required for Th17 cell differentiation.

Th17 cells are responsible for clearance of pathogens that require a massive inflammatory response for which Th1 or Th2 immune responses are not adequate. Gram positive, Gram negative, fungi-like, and of course tissue inflammation all trigger robust Th17 responses[52-54]. The involvement of Th17 cells in disease has been linked to their secretion of the pro-inflammatory cytokines IL-17A, IL-17F, IL-21, IL-22, and TNF[55,56] (Fig 1.2). Additionally Th17 cells are involved in the generation of chemokines that recruit neutrophils to mucosal surfaces[57,58]. Experimental models and human studies have implicated Th17 cells in autoimmune diseases, transplantation reactions, tumor development, and allergy.

T Helper 22 Cells

Interleukin 22 (IL-22) was originally thought to be a Th1 associated cytokine[59]. However its expression in IL-17 producing cells linked it with Th17 cells. More recently two independent groups analyzing patients with psoriasis and atopic dermatitis identified a T cell population in human samples that produced IL-22 but not IL-17 or IFN-γ[60,61]. These cells were further classified by their expression of the skin homing receptors C-C chemokine receptor type 6 (CCR6), CCR4, and CCR10. The production of IL-22 correlated with the

expression of CCR10, while higher IL-17 production was seen in the absence of CCR10, distinguishing Th22 cells from Th17 cells[60]. Expression of IL-22 by Th17 cell is highly dependent on the transcription factor RORγt. However, the expression of RORγt was absence in this newly discovered population[61]and the aryl hydrocarbon receptor (AHR), a transcription factor that may be associated with IL-22 production, was implicated as a regulator of Th22 cells[60]. Distinguishing Th22 cells from Th17 cells, Th22 cells produce chemokine (C-C motif) ligand (CCL7) and CCL15 while CCL20 is found on Th17 cells[62]. Furthermore IL-6 and TNF were also critical for the differentiation of Th22 cells[60]. The transcription factors baconuclin 2 (BCN2) and foxhead box protein O4 (FOXO4) were expressed at high levels in Th22 clones suggesting the involvement of multiple transcription factors in the differentiation of Th22 cells[60].

In addition to IL-22 Th22 cells also produced the proinflammatory cytokines IL-10 and TNF. Th22 cells have been implicated in numerous diseases including Rheumatoid arthritis, Crohn's disease, and eczema[56,63,64].

Regulatory T Cells

Regulatory T cells (T_{regs}) were first identified as immune suppressors in the 1970[65]. The lack of markers to identify regulatory T cells as a distinct T cell subset caused many researchers to question their existence. In the mid 1990s Sakaguchi identified the alpha chain of the IL-2 receptor, CD25, as a marker for T_{regs}[66]. Later researchers identified the forkhead box transcription factor, Foxp3, gene in *scurfy* mice that displayed defects in autoimmune disease due to uncontrolled T cell proliferation [24]. The expression of Foxp3 was linked to regulatory T cells and used as a primary marker for this cell population.

Conventional T_{regs} are now identified by the expression of CD4, CD25, and Foxp3. More recently a number of T_{reg} subsets have been identified with varying markers for identification. The current list of T_{reg} populations includes inducible T_{regs}, natural T_{regs}, type 1 regulatory T cells, and T helper 3[67]. $CD8^+$ regulatory T cells have also demonstrated suppressive function similar to conventional $CD4^+$ T_{regs}[68]. Although T_{regs} were not the focus of these studies, our group and others have demonstrated their importance in aGvHD in murine models and clinically[6,69,70].

T Cells and aGvHD

Patients and murine models of aGvHD have shown high levels of the Th1 cytokines, TNF and IFN-γ, causing some researchers to believe that Th1 cells mediate aGvHD, specifically tissue pathology[21,71]. IFN-γ has been shown to be important for the manifestation of aGvHD however, in the absence of IFN-γ aGvHD is exacerbated[72]. Nikolic et al. showed that Th1 and Th2 cells contribute to gastrointestinal tract pathology in aGvHD however, hepatic and skin manifestations were attributed to Th2 cells[73]. Controversial data has been found with Th17 cells in aGvHD as well. *In vitro* polarized Th17 cells were shown to mediate lung and skin pathogenesis[74] yet, the absence of Th17 cells exacerbated aGvHD[75]. The involvement of Th22 cells in GvHD has not been investigated however, the role of IL-22 in skin manifestations suggests a skin specific role for this cell population.

1.4 Mouse Models of Acute GvHD

Experimental models of aGvHD are essential for expanding the knowledge and understanding of the disease. T cell alloreactivity, an early event in aGvHD pathogenesis, was identified using mouse models of the disease[16]. The majority of mouse models of aGvHD involved the transfer of T cell depleted bone marrow cells with varying numbers of specific T cells given intravenously to lethally irradiated recipients. Donor and recipient genotype as well as recipient radiation dose affect the severity and organ involvement in disease onset.

Depletion of T cells from donor bone marrow allows for standardizing the number of donor T cells given to each recipient, while the bone marrow cells are necessary for hematopoietic reconstitution following irradiation. Disease severity and tissues affected using mouse models of aGvHD is dependent of a number of factors. The degree of MHC protein and peptide disparity between mouse strains is directly proportional to disease severity. Additionally, donor T cell subsets and dose of T cells administered affect the severity of disease[76,77]. Surprisingly the variations between environmental pathogens amongst different laboratories can also affect the pathogenesis of aGvHD[78].

Regardless of the degree of genetic disparity, all MHC-mismatched models of aGvHD use some dose of conditioning therapy whether myeloablative or non-myeloablative. Most MHC-mismatched models require both $CD4^+$ and $CD8^+$ T cells. However, $CD8^+$ T cell specific activity (MHC Class I response) can be evaluated using B6.C-$H2^{bm1}$ (bm1) mouse, a transgenic mouse containing a mutant alpha helix in the H-$2K^b$ class I MHC molecule. Similarly $CD4^+$ T cell specific pathogenesis can be analyzed using B6.C-$H2^{bm12}$ (bm12), a

mutant MHC II mouse strain. aGvHD pathogenesis using $CD8^+$ T cells involves perforin, Fas ligand, and granzyme after T cell receptor engagement[79,80]. The effects of $CD4^+$ alloreactive T cells, conversely, are mediated by TNF[81].

Both major and minor histocompatibility (miHA) mismatched murine models of GvHD are commonly used. Although MHC mismatch transplants are rarely performed in the patient population because of increased risk for GvHD, these models provide insight into disease development and potential treatment options.

Our studies used murine transplantation models to examine the effectiveness of specific T cell populations and knockout phenotypes. These MHC mismatched models include the parent into F1, C57BL/6 into B6D2F1 and the complete MHC mismatched, C57BL/6 into BALB/c. Murine models are also used to study the impact of miHA in transplantation. Additionally our studies use the C57BL/6 into BALB.b minor mismatch model for miHA analysis[82]. All three models are well established and provide insight into clinical manifestation and potential treatment options for aGvHD.

C57BL/6 into BALB.b Minor Mismatch GvHD Model

The most clinically relevant mouse model of GvHD is the minor mismatch model. These models display less systemic GvHD symptoms however GvHD lethally is still seen in recipient mice. Although multiple models exist our laboratory uses the C57BL/6 into BALB.b miHA GvHD model. This model, that is mainly dependent on $CD4^+$ T cells, uses lethal irradiation of recipient animals with concurrent infusion of donor T cells and T cell depleted bone marrow cells[82]. GvHD induced damage to the liver, gastrointestinal tract, and skin are seen in recipient mice.

Minor HA mismatch models are used on occasion in our laboratory. However our studies have focused on haploidentical MHC mismatched transplantation, a scenario seen more with the increased need for transplant donors.

C57BL/6 into B6D2 Haploidentical MHC Mismatch GvHD Mouse Model

Hematopoietic stem cell transplantation is often the only option for patients with otherwise incurable diseases. HLA-match siblings are often ideal donors for patients however, only a small percentage of patients have siblings as a donor option, leaving an unrelated match as the only alternative. The expansion of the worldwide donor program has made finding a suitable donor easier for transplant patients although many other challenges remain. Donor populations in ethnic minorities are small and the probability of finding a HLA match is often less than 10 percent[83]. Furthermore the process of finding a match and harvesting samples can take up to 4 months. Time is of the essence for these patients and often more complications or death occur during this extended waiting period. HLA-mismatch transplants are not ideal and carry their own set of complications however, the availability of this population often makes them a popular choice.

A commonly used model in which the donor and recipient are partially matched is the parent into F1 transplantation model. This model typically uses lethal irradiation of recipient mice and is dependent on both donor $CD4^+$ and $CD8^+$ T cells combined with T cell depleted bone marrow cells[84]. Donor mice are C57BL/6 while lethally irradiated recipients are the F1 generation of a C57BL/6 mouse crossed with DBA/2 mouse, more commonly known as B6D2. Using this transplantation model, recipient mice succumb to disease by day 30 with irradiation. Other groups have also used this model without irradiation to study aGvHD

effects during reduced intensity conditioning or nonmyeloablative treatment regimes[85]. In the absence of irradiation disease symptoms appear between day 30 and day 50 post transplantation[84]. Pathophysiology using the parent into F1 model with irradiation includes weight loss due to gastrointestinal involvement and skin manifestations.

C57BL/6 into BALB/c Complete MHC Mismatch GvHD Mouse Model

Due to the high treatment related mortality and post transplantation complications complete HLA-mismatched transplantations are not completed clinically. However, a well established murine transplantation model uses a completely mismatched MHC protein to induce GvHD. Although less relevant to the clinical transplant setting, this model provides insight into the contribution of specific cell populations, treatment options, and potential patient responses.

The C57BL/6 into BALB/c transplant models uses lethally irradiated recipients with mismatched donor cells. Donor cells are transplanted concurrently with T cell depleted bone marrow cells into recipient mice. Disease onset occurs between day 10 and day 30. Characteristics of disease development in using this model include severe weight loss, ruffled fur, and decreased activity. Further histological analysis shows lymphocyte infiltration in target GvHD organs with hydrophic degeneration, Civatte bodies, and keratosis in the skin[86].

Mouse models are beneficial for determining treatment options and outcomes. As treatment options remain limited for GvHD patients these resources are essential for GvHD research.

1.5 T Cell Migration and GvHD

The migration of T cells after transplantation has been determined by our group using enhanced green fluorescent protein labeled T cells[87]. Within hours of transplantation donor T cells migrate to lymphoid organs. Between 48 and 72 hours these cells are activated by antigen presenting cells found in lymphoid tissue and proliferate. In the first week these activated T cells migrate to GvHD target organs including the liver, gastrointestinal tract, lung, and skin where they cause damage through the generation of cytokines and via cytolytic activity, a finding that has also been confirmed by other groups[88].

Most recently our group has investigated the role of migratory proteins in T cell migration in GvHD. The contribution of C-C chemokine receptor expression and selectins have previously been determined by our group and others[6,70,89]. Our investigation of T cell migration post transplantation was further extended with the identification of actin interacting proteins, known as Coronins, that are important for cytoskeleton dynamics.

1.6 Actin Cytoskeleton Protein Dynamics

Actin Cytoskeleton Dynamics

Proper function and organization of the cytoskeleton is required for many cellular activities including cell migration, motility, and trafficking. In cells actin exists in two states, monomeric and filamentous (F-actin). Regulation of cytoskeleton dynamics are maintained through the homeostatic balance of these two actin forms. As an ATPase nucleotide hydrolysis allows the transition between G actin and F actin states[90]. The asymmetric structure of actin is determined by nucleotide dependent changes that alter the stability of the filament[91]. Chemical messengers sent via chemokines, growth factors, and hormones are

believed to trigger cytoskeleton rearrangement however the mechanism by which these factors mediate their effects remains elusive[90].

Origin of Coronins

In 1991 a protein specific for the projections of growing *Dictyostelium discoideum* was identified[92]. This protein was later termed 'coronin' for its crown shaped appearance when stained with antibody. Mutant *Dictyostelium discoideum* lacking Coronin showed reduced phagocytosis and motility, processes that require F-actin. Furthermore, coronin was shown to cosediment with F-actin, leading to the classification of coronin as an actin binding protein[93].

The coronin family of proteins consists of 12 subfamilies with 6 being exclusive to vertebrates. Coronin 1A was the first member of the coronin family discovered by de Hostos et al.[92]. Shortly thereafter the two closely related proteins Coronin 1B and Coronin 1C, also termed Coronin 2 and Coronin 3 respectively, were identified[94-96]. Although phagocytosis was found to be evolutionarily conserved between the family of proteins their functions remained unclear[93]. Gerisch et al. later hypothesized that coronins were involved in actin regulation and polymerization[97]. Their hypothesis was correct but it wasn't until the discovery of the Arp2/3 complex that involvement of coronins in actin dynamics was more clearly defined.

Polymerization and depolymerization of actin is important for the regulation of the cytoskeleton. The rate-limiting step in actin polymerization is nucleation[93]. Identification and characterization of the Arp2/3 complex identified its primary mechanism as nucleating during F-actin polymerization[98,99]. The Arp2/3 complex is a family of seven proteins

involved in the synthesis of F-actin. The interaction of coronin proteins with the Arp2/3 complex was determined after coronin 1A was found in human neutrophil Arp2/3 samples[100]. These data were further confirmed in budding yeast demonstrating the interaction of coronin 1A with Arp2/3 and F-actin[101].

Interaction with Arp2/3 has not been limited to Coronin 1A. Researchers at the University of North Carolina at Chapel Hill showed disrupted interactions of Coronin 1B with the Arp2/3 complex through phosphorylation of protein kinase C (PKC)[102]. Reduced ruffling and altered mobility were seen in cells that contained a mutation in the serine 2 residue[102]. Modification of Arp2/3 complex interactions through the phosphorylation of serine 2 have also been seen for Coronin 1A[103]. A clear role for Coronins in actin regulation has been shown although the exact mechanism by which coronins facilitate these effects remains elusive.

Classes of Coronins

Currently, the mammalian family of coronins includes seven coronin genes. However nomenclature based on sequence similarity, function, or simply numbering has been a constant debate. In 2001 the Human Genome Organization nomenclature committee introduced nomenclature based on phylogeny of the coronin proteins[104]. This system divided the proteins into two classes: Type I and Type II.

There are three members of the type 1 coronins: Coronin 1A, Coronin 1B, and Coronin 1C. Coronin 1A, also known as tryptophan aspartate-coating coat protein (TACO)[105] and p57, was originally identified as a protein that co-purified with phospholipase activity[106]. Coronin 1A is expressed primarily in hematopoietic cells and to a lesser extent neural

tissue[104]. Coronin 1A has been shown to be important in the regulation of leukocyte specific events[105]. A ubiquitously expressed isoform, Coronin 1B is found at the leading edge of migrating fibroblasts[107]. The least studied of the type 1 coronins, Coronin 1C is also ubiquitously expressed with reduced expression compared to Coronin 1B[108]. Coronin 1B functions in cytokinesis, cell motility, and regulation of leading edge dynamics[93]. Coronin 1C is involved in cell proliferation, invasion, migration, filopodia growth, cytokinesis, and the secretion of norepinephrine[109,110].

Type 2 coronins vary structurally from type 1 coronins and are found only in vertebrates[104]. There are two members of the type 2 Coronins: Coronin 2A and Coronin 2B. Coronin 2A is found in the uterus, brain, testes, and ovary while Coronin 2B is expressed primarily in the brain[104]. Like many of the Coronins the functions of Coronin 2A and Coronin 2B remains unclear.

Coronins and Disease

For years scientist questioned the importance of actin cytoskeleton integrity and human disease. However, identifying the link between cytoskeleton rearrangement and disease proved to be elusive. The involvement of Coronin 1A in normal peripheral T cell development and its importance in actin cytoskeleton structure lead to research regarding its role in disease. Within the past decade numerous groups have compelling data suggesting a role for Coronin 1A in multiple diseases[111-113].

Shiow et al. used a mouse forward genetics approached to determine the cause of thymus migration defects and lymph node trafficking in the cataract Shionogi mouse strain[114]. This research was coupled with clinical studies to determined genotype and phenotype

correlations in patients with severe combined immunodeficiency (SCID) [111]. SCID patients are characterized by defects in T cells numbers and defects in T and B cell function which if left untreated can result in death. Analyses of 16 patients identified a single patient who had a 2 base pair deletion in the paternal Coronin 1A coding sequence and a de novo deletion in the maternal Coronin 1A coding sequence[111]. Additionally variations in copy number at this same chromosome region are linked to autism spectrum disorders, a condition that was also present in the patient[111]. Although the patient population was limited to one this study provided early insight into actin cytoskeleton dynamics and disease.

In another study Dwight Kono's group suggested a requirement for Coronin 1A in the development of systemic lupus erythematosus (SLE)[112]. Screening of multiple gene associated with the lupus-modifying locus reveled Coronin 1A as a potential regulatory gene. Mice containing the mutant Coronin 1A gene, created by a single nonsense mutation, were less susceptible to disease[112]. Decreased T cell numbers in the periphery and thymus coupled with accumulation of F-actin in T cells, and defects in calcium flux in mutant mice were consistent with previously published data[103,112,115].

1.7 Dissertation Aims

Bone marrow transplantation for blood disorders and cancers has been effective for many years. However, post transplantation complications specifically acute graft-versus-host disease continue to be a problem. Specific cell populations, cytokine receptors, and ligands have been studied to determine their role in aGvHD. Although these studies have been beneficial, identifying molecules as potential drug targets remains difficult.

The main goal of this study was to identify proteins that may contribute to pathogenesis of aGvHD. Our studies expanded upon previous work from our laboratory suggesting a role for Th17 cells in disease. Alteration of cell migration, motility, and function can often be attributed to actin cytoskeleton dynamics. Here we are the first to identify an actin cytoskeleton protein that is important for the induction and severity of aGvHD pathogenesis.

Figure 1.1

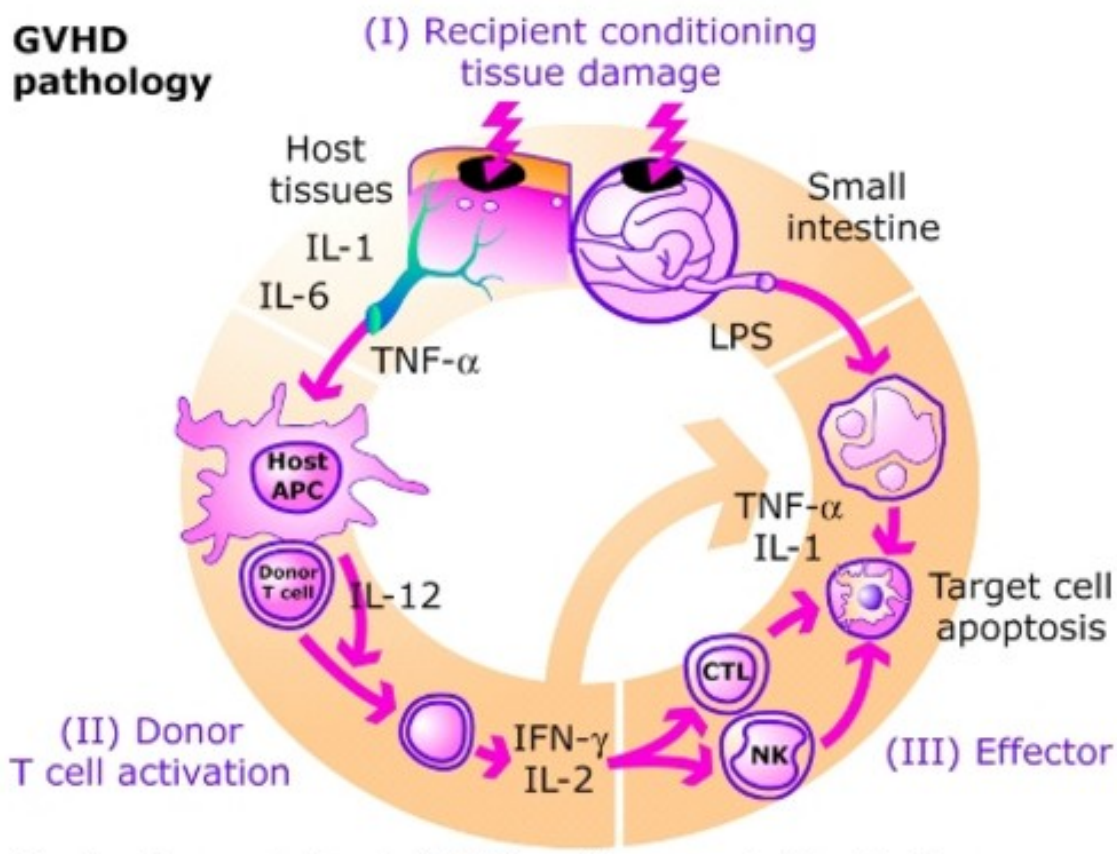

Figure 1.1 Pathophysiology of acute GvHD (From Ferrara et al[20]). During the initial precondition phases host tissues are damaged due to chemotherapy and irradiation. Host antigen presenting cells are also activated during this first step. Donor T cells that have contaminated bone marrow or stem cell inoculums proliferate, differentiate, and are activated in phase II. The final phase, the effector phase, is marked by tissue damage and inflammation due to cytokines and other effector molecules.

Figure 1.2

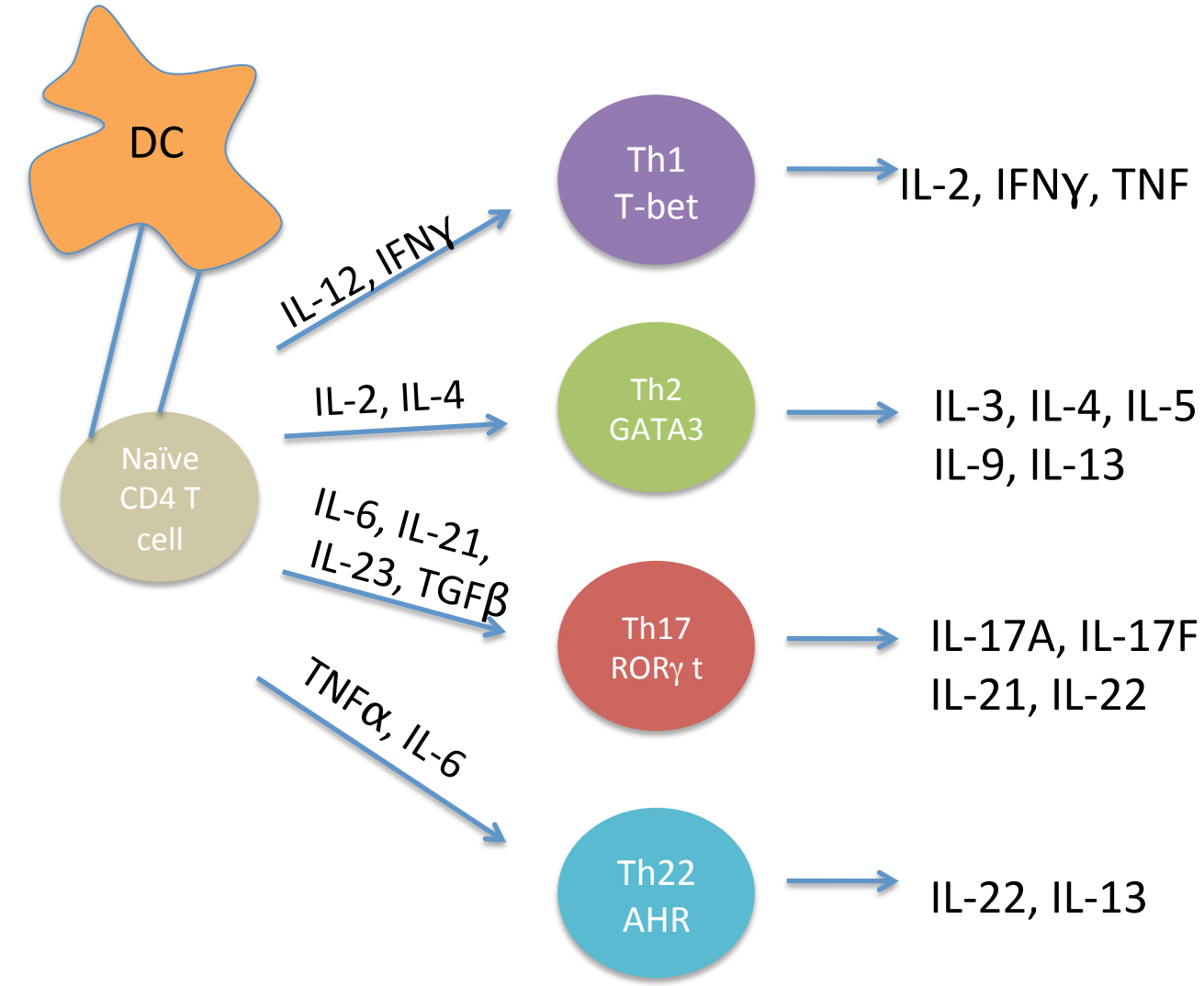

Figure 1.2 Naïve T Cell Differentiation. After activation from antigen presenting cells naïve T cells differentiate into different T cell subsets depending upon cytokines and transcriptions factors present.

References

1. BLAESE RM, MARTINEZ C, GOOD RA (1964) IMMUNOLOGIC INCOMPETENCE OF IMMUNOLOGICALLY RUNTED ANIMALS. J Exp Med 119: 211–224.

2. Billingham RE (1966) The biology of graft-versus-host reactions. Harvey Lect 62: 21–78.

3. Paul WE (2008) Fundamental Immunology. Lippincott Williams & Wilkins. 1 pp.

4. Socié G, Blazar BR (2009) Acute graft-versus-host disease: from the bench to the bedside. Blood 114: 4327–4336. doi:10.1182/blood-2009-06-204669.

5. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, Lucas PJ, Gress RE, et al. (2004) L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. Blood 104: 3804–3812. doi:10.1182/blood-2004-05-1850.

6. Carlson MJ, Fulton LM, Coghill JM, West ML, Burgents JE, et al. (2010) L-selectin is dispensable for T regulatory cell function postallogeneic bone marrow transplantation. Am J Transplant 10: 2596–2603. doi:10.1111/j.1600-6143.2010.03319.x.

7. La Nasa G, Giardini C, Argiolu F, Locatelli F, Arras M, et al. (2002) Unrelated donor bone marrow transplantation for thalassemia: the effect of extended haplotypes. Blood 99: 4350–4356.

8. Socié G, Stone JV, Wingard JR, Weisdorf D, Henslee-Downey PJ, et al. (1999) Long-term survival and late deaths after allogeneic bone marrow transplantation. Late Effects Working Committee of the International Bone Marrow Transplant Registry. N Engl J Med 341: 14–21. doi:10.1056/NEJM199907013410103.

9. van Besien K, Sobocinski KA, Rowlings PA, Murphy SC, Armitage JO, et al. (1998) Allogeneic bone marrow transplantation for low-grade lymphoma. Blood 92: 1832–1836.

10. Appelbaum FR (2001) Haematopoietic cell transplantation as immunotherapy. Nature 411: 385–389. doi:10.1038/35077251.

11. Toubai T, Sun Y, Reddy P (2008) GVHD pathophysiology: is acute different from chronic? Best Pract Res Clin Haematol 21: 101–117. doi:10.1016/j.beha.2008.02.005.

12. Kolb H-J (2008) Graft-versus-leukemia effects of transplantation and donor lymphocytes. Blood 112: 4371–4383. doi:10.1182/blood-2008-03-077974.

13. O'Reilly RJ (1983) Allogenic bone marrow transplantation: current status and future directions. Blood 62: 941–964.

14. Goker H, Haznedaroglu IC, Chao NJ (2001) Acute graft-vs-host disease: pathobiology and management. Exp Hematol 29: 259–277.

15. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, et al. (2002) Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science 295: 2097–2100. doi:10.1126/science.1068440.

16. Korngold R, Sprent J (1978) Lethal graft-versus-host disease after bone marrow transplantation across minor histocompatibility barriers in mice. Prevention by removing mature T cells from marrow. J Exp Med 148: 1687–1698.

17. Wysocki CA, Panoskaltsis-Mortari A, Blazar BR, Serody JS (2005) Leukocyte migration and graft-versus-host disease. Blood 105: 4191–4199. doi:10.1182/blood-2004-12-4726.

18. Shlomchik WD, Couzens MS, Tang CB, McNiff J, Robert ME, et al. (1999) Prevention of graft versus host disease by inactivation of host antigen-presenting cells. Science 285: 412–415.

19. Reddy P (2003) Pathophysiology of acute graft-versus-host disease. Hematol Oncol 21: 149–161. doi:10.1002/hon.716.

20. Ferrara JLM, Levine JE, Reddy P, Holler E (2009) Graft-versus-host disease. Lancet 373: 1550–1561. doi:10.1016/S0140-6736(09)60237-3.

21. Antin JH, Ferrara JL (1992) Cytokine dysregulation and acute graft-versus-host disease. Blood 80: 2964–2968.

22. Takahashi T, Kuniyasu Y, Toda M, Sakaguchi N, Itoh M, et al. (1998) Immunologic self-tolerance maintained by CD25+CD4+ naturally anergic and suppressive T cells: induction of autoimmune disease by breaking their anergic/suppressive state. Int Immunol 10: 1969–1980.

23. van der Vliet HJJ, Nieuwenhuis EE (2007) IPEX as a result of mutations in FOXP3. Clin Dev Immunol 2007: 89017. doi:10.1155/2007/89017.

24. Godfrey VL, Wilkinson JE, Rinchik EM, Russell LB (1991) Fatal lymphoreticular disease in the scurfy (sf) mouse requires T cells that mature in a sf thymic environment: potential model for thymic education. Proc Natl Acad Sci USA 88: 5528–5532.

25. Corthay A (2009) How do regulatory T cells work? Scand J Immunol 70: 326–336. doi:10.1111/j.1365-3083.2009.02308.x.

26. Curotto de Lafaille MA, Muriglan S, Sunshine MJ, Lei Y, Kutchukhidze N, et al.

(2001) Hyper immunoglobulin E response in mice with monoclonal populations of B and T lymphocytes. J Exp Med 194: 1349–1359.

27. Asseman C, Fowler S, Powrie F (2000) Control of experimental inflammatory bowel disease by regulatory T cells. Am J Respir Crit Care Med 162: S185–S189.

28. Karlsson MR, Rugtveit J, Brandtzaeg P (2004) Allergen-responsive CD4+CD25+ regulatory T cells in children who have outgrown cow's milk allergy. J Exp Med 199: 1679–1688. doi:10.1084/jem.20032121.

29. Doyle D (2006) William Hewson (1739-74): the father of haematology. 7 pp. doi:10.1111/j.1365-2141.2006.06037.x.

30. Mitchell GF, Miller JF (1968) Cell to cell interaction in the immune response. II. The source of hemolysin-forming cells in irradiated mice given bone marrow and thymus or thoracic duct lymphocytes. J Exp Med 128: 821–837.

31. Cantor H, Boyse EA (1975) Functional subclasses of T-lymphocytes bearing different Ly antigens. I. The generation of functionally distinct T-cell subclasses is a differentiative process independent of antigen. J Exp Med 141: 1376–1389.

32. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136: 2348–2357.

33. Cherwinski HM, Schumacher JH, Brown KD, Mosmann TR (1987) Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J Exp Med 166: 1229–1244.

34. Zhu J, Paul WE (2008) CD4 T cells: fates, functions, and faults. Blood 112: 1557–1569. doi:10.1182/blood-2008-05-078154.

35. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, et al. (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell 100: 655–669.

36. Zheng W, Flavell RA (1997) The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. Cell 89: 587–596.

37. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, et al. (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 6: 1123–1132. doi:10.1038/ni1254.

38. Gran B, Zhang G-X, Yu S, Li J, Chen X-H, et al. (2002) IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. J Immunol 169: 7104–7110.

39. Krakowski M, Owens T (1996) Interferon-gamma confers resistance to experimental allergic encephalomyelitis. Eur J Immunol 26: 1641–1646. doi:10.1002/eji.1830260735.

40. Gutcher I, Urich E, Wolter K, Prinz M, Becher B (2006) Interleukin 18-independent engagement of interleukin 18 receptor-alpha is required for autoimmune inflammation. Nat Immunol 7: 946–953. doi:10.1038/ni1377.

41. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, et al. (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity 13: 715–725.

42. Cua DJ, Sherlock J, Chen Y, Murphy CA, Joyce B, et al. (2003) Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. Nature 421: 744–748. doi:10.1038/nature01355.

43. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, et al. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 201: 233–240. doi:10.1084/jem.20041257.

44. Park H, Li Z, Yang XO, Chang SH, Nurieva R, et al. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 6: 1133–1141. doi:10.1038/ni1261.

45. Gorelik L, Fields PE, Flavell RA (2000) Cutting edge: TGF-beta inhibits Th type 2 development through inhibition of GATA-3 expression. J Immunol 165: 4773–4777.

46. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, et al. (2006) Transforming growth factor-beta induces development of the T(H)17 lineage. Nature 441: 231–234. doi:10.1038/nature04754.

47. Korn T, Bettelli E, Gao W, Awasthi A, Jäger A, et al. (2007) IL-21 initiates an alternative pathway to induce proinflammatory TH17 cells. Nature 448: 484–487. doi:10.1038/nature05970.

48. Manel N, Unutmaz D, Littman DR (2008) The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat Immunol 9: 641–649. doi:10.1038/ni.1610.

49. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, et al. (2008) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity 28: 29–39. doi:10.1016/j.immuni.2007.11.016.

50. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, et al. (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126: 1121–1133. doi:10.1016/j.cell.2006.07.035.

51. Mathur AN, Chang H-C, Zisoulis DG, Stritesky GL, Yu Q, et al. (2007) Stat3 and Stat4 direct development of IL-17-secreting Th cells. J Immunol 178: 4901–4907.

52. Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, et al. (2001) Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med 194: 519–527.

53. Khader SA, Bell GK, Pearl JE, Fountain JJ, Rangel-Moreno J, et al. (2007) IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. Nat Immunol 8: 369–377. doi:10.1038/ni1449.

54. Rudner XL, Happel KI, Young EA, Shellito JE (2007) Interleukin-23 (IL-23)-IL-17 cytokine axis in murine Pneumocystis carinii infection. Infect Immun 75: 3055–3061. doi:10.1128/IAI.01329-06.

55. Bucher C, Koch L, Vogtenhuber C, Goren E, Munger M, et al. (2009) IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. Blood 114: 5375–5384. doi:10.1182/blood-2009-05-221135.

56. Ma H-L, Liang S, Li J, Napierata L, Brown T, et al. (2008) IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. J Clin Invest 118: 597–607. doi:10.1172/JCI33263.

57. Liang SC, Long AJ, Bennett F, Whitters MJ, Karim R, et al. (2007) An IL-17F/A heterodimer protein is produced by mouse Th17 cells and induces airway neutrophil recruitment. J Immunol 179: 7791–7799.

58. Pelletier M, Maggi L, Micheletti A, Lazzeri E, Tamassia N, et al. (2010) Evidence for a cross-talk between human neutrophils and Th17 cells. Blood 115: 335–343. doi:10.1182/blood-2009-04-216085.

59. Wolk K, Witte E, Witte K, Warszawska K, Sabat R (2010) Biology of interleukin-22. Semin Immunopathol 32: 17–31. doi:10.1007/s00281-009-0188-x.

60. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H (2009) Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. Nat Immunol 10: 864–871. doi:10.1038/ni.1770.

61. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F (2009) Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. Nat Immunol 10: 857–863. doi:10.1038/ni.1767.

62. Coghill JM, Sarantopoulos S, Moran TP, Murphy WJ, Blazar BR, et al. (2011) Effector CD4+ T cells, the cytokines they generate, and GVHD: something old and

something new. Blood 117: 3268–3276. doi:10.1182/blood-2010-12-290403.

63. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Karow M, et al. (2007) Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. Immunity 27: 647–659. doi:10.1016/j.immuni.2007.07.023.

64. Zhang N, Pan H-F, Ye D-Q (2011) Th22 in inflammatory and autoimmune disease: prospects for therapeutic intervention. Mol Cell Biochem 353: 41–46. doi:10.1007/s11010-011-0772-y.

65. Gershon RK, Kondo K (1970) Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology 18: 723–737.

66. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 155: 1151–1164.

67. Sakaguchi S, Yamaguchi T, Nomura T, Ono M (2008) Regulatory T Cells and Immune Tolerance. Cell 133: 775–787. doi:10.1016/j.cell.2008.05.009.

68. Cosmi L, Liotta F, Lazzeri E, Francalanci M, Angeli R, et al. (2003) Human CD8+CD25+ thymocytes share phenotypic and functional features with CD4+CD25+ regulatory thymocytes. Blood 102: 4107–4114. doi:10.1182/blood-2003-04-1320.

69. Di Ianni M, Falzetti F, Carotti A, Terenzi A, Castellino F, et al. (2011) Tregs prevent GVHD and promote immune reconstitution in HLA-haploidentical transplantation. Blood 117: 3921–3928. doi:10.1182/blood-2010-10-311894.

70. Coghill JM, Carlson MJ, Panoskaltsis-Mortari A, West ML, Burgents JE, et al. (2010) Separation of graft-versus-host disease from graft-versus-leukemia responses by targeting CC-chemokine receptor 7 on donor T cells. Blood 115: 4914–4922. doi:10.1182/blood-2009-08-239848.

71. Remberger M, Ringden O, Markling L (1995) TNF alpha levels are increased during bone marrow transplantation conditioning in patients who develop acute GVHD. Bone Marrow Transplant 15: 99–104.

72. Murphy WJ, Welniak LA, Taub DD, Wiltrout RH, Taylor PA, et al. (1998) Differential effects of the absence of interferon-gamma and IL-4 in acute graft-versus-host disease after allogeneic bone marrow transplantation in mice. J Clin Invest 102: 1742–1748. doi:10.1172/JCI3906.

73. Nikolic B, Lee S, Bronson RT, Grusby MJ, Sykes M (2000) Th1 and Th2 mediate acute graft-versus-host disease, each with distinct end-organ targets. J Clin Invest 105: 1289–1298. doi:10.1172/JCI7894.

74. Carlson MJ, West ML, Coghill JM, Panoskaltsis-Mortari A, Blazar BR, et al. (2009) In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. Blood 113: 1365–1374. doi:10.1182/blood-2008-06-162420.

75. Yi T, Zhao D, Lin C-L, Zhang C, Chen Y, et al. (2008) Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. Blood 112: 2101–2110. doi:10.1182/blood-2007-12-126987.

76. Sprent J, Schaefer M, Gao EK, Korngold R (1988) Role of T cell subsets in lethal graft-versus-host disease (GVHD) directed to class I versus class II H-2 differences. I. L3T4+ cells can either augment or retard GVHD elicited by Lyt-2+ cells in class I different hosts. J Exp Med 167: 556–569.

77. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, et al. (2003) CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med 9: 1144–1150. doi:10.1038/nm915.

78. Nestel FP, Price KS, Seemayer TA, Lapp WS (1992) Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor alpha during graft-versus-host disease. J Exp Med 175: 405–413.

79. Via CS, Nguyen P, Shustov A, Drappa J, Elkon KB (1996) A major role for the Fas pathway in acute graft-versus-host disease. J Immunol 157: 5387–5393.

80. Maeda Y, Levy RB, Reddy P, Liu C, Clouthier SG, et al. (2005) Both perforin and Fas ligand are required for the regulation of alloreactive CD8+ T cells during acute graft-versus-host disease. Blood 105: 2023–2027. doi:10.1182/blood-2004-08-3036.

81. Schroeder MA, DiPersio JF (2011) Mouse models of graft-versus-host disease: advances and limitations. Dis Model Mech 4: 318–333. doi:10.1242/dmm.006668.

82. Berger M, Wettstein PJ, Korngold R (1994) T cell subsets involved in lethal graft-versus-host disease directed to immunodominant minor histocompatibility antigens. Transplantation 57: 1095–1102.

83. Huang X-J (2008) Current status of haploidentical stem cell transplantation for leukemia. J Hematol Oncol 1: 27. doi:10.1186/1756-8722-1-27.

84. Via CS, Rus V, Nguyen P, Linsley P, Gause WC (1996) Differential effect of CTLA4Ig on murine graft-versus-host disease (GVHD) development: CTLA4Ig prevents both acute and chronic GVHD development but reverses only chronic GVHD. J Immunol 157: 4258–4267.

85. Hakim FT, Sharrow SO, Payne S, Shearer GM (1991) Repopulation of host lymphohematopoietic systems by donor cells during graft-versus-host reaction in unirradiated adult F1 mice injected with parental lymphocytes. J Immunol 146:

2108–2115.

86. van Leeuwen L, Guiffre A, Atkinson K, Rainer SP, Sewell WA (2002) A two-phase pathogenesis of graft-versus-host disease in mice. Bone Marrow Transplant 29: 151–158. doi:10.1038/sj.bmt.1703328.

87. Panoskaltsis-Mortari A, Price A, Hermanson JR, Taras E, Lees C, et al. (2004) In vivo imaging of graft-versus-host-disease in mice. Blood 103: 3590–3598. doi:10.1182/blood-2003-08-2827.

88. Ichiba T (2003) Early changes in gene expression profiles of hepatic GVHD uncovered by oligonucleotide microarrays. Blood 102: 763–771. doi:10.1182/blood-2002-09-2748.

89. Wysocki CA, Jiang Q, Panoskaltsis-Mortari A, Taylor PA, McKinnon KP, et al. (2005) Critical role for CCR5 in the function of donor CD4+CD25+ regulatory T cells during acute graft-versus-host disease. Blood 106: 3300–3307. doi:10.1182/blood-2005-04-1632.

90. Lee SH, Dominguez R (2010) Regulation of actin cytoskeleton dynamics in cells. Mol Cells 29: 311–325.

91. Belmont LD, Orlova A, Drubin DG, Egelman EH (1999) A change in actin conformation associated with filament instability after Pi release. Proc Natl Acad Sci USA 96: 29–34.

92. de Hostos EL, Bradtke B, Lottspeich F, Guggenheim R, Gerisch G (1991) Coronin, an actin binding protein of Dictyostelium discoideum localized to cell surface projections, has sequence similarities to G protein beta subunits. EMBO J 10: 4097–4104.

93. Clemen CS, Rybakin V, Eichinger L (2008) The coronin family of proteins. Subcell Biochem 48: 1–5. doi:10.1007/978-0-387-09595-0_1.

94. Zaphiropoulos PG, Toftgård R (1996) cDNA cloning of a novel WD repeat protein mapping to the 9q22.3 chromosomal region. DNA Cell Biol 15: 1049–1056.

95. Terasaki AG, Ohnuma M, Mabuchi I (1997) Identification of actin-binding proteins from sea urchin eggs by F-actin affinity column chromatography. J Biochem 122: 226–236.

96. Okumura M, Kung C, Wong S, Rodgers M, Thomas ML (1998) Definition of family of coronin-related proteins conserved between humans and mice: close genetic linkage between coronin-2 and CD45-associated protein. DNA Cell Biol 17: 779–787.

97. Gerisch G, Albrecht R, Heizer C, Hodgkinson S, Maniak M (1995) Chemoattractant-controlled accumulation of coronin at the leading edge of Dictyostelium cells

monitored using a green fluorescent protein-coronin fusion protein. Curr Biol 5: 1280–1285.

98. Mullins RD, Pollard TD (1999) Structure and function of the Arp2/3 complex. Curr Opin Struct Biol 9: 244–249.

99. Welch MD, Iwamatsu A, Mitchison TJ (1997) Actin polymerization is induced by Arp2/3 protein complex at the surface of Listeria monocytogenes. Nature 385: 265–269. doi:10.1038/385265a0.

100. Machesky LM, Reeves E, Wientjes F, Mattheyse FJ, Grogan A, et al. (1997) Mammalian actin-related protein 2/3 complex localizes to regions of lamellipodial protrusion and is composed of evolutionarily conserved proteins. Biochem J 328 (Pt 1): 105–112.

101. Humphries CL, Balcer HI, D'Agostino JL, Winsor B, Drubin DG, et al. (2002) Direct regulation of Arp2/3 complex activity and function by the actin binding protein coronin. J Cell Biol 159: 993–1004. doi:10.1083/jcb.200206113.

102. Cai L, Makhov AM, Bear JE (2007) F-actin binding is essential for coronin 1B function in vivo. J Cell Sci 120: 1779–1790. doi:10.1242/jcs.007641.

103. Föger N, Rangell L, Danilenko DM, Chan AC (2006) Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. Science 313: 839–842. doi:10.1126/science.1130563.

104. Uetrecht AC, Bear JE (2006) Coronins: the return of the crown. Trends Cell Biol 16: 421–426. doi:10.1016/j.tcb.2006.06.002.

105. Ferrari G, Langen H, Naito M, Pieters J (1999) A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell 97: 435–447.

106. Suzuki K, Nishihata J, Arai Y, Honma N, Yamamoto K, et al. (1995) Molecular cloning of a novel actin-binding protein, p57, with a WD repeat and a leucine zipper motif. FEBS Lett 364: 283–288.

107. Cai L, Holoweckyj N, Schaller MD, Bear JE (2005) Phosphorylation of coronin 1B by protein kinase C regulates interaction with Arp2/3 and cell motility. J Biol Chem 280: 31913–31923. doi:10.1074/jbc.M504146200.

108. Cai L, Makhov AM, Schafer DA, Bear JE (2008) Coronin 1B antagonizes cortactin and remodels Arp2/3-containing actin branches in lamellipodia. Cell 134: 828–842. doi:10.1016/j.cell.2008.06.054.

109. Rosentreter A, Hofmann A, Xavier C-P, Stumpf M, Noegel AA, et al. (2007) Coronin 3 involvement in F-actin-dependent processes at the cell cortex. Exp Cell Res 313: 878–895. doi:10.1016/j.yexcr.2006.12.015.

110. Thal D, Xavier C-P, Rosentreter A, Linder S, Friedrichs B, et al. (2008) Expression of coronin-3 (coronin-1C) in diffuse gliomas is related to malignancy. J Pathol 214: 415–424. doi:10.1002/path.2308.

111. Shiow LR, Paris K, Akana MC, Cyster JG, Sorensen RU, et al. (2009) Severe combined immunodeficiency (SCID) and attention deficit hyperactivity disorder (ADHD) associated with a Coronin-1A mutation and a chromosome 16p11.2 deletion. Clin Immunol 131: 24–30. doi:10.1016/j.clim.2008.11.002.

112. Haraldsson MK, Louis-Dit-Sully CA, Lawson BR, Sternik G, Santiago-Raber M-L, et al. (2008) The lupus-related Lmb3 locus contains a disease-suppressing Coronin-1A gene mutation. Immunity 28: 40–51. doi:10.1016/j.immuni.2007.11.023.

113. Moriceau S, Kantari C, Mocek J, Davezac N, Gabillet J, et al. (2009) Coronin-1 is associated with neutrophil survival and is cleaved during apoptosis: potential implication in neutrophils from cystic fibrosis patients. J Immunol 182: 7254–7263. doi:10.4049/jimmunol.0803312.

114. Shiow LR, Roadcap DW, Paris K, Watson SR, Grigorova IL, et al. (2008) The actin regulator coronin 1A is mutant in a thymic egress-deficient mouse strain and in a patient with severe combined immunodeficiency. Nat Immunol 9: 1307–1315. doi:10.1038/ni.1662.

115. Mueller P, Massner J, Jayachandran R, Combaluzier B, Albrecht I, et al. (2008) Regulation of T cell survival through coronin-1-mediated generation of inositol-1,4,5-trisphosphate and calcium mobilization after T cell receptor triggering. Nat Immunol 9: 424–431. doi:10.1038/ni1570.

CHAPTER TWO

ATTENUATION OF ACUTE GVHD IN THE ABSENCE OF THE TRANSCRIPTION FACTOR RORγT*

2.1 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].

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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 [17-19]. Th17 cells produce proinflammatory cytokines such as TNF, IL-21, and IL-22 [20-22]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[20] while IL-22 has been found to be important for the induction of psoriasis in experimental models [23]. 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 [24].

Keppel *et al* using IL-17A knockout (-/-) $CD4^+$ T cells demonstrated that IL-17 contributes to aGVHD[25]. In contrast, Yi *et al* has shown that IL-$17A^{-/-}$ T cells exacerbated aGVHD due to augmented release of IFN-γ [26]. Recent studies in our laboratory demonstrated that *in vitro* differentiated Th17 cells generated substantial cutaneous and pulmonary pathology in murine models of aGvHD[27] 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.

2.2 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[28]. 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[29]. Histopathology specimens were generated as described[30] and analyzed by one of us (APM) blinded to the genotype of donor used. Scoring of tissues was performed per our previous method[31].

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[27]. 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[32].

GvL Analysis

Recipient mice were infused with 1 x 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 [33]. Differences in GvHD clinical and pathology scores were determined using Mann-Whitney test. P values ≤ 0.05 were considered statistically significant.

2.3 Results

2.3.1 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 2.1A). Using a semi-quantitative scoring system we evaluated the clinical manifestations of aGvHD[34] 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 2.1B).

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 2.1C) with a diminished GvHD score (Fig 2.1D) 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 (Supplemental Fig 2.1). 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.

2.3.2 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 2.2). 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.

2.3.3 *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 2.3A). A substantial decrease in IL-17 and TNF were seen in the serum of recipients *RORC*$^{-/-}$ Tconv compared to WT Tconv recipients (Fig 2.3A). 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 2.3B).

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.

2.3.4. *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[35]. 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 2.4A). 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 2.4B). 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.

2.3.5 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 x 10^{6} *RORC*$^{-/-}$ or WT CD4^{+} T cells with 3 x 10^{6} WT TCD BM cells while lethally irradiated BALB/c recipients were infused with 5 x 10^{5} *RORC*$^{-/-}$ or WT CD4^{+} T cells supplemented

with 5 x 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 2.4C), 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 2.4D). 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 2.4D) 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 x 10^6 WT or *RORC*$^{-/-}$ CD4$^+$ T cells with 3 x 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 2.4D). 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.

2.3.6 *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 x 10^6 $RORC^{-/-}$ $CD4^+$ T cells with 2 x 10^6 WT $CD8^+$ T cells supplemented with 3 x 10^6 TCD BM cells. A second group received 2 x 10^6 WT $CD4^+$ T cells with $RORC^{-/-}$ $CD8^+$ T cells supplemented with 3 x 10^6 WT TCD BM cells. A final group received only 3 x 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 2.5A). 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 2.5B). 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.

2.3.7 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 2.6A). 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 2.6B). A trend for decreased pathology was also seen in the colon ($p = 0.08$, Fig 2.6B). 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[36].

2.3.8 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 2.7). 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).

2.4 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[37]. 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[38]. 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 (Supplemental Fig 2.2). 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[39]. 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.

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Figure 2.1

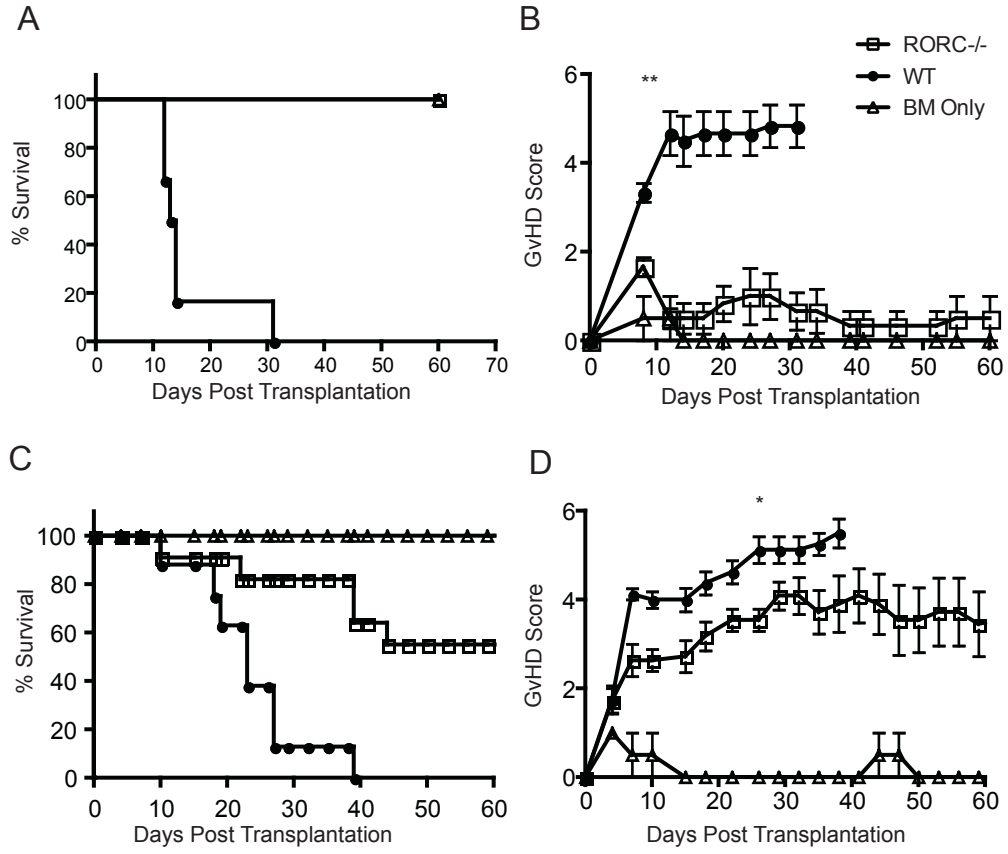


Figure 2.1. Survival and GvHD scores of B6D2 recipients. (A-B) B6D2 recipients were lethally irradiated (950 cGy) on day -1. One day following irradiation, 4 x 10^6 WT or *RORC*$^{-/-}$ CD25- Tconv cells supplemented with 3 x 10^6 TCD BM were injected intravenously into recipient mice. Recipient mice 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 x 10^5 WT or *RORC*$^{-/-}$ CD25- T cells supplemented with 5 x 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 2.2

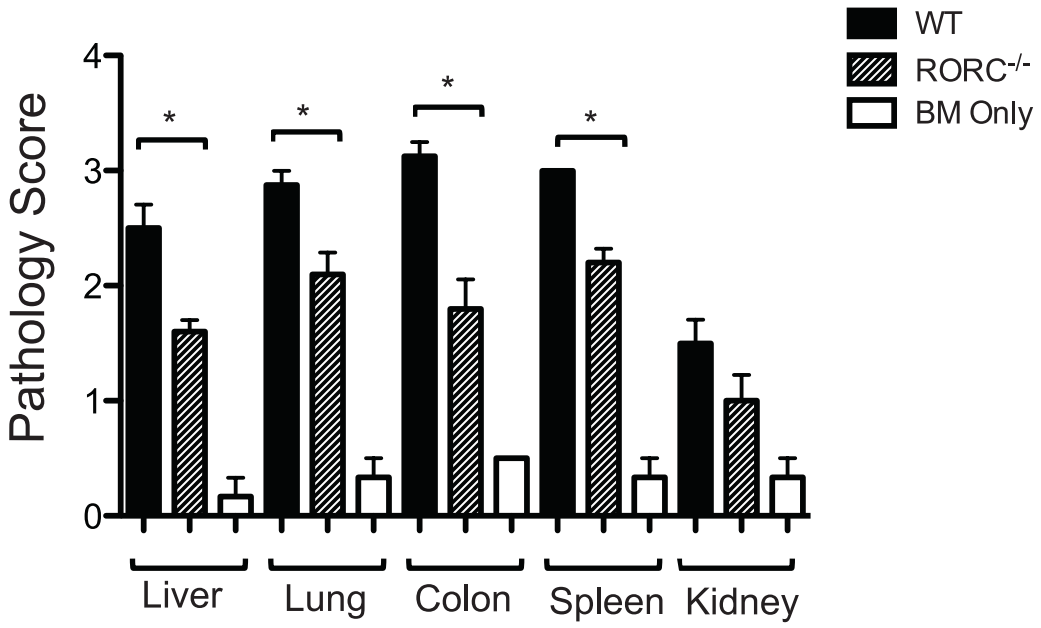


Figure 2.2. Decreased tissue pathology in recipient mice given *RORC*$^{-/-}$ donor T cells. 4 x 10^6 ($CD25^-$) Tconv cells from *RORC*$^{-/-}$ or WT mice with WT TCD BM were transplanted into lethally irradiated (950 cGy) B6D2 F1 recipients. Organs were harvested on day 15 post-transplantation and 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 2.3

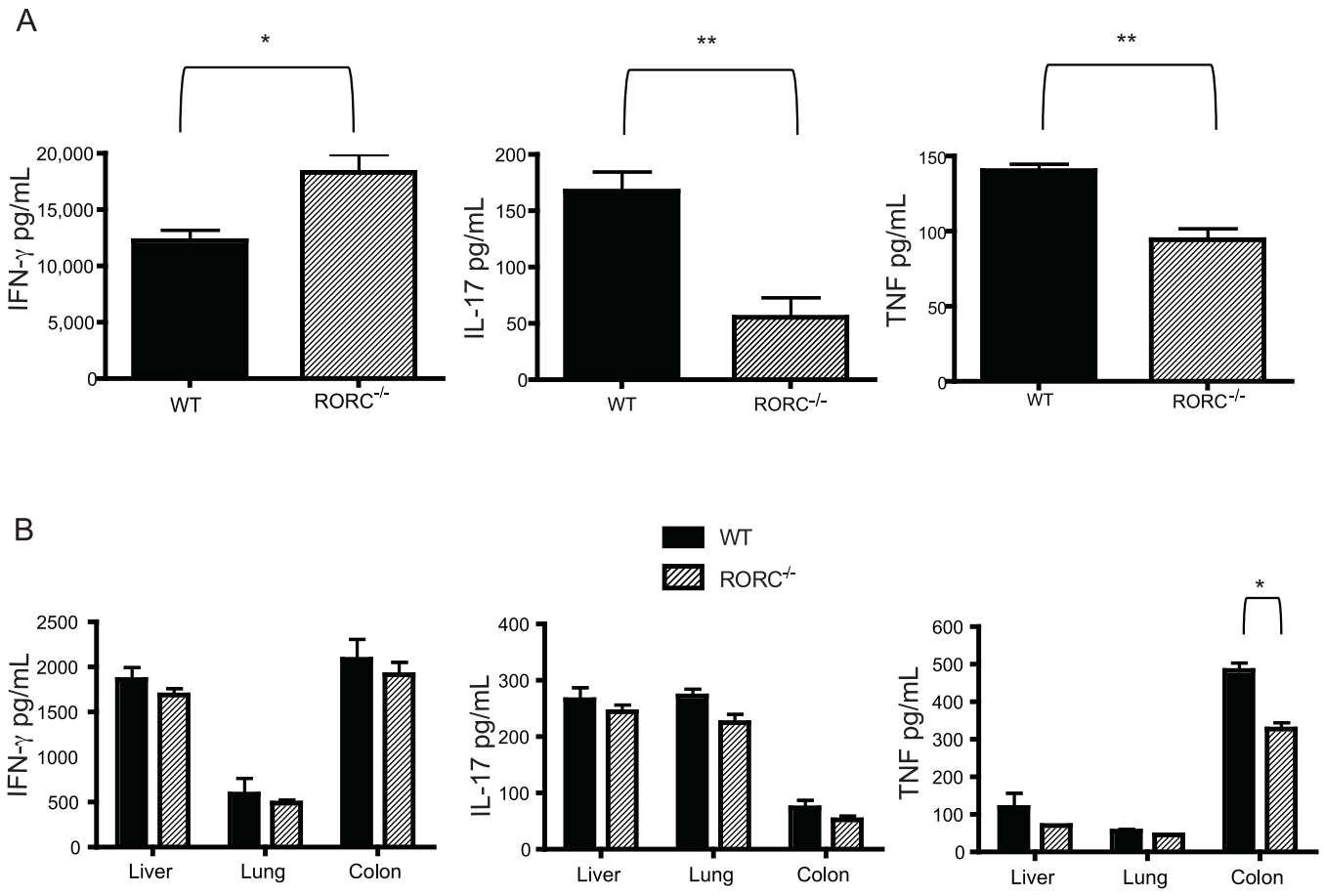


Figure 2.3. Increased serum IFN-γ and decreased TNF expression *RORC*$^{-/-}$ recipients. WT TCD BM and *RORC*$^{-/-}$ or WT Tconv were transplanted into lethally irradiated B6D2 mice. 14 days post transplantation (A) serum and (B) organs were collected from B6D2 F1 recipients and analyzed by ELISA for 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 2.4

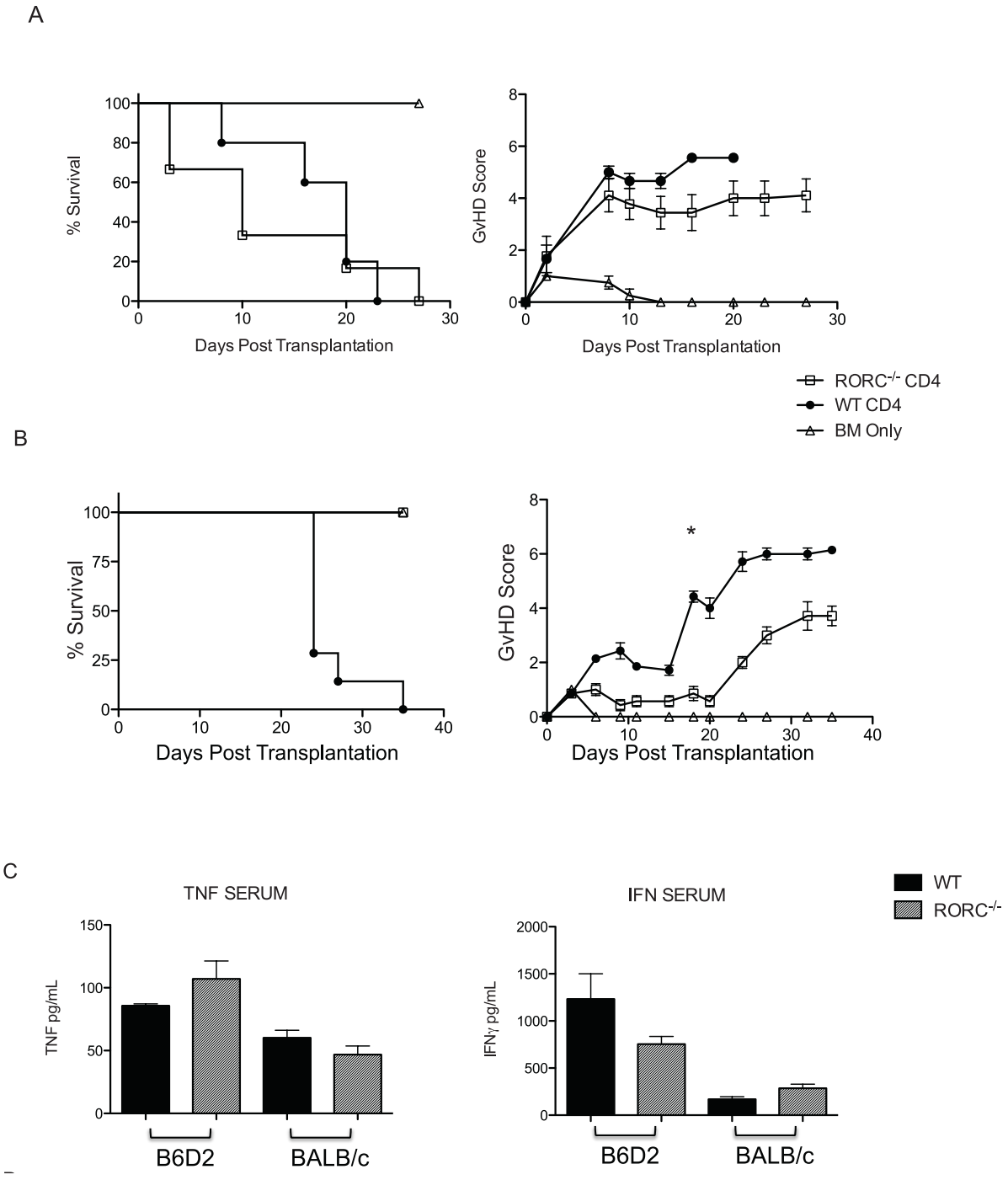

A
% Survival
Days Post Transplantation
GvHD Score
Days Post Transplantation
RORC-/- CD4
WT CD4
BM Only
B
% Survival
Days Post Transplantation
GvHD Score
*
Days Post Transplantation
C
TNF SERUM
TNF pg/mL
B6D2
BALB/c
IFN SERUM
IFNγ pg/mL
B6D2
BALB/c
WT
RORC-/-

Figure 2.4 contd.

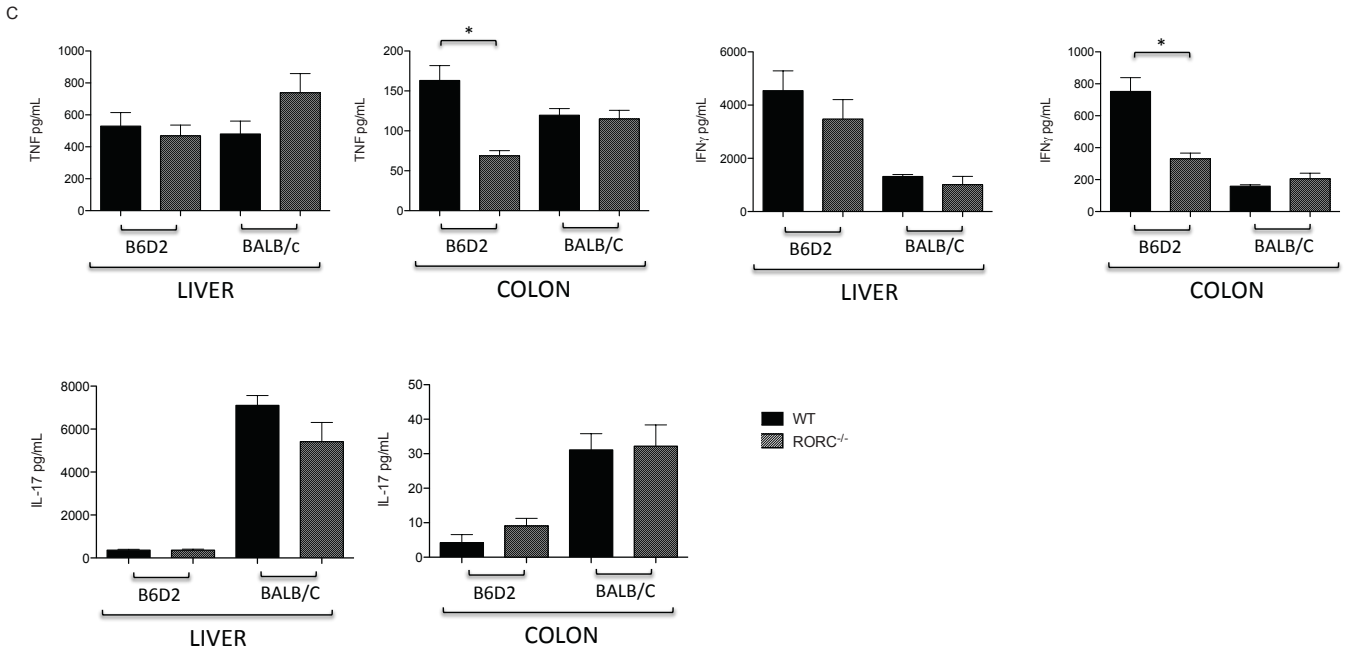


Figure 2.4. Function of *RORC* in Donor CD4$^+$ T cells is Model Dependent. (A) Lethally irradiated BALB/c recipients were injected with 5 x 10^5 *RORC*$^{-/-}$ CD4$^+$ or WT CD4$^+$ T cells supplemented with 5 x 10^6 WT TCD BM. Survival and GvHD scores are shown. n= 9 for *RORC*$^{-/-}$ recipients. n=9 for WT recipients, n=4 for bone marrow only recipients. (B) Lethally irradiated B6D2 F1 recipients were injected with 2 x 10^6 *RORC*$^{-/-}$ CD4$^+$ T cells or WT CD4$^+$ T cells supplemented with 3 x 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 2.5

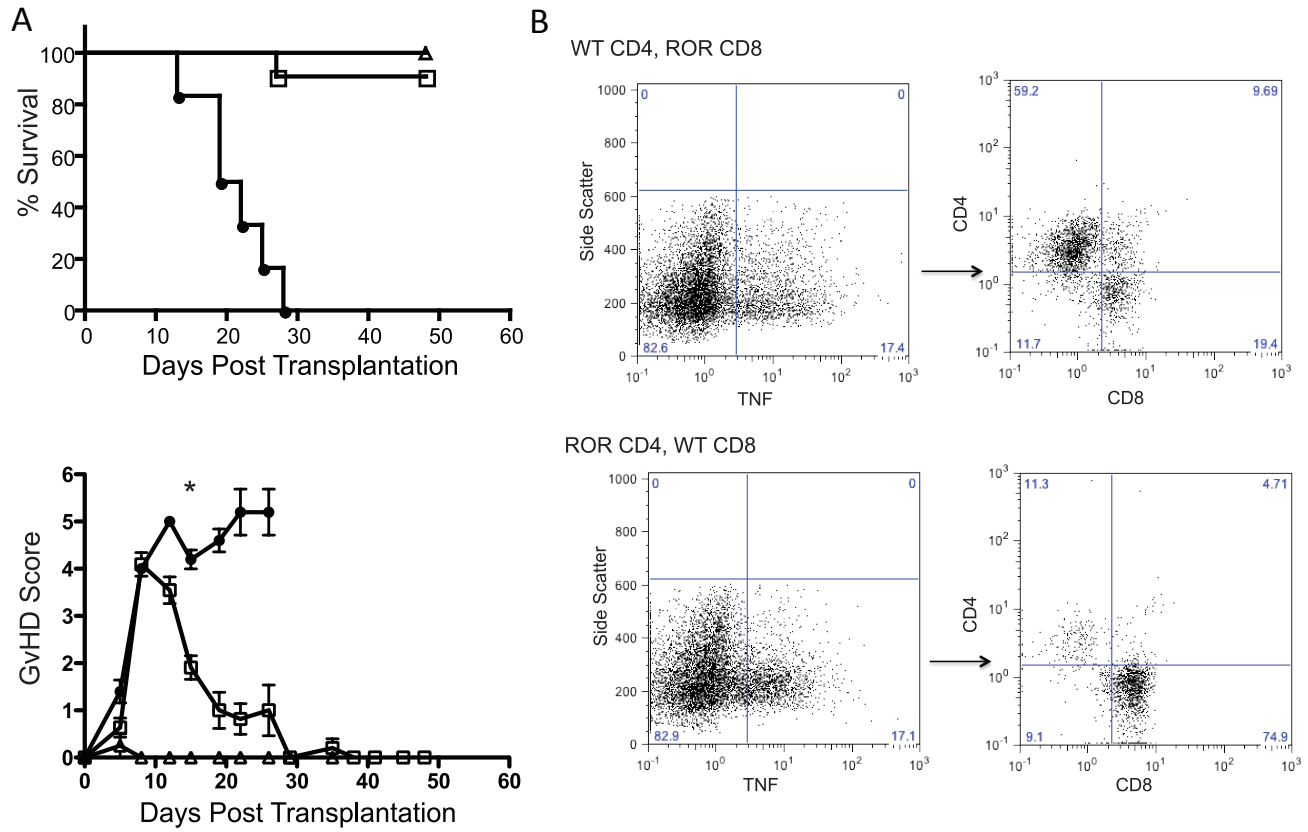


Figure 2.5. Attenuated GvHD using *RORC*$^{-/-}$ Tconv cells is mediated by CD4^{+} T cells. (A) Lethally irradiated B6D2 F1 mice were injected with 3 x 10^6 TCD BM. In addition to BM one group received 2 x 10^6 *RORC*$^{-/-}$ CD4^{+} T cells and 2 x 10^6 WT CD8^{+} T cells, one group received 2 x 10^6 WT CD4^{+} T cells and 2 x 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 2.6

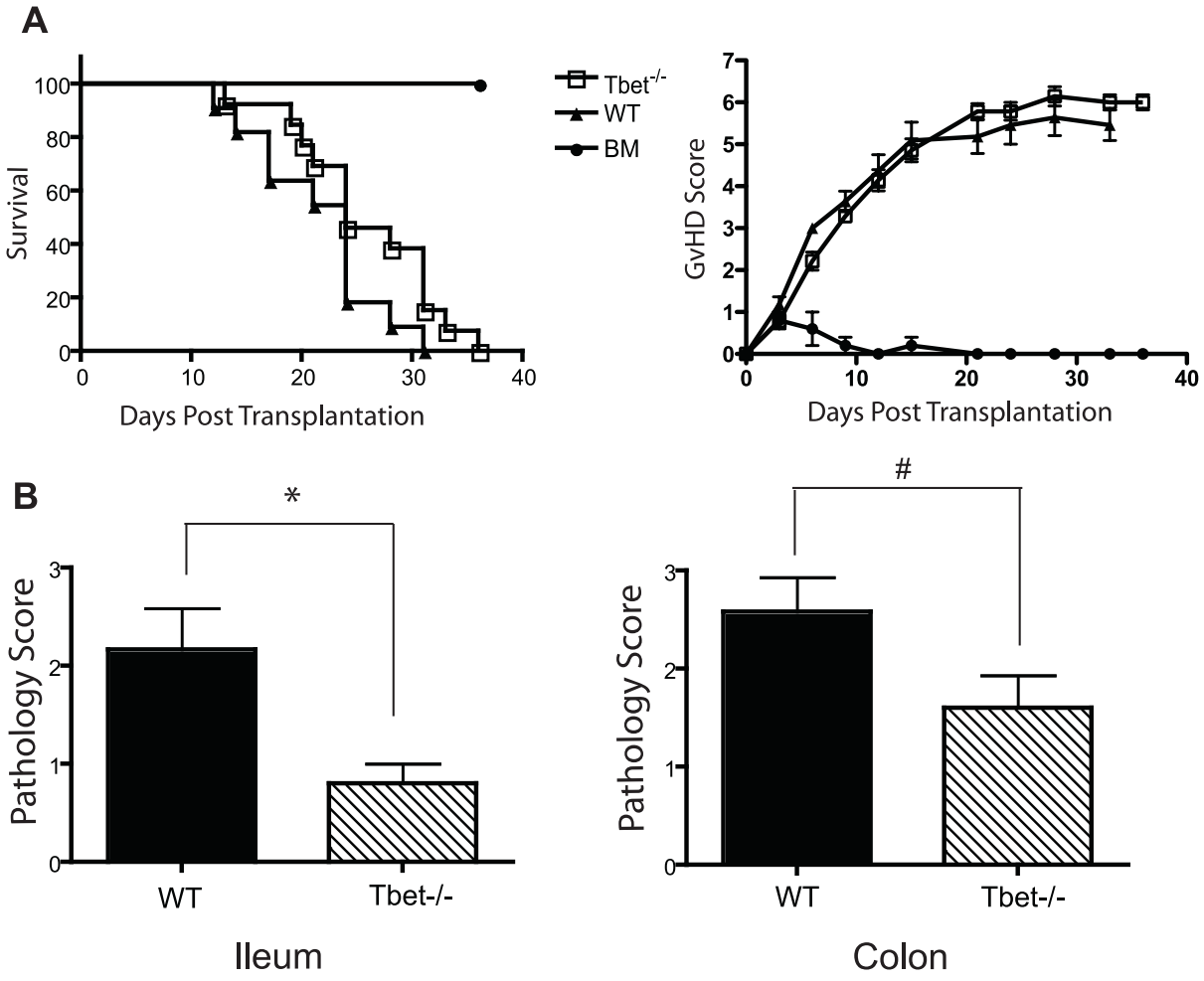


Figure 2.6. T-bet$^{-/-}$ Tconv cells decrease pathology in the GI tract but do not attenuate GvHD. (A) B6D2 F1 recipient mice were lethally irradiated (950 cGy) on day -1. Following irradiation on day 0 mice were injected intravenously with 4 x 10^6 WT or T-bet$^{-/-}$ Tconv cells supplemented with 3 x 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 2.7

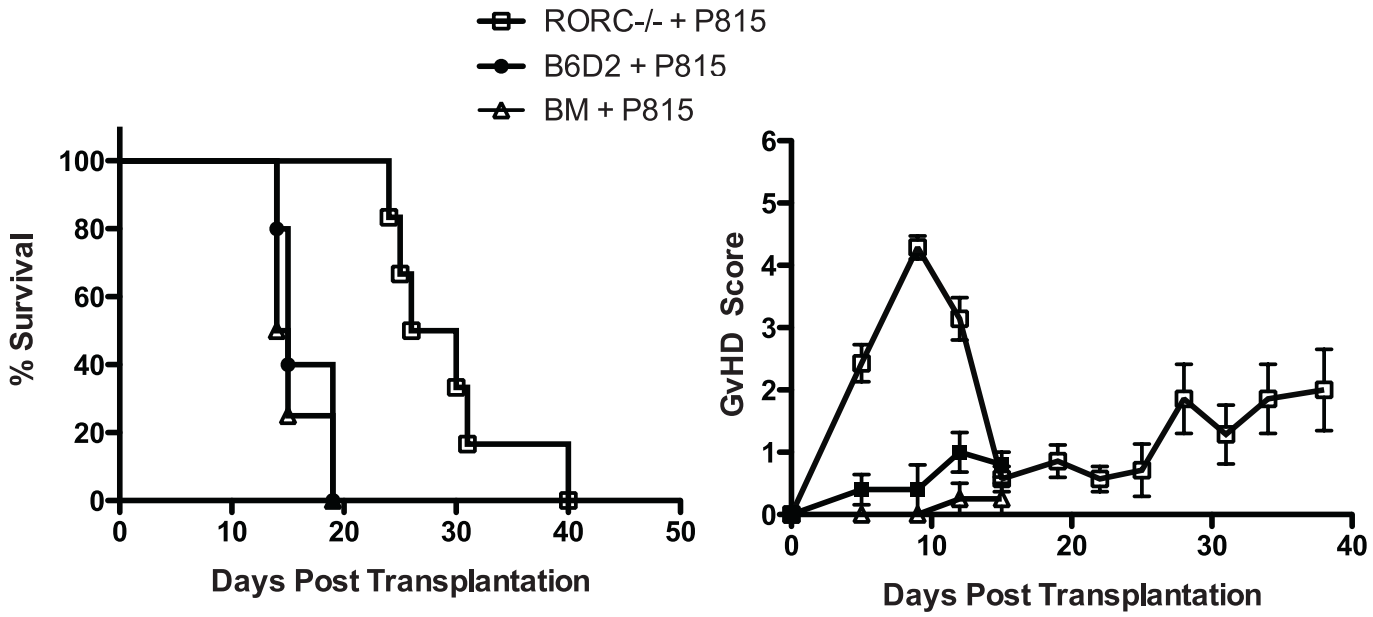
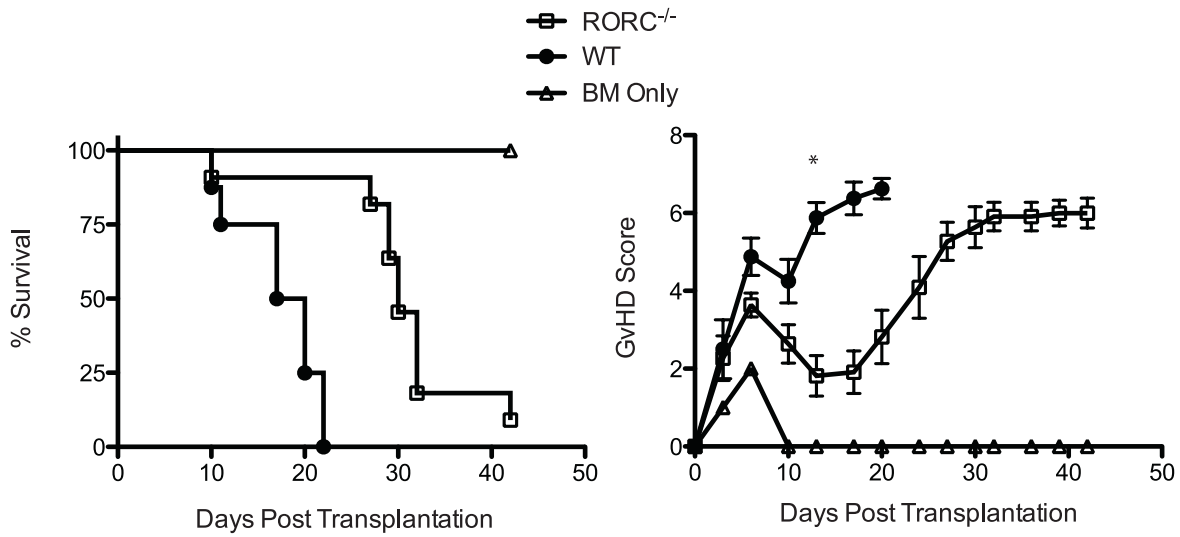


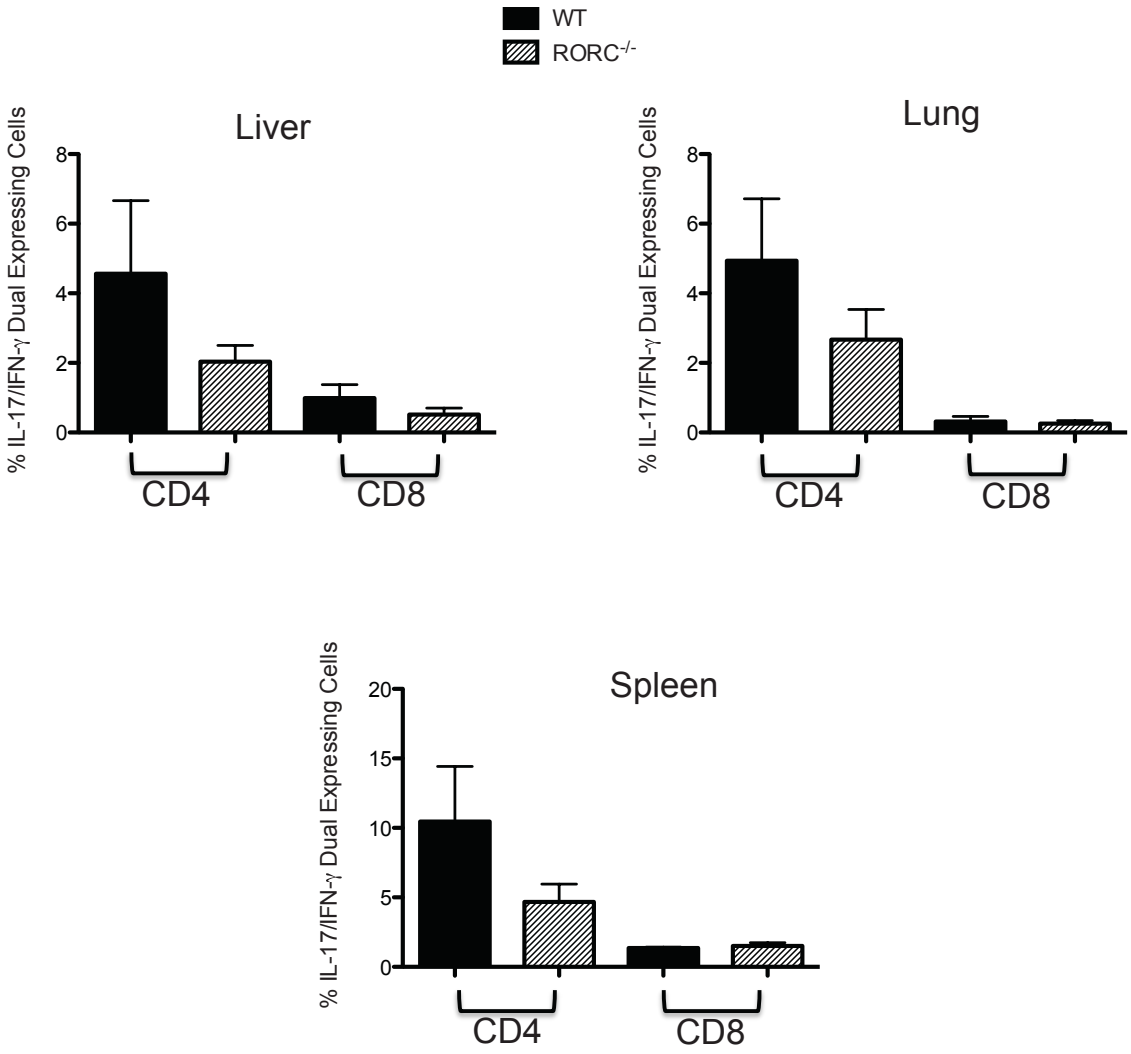
Figure 2.7. Improved anti-tumor responses in the absence of *RORC*. Lethally irradiated B6D2 F1 mice were injected with 3 x 10^6 TCD BM with or without 4 x 10^6 WT B6D2 or *RORC*$^{-/-}$ Tconv cells. Additionally all recipient mice received 1 x 10^4 P815 cells with the BM inoculum. Survival was determined 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.

Supplementary Figure 2.1



Supplementary Figure 2.1. Attenuated GvHD using *RORC*$^{-/-}$ Tconv cells in minor mismatch MHC I GvHD model. Lethally irradiated BALB.B recipients were injected intravenously with 3 x 10^6 *RORC*$^{-/-}$ Tconv cells or WT Tconv cells supplemented with 5 x 10^6 WT TCD BM. $P<0.05$ difference in median survival using log rank test. GvHD score statistic determined using Mann-Whitney test. n=11 recipients receiving *RORC*$^{-/-}$ T cell recipients, n=8 for recipients of WT T cells, n=3 for bone marrow only recipients. *$p<0.05$

Supplementary Figure 2.2



Supplementary Figure 2.2. IL-17/IFN-γ Dual producing cells in GvHD target organs. The spleen, lung and liver were removed from B6D2 mice transplanted with WT TCD BM plus Tconv cells from WT or *RORC*$^{-/-}$ donors 12 days post transplantation and analyzed for the production of IL-17A and IFN-γ. n= 3 mice evaluated per each transplant group. Graphs indicate the percentage of cells producing IFN-γ and IL-17.

References

1. Forman SJ (1998) Stem cell transplantation in acute leukemia. Curr Opin Oncol 10: 10–16.

2. Khouri IF, Champlin RE (2004) Nonmyeloablative stem cell transplantation for lymphoma. Semin Oncol 31: 22–26.

3. Tsai TW, Freytes CO (1997) Allogenic bone marrow transplantation for leukemias and aplastic anemia. Adv Intern Med 42: 423–451.

4. Socié G, Blazar BR (2009) Acute graft-versus-host disease: from the bench to the bedside. Blood 114: 4327–4336. doi:10.1182/blood-2009-06-204669.

5. Beilhack A, Schulz S, Baker J, Beilhack GF, Wieland CB, et al. (2005) In vivo analyses of early events in acute graft-versus-host disease reveal sequential infiltration of T-cell subsets. Blood 106: 1113–1122. doi:10.1182/blood-2005-02-0509.

6. Dean RM, Bishop MR (2003) Graft-versus-host disease: emerging concepts in prevention and therapy. Curr Hematol Rep 2: 287–294.

7. Wysocki CA, Panoskaltsis-Mortari A, Blazar BR, Serody JS (2005) Leukocyte migration and graft-versus-host disease. Blood 105: 4191–4199. doi:10.1182/blood-2004-12-4726.

8. Shlomchik WD (2007) Graft-versus-host disease. Nat Rev Immunol 7: 340–352. doi:10.1038/nri2000.

9. Carayol G, Bourhis JH, Guillard M, Bosq J, Pailler C, et al. (1997) Quantitative analysis of T helper 1, T helper 2, and inflammatory cytokine expression in patients after allogeneic bone marrow transplantation: relationship with the occurrence of acute graft-versus-host disease. Transplantation 63: 1307–1313.

10. Imamura M, Hashino S, Kobayashi H, Kubayashi S, Hirano S, et al. (1994) Serum cytokine levels in bone marrow transplantation: synergistic interaction of interleukin-6, interferon-gamma, and tumor necrosis factor-alpha in graft-versus-host disease. Bone Marrow Transplant 13: 745–751.

11. Yang YG, Dey BR, Sergio JJ, Pearson DA, Sykes M (1998) Donor-derived interferon gamma is required for inhibition of acute graft-versus-host disease by interleukin 12. J Clin Invest 102: 2126–2135. doi:10.1172/JCI4992.

12. Murphy WJ, Welniak LA, Taub DD, Wiltrout RH, Taylor PA, et al. (1998) Differential effects of the absence of interferon-gamma and IL-4 in acute graft-versus-host disease after allogeneic bone marrow transplantation in mice. J Clin Invest 102: 1742–1748. doi:10.1172/JCI3906.

13. Jasperson LK, Bucher C, Panoskaltsis-Mortari A, Taylor PA, Mellor AL, et al. (2008)

Indoleamine 2,3-dioxygenase is a critical regulator of acute graft-versus-host disease lethality. Blood 111: 3257–3265. doi:10.1182/blood-2007-06-096081.

14. Mus AMC, Cornelissen F, Asmawidjaja PS, van Hamburg JP, Boon L, et al. (2010) Interleukin-23 promotes Th17 differentiation by inhibiting T-bet and FoxP3 and is required for elevation of interleukin-22, but not interleukin-21, in autoimmune experimental arthritis. Arthritis Rheum 62: 1043–1050. doi:10.1002/art.27336.

15. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, et al. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 441: 235–238. doi:10.1038/nature04753.

16. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, et al. (2006) Transforming growth factor-beta induces development of the T(H)17 lineage. Nature 441: 231–234. doi:10.1038/nature04754.

17. Nestel FP, Price KS, Seemayer TA, Lapp WS (1992) Macrophage priming and lipopolysaccharide-triggered release of tumor necrosis factor alpha during graft-versus-host disease. J Exp Med 175: 405–413.

18. Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, et al. (2008) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. Immunity 28: 29–39. doi:10.1016/j.immuni.2007.11.016.

19. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, et al. (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 126: 1121–1133. doi:10.1016/j.cell.2006.07.035.

20. Bucher C, Koch L, Vogtenhuber C, Goren E, Munger M, et al. (2009) IL-21 blockade reduces graft-versus-host disease mortality by supporting inducible T regulatory cell generation. Blood 114: 5375–5384. doi:10.1182/blood-2009-05-221135.

21. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, et al. (2009) Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. J Clin Invest 119: 3573–3585. doi:10.1172/JCI40202.

22. Wolk K, Witte E, Witte K, Warszawska K, Sabat R (2010) Biology of interleukin-22. Semin Immunopathol 32: 17–31. doi:10.1007/s00281-009-0188-x.

23. Ma H-L, Liang S, Li J, Napierata L, Brown T, et al. (2008) IL-22 is required for Th17 cell-mediated pathology in a mouse model of psoriasis-like skin inflammation. J Clin Invest 118: 597–607. doi:10.1172/JCI33263.

24. Kolls JK, Khader SA (2010) The role of Th17 cytokines in primary mucosal immunity. Cytokine Growth Factor Rev 21: 443–448. doi:10.1016/j.cytogfr.2010.11.002.

25. Kappel LW, Goldberg GL, King CG, Suh DY, Smith OM, et al. (2009) IL-17

contributes to CD4-mediated graft-versus-host disease. Blood 113: 945–952. doi:10.1182/blood-2008-08-172155.

26. Yi T, Zhao D, Lin C-L, Zhang C, Chen Y, et al. (2008) Absence of donor Th17 leads to augmented Th1 differentiation and exacerbated acute graft-versus-host disease. Blood 112: 2101–2110. doi:10.1182/blood-2007-12-126987.

27. Carlson MJ, West ML, Coghill JM, Panoskaltsis-Mortari A, Blazar BR, et al. (2009) In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. Blood 113: 1365–1374. doi:10.1182/blood-2008-06-162420.

28. Sun K, Welniak LA, Panoskaltsis-Mortari A, O'Shaughnessy MJ, Liu H, et al. (2004) Inhibition of acute graft-versus-host disease with retention of graft-versus-tumor effects by the proteasome inhibitor bortezomib. Proc Natl Acad Sci USA 101: 8120–8125. doi:10.1073/pnas.0401563101.

29. Coghill JM, Carlson MJ, Panoskaltsis-Mortari A, West ML, Burgents JE, et al. (2010) Separation of graft-versus-host disease from graft-versus-leukemia responses by targeting CC-chemokine receptor 7 on donor T cells. Blood 115: 4914–4922. doi:10.1182/blood-2009-08-239848.

30. Serody JS, Burkett SE, Panoskaltsis-Mortari A, Ng-Cashin J, McMahon E, et al. (2000) T-lymphocyte production of macrophage inflammatory protein-1alpha is critical to the recruitment of CD8(+) T cells to the liver, lung, and spleen during graft-versus-host disease. Blood 96: 2973–2980.

31. Blazar BR, Taylor PA, McElmurry R, Tian L, Panoskaltsis-Mortari A, et al. (1998) Engraftment of severe combined immune deficient mice receiving allogeneic bone marrow via In utero or postnatal transfer. Blood 92: 3949–3959.

32. van Den Brink MR, Moore E, Horndasch KJ, Crawford JM, Hoffman J, et al. (2000) Fas-deficient lpr mice are more susceptible to graft-versus-host disease. J Immunol 164: 469–480.

33. Kaplan EL, Meier P (2012) Nonparametric Estimation from Incomplete Observations. Journal of the American Statistical Association 53: 457–481. doi:doi: 10.1080/01621459.1958.10501452.

34. Wysocki CA, Burkett SB, Panoskaltsis-Mortari A, Kirby SL, Luster AD, et al. (2004) Differential roles for CCR5 expression on donor T cells during graft-versus-host disease based on pretransplant conditioning. J Immunol 173: 845–854.

35. Iclozan C, Yu Y, Liu C, Liang Y, Yi T, et al. (2010) T helper17 cells are sufficient but not necessary to induce acute graft-versus-host disease. Biol Blood Marrow Transplant 16: 170–178. doi:10.1016/j.bbmt.2009.09.023.

36. Hill GR, 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.

37. Rangachari M, Mauermann N, Marty RR, Dirnhofer S, Kurrer MO, et al. (2006) T-bet negatively regulates autoimmune myocarditis by suppressing local production of interleukin 17. J Exp Med 203: 2009–2019. doi:10.1084/jem.20052222.

38. Yu Y, Wang D, Liu C, Kaosaard K, Semple K, et al. (2011) Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORγt in mice. Blood 118: 5011–5020. doi:10.1182/blood-2011-03-340315.

39. Hori K, Ehrke MJ, Mace K, Mihich E (1987) Effect of recombinant tumor necrosis factor on tumoricidal activation of murine macrophages: synergism between tumor necrosis factor and gamma-interferon. Cancer Res 47: 5868–5874.

CHAPTER THREE

ALTERED T CELL ENTRY AND EGRESS IN THE ABSENCE OF CORO 1A ATTENUATES MURINE ACUTE GRAFT VERSUS HOST DISEASE[1]

3.1 Introduction

Acute graft-versus-host disease (aGvHD), a disease of selective epithelial damage, is a severe complication of allogeneic stem cell transplantation. aGvHD occurs when mature donor T cells recognize host alloantigen and initiate an immune response[1]. Work from our group and others has shown that prior to tissue destruction donor T cells must migrate to secondary lymphoid tissue where they are activated by host antigen presenting cells (APCs). Upon activation, donor T cells migrate to target organs primarily the liver, gastrointestinal tract, and lung, where they cause tissue damage and destruction characteristic of aGvHD[2].

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Migration of lymphocytes to target organs involves selectins, integrins, and small chemotactic proteins known as chemokines[3]. Chemokines bind a family of G-protein coupled receptors, C-C chemokine receptors, that direct the migration of lymphocytes to target locations. Our group has demonstrated the importance of migration of donor T cells and their interaction with APCs in GvHD pathogenesis[4]. Furthermore the importance of secondary lymphoid tissue (SLT) in GvHD pathogenesis has been demonstrated as animals lacking all SLT including the spleen display markedly attenuated GvHD[5,6].

Numerous biological processes are regulated by the actin cytoskeleton and its associated proteins. The coronin family of actin-associated proteins has been shown to be involved in cell migration, motility, and cell survival[7]. Coronins bind F-actin and interact with the Arp2/3 complex[8] where they are critical in preventing nucleation of the branched F-actin chain. Coronin 1A (Coro 1A) was the first of the seven family members identified and is the most understood. Coro 1A is expressed primarily in hematopoietic cells and co-localizes with F-actin[9]. Expression of Coro 1A in T lymphocytes is important for cytoskeleton rearrangement[10-12]. Several groups have evaluated the function of immune cells from mice lacking Coro 1A. These studies have indicated that T cells from Coro 1A knockout mice do not function normally, although the mechanisms for this finding are still somewhat unclear and focus either on proximal signaling events after activation of the T cell receptor and/or the induction of apoptosis due to impaired generation of F-actin[11,12]. In addition, a third group evaluated the migration of thymocytes using mice with a point mutation in Coro 1A that led to hypomorphic function for Coro 1A. They demonstrated impaired migration of thymocytes from these mice in response to sphingosine 1 phosphate

leading to impaired thymic egress[13].

Reorganization of the actin cytoskeleton is an early response to chemokine receptor stimulation[14]. More recently chemokine receptors have been shown to regulate signaling molecules[14]. These molecules have been shown to be important for regulation of chemotaxis in lymphocytes and other cells[15,16]. More interestingly, the transcription factor nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) has been shown to be involved in the interaction between activation of T cells and changes in the cytoskeleton allowing for alterations in mobility[17]. However, the mechanisms by which these processes are linked is still not clear.

Currently, while multiple investigators have indicated that the complete absence of SLT including the spleen eliminated acute GvHD, it is not clear if GvHD would be impacted by the inability of donor T cells to egress from SLT. The previous work suggesting that the absence of Coro 1A led to impaired migration led us to investigate the biology of acute GvHD in a system where T cells could not enter or exit SLT. Here we show that aGvHD is completely eliminated by the inability of donor T cells to exit SLT and migrate to GvHD target organs. This is mediated by alterations in the activation of NF-κB in the absence of Coro 1A, which leads to significantly decreased expression of the critically important migratory proteins S1Pr1 and CCR7.

3.2 Methods

Mice

C57BL/6J ($H2^b$) (termed WT), BALB/c, and C57BL/6J x DBA/2 F1 (termed B6D2) were purchased from The Jackson Laboratory. The generation of enhanced green fluorescent protein expressing (GFP) C57BL/6 mice has been described previously[2]. Coro 1A deficient (Coro $1A^{-/-}$) C57BL/6 mice were obtained from Niko Foger and generated as described[11,18]. Coro $1A^{-/-}$ GFP mice were generated by crossing Coro $1A^{-/-}$ mice with GFP C57BL/6 mice. All experiments were performed in accordance with protocols approved by the University of North Carolina Institutional Animal Care and Use Committee.

Transplantation Models

T cell depleted bone marrow (TCD BM) was prepared as previously described[19]. CD25 depleted T cells were prepared using a total T cell isolation kit (Cedarlane Laboratories) followed by antibody depletion and magnetic cell separation as previously described[6]. Histopathology analyses were prepared as previously described and analyzed by one of us (A.P.M.) blinded to the genotype of the donor[20].

Stereomicroscopy

Organs from anesthetized animals were imaged with a Zeiss Stereo Lumar V12 microscope with GFP bandpass filter (Carl Zeiss MicroImaging, Inc.) at room temperature. AxioVision (Carl Zeiss) software was used to determine GFP intensities. WT GFP and Coro $1A^{-/-}$ GFP recipient organs were imaged using the identical magnification (mag) and exposure (exp) times for each time point. Day +3: PP-exp 976ms, mag 32X MLN-exp 2.5s, mag 15X Day +14: PP-exp 1s, mag 30X MLN-exp 1s, mag 20X Colon-exp 4s, mag 13X Liver-exp 2s,

mag 40X Lung-exp 4s, mag 18X Day +28: PP-exp 750ms, mag 30X MLN 600ms, mag 20X Colon-exp 3s, mag 13X Liver-exp 3s, mag 40X

Organ GPF Quantification

Organs from recipient animals were homogeneized and absolute GFP levels determine by ELISA (Cell Biolabs). Detailed experimental procedures were conducted as described previously[6].

***In Vivo* Competitive Migration Assay**

CD25 negative total T cells were isolated as described above from Coro $1A^{-/-}$ GFP and Thy 1.1^{+} WT mice. Recipient B6D2 mice were injected intravenously with equal amounts of Coro $1A^{-/-}$ GFP and WT Thy 1.1^{+} donor T cells. 16 hours post transplantation the mesenteric lymph node, inguinal lymph node, and spleen were stained and analyzed by flow cytometry.

Real Time PCR Analysis

Real time PCR was performed as previously described[20]. Gene expression was normalized to the housekeeping gene GusB before determining fold induction. Taqman expression assay probes for S1Pr1, S1Pr3, S1Pr5, and CCR7 were purchased from Applied Biosystems.

Chemotaxis Analysis

Naïve total T cells were isolated using Cedarlane total T cell isolation kit following by antibody depletion coupled with negative selection. Following isolation the cells were washed twice with PBS. 5×10^5 or 2×10^5 total T cells in 100µL were added to the upper chamber of a PVP treated 5µM pore polycarbonate membrane inside of a

ChemoTx® chamber system (Neuroprobe). The bottom chamber was filled with the indicated concentrations of sphingosine-1 phosphate (Sigma) or C-C motif chemokine 19 (Peprotech) and incubated for 3 hours at 37^{o}C. CyQuant cell quantification kit (Invitrogen) was used to determine cell migration from the upper chamber to the lower chamber.

Western Blot Analysis

Freshly isolated naïve T cells were lysed in RIPA (Invitrogen) buffer supplemented with protease and phosphatase inhibitors (Roche). Lysates were separated by SDS-PAGE on a 4-

12% Bis-Tris gel (Life Technologies), transferred onto a nitrocellulose membrane and incubated in 5% non-fat dry milk to block non-specific binding. Membranes were incubated with the following antibodies purchased from Cell Signaling Technology: phospho NF-||B p65 (Ser536), NF-||B2 p100/p52. GAPDH antibody was purchased from Santa Cruz Biotechnology. Proteins were detected using anti-rabbit IgG HRP (Promega) and the ECL western blotting detection kit according to manufacturer's instructions (GE Healthcare).

***In Vivo* Proliferation**

Lethally irradiated B6D2 recipients were transplanted with equal amounts of Coro 1A$^{-/-}$ GFP and WT Thy 1.1^{+} donor total T cells concurrently with WT TCD BM. 10 days post transplantation recipient mice were injected intraperitoneally with BrdU labeling reagent (Invitrogen). 4 hours after injection the spleens were harvested and stained for BrdU (Invitrogen) and the following antibodies from eBioscience: CD45, CD44, CD62L, Thy 1.1.

GvHD Scoring

Mice were observed twice weekly for clinical GVHD signs and symptoms based on a previously established clinical scoring system[21].

Statistical Analysis

Survival curves were constructed using the Kaplan Meier method. Median survival was determined using the log rank test. Continuous values including cytokine levels, total cell numbers, and GFP expression were determined using two-tailed Student's T test. P values less that 0.05 were considered significant. Error bars represent standard error of the mean.

3.3 Results

3.3.1 Attenuated GvHD in Multiple Mouse Models Using Coro $1A^{-/-}$ T Cells

The expression of Coronin 1A (Coro 1A) has been shown to be important for T cell trafficking[12] however the contribution of Coro 1A to disease pathophysiology remains unclear[13]. To address the importance of Coro 1A in aGvHD, conventional T cells (T_{cons}) from Coro $1A^{-/-}$ or C57BL/6 (WT) donors supplemented with WT T cell depleted bone marrow (TCD BM) cells were transplanted into lethally irradiated B6D2 F1 recipients. As shown in figure 1A, recipients of Coro $1A^{-/-}$ T_{cons} had significantly improved survival compared to recipients of WT donor T_{cons}. Clinical GvHD score confirmed survival data demonstrating decreased clinical symptoms in recipients of Coro $1A^{-/-}$ compared WT T cell recipients (Fig 3.1A).

We further evaluated the importance of Coro 1A in GvHD using a complete mismatch (B6 into BALB/c) model. Lethally irradiated BALB/c recipients were administered T_{cons} from Coro $1A^{-/-}$ or WT donors with WT TCD BM. Similar to the haploidentical model, recipients of Coro $1A^{-/-}$ T_{cons} showed increased survival with minimal clinical manifestations of GvHD (Fig 3.1B). Histopathology analysis of GvHD target organs revealed decreased pathology in Coro $1A^{-/-}$ recipients compared to WT recipients (Fig 3.2) with a significant difference in the liver and spleen. Coro $1A^{-/-}$ T_{con} recipients also displayed decreased organ cytokine production on day 14 with the values for pro-inflammatory cytokine production in the liver being statistically significant (Sup Fig 3.1A). Thus, the absence of Coro 1A from donor T cells led to a profound decrease in the generation of acute GvHD in two different models.

3.3.2 *In Vivo* Activation of Coro 1A$^{-/-}$ T Cells

Next we wished to determine mechanisms for the diminished GvHD found after the infusion of donor T cells lacking Coro 1A. Previous work suggested two potential mechanisms. T cells deficient in Coro 1A may be impaired in activation mediated by engagement of the T cell receptor[11], or Coro 1A$^{-/-}$ T cells may be impaired in the ability to migrate in and out of lymphoid tissue[13]. Thus, we analyzed T cell proliferation using BrdU and the expression of CD62L and CD44 from Coro 1A$^{-/-}$ or WT donor T cells isolated from lethally irradiated B6D2 recipients. There was no difference in the proliferation of donor WT versus Coro 1A$^{-/-}$ T cells isolated from the spleen as assessed on day 10 post transplant (Sup Fig 3.1B). However, we did find differences in the percentage of activated donor T cells in the mesenteric lymph node (MLN) and Peyer's Patch (PP) (Fig 3.3). Expression of CD44 was decreased in the MLN of Coro 1A$^{-/-}$ recipients compared to WT T cell recipients although no difference was seen in T cells isolated from the PPs of recipient animals. Similarly the percentage of donor T cells that had downregulated CD62L was significantly less from B6D2 mice given donor Coro 1A null compared to WT T_{cons} cells.

Next, to determine if the difference in the expression of CD62L was due to intrinsic differences in T cell activation, we evaluated T cell activation and proliferation *in vitro*. Interestingly, we did not find a difference in the expression of CD62L, the dilution of CFSE or the generation of IFN-© by T cells from WT compared to Coro 1A null mice (data not shown) activated using either allogeneic APCs or antibodies specific for CD3 and CD28. These data suggest that there is an extrinsic problem with the activation of donor T cells in the absence of Coro 1A.

3.3.3 Accumulated Coro 1A$^{-/-}$ T Cells in Gastrointestinal Tract Lymph Nodes

Donor T cell activation requires the migration of donor cells into the spleen and secondary lymphoid tissue of the host. One hypothesis for the inability to activate donor T cells from Coro 1A null donors is the inability of those cells to migrate to SLT. To determine if migration defects contributed to attenuated GvHD using Coro 1A$^{-/-}$ donor T cells we crossed Coro 1A$^{-/-}$ mice with mice that constitutively express enhanced green fluorescent protein (GFP). Using Coro 1A$^{-/-}$ GFP and WT GFP mice as donors lethally irradiated B6D2 recipients were administered T_{cons} with WT (non GFP) TCD BM. 3 days post transplantation the mesenteric MLN and PP were imaged by stereomicroscopy. Increased GFP expression in the MLN was seen in WT GFP recipients as compared to Coro 1A$^{-/-}$ GFP recipients, suggesting a delay in entry into lymph nodes by Coro 1A$^{-/-}$ T cells (Fig 3.4A). Surprisingly, accumulation of Coro 1A$^{-/-}$ T cells was seen in the MLN and PP of B6D2 recipients 14 days post transplantation which continued through 28 days post transplantation (Fig 3.4A). Consistent with the accumulation seen in the MLN and PP there was a decrease in donor T cells in the liver and colon of Coro 1A$^{-/-}$ T cells as measured by stereomicroscopy and GFP ELISA (Fig 3.4A and B). Migration defects displayed by microscopy were complemented by blood analysis on day 14 post transplantation that revealed a decrease in circulating T cells in B6D2 recipients given T cells from Coro 1A$^{-/-}$ GFP donors compared to WT GFP T cell donors (Fig 3.4C). These data were consistent with impaired entry and egress into lymph nodes by Coro 1A$^{-/-}$ T cells.

To further confirm a defect in entry into and out of lymphoid organs we performed an *in vivo* competitive migration assay. Equal amounts of Coro 1A$^{-/-}$ GFP and WT Thy 1.1^{+} T

cells were injected into lethally irradiated B6D2 recipients. 16 hours post transplantation the MLN and inguinal lymph nodes (ILN) were harvested and analyzed by flow cytometry. As demonstrated in figure 3.4D, even at this early time point Coro $1A^{-/-}$ T cells were markedly less efficient in entering the MLN and ILN as compared to WT T cells.

3.3.4 Decreased SLT Ingress and Egress Receptors In Coro $1A^{-/-}$ T Cells

Numerous researchers have shown that the C-C chemokines receptor type 7 (CCR7) is important for entry of T cells into secondary lymphoid organs[22]. Furthermore, data from our laboratory has highlighted the importance of CCR7 in migration and GvHD pathogenesis[6]. As Coro $1A^{-/-}$ T cells displayed defects in lymph node entry we questioned if Coro $1A^{-/-}$ T cells had decreased CCR7 expression. To address this question real time analysis was performed on freshly isolated Coro $1A^{-/-}$ and WT T cells. Surprisingly, Coro $1A^{-/-}$ T cells expressed 2 fold less CCR7 than WT T cells (Fig 3.5A). This decrease in CCR7 was further confirmed using an *in vitro* chemotaxis assay to the CCR7 ligand, CCL19. Similar to the real time data results, Coro $1A^{-/-}$ T cells displayed impaired migration to CCL19 (Fig 3.5A). Responsiveness of Coro $1A^{-/-}$ T cells to a chemoattractant was confirmed using the supernatant from stimulated allogeneic dendritic cells (Sup Fig 3.1C).

In addition to the impaired migration into secondary lymphoid tissue, Coro 1A deficient T cells were unable to egress out of lymphoid tissue similar to WT T cells. Sphingosine-1 phosphate (S1P) is a signaling sphingolipid that is produced by hematopoietic cells that has been shown to be important for lymphocyte egress from SLT. Of the 5 S1P receptors S1Pr1 has been shown to be important for lymphocyte egress[23]. To evaluate S1P receptor expression in Coro $1A^{-/-}$ T cells we used quantitative real time PCR analysis. Coro

1A$^{-/-}$ naïve T cells displayed decreased S1Pr1 expression compared to WT naïve T cells (Fig 3.5C). However, no difference was found in the expression of the other S1P receptors (Fig 3.5b and data not shown) on Coro 1A deficient T cells. Additionally, we demonstrated a marked impairment in the migration of Coro 1A$^{-/-}$ T cells to S1P compared to WT T cells indicating that this difference in expression led to functional differences in response to the ligand. These data indicate that decreased CCR7 and S1Pr1 expression on Coro 1A$^{-/-}$ T cells correlated with the decreased migration into and out of lymphoid organs.

3.3.5 Disruption of the NF-κB Pathway in the Absence of Coro 1A

To investigate the mechanism for the diminished expression of CCR7 and S1Pr1 by Coro 1A$^{-/-}$ T cells, we analyzed signaling pathways in naïve T cells. Regulation of the integrity of the actin cytoskeleton is important for numerous signaling pathways including the NF-κB and the mitogen-activated protein kinase (MAPK) pathways[24,25]. Decreased phosphorylated p65 was found under stimulating and non-stimulating conditions in Coro 1A$^{-/-}$ naïve T cells (Fig 3.6A). Alterations in the NF-κB pathway were specific to the canonical pathway as no changes in the p100 subunit were observed in WT or Coro 1A$^{-/-}$ T cells (Fig 3.6A). We found no difference in activation using TNF or anti-CD3/anti-CD28 mAb of p38 in Coro 1A null compared to WT T cells as evaluated by Western blot (Supp Fig 3.2). Thus, the diminished expression of CCR7 and S1P1r correlated with impaired activation of the canonical NF-κB pathway[26].

3.3.6 Modest GvL Response using Coro $1A^{-/-}$ T_{cons}

T cells responsible for the pathogenesis of aGvHD are also responsible for the graft-versus-leukemia effect (GvL) that eliminates residual tumor cells in host recipients. Knowingly, we investigated whether Coro $1A^{-/-}$ T_{cons} maintain GvL effects. WT TCD BM with P815 murine mastocytoma cells were transplanted into lethally irradiated B6D2 recipients with either Coro $1A^{-/-}$ T_{cons} or WT T_{cons}. There was a modest improvement in overall survival in B6D2 recipients given T cells from Coro $1A^{-/-}$ donors. While all mice given WT donor T cells succumbed to GvHD on day 20 post transplant, recipients of Coro $1A^{-/-}$ T cells died from tumor progression by day 30 post transplant. All mice given bone marrow alone also died by day 20 of tumor progression, demonstrating a statistically significant improvement in survival for recipients of Coro 1A deficient T cells ($p = 0.008$)(Fig 3.7).

3.4 Discussion

The contribution of T cell migration to aGvHD pathogenesis has been well studied[6,27]. Our group and others have shown that chemokines and their receptors, integrins, and selectins all play a critical role in T cell migration during aGvHD[4,28,29]. Here we extend these findings demonstrating that cells deficient in the F-actin associating protein, Coronin 1A, are markedly impaired in their ability to mediate acute GvHD across different MHC mismatched models. The diminished GvHD using T cells deficient in Coro 1A correlated with decreased tissue pathology in the GI tract and liver of recipient mice given T cells lacking Coro 1A. Coro 1A null T cells were impaired in activation *in vivo*, which correlated with the diminished ability to migrate into and then later exit SLT. Finally, we show that signaling cascades downstream of the TCR are impaired in the absence of Coro 1A. The reduction in the expression and function of CCR7 and S1Pr1 correlated with impaired activation of the canonical NF-κB pathway. Thus, these data suggest that one method of blocking acute GvHD is to prevent the migration of donor lymphocytes in and out of SLT.

The function of SLT in the biology of acute GvHD has been studied elegantly by several different investigators predominately using genetic approaches. These data indicated that secondary lymphoid tissue was critical to the induction of acute GVHD[5,30]. However, this activity was redundant with all secondary lymphoid tissue and the spleen capable of initiating acute GvHD. Thus, it has not been clear if this process would be amenable to clinical intervention. Here, we show that acute GvHD can be prevented by blocking the

migration of T cells into and out of secondary lymphoid tissue, which correlated with the impaired function of CCR7 and S1Pr1. Both CCR7 and S1Pr1 are potential targets for the prevention of aGvHD.

Several laboratories, but most specifically the Cyster laboratory, have shown in a number of elegant manuscripts the requirement for S1Pr1 expression on T cells for migration of those cells out of lymph nodes via the efferent lymph system[31]. The function of S1Pr1 is not limited to lymphocyte migration as S1Pr1 has also been shown to be important in inflammatory responses in other immune cells[32]. Similarly other S1P receptors have been suggested to function in T cell chemotaxis and migration[33]. Real time analysis of Coro $1A^{-/-}$ T cells confirmed decreased expression of S1Pr1 but not S1Pr3 or S1Pr5 in Coro $1A^{-/-}$ T cells. Interestingly, our group has previously evaluated the function of FTY720, which is an agonist of S1P that in models prevents acute GvHD pathogenesis[34]. While we were able to indicate that FTY720 administration could abrogate acute GvHD, this did not correlate with impaired egress of donor T cells from SLT. Thus, the current data are the first to indicate that egress out of SLT is important for the function of donor T cells during acute GvHD[35].

The importance of the chemokine receptor CCR7 in T lymphocyte migration has been well established. Data from our group demonstrated impaired donor T cell migration to secondary lymphoid organs of donor T cells lacking CCR7[6]. However, in our previous work we were unable to completely block acute GvHD in the major mismatch model by infusing T cells lacking CCR7. This indicates that the profound decrease in GvHD found after the infusion of T cells lacking Coro 1A in BALB/c recipients is not solely due to the

absence of CCR7. This would suggest that blocking migration in and out of secondary lymphoid tissue has a more profound effect than blocking the initial interaction of donor T cells with APCs.

One critical question from these evaluations is whether the impaired activation of donor Coro 1A deficient T cells is due to cell intrinsic or extrinsic factors or both. This was difficult for us to evaluate *in vivo* and we specifically sought *in vitro* evidence of cell intrinsic activation problems. However, using allogeneic APCs to stimulate Coro 1A deficient or WT T cells, we were not able to show impaired initial activation, proliferation or cytokine production by Coro $1A^{-/-}$ T cells (data not shown). Interestingly, there was a modest effect on T cell activation in the presence of anti-CD3 mAb suggesting that signaling downstream of the TCR may be altered in the absence of Coro 1A. Nevertheless, our data indicated that Coro 1A deficient T cells activated by APCs *in vitro* were able to proliferate and differentiate suggesting that impaired activation from these cells *in vivo* was cell extrinsic. How the absence of Coro 1A affects TCR signaling is not entirely clear and the focus of significant work in our and other laboratories.

Blocking of GvHD that mitigates the GvL response is not a successful strategy for improving allogeneic SCT. Loss of Coro 1A led to some retention of the GvL response although this was not similar to that found after allogeneic SCT. The targeting of Coro 1A may be difficult despite its presence solely in the hematopoietic compartment. However, CCR7 and S1P1r are viable pharmacological targets. It is our contention that the targeting of

these proteins may allow for a more robust anti-tumor response compared to blocking the function of Coro 1A.

In summary, we have found that the absence of Coro 1A in donor T cells markedly diminished the incidence and severity of acute GvHD. We demonstrate that Coro $1A^{-/-}$ T cells have impaired migration into and out of secondary lymphoid tissue, which correlated with diminished expression of CCR7 and S1P1r. These data indicate that approaches that prevent the migration of T cells into AND out of secondary lymphoid tissue may significantly impact the occurrence of acute GvHD.

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Authorship

Contribution: L.M.F designed and performed research and wrote the manuscript. J.M.C., M.L.W., and A.P.-M. performed experiments. N.F. provided reagents and reviewed the manuscript. J.E.B. and A.S.B. designed experiments and reviewed the manuscript. J.S.S. designed the research and wrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interest.

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Figure 3.1

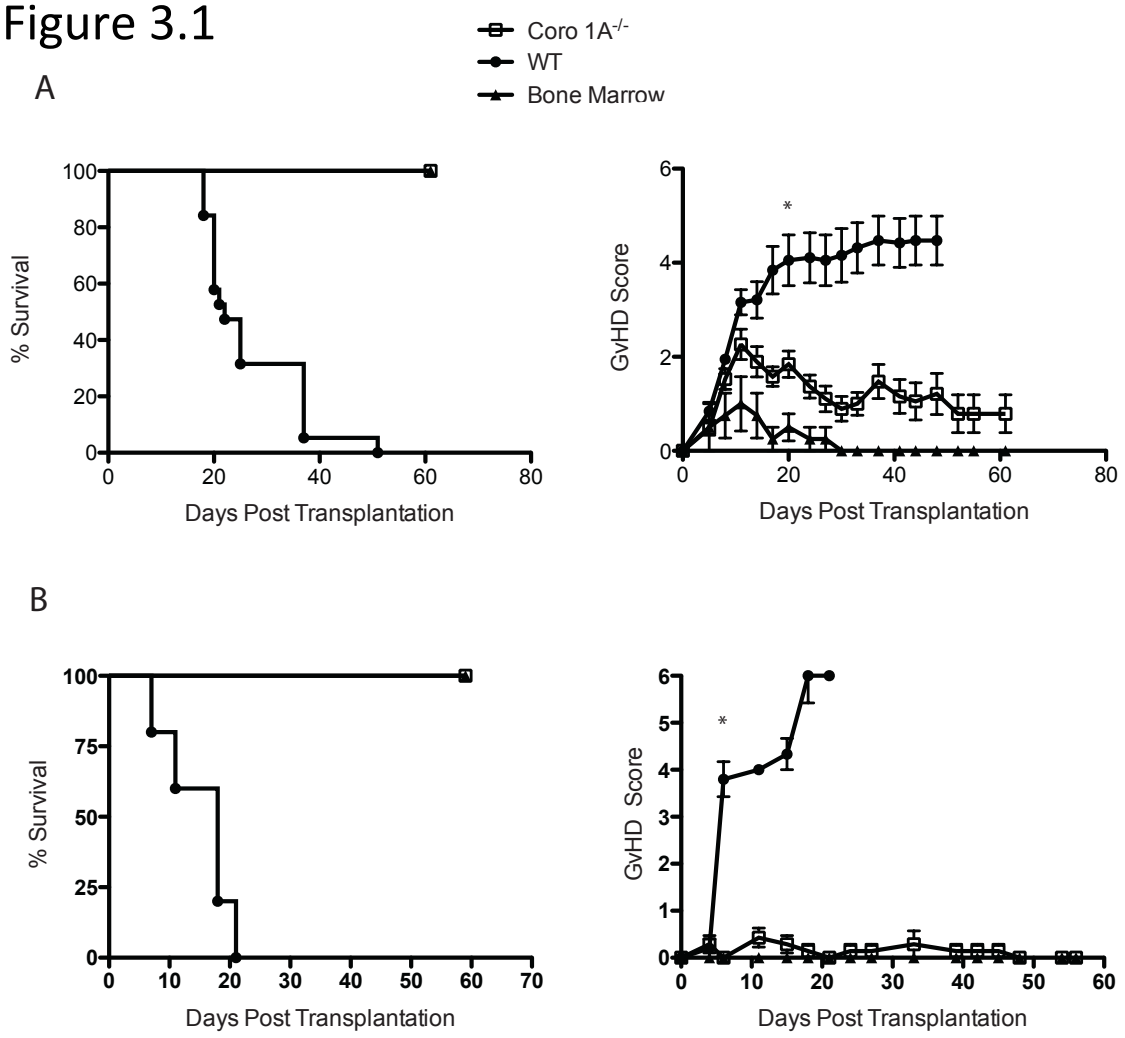


Figure 3.1. Attenuated GvHD in the Absence of Coro 1A. (A) 4 x 10^6 Coro $1A^{-/-}$ T cells (T_{cons}) or WT T cells supplemented with 3 x 10^6 WT T cell depleted bone marrow cells (TCD BM) were injected into lethally irradiated B6D2 recipients. n=14 for Coro $1A^{-/-}$ and WT T cell recipients. n=4 for bone marrow only. Data are pooled from 3 individual experiments (B) Lethally irradiated BALB/c recipients were infused with 5 x 10^5 Coro $1A^{-/-}$ or WT T_{cons} with 5 x 10^6 WT TCD BM cells. Following transplantation mice were monitored for survival and clinical GvHD development. n=19 for Coro $1A^{-/-}$ and WT T cell recipients. n=6 for bone marrow only. Data are pooled from 3 individual experiments. *$p<0.001$

Figure 3.2

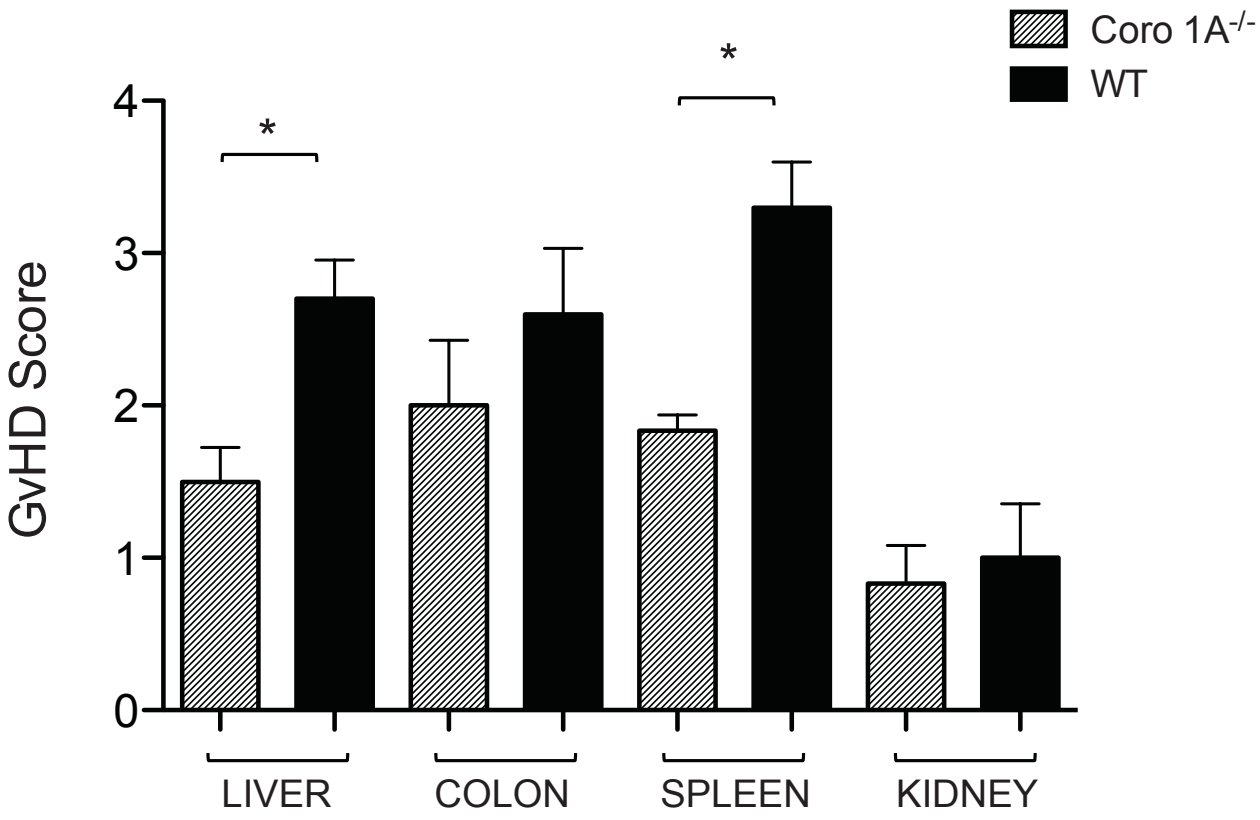


Figure 3.2. Histopathology of B6D2 recipients after transplantation of Coro 1A$^{-/-}$ or WT T Cells. Lethally irradiated B6D2 mice were transplanted with 4 x 10^6 Coro 1A$^{-/-}$ or WT T_{cons} with 3 x 10^6 TCD BM. 14 days post transplantation organs were harvested for pathology analyses. n=6 for Coro 1A$^{-/-}$ and WT recipients. n=4 for bone marrow controls. $p<0.05$

Figure 3.3

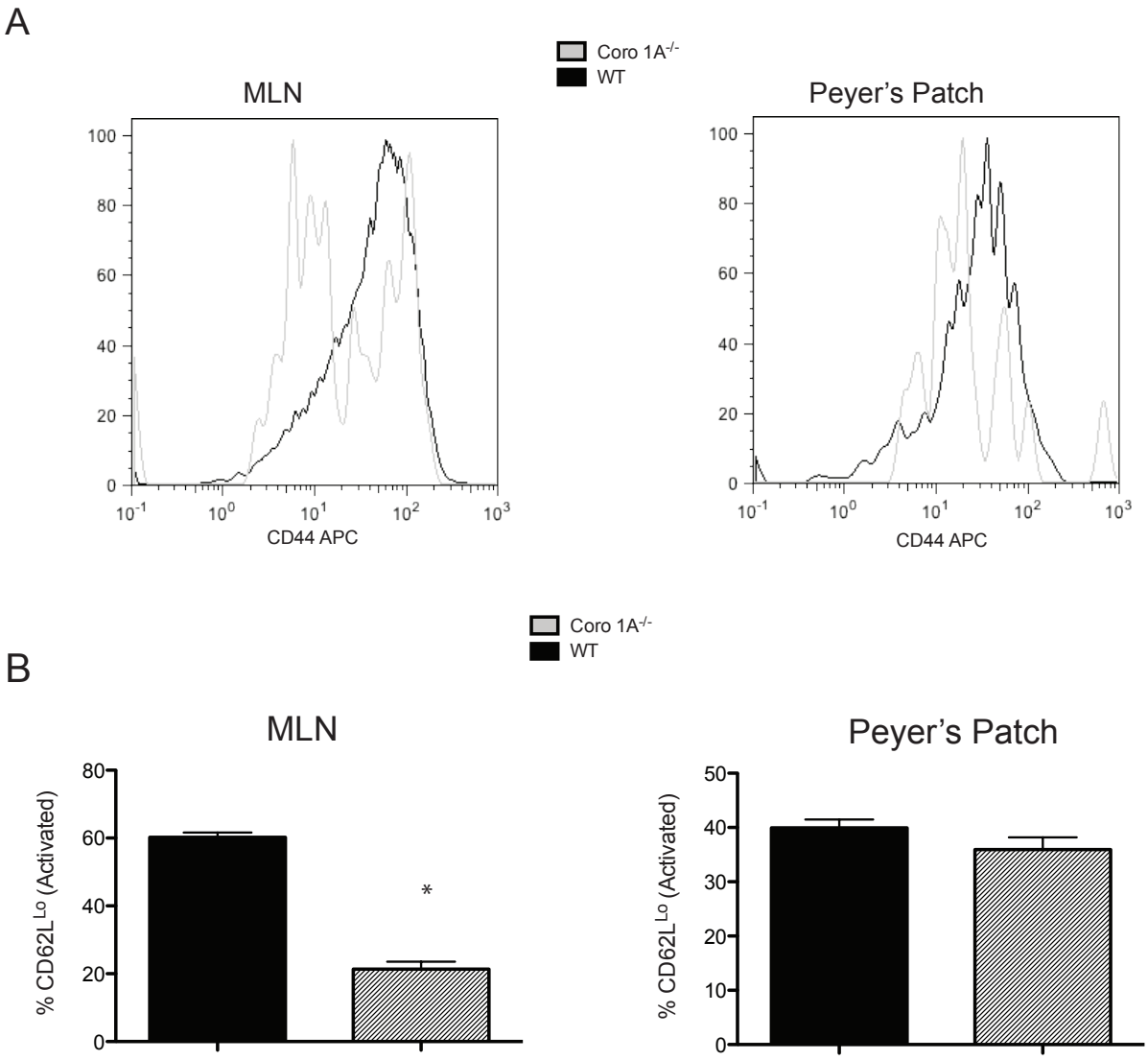
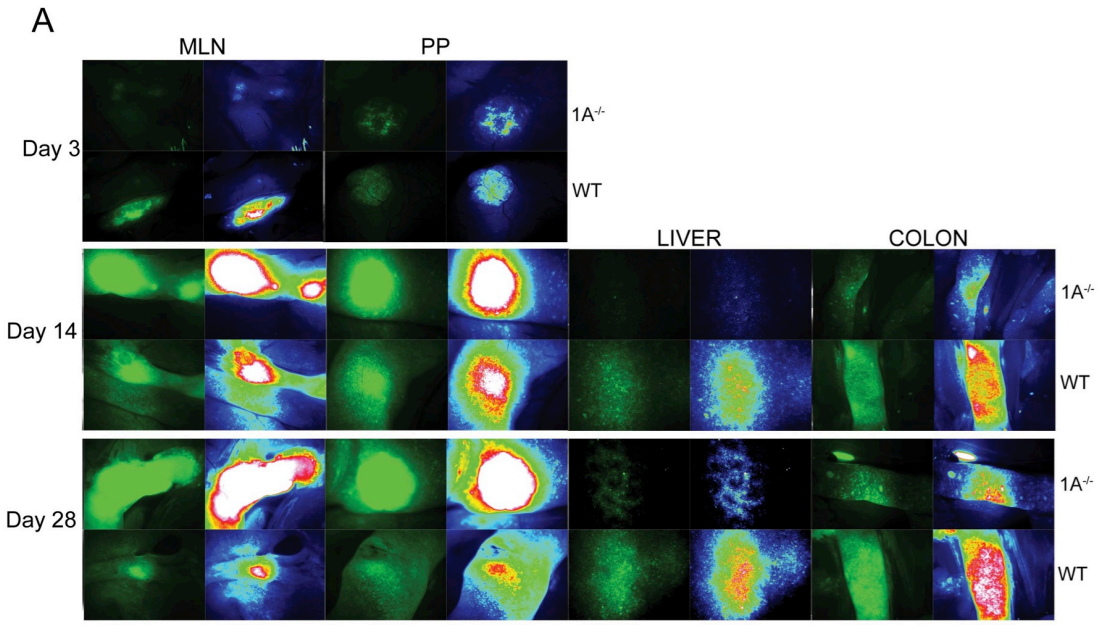


Figure 3.3. Decreased Activation *In Vivo* in the Absence of Coro 1A. Coro 1A$^{-/-}$ GFP or WT GFP T_{cons} cells were injected into lethally irradiated B6D2 mice. Mesenteric lymph nodes and Peyer's patches from WT and Coro 1A$^{-/-}$ recipients were harvested 3 days post transplantation. Donor (GFP^{+}) T cells were analyzed by flow cytometry for the expression of (A) CD44 and (B) CD62L. n=3 for Coro 1A$^{-/-}$ and WT recipients. *$p<0.05$

Figure 3.4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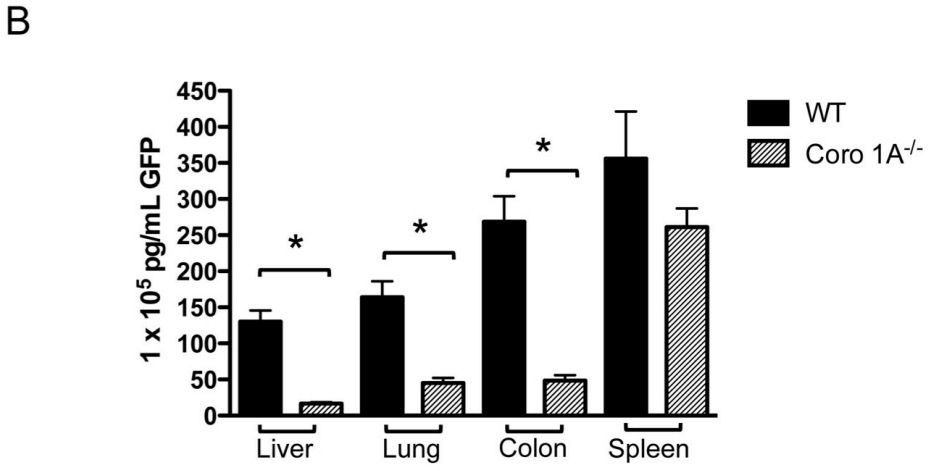
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Coro 1A-/-

Figure 3.4 cont

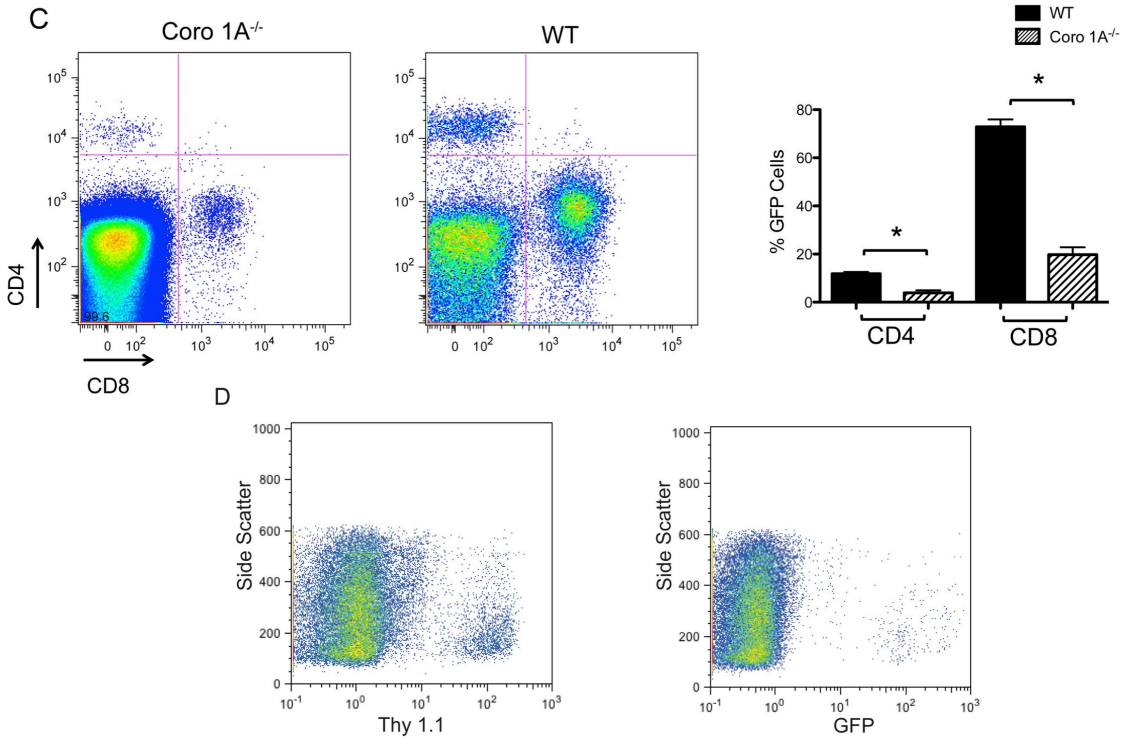


Figure 3.4. Delayed Entry and Impaired Egress out of Secondary Lymphoid Organs by Coro 1A$^{-/-}$ T Cells. Coro 1A$^{-/-}$ GFP or WT GFP T_{cons} supplemented with T cell depleted bone marrow cells were infused into lethally irradiated B6D2 recipients. (A) Migration of the cells to the liver, colon, mesenteric lymph node and Peyer's patches was determined using stereomicroscopy. Images were collected 3, 14 and 28 days post transplantation. Left panels display GFP expression while right panels reflex intensity. Data are representative of 6-8 B6D2 recipients given either Coro 1A$^{-/-}$ GFP or WT GFP recipients. (B) GFP ELISA was used to quantify GFP expression in B6D2 recipients 14 days post transplantation. n=5 for WT GFP or Coro 1A$^{-/-}$ GFP recipient. (C) Peripheral blood was collected from WT or Coro 1A$^{-/-}$ recipients 14 days post transplantation. T cells in the blood were evaluated by flow cytometry using CD4 and CD8. n=4 for Coro 1A$^{-/-}$ GFP recipients, n=3 for WT GFP recipients. *In vivo* competitive migration using Coro 1A$^{-/-}$ and WT (Thy 1.1) cells were performed as detailed in methods. (D) Flow cytometry analysis of Coro 1A$^{-/-}$ GFP and WT (Thy 1.1) T_{effs} 16 hours post transplantation.

Figure 3.5

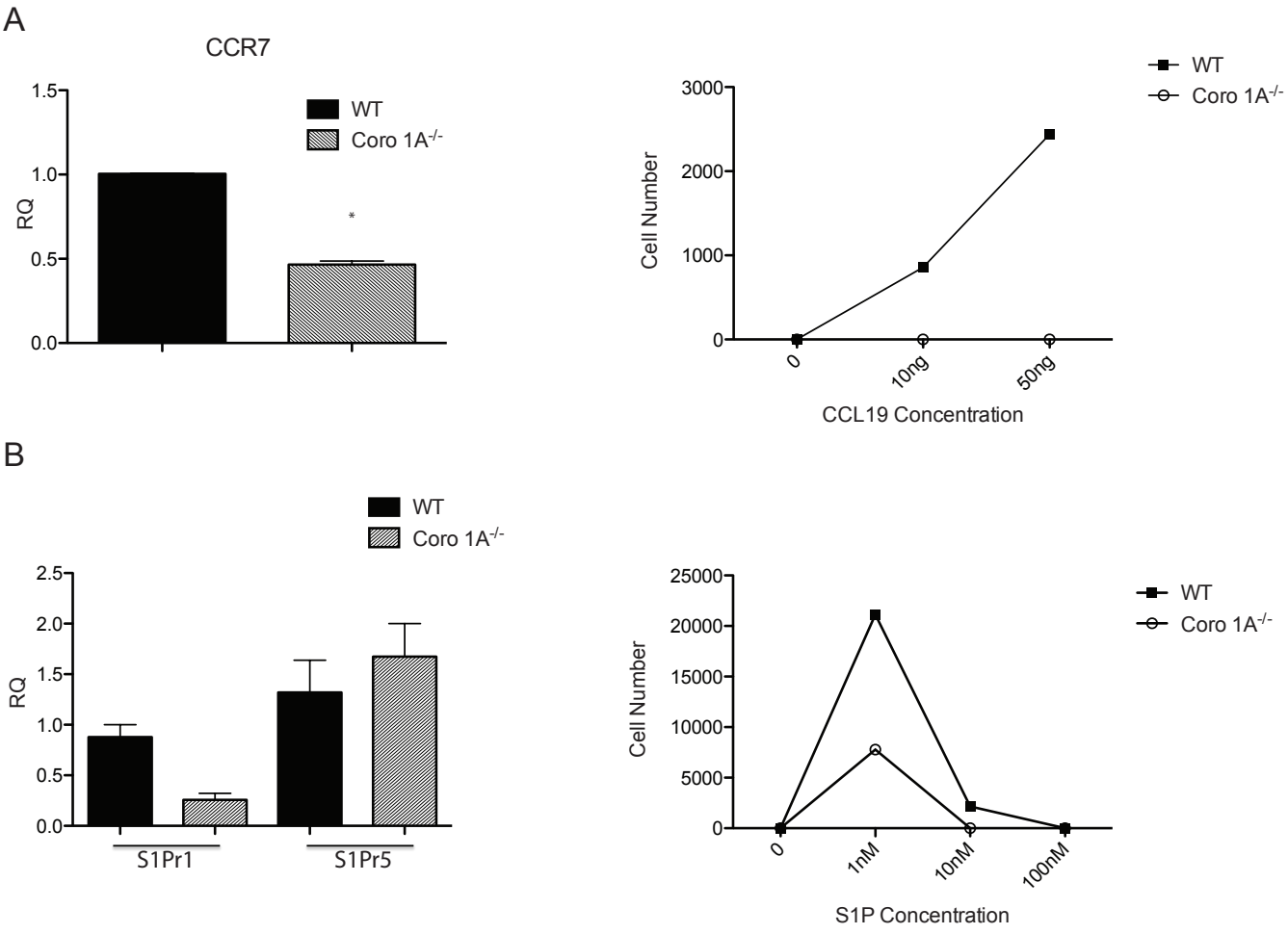


Figure 3.5. Decreased Receptor Expression and Impaired Chemotaxis in the Absence of Coro 1A. (A) Real time expression of CCR7 and chemotaxis to CCL19 of naïve WT or Coro $1A^{-/-}$ T cells (B) Real time expression of S1Pr1 and S1Pr5 and chemotaxis to S1P in WT and Coro $1A^{-/-}$ naïve T cells. *$p<0.05$. Data are representation of 2 individual experiments.

Figure 3.6

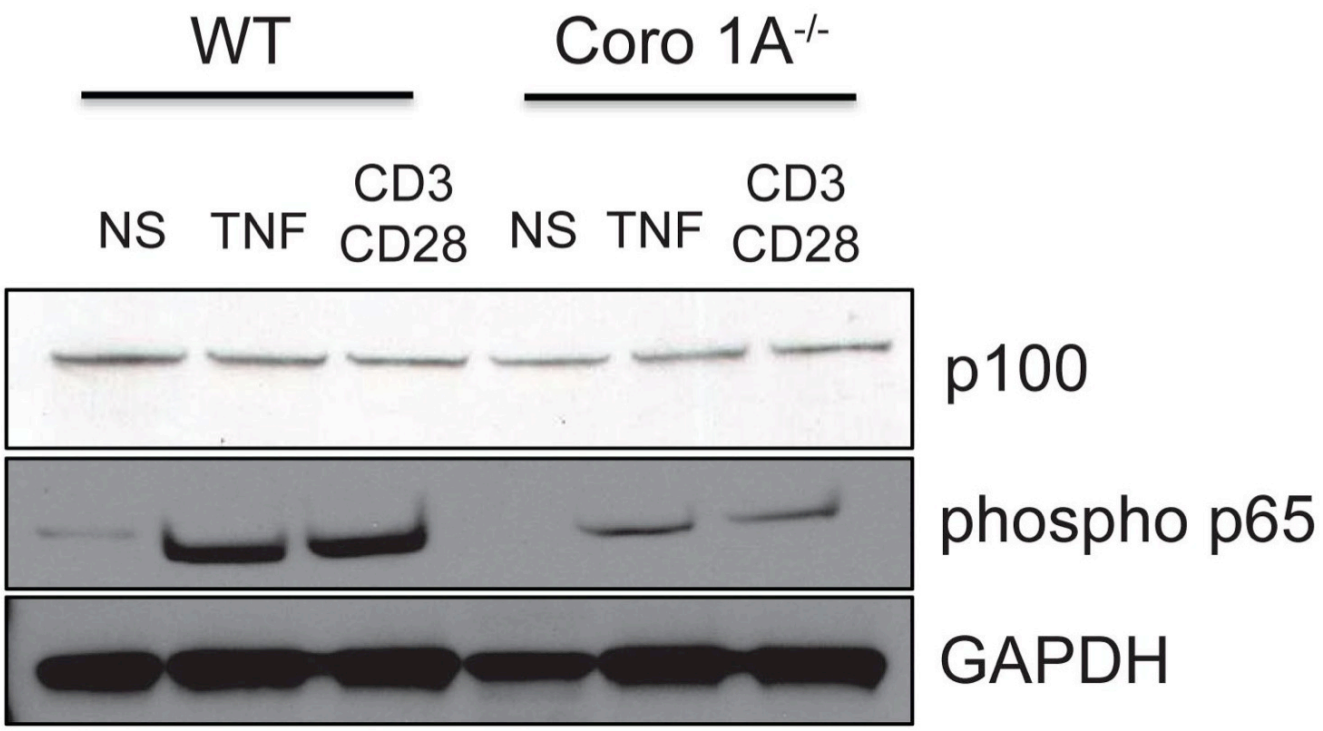


Figure 3.6. Decreased Activation of NF-κB in Coro 1A$^{-/-}$ T cells. 3 x 10^6 WT or Coro 1A$^{-/-}$ T cells were stimulated for 30 minutes with either 20ng/mL of TNF or 20ng/mL of anti-CD3 and 10ng/mL of anti-CD28. T cells were harvested and analyzed by western blot: p100 and phospho p65. Data are representative of 3 individual experiments.

Figure 3.7

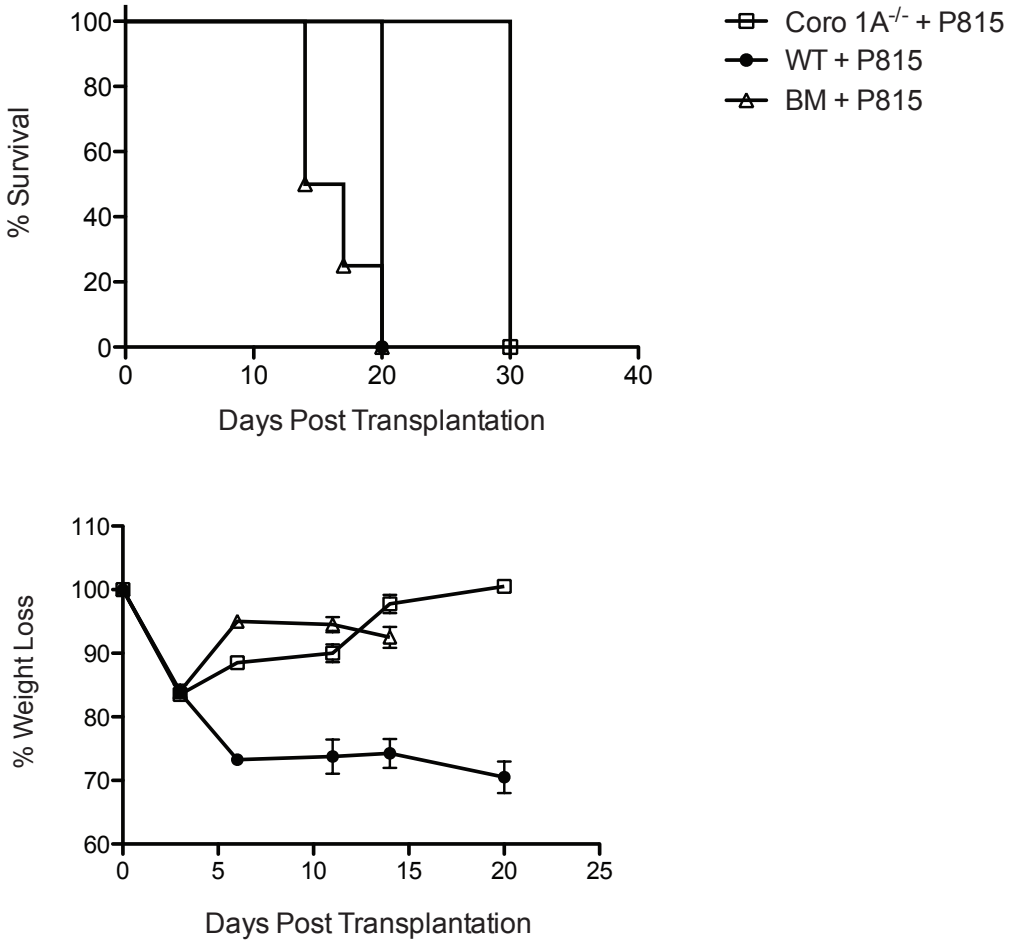
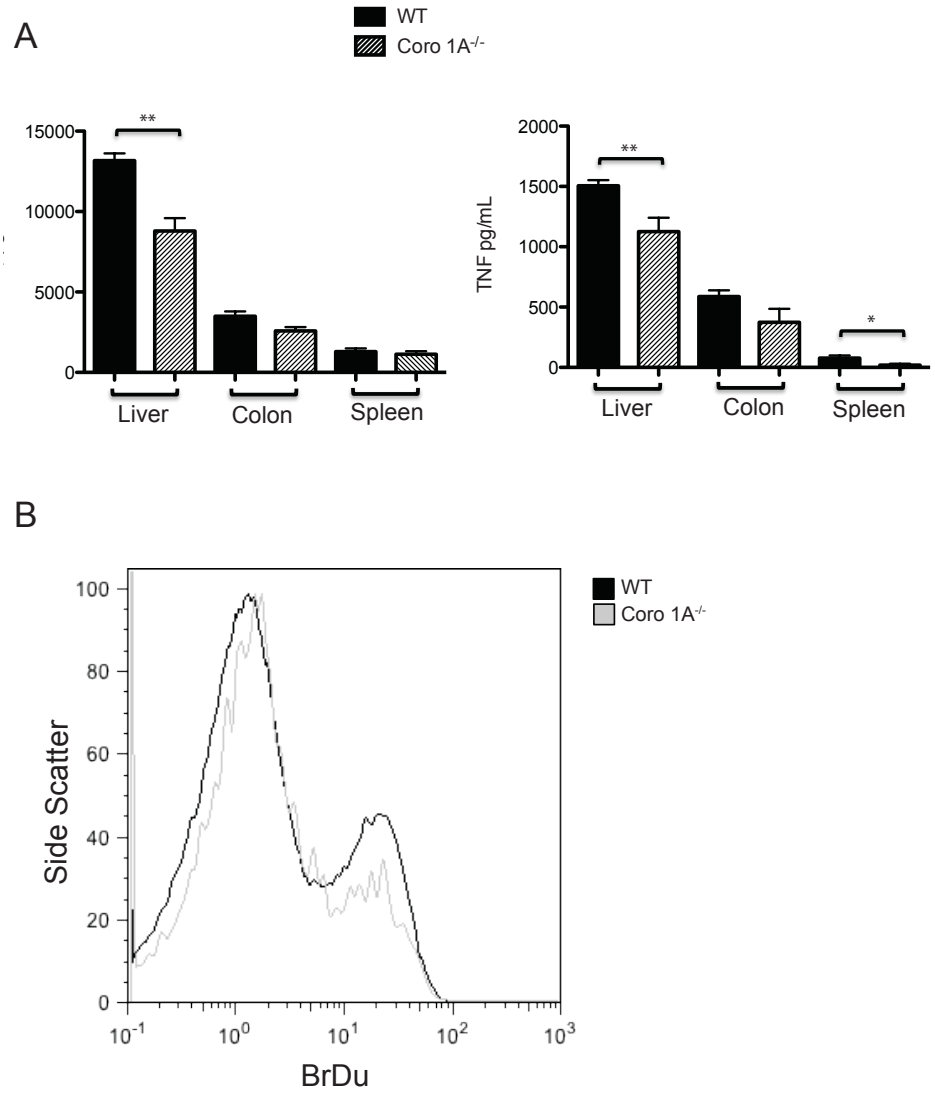
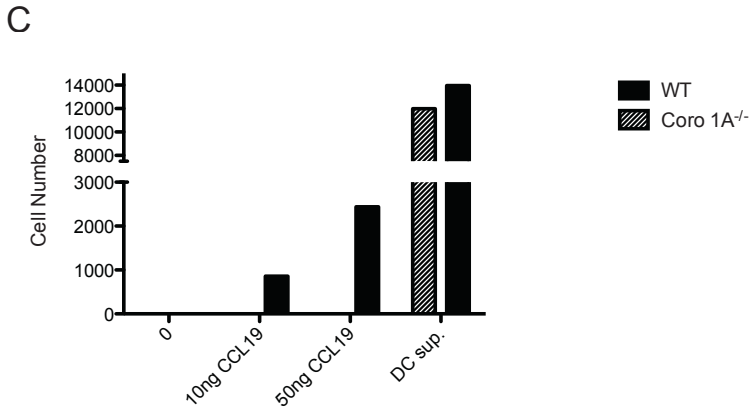


Figure 3.7. GvL Response using Coro 1A$^{-/-}$ T Cells. 2.5 x 10^4 P815 murine mastocytoma cells supplemented with TCD bone marrow cells were injected into lethally irradiated B6D2 recipients. Mice were also injected with either Coro 1A$^{-/-}$ or WT T_{cons}. Mice were monitored for survival and tumor infiltration following transplantation. n=8 for Coro 1A$^{-/-}$ or WT T cell recipient. n=7 for P815 + bone marrow. Data are pooled from two individual experiments.

Supplementary Figure 3.1

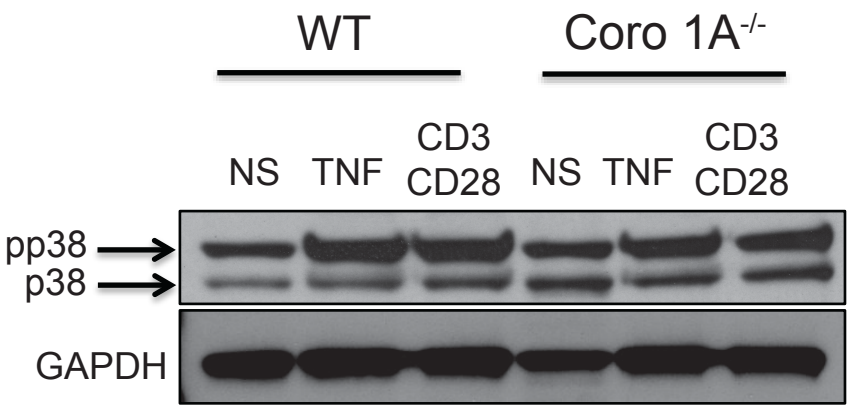


Supplementary Figure 3.1 contd



Supplementary Figure 3.1. Cytokine Production, Proliferation, and Chemotaxis of Coro 1A$^{-/-}$ T cells. Lethally irradiated B6D2 recipients were injected with T_{cons} from Coro 1A$^{-/-}$ or WT donors supplemented with WT TCD BM. (A) 14 days post transplantation animals were perfused and organs were harvested and homogenized for cytokine production by ELISA. n=5 for Coro 1A$^{-/-}$ or WT (B) 10 days post transplantation mice were injected with BrdU. 4 hours after injection spleens were harvested and analyzed by flow cytometery for BrdU uptake (C) Freshly isolated T cells from Coro 1A$^{-/-}$ mice were placed inside a chemotaxis chamber with indicated amounts of CCL19 or supernatant from bone marrow derived B6D2 dendritic cells.

Supplementary Figure 3.2



Supplementary Figure 3.2. p38 Expression in Coro 1A$^{-/-}$ T Cells. 3 x 10^6 Coro 1A$^{-/-}$ or WT T cells were stimulated for 30 minutes with either 20ng of soluble TNF or 20μg of anti-CD3 and 10μg of anti-CD28 antibodies. Following stimulation the cells were harvested and western blots performed as described in 'Methods'.

References

1. Reddy P (2003) Pathophysiology of acute graft-versus-host disease. Hematol Oncol 21: 149–161. doi:10.1002/hon.716.

2. Panoskaltsis-Mortari A, Price A, Hermanson JR, Taras E, Lees C, et al. (2004) In vivo imaging of graft-versus-host-disease in mice. Blood 103: 3590–3598. doi:10.1182/blood-2003-08-2827.

3. Moser B, Wolf M, Walz A, Loetscher P (2004) Chemokines: multiple levels of leukocyte migration control. Trends Immunol 25: 75–84. doi:10.1016/j.it.2003.12.005.

4. Wysocki CA, Jiang Q, Panoskaltsis-Mortari A, Taylor PA, McKinnon KP, et al. (2005) Critical role for CCR5 in the function of donor CD4+CD25+ regulatory T cells during acute graft-versus-host disease. Blood 106: 3300–3307. doi:10.1182/blood-2005-04-1632.

5. Beilhack A, Schulz S, Baker J, Beilhack GF, Nishimura R, et al. (2008) Prevention of acute graft-versus-host disease by blocking T-cell entry to secondary lymphoid organs. Blood 111: 2919–2928. doi:10.1182/blood-2007-09-112789.

6. Coghill JM, Carlson MJ, Panoskaltsis-Mortari A, West ML, Burgents JE, et al. (2010) Separation of graft-versus-host disease from graft-versus-leukemia responses by targeting CC-chemokine receptor 7 on donor T cells. Blood 115: 4914–4922. doi:10.1182/blood-2009-08-239848.

7. Pieters J (2008) Coronin 1 in innate immunity. Subcell Biochem 48: 116–123. doi:10.1007/978-0-387-09595-0_11.

8. Uetrecht AC, Bear JE (2006) Coronins: the return of the crown. Trends Cell Biol 16: 421–426. doi:10.1016/j.tcb.2006.06.002.

9. Nal B, Carroll P, Mohr E, Verthuy C, Da Silva M-I, et al. (2004) Coronin-1 expression in T lymphocytes: insights into protein function during T cell development and activation. Int Immunol 16: 231–240.

10. Ferrari G, Langen H, Naito M, Pieters J (1999) A coat protein on phagosomes involved in the intracellular survival of mycobacteria. Cell 97: 435–447.

11. Föger N, Rangell L, Danilenko DM, Chan AC (2006) Requirement for coronin 1 in T lymphocyte trafficking and cellular homeostasis. Science 313: 839–842. doi:10.1126/science.1130563.

12. Mueller P, Massner J, Jayachandran R, Combaluzier B, Albrecht I, et al. (2008) Regulation of T cell survival through coronin-1-mediated generation of inositol-1,4,5-trisphosphate and calcium mobilization after T cell receptor triggering. Nat Immunol 9: 424–431. doi:10.1038/ni1570.

13. Shiow LR, Roadcap DW, Paris K, Watson SR, Grigorova IL, et al. (2008) The actin regulator coronin 1A is mutant in a thymic egress-deficient mouse strain and in a patient with severe combined immunodeficiency. Nat Immunol 9: 1307–1315. doi:10.1038/ni.1662.

14. Wong MM, Fish EN (2003) Chemokines: attractive mediators of the immune response. Semin Immunol 15: 5–14.

15. Sotsios Y, Ward SG (2000) Phosphoinositide 3-kinase: a key biochemical signal for cell migration in response to chemokines. Immunol Rev 177: 217–235.

16. Curnock AP, Logan MK, Ward SG (2002) Chemokine signalling: pivoting around multiple phosphoinositide 3-kinases. Immunology 105: 125–136.

17. Baldwin AS (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 14: 649–683. doi:10.1146/annurev.immunol.14.1.649.

18. Föger N, Jenckel A, Orinska Z, Lee K-H, Chan AC, et al. (2011) Differential regulation of mast cell degranulation versus cytokine secretion by the actin regulatory proteins Coronin1a and Coronin1b. J Exp Med 208: 1777–1787. doi:10.1084/jem.20101757.

19. Wysocki CA, Burkett SB, Panoskaltsis-Mortari A, Kirby SL, Luster AD, et al. (2004) Differential roles for CCR5 expression on donor T cells during graft-versus-host disease based on pretransplant conditioning. J Immunol 173: 845–854.

20. Fulton LM, Carlson MJ, Coghill JM, Ott LE, West ML, et al. (2012) Attenuation of Acute Graft-versus-Host Disease in the Absence of the Transcription Factor RORγt. J Immunol. doi:10.4049/jimmunol.1200858.

21. van Den Brink MR, Moore E, Horndasch KJ, Crawford JM, Hoffman J, et al. (2000) Fas-deficient lpr mice are more susceptible to graft-versus-host disease. J Immunol 164: 469–480.

22. Förster R, Schubel A, Breitfeld D, Kremmer E, Renner-Müller I, et al. (1999) CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 99: 23–33.

23. Cyster JG, Schwab SR (2012) Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. Annu Rev Immunol 30: 69–94. doi:10.1146/annurev-immunol-020711-075011.

24. Wang Q, Doerschuk CM (2001) The p38 mitogen-activated protein kinase mediates cytoskeletal remodeling in pulmonary microvascular endothelial cells upon intracellular adhesion molecule-1 ligation. J Immunol 166: 6877–6884.

25. Fazal F, Minhajuddin M, Bijli KM, McGrath JL, Rahman A (2007) Evidence for actin cytoskeleton-dependent and -independent pathways for RelA/p65 nuclear

translocation in endothelial cells. J Biol Chem 282: 3940–3950. doi:10.1074/jbc.M608074200.

26. Kuwabara T, Tanaka Y, Ishikawa F, Kondo M, Sekiya H, et al. (2012) CCR7 ligands up-regulate IL-23 through PI3-kinase and NF-κ B pathway in dendritic cells. J Leukoc Biol 92: 309–318. doi:10.1189/jlb.0811415.

27. Wysocki CA, Panoskaltsis-Mortari A, Blazar BR, Serody JS (2005) Leukocyte migration and graft-versus-host disease. Blood 105: 4191–4199. doi:10.1182/blood-2004-12-4726.

28. Duffner U, Lu B, Hildebrandt GC, Teshima T, Williams DL, et al. (2003) Role of CXCR3-induced donor T-cell migration in acute GVHD. Exp Hematol 31: 897–902.

29. Carlson MJ, Fulton LM, Coghill JM, West ML, Burgents JE, et al. (2010) L-selectin is dispensable for T regulatory cell function postallogeneic bone marrow transplantation. Am J Transplant 10: 2596–2603. doi:10.1111/j.1600-6143.2010.03319.x.

30. Anderson BE, Taylor PA, McNiff JM, Jain D, Demetris AJ, et al. (2008) Effects of donor T-cell trafficking and priming site on graft-versus-host disease induction by naive and memory phenotype CD4 T cells. Blood 111: 5242–5251. doi:10.1182/blood-2007-09-107953.

31. Cyster JG (2005) Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol 23: 127–159. doi:10.1146/annurev.immunol.23.021704.115628.

32. Rosen H, Sanna MG, Cahalan SM, Gonzalez-Cabrera PJ (2007) Tipping the gatekeeper: S1P regulation of endothelial barrier function. Trends Immunol 28: 102–107. doi:10.1016/j.it.2007.01.007.

33. Rivera J, Proia RL, Olivera A (2008) The alliance of sphingosine-1-phosphate and its receptors in immunity. Nat Rev Immunol 8: 753–763. doi:10.1038/nri2400.

34. Taylor PA, Ehrhardt MJ, Lees CJ, Tolar J, Weigel BJ, et al. (2007) Insights into the mechanism of FTY720 and compatibility with regulatory T cells for the inhibition of graft-versus-host disease (GVHD). Blood 110: 3480–3488. doi:10.1182/blood-2007-05-087940.

35. Schwab SR, Cyster JG (2007) Finding a way out: lymphocyte egress from lymphoid organs. Nat Immunol 8: 1295–1301. doi:10.1038/ni1545.

CHAPTER FOUR

L-SELECTIN IS DISPENSABLE FOR T REGULATORY CELL FUNCTION POST ALLOGENEIC BONE MARROW TRANSPLANTATION[1]

4.1 Introduction

Allogeneic hematopoeitic stem cell transplantation (allo-HSCT) is a potentially curative therapy for numerous blood born malignant and non-malignant disorders[1,2]. Although allo-HSCT holds much promise, the prevalence of graft-versus-host disease (GvHD) limits its widespread use [3]. $CD4^+/CD25^+$ naturally occurring T regulatory (T_{reg}) cells offer a potential solution to the prevention of GvHD. Importantly, T_{regs} can suppress allo-reactive T cell responses, including those involved in solid organ and skin allograft rejection [4]. Multiple groups, including our own, have demonstrated that T_{regs} are capable of inhibiting GvHD without impacting the GvL response [5,6].

L-selectin (CD62L) is a member of the selectin family that is involved in leukocyte homing [7]. CD62L is constitutively expressed by myeloid cells, naïve lymphocytes and

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central memory T cells [7,8]. We, and others, have shown the importance of T_{reg} homing molecule expression in preventing GvHD [5,9,10]. These studies provided evidence that the phenotype of the $CD62L^{Hi}$ T_{reg} population was responsible for inhibition of GvHD, however, they did not directly assess the role of CD62L in this process. Decreased expression of CD62L may be indicative of a T_{reg} subset that is biologically distinct from $CD62L^{hi}$ T_{regs} independent of the function of CD62L.

Using a clinically relevant model of allo-HSCT we now show that CD62L expression by T_{regs} was not required for the inhibition of GvHD as $CD62L^{-/-}$ T_{regs} provided similar protection from lethal acute GvHD compared to WT T_{regs}. In addition, CD62L expression was not critical for T_{reg} migration to GvHD target organs. However, the expression of CD62L was important for the prompt migration of T_{regs} to PLNs.

4.2 Methods

Mice

Donor mice consisted of male C57BL/6J (B6), ($H\text{-}2^{b}$; The Jackson Laboratory, Bar Harbor, ME), $Thy1.1^{+}$ mice ($H\text{-}2^{b}$; The Jackson Laboratory) and $CD62L^{-/-}$ mice, which have been described previously[11]. $CD62L^{-/-}$ mice were crossed with B6 mice expressing the enhanced GFP (eGFP) protein to generate eGFP expressing $CD62L^{-/-}$ mice. The generation of B6-eGFP mice has been described [12]. In some experiments T_{reg} cells were isolated from FIR mice (expressing red fluorescent protein (RFP) under the FoxP3 promoter) as described[13]. Recipient mice were male (C57BL/6JXDBA/2) FI mice, (B6D2) ($H\text{-}2^{bxd}$; The Jackson Laboratory). Within each experiment, all recipient and donor mice were male mice

ranging from 9-14 wk. All animal experiments were performed in accordance with protocols approved by the University of North Carolina Institutional Animal Care and Use Committee.

Antibodies and Flow Cytometry

Antibodies with the following specificities were purchased from eBiosciences (San Diego, CA): anti-CD4 (RM 4.5), CD62L (Mel-14), CD25 (PC61), CD8 (53-6-7), Thy1.1 (HIS51), and FoxP3 (FJK-16s). Acquisition was performed on a FACSCalibur using CellQuest software (BD Biosciences; San Jose, CA). Analysis was performed using FlowJo (Treestar Inc., Ashland, OR) software.

Preparation of cells for transplant and bone marrow transplants

T cell depleted bone marrow (TCD BM) cells, T_{eff} cells and T_{regs} were isolated and infused as described[14].

T_{reg} cell expansion

$CD4^+/CD25^+/RFP^+/CD62L^{high}$ cells were sorted on a MoFlo cell sorter (Dako A/S, Glostrup, Denmark) from the spleens of RFP-FoxP3 mice. Sorted cells were expanded with plate-bound anti-CD3 (145-2C11, 15mg/ml; eBioscience) and CD28 (37.51, 10mg/ml; eBioscience) supplemented with IL-2 (500 units/ml; Peprotech; Rocky Hill, NJ) for 12 days. After 12 days, cells were harvested, stained for CD62L and sorted. Sorted cells were always >95% $CD4^+/mRFP^+/CD62L^{Lo}$.

***In vitro* suppression assay**

In vitro suppression assays were performed as previously described[10].

GvHD grading

Mice were observed twice weekly for signs of GvHD using previously described clinical scoring system[15].

Fluorescence microscopy

Animals were anesthetized with avertin and organs were imaged with a Zeiss SteREO Lumar V12 microscope with eGFP bandpass filter (Carl Zeiss MicroImaging, Inc., Thornwood, NY) as described [14].

Competitive T_{reg} Migration Assay

Competitive migration of WT versus CD62L -/- T_{reg} cells was done as described [14].

Histopathology

The sections were scored by one of us (A.P.-M.) who was blinded to the treatment given using a previous described method[14] .

Quantitation of chemokine receptor transcripts

RNA was isolated from sort-purified T_{regs} using the Qiagen RNeasy Kit (Qiagen; Valencia, CA). Quantitative RT-PCR for chemokine receptor transcripts was performed using primers and probes to CCR1, CCR2, CCR4, CCR5, CCR7, CCR8, CCR9, CCR10, CXCR3, and CXCR4 (Applied Biosystems; Corvalis, OR). The ΔCt method was used to normalize transcripts to 18S RNA and to calculate fold induction.

Measurement of serum IFN-g

Serum samples were obtained from mice receiving whole naïve T cells, with or without WT T_{regs}, $CD62L^{-/-}$ T_{regs}, $CD62L^{Lo}$ T_{regs} or BM only. Samples were harvested when animals

reached a clinical GvHD score of 4. IFN-g concentrations were determined according to the manufacturer's instructions using ELISA (Biolegend San Diego CA).

Statistical analysis

For GvHD scoring, we used the Student's t-test; for overall survival we used Fisher's exact test, and for median survival we used the Mann-Whitney log rank test. P values ≤ 0.05 were considered significant.

4.3 Results

4.3.1 CD62L$^{-/-}$ T_{regs} mediate protection against lethal GvHD

To determine the precise requirement for CD62L expression in T_{reg}-mediated protection during GvHD, we isolated fresh CD4^{+}/CD25^{+} cells from WT or CD62L deficient animals (CD62L$^{-/-}$). Unexpectedly, we did not observe a significant difference in the overall survival (P=1.0) or median survival time (P=0.86) in recipient mice given WT compared to CD62L$^{-/-}$ T_{regs} (Figure 4.1A). Both WT and CD62L$^{-/-}$ T_{regs} recipients had significantly improved overall survival (P<0.001) compared to recipients of WT T cells alone. Our previous work has demonstrated that *in vitro* expanded CD62L^{Lo} T_{regs} were unable to ameliorate GvHD pathology[5], however our subsequent analysis of expanded CD4^{+}/CD25^{+} cells has revealed considerable contamination by FoxP3^{-} cells in the CD62L^{Lo} fraction (M. Carlson, J. Serody; unpublished observation). We therefore isolated cells from FIR mice in which the red fluorescent protein is expressed under control of the FoxP3 promoter[13] and thus, T_{regs} can be identified from the CD4^{+}/CD25^{+} fraction by their expression of mRFP. Recipients of *ex vivo* expanded mRFP^{+}/CD62L^{Lo} T_{regs} displayed only a very modest

improved overall (P=0.09) and median survival time (P=0.12) relative to animals receiving T cells alone (Figure 4.1A). These results demonstrated that $CD62L^{-/-}$ T_{regs} were capable of providing protection from lethal acute and GvHD. These data also demonstrate that contamination of $CD62^{Lo}$ T_{reg} cells with effector cells was not an explanation for the lack of activity of $CD62L^{Lo}$ T_{regs} in the current study. The paucity of $CD62L^{Lo}$ T_{regs} present in FIR mice precluded the evaluation of this population of cells without *ex vivo* expansion.

Next, we determined disease severity using a defined clinical scoring system[15]. Although the survival outcomes were not significantly different, WT T_{regs} did afford reduced clinical GvHD scores compared to $CD62L^{-/-}$ T_{regs} during the first 21 days post-transplant ($P<0.04$ for days 7 to 21) (Figure 4.1B). Starting on day 24, and for the duration of the experiment, GvHD scores were not significantly different ($P>0.05$) in recipients given either WT or $CD62L^{-/-}$ T_{regs}. Consistent with no improvement in overall or median survival, $CD62L^{Lo}$ T_{regs} did not reduce clinical manifestations of GvHD as compared to T cells alone (Figure 4.1B). Collectively, these data demonstrated that $CD62L^{-/-}$ T_{regs} were able to protect animals from lethal GvHD, albeit they did not suppress clinical GvHD manifestations as well as WT T_{reg} cells in the first three weeks post transplant. In addition, $CD62L^{-/-}$ T_{regs} functioned more efficiently to prevent GvHD than $mRFP^{+}/CD62L^{Lo}$ T_{regs} post-transplantation.

4.3.2 CD62L$^{-/-}$ T_{regs} function normally to suppress T cell responses to allo-antigen *in vitro*

Because we observed significant differences early post-transplant in the clinical appearance of GvHD between recipients of WT and CD62L$^{-/-}$ T_{regs}, we sought to determine the ability of CD62L$^{-/-}$ T_{regs} to inhibit effector T cell responses to allo-antigen. To address this question, freshly isolated CD4^{+}/CD25^{+} cells from WT and CD62L$^{-/-}$ mice were co-cultured with WT CD4^{+}/CD25$^{—}$ responder cells stimulated with irradiated B6D2 splenocytes. CD62L$^{-/-}$ and WT T_{regs} displayed equivalent suppression of WT effector T cells up to a 1:8 T_{reg}:Effector cell ratio (Figure 4.1C). Therefore, the early elevated GvHD scores of animals given CD62L$^{-/-}$ T_{regs} was not due to an intrinsic defect in their suppressive function. As described, CD62L^{Lo} T_{regs} were potent suppressors of allo-reactive T cells *in vitro* up to a ratio of 1:32 T_{regs}: Effector cells (Supplemental Figure 4.1).

4.3.3 GvHD target organ histopathology

Given the differences observed in clinical GvHD scores, we were interested in determining the impact that phenotypically different T_{regs} had on individual organ pathology. Histopathology scores in the colon were not statistically different between any of the groups (Figure 4.2A). Recipients of WT T_{regs} demonstrated less pathological damage in the lung as compared to recipients of CD62L$^{-/-}$ T_{regs} ($P=0.05$) (Figure 4.2B). Examination of the liver demonstrated that both WT and CD62L$^{-/-}$ T_{regs} significantly inhibited GvHD pathology ($P<0.03$) compared to recipients of effector T cells alone (Figure 4.2C). Interestingly, despite the modest difference in tissue pathology, there were significant difference in serum

IFN-γ levels in mice given effector T cells alone compared to WT or CD62L$^{-/-}$ T_{regs} ($P<0.01$) (Figure 4.2D). Overall, these results demonstrated that with the exception of worsened lung pathology, CD62L$^{-/-}$ T_{regs} functioned as well as WT T_{regs} to prevent GvHD, while both were potent in their ability to inhibit systemic IFN-g production.

4.3.4 CD62L$^{-/-}$ T_{regs} traffic to secondary lymphoid tissues and GvHD target organs

Because we observed differences early on in the clinical manifestation of GvHD between recipients of WT and CD62L$^{-/-}$ T_{regs}, we were interested in determining the trafficking pattern of these T_{regs}. To evaluate *in vivo* T_{reg} trafficking, we used a competitive lymphocyte migration assay[16]. As illustrated in Figure 4.3A, six days after transfer, CD62L$^{-/-}$ T_{regs} were found at a similar frequency as WT T_{regs} in the liver, lung, spleen, bone marrow, and mesenteric lymph node (MLN), although as expected, there were substantially fewer CD62L$^{-/-}$ T_{regs} in the PLNs of recipient animals. Further analysis 16 days post- T_{reg} transfer showed no difference between WT and CD62L$^{-/-}$ T_{reg} migration to liver, spleen, bone marrow, or MLN (Figure 4.3B). However, although not statistically different, there were fewer CD62L$^{-/-}$ T_{regs} detected in the lung and PLN on day 16 compared to WT T_{regs}, which correlated with the enhanced GvHD in the lung of recipient animals receiving CD62L$^{-/-}$ T_{regs} (Figure 4.3B).

To confirm our findings regarding the function of CD62L in the migration of T_{regs} in a lymphopenic environment, we performed *in vivo* imaging using fluorescence stereomicroscopy. In the MLN (Figure 4.3C) and spleen (Figure 4.3D) we found similar distribution and GFP signal intensity by WT and CD62L$^{-/-}$ T_{regs}, indicating that the migration

and accumulation of CD62L$^{-/-}$ T_{regs} was indistinguishable from WT T_{regs} in these organs sixteen days post-transplantation. Interestingly, we observed fewer GFP^{+} CD62L$^{-/-}$ T_{regs} in the lung (Figure 4.3E) and PLN (Figure 4.3F) at this time point. Taken together, these observations illustrated that CD62L$^{-/-}$ T_{regs} home to GvHD target organs, similar to WT T_{regs}, with the exception of a modest impairment in migration to the lung. Differences in the migration of WT compared to CD62L$^{-/-}$ T_{regs} to PLNs were found in the first week post-transplantation demonstrating the importance of CD62L in the initial migration of T_{regs} to PLNs. However, at day 16 these differences were minimized indicating that CD62L was not absolutely required for the eventual migration of T_{regs} to PLNs.

4.3.5 CD62L^{Hi}, CD62L$^{-/-}$, and CD62L^{Lo} T_{regs} display differential expression of chemokine receptors

The finding of CD62L$^{-/-}$ T_{regs} in the PLNs of irradiated recipients was somewhat surprising given the role that CD62L plays in T cell rolling and homing to lymph nodes. This observation suggests that in the absence of CD62L other proteins important for T cell migration may serve a similar function. To this end we examined the phenotypic profile of T_{regs} based on CD62L expression (Figures 4.4A-4.4C). As shown in Figure 4D, CD62L^{Hi} and CD62L$^{-/-}$ T_{regs} have increased expression of CCR7 mRNA relative to CD62L^{Lo} T_{regs}. We then compared the three T_{reg} types to naïve CD4^{+}/mRFP^{-} cells in their mRNA expression of other chemokine receptors. CD62L$^{-/-}$ T_{regs} resembled CD62L^{Lo} T_{regs} in the expression of CCR2, CCR4, and CXCR3, and resembled CD62L^{Hi} T_{regs} in the expression of CCR9. CD62L$^{-/-}$ T_{regs} had intermediate levels of CCR5 and CCR8, with distinctive expression of CCR1 and CCR10. Collectively, these data demonstrated that the migratory profile of

$CD62L^{-/-}$ T_{regs} was that of an intermediate activated phenotype with higher expression of pro-inflammatory chemokine receptors compared to $CD62L^{Hi}$ T_{regs} and much greater expression of CCR7 compared to $CD62L^{Lo}$ T_{regs}.

4.4 Discussion

In the current work, we were interested in determining whether CD62L itself was critical for T_{reg} function and migration into lymphoid tissue. We demonstrated that CD62L was not critical for T_{reg} function to prevent GvHD lethality as $CD62L^{-/-}$ T_{regs} afforded substantial protection from lethal acute GvHD in the clinically relevant model employed. WT T_{regs} yielded reduced clinical scores compared to $CD62L^{-/-}$ T_{regs} during the first three weeks post-transplant, which correlated with delayed migration of $CD62L^{-/-}$ T_{regs} to PLNs. Histopathological analysis of GvHD target organs correlated with the clinical scores, as recipients of WT T_{regs} showed improved pathology in the lung and similar pathology in the colon and liver compared to $CD62L^{-/-}$ T_{regs}. Lastly, we demonstrated differential chemokine receptor expression of T_{regs} based on CD62L expression, where the $CD62L^{-/-}$ T_{regs} displayed a phenotype that appeared to be an intermediate between the naïve $CD62L^{Hi}$ and activated $CD62L^{Lo}$.

Previous reports examining the role of CD62L in T_{reg}-mediated inhibition of GvHD suggested either that[1] CD62L itself was critically important in the function of T_{regs} or that [2] the $CD62L^{Hi}$ phenotype functioned differently than $CD62L^{Lo}$ T_{regs} but that CD62L itself was not critical [5,9]. Our data demonstrated that CD62L itself was not critically required for the prevention of GvHD lethality or for the ability to migrate into LN post transplantation. While there was no difference in either overall or median survival time, our data indicated that CD62L did serve as an accessory molecule given the statistical difference in clinical scores between WT and $CD62L^{-/-}$ T_{regs} during the first three weeks post-transplantation. Of

interest, we also observed no statistical difference in clinical scores between $CD62L^{-/-}$ and $CD62L^{Lo}$ T_{regs} for the first two weeks post-transplantation suggesting CD62L serves early on to promote T_{reg} inhibition of GvHD most likely by enhancing the migration of T_{regs} into lymphoid tissue.

One concern in our previous studies in which we expanded $CD4^{+}/CD25^{+}$ T cells to obtain a $CD62L^{Lo}$ population was the difficulty in eliminating $CD25^{+}$ effector cells from the T_{reg} infusion[5]. Here, we have circumvented this concern by using T_{regs} from FIR mice in which mRFP is under control of the FoxP3 promoter and thus cells expressing FoxP3 can be detected using flow cytometry[13]. Our data confirm previous observations that $CD62L^{Lo}$ T_{regs} were not sufficient to prevent GvHD in the overwhelming majority of transplanted recipients. The possibility of impaired suppressive function of these cells was ruled out by *in vitro* analysis in which $CD62L^{Lo}$ T_{regs} were more proficient suppressors of T cell responses to allo-antigen, consistent with previously published data[17]. Therefore, the inability of $CD62L^{Lo}$ T_{regs} to provide protection against GvHD could not be explained by impaired function but may be due to impaired homing to lymphoid tissue or diminished survival after infusion.

Examination of the pathology in individual organs revealed that WT and $CD62L^{-/-}$ T_{regs} ameliorated disease in the liver, whereas WT T_{reg} recipients displayed reduced pathology in the lung as compared to $CD62L^{-/-}$ T_{reg} recipients. The increased lung pathology correlated with modestly impaired $CD62L^{-/-}$ T_{reg} migration to the lung. The accumulation of IFN-g in the serum has been shown to be a predictor of GvHD mortality[18]. We also

documented a substantial reduction in the level of IFN-g in the serum of animals receiving either WT or CD62L$^{-/-}$ T_{regs} an effect not seen in recipients of mRFP^{+}/CD62L^{Lo} T_{regs} (data not shown). Here again, a functional distinction was made between CD62L$^{-/-}$ and CD62L^{Lo} T_{regs}.

While it is clear that T_{regs} do inhibit effector T cell expansion and suppress effector functions, it is less clear as to whether the inhibition is in lymphoid tissues or GvHD target organs. In the current report, we demonstrated that CD62L$^{-/-}$ T_{regs} migrate to GvHD target organs with similar efficiency as WT T_{regs}, however their accumulation within the PLNs was delayed. It is interesting to note that this delay corresponded with increased clinical GvHD scores, thus supporting the hypothesis that entry into lymph nodes by T_{regs} was important in inhibiting the initial expansion of donor T cells. The inability of CD62L^{Lo} T_{regs} to inhibit GvHD has been attributed to ineffective trafficking to secondary lymphoid tissues[9]. Normal trafficking seen in CD62L$^{-/-}$ T_{regs} provides another distinction between CD62L^{Lo} and CD62L$^{-/-}$ phenotypes.

Other studies have examined chemokine receptor expression on T_{reg} subsets, including the CD62L^{Hi} and CD62L^{Lo} populations[19,20]. Our data is in agreement that the CD62L^{Hi} fraction expressed high levels of the lymph node homing chemokine receptor CCR7. Of interest, the CD62L$^{-/-}$ population also expressed high levels of CCR7, providing a plausible mechanism for their migration to secondary lymphoid tissues. In keeping with an activated status the CD62L^{Lo} T_{regs} expressed high levels of CCR5 and CCR8 while the CD62L$^{-/-}$ T_{regs} displayed intermediate expression.

In summary, our data demonstrate that post-transplant, CD62L was dispensable for T_{reg} inhibition of GvHD lethality.

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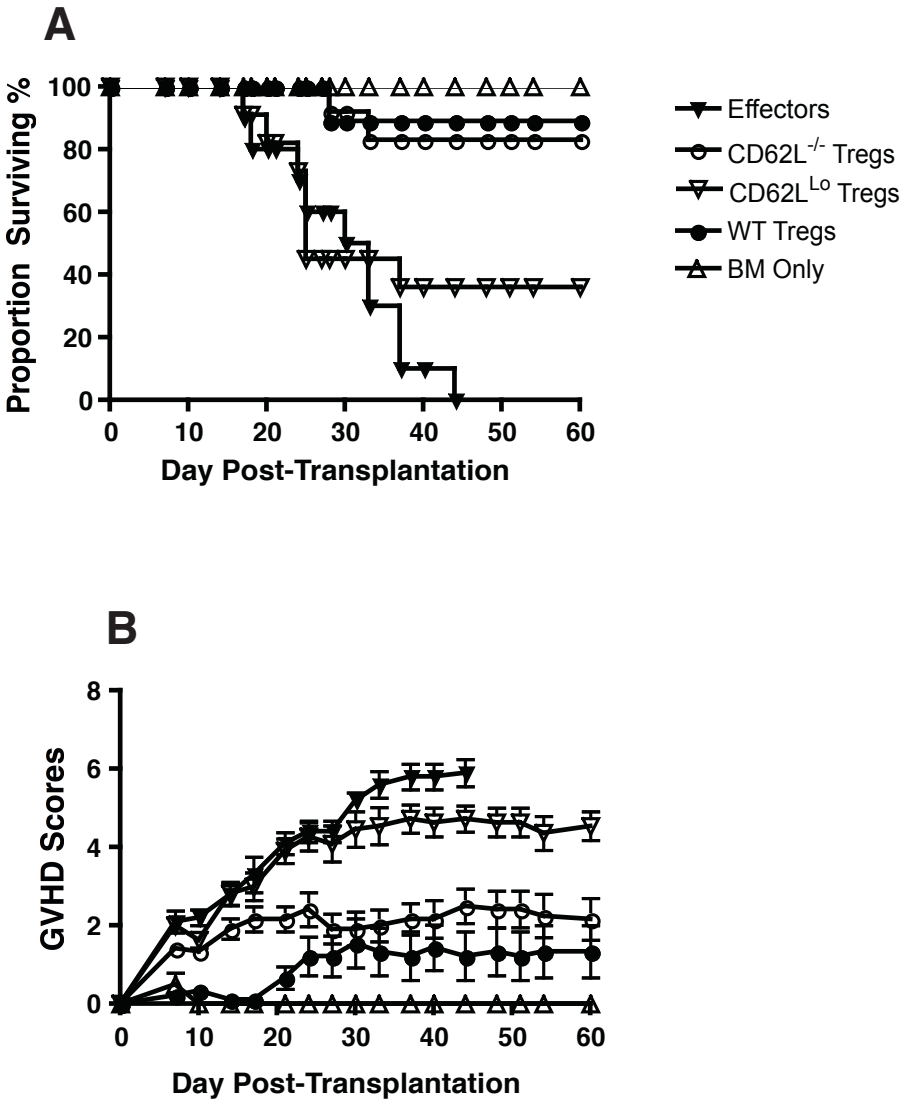
Disclosure

The authors of this manuscript have no conflict of interest to disclose as described by the American Journal of Transplantation.

Figure 4.1


A
Proportion Surviving %
100
80
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40
20
0
0
10
20
30
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50
60
Day Post-Transplantation
Effectors
CD62L-/- Tregs
CD62LLo Tregs
WT Tregs
BM Only
B
GVHD Scores
8
6
4
2
0
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Day Post-Transplantation

Figure 4.1 contd

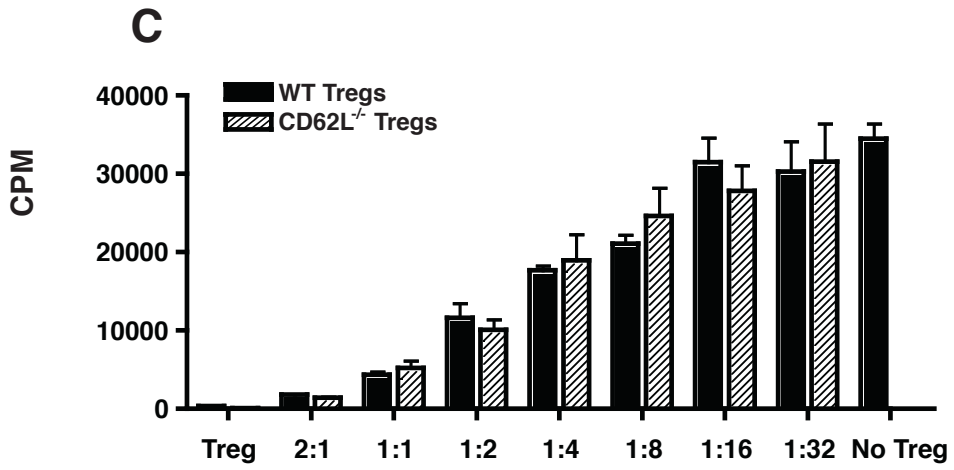


Figure 4.1. CD62L$^{-/-}$ T$_{regs}$ protect from lethal acute GvHD and are potent suppressers *in vitro*. 1.25x10^6 WT (of which approximately 80% expressed CD62L and 67% had high levels of expression of CD62L), CD62L^{Lo} T$_{regs}$, or CD62L$^{-/-}$ T$_{regs}$ were transferred with 3x10^6 TCD BM cells into lethally irradiated B6D2 recipients on day 0. 4x10^6 whole splenic T cells from WT mice were then transferred on day +2 (n=9 WT T$_{regs}$, n=11 CD62L^{Lo} T$_{regs}$, n=12 CD62L$^{-/-}$ T$_{regs}$, n=10 Effectors alone, n=4 BM only). Animals were monitored for (A) survival and (B) signs of GvHD. Data represent mean score ± SEM at each time point. (C) Suppression of WT responder cell (CD4$^+$/ CD25$^-$) proliferation in response to B6D2 alloantigen by WT (▉) or CD62L$^{-/-}$ T$_{regs}$ (▨) was determined as described in 'Materials and Methods'.

Figure 4.2

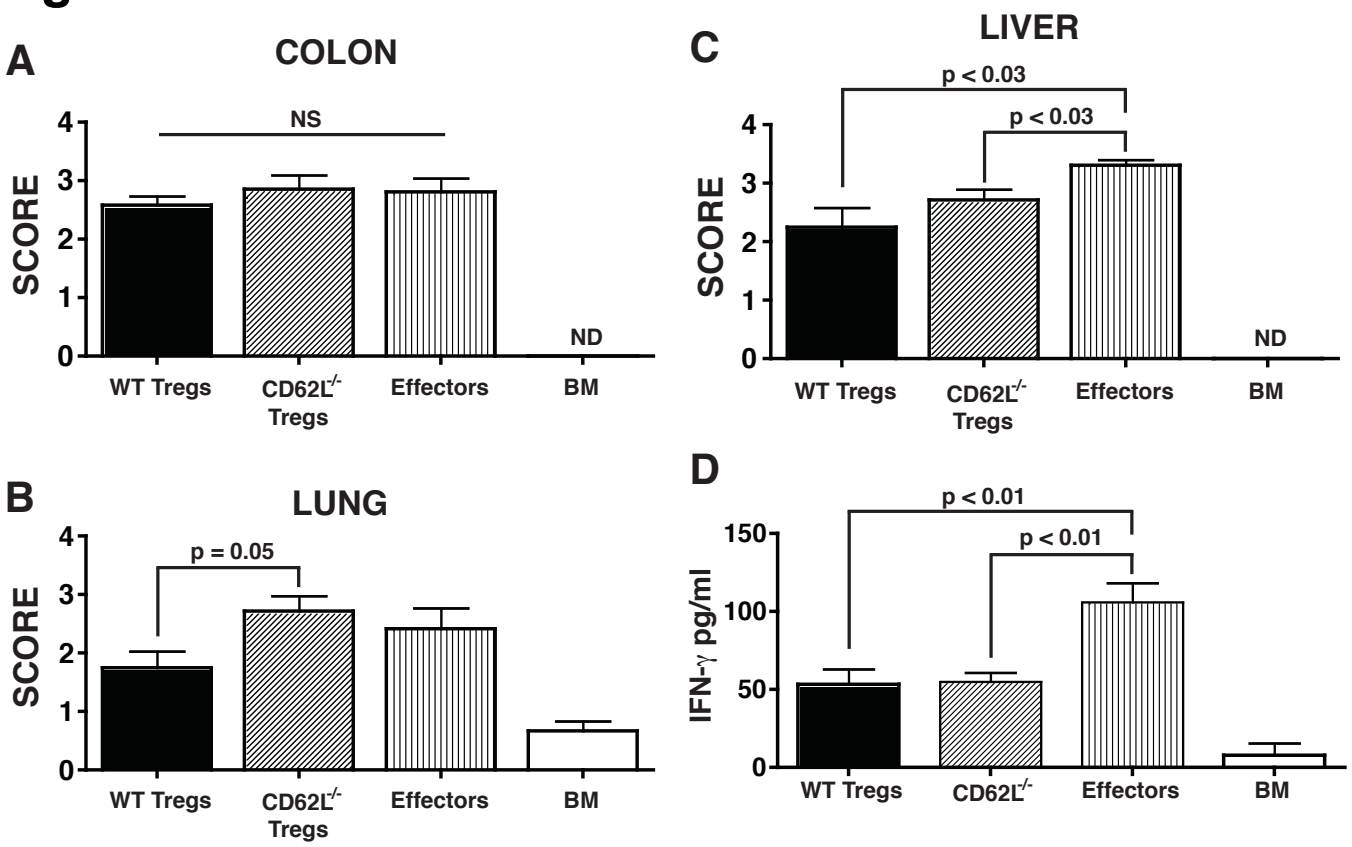


Figure 4.2. CD62L$^{-/-}$ and WT T_{regs} suppress liver pathology. 1.25x10^6 WT or CD62L$^{-/-}$ T_{regs} were transferred with 3x10^6 TCD BM cells into lethally irradiated B6D2 recipients on day 0. 4x10^6 whole splenic T cells from WT mice were then transferred on day +2 (n=6 WT T_{regs} (▉), n=7 CD62L$^{-/-}$ T_{regs}, (▤), n=8 Effectors alone (▥), n=3 BM only (⬭). Animals were harvested when clinical scores reached a total of >4. Animals that did not reach a score of 4 were harvested on days 25-27 post-transplant. Histopathological assessment of the (A) colon, (B) lung, (C) liver. (D) Serum was harvested from animals at the time of histopathology assessment and analyzed by ELISA for levels of IFN-γ.

Figure 4.3

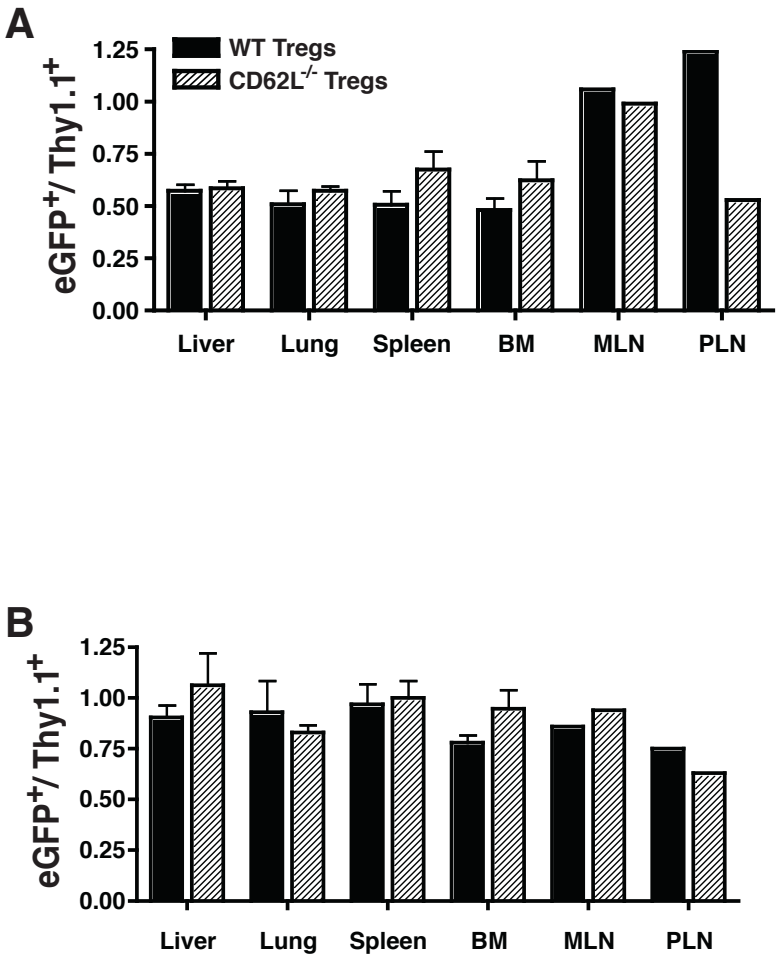

A
WT Tregs
CD62L-/- Tregs
eGFP+/ Thy1.1+
1.25
1.00
0.75
0.50
0.25
0.00
Liver
Lung
Spleen
BM
MLN
PLN
B
eGFP+/ Thy1.1+
1.25
1.00
0.75
0.50
0.25
0.00
Liver
Lung
Spleen
BM
MLN
PLN

Figure 4.3 contd

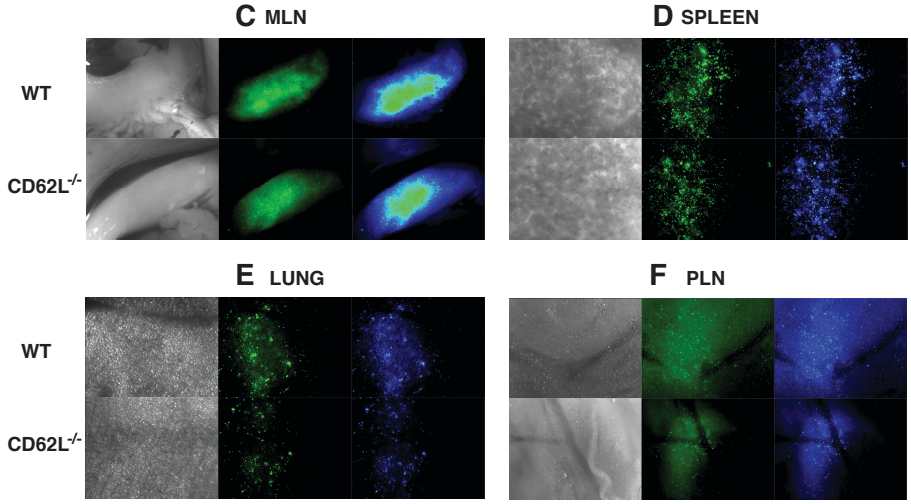


Figure 4.3. CD62L$^{-/-}$ and WT T_{reg} trafficking. 5x10^5 WT-GFP (▉) or CD62L$^{-/-}$-GFP (▨) T_{regs} were transferred with 5x10^5 Thy1.1$^+$ T_{regs} along with 3x10^6 TCD BM cells into lethally irradiated B6D2 recipients on day 0. 2x10^6 whole splenic T cells from WT mice were then transferred on day +2. On days 6 (A) and 16 (B) post-T_{reg} transfer, lymphocytes were isolated from GvHD target organs and secondary lymphoid tissues as described in 'Materials and Methods'. The ratio of eGFP$^+$/FoxP3$^+$:Thy1.1$^+$/FoxP3$^+$ cells are shown (n=4 for each group). N=4 animals/time point (MLNs were pooled for each group, and PLNs were pooled for each group). 1.0x10^6 WT-GFP (*top*) or CD62L$^{-/-}$-GFP (*bottom*) T_{regs} were transferred along with 3x10^6 TCD BM cells into lethally irradiated B6D2 recipients on day 0. 2x10^6 whole splenic T cells from WT mice were then transferred on day +2. 16 days post-T_{reg} transfer animals were anesthetized with avertin and organs were imaged with a Zeiss SteREO Lumar.V12 microscope with eGFP bandpass filter. Brightfield images (*left*), and GFP images (*middle*) were taken for each organ. GFP intensities (*right*) were determined by software analysis. (C) MLN (D) spleen (E) lung (F) peripheral lymph node. Original magnification: lung = 25X, spleen = 40X, MLN = 45X, ILN = 45X. Data represent mean score ± SEM for each organ.

Figure 4.4

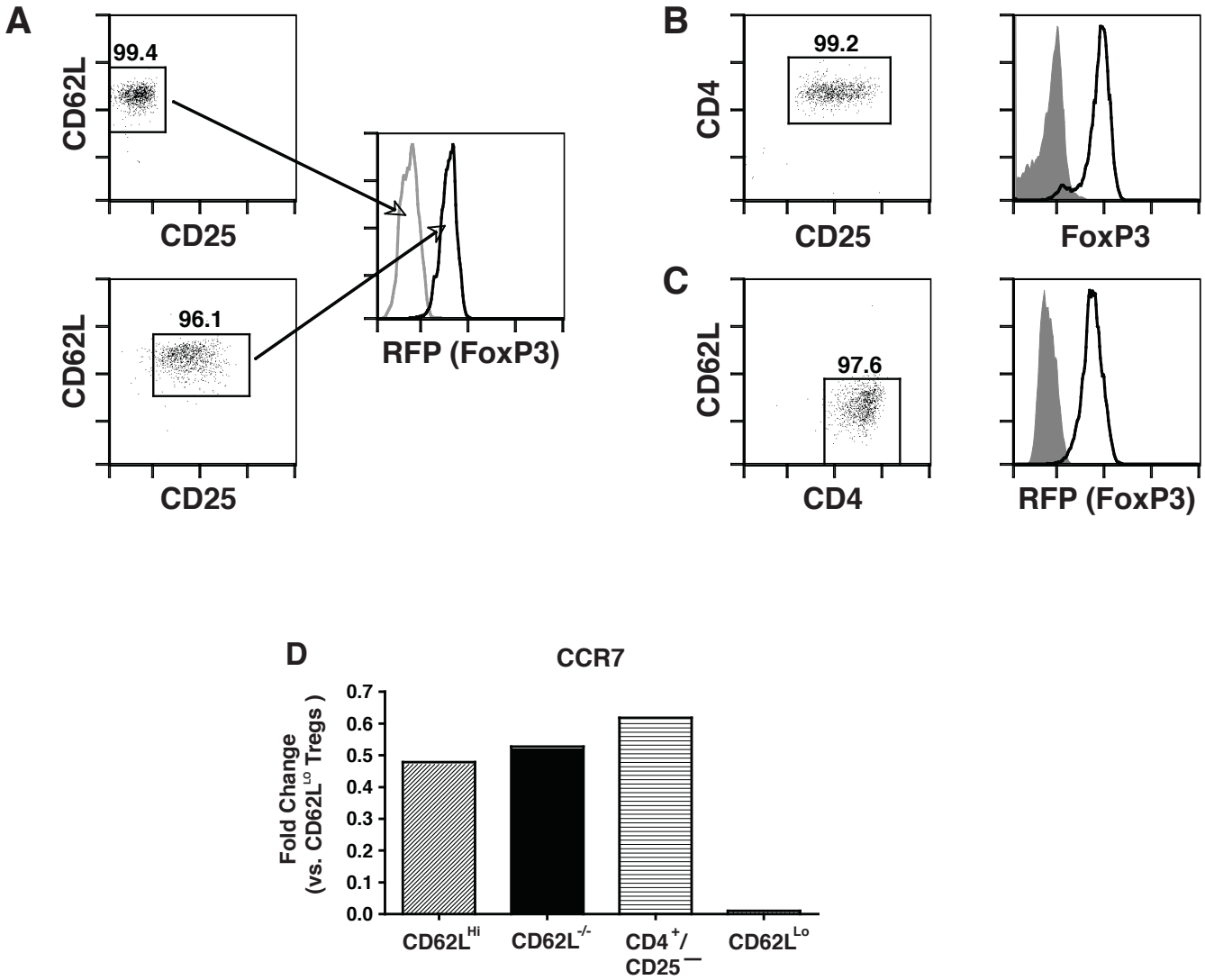

A
99.4
CD62L
CD25
96.1
CD62L
CD25
RFP (FoxP3)
B
99.2
CD4
CD25
FoxP3
C
97.6
CD62L
CD4
RFP (FoxP3)
D
CCR7
Fold Change (vs. CD62L^LO Tregs)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CD62L^Hi
CD62L^-/-
CD4^+/ CD25^—
CD62L^Lo

Figure 4.4 contd

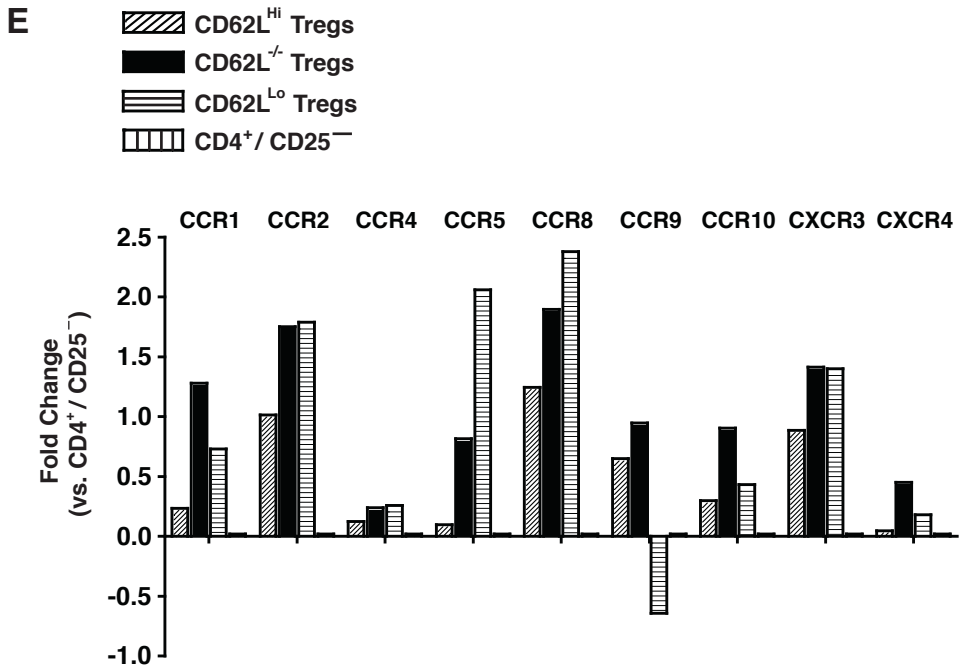
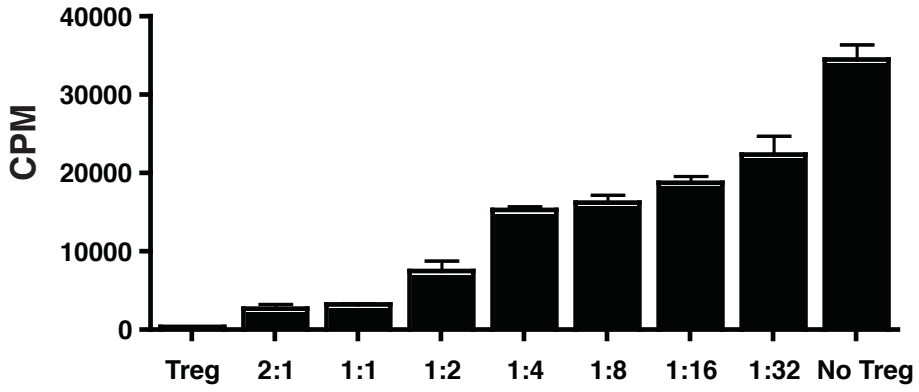


Figure 4.4. T_{reg} chemokine receptor expression based on CD62L expression. Sort purification of cells for quantitative real-time PCR analysis. (A) $CD4^+/CD25^+/mRFP^+/CD62L^{Hi}$ T_{regs} (*top*) and $CD4^+/CD25^-/mRFP^-/CD62L^{Hi}$ naïve T cells (*bottom*) were sort purified from mRFP-FoxP3 mice. (B) $CD4^+/CD25^+$ T_{regs} were sort purified from $CD62L^{-/-}$ mice. (C) $CD4^+/mRFP^+/CD62L^{Lo}$ T_{regs} were sort purified following *in vitro* expansion. RNA was extracted and real-time PCR performed as described in materials and methods. (D) CCR7 expression on $mRFP^+/CD62L^{Hi}$ T_{regs} (▨), $CD4^+/CD25^+/CD62L^{-/-}$ T_{regs} (■), naïve $CD4^+/CD25^-$ T cells (▤), and $mRFP^+/CD62L^{Lo}$ Tregs (▥). Data is shown as relative change in expression (logarithmic scale) compared to $CD4^+/mRFP^+/CD62L^{Lo}$ T_{regs}. E. Chemokine receptor expression on $mRFP^+/CD62L^{Hi}$ T_{regs} (▨), $CD4^+/CD25^+/CD62L^{-/-}$ T_{regs} (■), $mRFP^+/CD62L^{Lo}$ T_{regs} (▤), and naïve $CD4^+/CD25^-$ T cells (▥). Data is shown as relative change in expression (logarithmic scale) compared to naïve $CD4^+/CD25^-$ T cells. Data are representative of 3 independent experiments.

Supplementary Figure 4.1



Supplemental Figure 4.1. CD62L^{Lo} T$_{regs}$ are potent suppressers *in vitro*. Suppression of WT responder cell (CD4^{+}/CD25$^{—}$) proliferation in response to B6D2 alloantigen by CD62L^{Lo} T$_{regs}$ (■) was determined as described in 'Materials and Methods'.

References

1. Appelbaum FR (2001) Haematopoietic cell transplantation as immunotherapy. Nature 411: 385–389. doi:10.1038/35077251.

2. Armitage JO (1994) Bone marrow transplantation. N Engl J Med 330: 827–838. doi:10.1056/NEJM199403243301206.

3. Ferrara JL, Cooke KR, Pan L, Krenger W (1996) The immunopathophysiology of acute graft-versus-host-disease. Stem Cells 14: 473–489. doi:10.1002/stem.140473.

4. Kingsley CI, Karim M, Bushell AR, Wood KJ (2002) CD25+CD4+ regulatory T cells prevent graft rejection: CTLA-4- and IL-10-dependent immunoregulation of alloresponses. J Immunol 168: 1080–1086.

5. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, Lucas PJ, Gress RE, et al. (2004) L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. Blood 104: 3804–3812. doi:10.1182/blood-2004-05-1850.

6. Trenado A, Charlotte F, Fisson S, Yagello M, Klatzmann D, et al. (2003) Recipient-type specific CD4+CD25+ regulatory T cells favor immune reconstitution and control graft-versus-host disease while maintaining graft-versus-leukemia. J Clin Invest 112: 1688–1696. doi:10.1172/JCI17702.

7. Kansas GS (1996) Selectins and their ligands: current concepts and controversies. Blood 88: 3259–3287.

8. Boyman O, Létourneau S, Krieg C, Sprent J (2009) Homeostatic proliferation and survival of naïve and memory T cells. Eur J Immunol 39: 2088–2094. doi:10.1002/eji.200939444.

9. Ermann J, Hoffmann P, Edinger M, Dutt S, Blankenberg FG, et al. (2005) Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. Blood 105: 2220–2226. doi:10.1182/blood-2004-05-2044.

10. Wysocki CA, Jiang Q, Panoskaltsis-Mortari A, Taylor PA, McKinnon KP, et al. (2005) Critical role for CCR5 in the function of donor CD4+CD25+ regulatory T cells during acute graft-versus-host disease. Blood 106: 3300–3307. doi:10.1182/blood-2005-04-1632.

11. Arbonés ML, Ord DC, Ley K, Ratech H, Maynard-Curry C, et al. (1994) Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. Immunity 1: 247–260.

12. Serody JS, Burkett SE, Panoskaltsis-Mortari A, Ng-Cashin J, McMahon E, et al. (2000) T-lymphocyte production of macrophage inflammatory protein-1alpha is critical to the recruitment of CD8(+) T cells to the liver, lung, and spleen during graft-

versus-host disease. Blood 96: 2973–2980.

13. Wan YY, Flavell RA (2005) Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. Proc Natl Acad Sci USA 102: 5126–5131. doi:10.1073/pnas.0501701102.

14. Coghill JM, Carlson MJ, Panoskaltsis-Mortari A, West ML, Burgents JE, et al. (2010) Separation of graft-versus-host disease from graft-versus-leukemia responses by targeting CC-chemokine receptor 7 on donor T cells. Blood 115: 4914–4922. doi:10.1182/blood-2009-08-239848.

15. van Den Brink MR, Moore E, Horndasch KJ, Crawford JM, Hoffman J, et al. (2000) Fas-deficient lpr mice are more susceptible to graft-versus-host disease. J Immunol 164: 469–480.

16. Sheng-Tanner X, McKerlie C, Spaner D (2000) Characterization of graft-versus-host disease in SCID mice and prevention by physicochemical stressors. Transplantation 70: 1683–1693.

17. Chai J-G, Coe D, Chen D, Simpson E, Dyson J, et al. (2008) In vitro expansion improves in vivo regulation by CD4+CD25+ regulatory T cells. J Immunol 180: 858–869.

18. Krenger W, Hill GR, Ferrara JL (1997) Cytokine cascades in acute graft-versus-host disease. Transplantation 64: 553–558.

19. Huehn J, Siegmund K, Lehmann JCU, Siewert C, Haubold U, et al. (2004) Developmental stage, phenotype, and migration distinguish naive- and effector/memory-like CD4+ regulatory T cells. J Exp Med 199: 303–313. doi:10.1084/jem.20031562.

20. Szanya V, Ermann J, Taylor C, Holness C, Fathman CG (2002) The subpopulation of CD4+CD25+ splenocytes that delays adoptive transfer of diabetes expresses L-selectin and high levels of CCR7. J Immunol 169: 2461–2465.

CHAPTER FIVE

DISCUSSION

Previous work from our laboratory demonstrated that *in vitro* derived Th17 cells were responsible for pulmonary and skin manifestations in acute GvHD[1]. Extending these studies we evaluated the contribution of the transcription factor required for the differentiation of Th17 cells, RORγt (*RORC*), in the pathogenesis of aGvHD. Using multiple murine models we determined that *RORC* $CD4^+$ T cell expression was important for acute GvHD development. $CD4^+$ T cells deficient in *RORC* attenuated aGvHD in a haploidentical model but did not alter aGvHD pathogenesis in a complete mismatch BALB/c transplant model. As expected the production of IL-17 was significantly diminished in mice receiving cells deficient in *RORC* using the haploidentcal model. However IL-17 production was not altered in recipients of *RORC* knockout cells using the BALB/c transplant model likely due to the production of IL-17 by other donor or host cells. While it is typically believed that the degree of MHC or miHA disparity is the major difference between murine transplant models our data suggests that cytokine production in different models may also alter aGvHD pathogenesis.

Migration of T cells to target organs is essential for GvHD pathogenesis. The involvement of the actin cytoskeleton in T cell migration has been investigated however research connecting actin cytoskeleton dynamics and disease has been limited. We

investigated the role of the actin cytoskeleton proteins Coronins in the pathogenesis of acute GvHD. We found that migration of T cells into and out of lymphoid organs was dependent on the expression of Coronin 1A. Furthermore T cells deficient in Coronin 1A accumulated in the mesenteric lymph nodes and Peyer's patches, a finding that could be attributed to decreased receptor expression for the signaling sphingolipid S1P. Our data suggests targeting actin cytoskeleton components may offer an alternative method for the treatment and prevention of acute GvHD.

5.1 GvHD Prophylaxis and Treatment

The specificity of transcription factors to T helper cell populations makes them an ideal target for immunotherapy. Th17 cells self-renew through an autocrine feedback loop and produce cytokines that recruit leukocytes to sites of inflammation. It is possible that the rate of disease progression is important for attenuated aGvHD in the absence of *RORC* on $CD4^+$ T cells. aGvHD pathogenesis in the haploidential transplant model occurs over a longer course compared to the completely mismatched MHC transplant model, allowing for other immune cells to reach sites of inflammation and mitigate damage. However during rapid and robust disease progression, as seen using a complete MHC mismatch transplantation model, recruitment of immune cells may be too slow to prevent or attenuate disease. This hypothesis could be evaluated by decreasing the number of $RORC^{-/-}$ $CD4^+$ T cells administered to recipient mice using our completely mismatched MHC transplantation model. Recruitment of immune cells to sites of inflammation would be best analyzed using intravital microscopy although experimental this may be challenging.

$CD4^+$ naïve T cells through stimulation of transcription factors and cytokines differentiate into multiple T helper cell populations. $CD8^+$ T cell differentiation is less elaborate as they become terminal effectors or memory precursors. The expression of *RORC* on $CD4^+$ T cells was sufficient to attenuate GvHD however *RORC* is found on both $CD4^+$ and $CD8^+$ T cells, and the expression of *RORC* on $CD8^+$ T cells was dispensable for aGvHD pathogenesis. Our studies suggested a conversion of Th17 cells to Th1 cells later in disease as a large population of dual IL-17/IFN-γ expressing T cells with increased expression of serum TNF were found in $RORC^{-/-}$ transplant recipient animals (Sup Fig 2.2)[2]. Attenuated aGvHD in the absence of *RORC* on $CD4^+$ T cells is potential due to the lost of early IL-17, TNF and IL-21 that would normal be produced by Th17 cells. Anti-TNF treatment has been pursued by multiple groups as aGvHD is still often thought of as a Th1 mediated disease. While TNF antibody treatment alone has been beneficial for some patients the involvement of other cytokines is demonstrated in patients whose symptoms persists following antibody treatment[3,4]. The involvement of IL-17 in aGvHD has been demonstrated both here and in other publications[1,5,6]. Inhibiting both IL-17 and TNF early after transplantation with antibody administration may be an effective prophylaxis or treatment for aGvHD.

Effective evaluation of Th17 cells in aGvHD is necessary to determine if there is truly a conversion from Th17 cells to Th1 cells although this conversion has been suggested in other disease models[7]. Using Cre/Lox recombination, mice that express enhanced yellow fluorescent protein (eYFP) under the control of the IL-17 promoter can be used to evaluate the conversion of Th17 cells to Th1 cells. IL-17 producing Th17 cells that express eYFP would be used as donors for transplantation. A Th1 cell phenotype can be determined by transcription factor and cytokine production analyses. eYFP expression in donor Th17 cells

will persist even if the production of IL-17 is terminated demonstrating the conversion from Th17 to Th17 and/or a switch from the production of IL-17 to the production of IFN-γ. Trafficking to sites of inflammation, particularly target organs, should also be assessed in mice transplanted using Th17 eYFP cells.

5.2 Targeting T Cell Migratory Proteins

In chapter 4 we discussed the effects of elimination of the hematopoietic specific, actin associating protein Coro 1A and attenuation of GvHD primarily through migratory and trafficking pathway components. Cells deficient in Coro 1A have delayed entry and are impaired in their ability to reach target organs with accumulation in secondary lymphoid organs. This delayed entry is potentially beneficial for transplant recipients as complete elimination of T cells increases the probability of infection and eliminates the potential for GvL response[8] however some researchers believe that limited T cell depletion may be beneficial for some patients who cannot tolerate high dose chemotherapy[9].

The specificity of Coro 1A to hematopoietic cells made it an ideal candidate for our bone marrow transplantation studies. While our transplants use only T cells to see the effect of GvHD, other cells of hematopoietic origin (B cells, macrophages, neutrophils) are also likely affected by the absence of Coro 1A. These cells, although not believed to be major contributors in GvHD, also play a role in disease pathogenesis[10,11]. Similarly Coro 1A is also expressed on microglia of the brain. Although the central nervous system (CNS) is not traditionally thought to be a target organ for GvHD there are multiple reports of inexplicable neurologic manifestations following bone marrow transplantation[12]. Minimizing the effects of T cells in bone marrow and stem cell inoculums is often the focus of healthcare providers however, knowing the effects of these adverse cell populations would also be beneficial for decreasing patient morbidity and mortality.

Although the majority of Coro 1A deficient T cells accumulated in mesenteric lymph nodes and Peyer's patches after transplantation some cells were able to migrate to target

organs. Once in target organs these cells maintained effector functions similar to wild type cells as measure by cytokine production and proliferation. Interestingly Coro 1A deficient cells that accumulated in lymph nodes, although they were activated and produced cytokines, did not cause damage and destruction to these lymph nodes. This could be due to decreases in signaling pathways involved in cytolytic damage. We noted that Coro 1A deficient cells upon activation had decreased expression of the canonical NF-κB pathway component p65. The transcription factor NF-κB regulates a number of processes including the expression of CCR7 that was of interest for our studies. Defects seen in the NF-κB signaling pathway may affect other functions of Coro 1A deficient cells that were not investigated here.

Research to determine the links between the actin cytoskeleton and TCR activation has been ongoing however many questions remain. Our studies demonstrated alterations in the activation of the NF-κB component p65 upon TCR activation in the absence of Coro 1A. However a direct interaction of Coro 1A to p65 was not observed through immunoprecipatation experiments (Fulton and Serody unpublished). Rho GTPases have been shown to be important in actin cytoskeleton dynamics[13,14]. More importantly Coro 1A has been shown to be involved in the regulation of Ras-related C3 botulinum toxin substrate 1 more commonly known as Rac 1 that is involved in multiple processes including cytoskeletal reorganization[15]. Transactivation of the p65 subunit of NF-κB is regulated by Rac 1 through IL-1[16]. We hypothesize that Coro 1A regulation of Rac 1 also regulates the expression of p65 (Fig 5.1). Using a pull down assay we can determine if Rac 1 is activated in the absence of Coro 1A. We expect that decreased Rac 1 activation would correlate with decreased p65 activation.

Identifying transcription factors that are potential targets for therapy is an easy task when working in murine models. However targeting intercellular proteins for the therapy would require the use of microRNA (miRNAs)[17] or small interfering RNA (siRNAs)[18]. Although Coro 1A is an intracellular protein we were able to link defects in ingress and egress by cells deficient in Coro 1A to the decreased expression of the chemokine receptor CCR7 and signaling lipid receptor S1Pr1. Both CCR7 and S1Pr1 are cell surface proteins that can be easily target for therapy.

5.3 CD62L and GvHD

Previous studies from our laboratory have investigated T cell migration in GvHD based on receptor expression[19]. Additionally infusion of regulatory T cells has shown promising effects in the prevention of GvHD[20,21]. Knowing the importance of the selectins in T cell homing we determined if the expression of CD62L (L-selectin) on Tregs was important for the prevention of GvHD. As discussed in chapter 4 Tregs lacking CD62L protected recipients from GvHD (Fig 4.1). Although these were not the results initially expected this was not completely surprising as multiple selectins and chemokine receptors are involved in T cell homing[22].

While we did not see a difference between $CD62L^{-/-}$ Tregs and WT Tregs in their ability to protect from aGvHD, $CD62L^{Lo}$ Tregs were impaired in their ability to protect against GvHD. The inability of $CD62L^{Lo}$ Tregs to protect from aGvHD is potentially explained by data demonstrating that activated T cells do not cause aGvHD likely due to altered functions and inability to traffic to secondary lymphoid organs[23]. The redundancy in function is one of the challenges when considering targeting chemokine receptors for

therapeutic treatments in the patient population. Interestingly Tregs deficient in CCR7 and CD62L ($CCR7^{-/-}CD62L^{-/-}$) do not protect from lethal acute GvHD. Interestingly recipient animals have delayed GvHD onset with hair loss and skin manifestations similar to those seen in chronic GvHD (Coghill and Serody unpublished).

5.4 Coronin 1B in GvHD

As there are multiple members of the Coronin family of proteins there is the potential for the involvement of other Coronin proteins in aGvHD. One of the largest distinguishing factors between Coronin proteins is their tissue specific expression, hence our reasoning for investigating the role of Coro 1A. However Coro 1B is ubiquitously expressed making it a potential protein to investigate in GvHD.

We first determined the contribution of Coro 1B to GvHD using our murine transplant models. Increased survival with decreased GvHD scores was observed in Coro $1B^{-/-}$ donor T recipients compared to WT T cell recipients using the parent into F1 haploidentical transplant model (Fig 5.2A). Interestingly and different from Coro $1A^{-/-}$ donor T cell recipients, Coro $1B^{-/-}$ donor T cell recipients succumbed to GvHD similar to WT T cell recipients using the completely mismatched BALB/c transplant model (Fig 5.2B). We also generated Coro $1B^{-/-}$ GFP mice and used stereomicroscopy to determine if Coro $1B^{-/-}$ donor T cells had the same accumulation patterns in gastrointestinal lymph nodes as Coro $1A^{-/-}$ donor T cells in the haploidentical transplant model. No difference in migration to lymphoid organs or target organs was seen in recipients of Coro $1B^{-/-}$ GFP donor T cells compared to those that received WT GFP donor T cells (Fulton, Serody, Dant unpublished). These data suggests defects in other mechanisms for attenuated GvHD by Coro $1B^{-/-}$ T cells. We also

examined *in vitro* proliferation in the presence of allogeneic stimulation using Coro $1B^{-/-}$ T cells. Surprisingly, Coro $1B^{-/-}$ T cells were more responsive and proliferated more than wild type T cells after 3 and 5 days of stimulation (Fig 5.3). While there appears to be a role for Coro 1B in GvHD experiments both *in vitro* and *in vivo* to determine the contribution of Coro 1B to disease are ongoing.

5.5 Concluding Remarks

Global Impact of GvHD Research

The main goals of our studies were to identify proteins that were important for aGvHD pathogenesis. More importantly we wanted to determine effective methods for targeting these proteins with the ultimate goal of drug development. Our investigation of migratory proteins revealed targetable proteins for drug development. As aGvHD continues to be worldwide problem we hope that our Coro 1A, *RORC*, and CD62L research is beneficial for cost efficient treatment.

Allogeneic stem cell transplantation is an effective treatment for many hematological malignancies. However, complications from graft-versus-host disease continues to cause morbidity and mortality in transplant patients. While mortality due to relapse has decreased over the past 20 years due to better techniques during transplantation, prophylactic and treatment drug development for GvHD remain stagnant. To date no single drug has been approved by the US Food and Drug Administration for the treatment or prevention of GvHD[24]. Clinical trials continue to focus on the use of steroids however many studies have been unsuccessful[25,26]. For decades we have know that T cells are the major player in the disease. While studies in murine models targeting T cell populations and cytokines

show promising results, translating these results to patients continues to pose a challenge[27-29].

The data presented here further highlight the importance of T cell populations in GvHD and offer potential targets for immunotherapy in disease treatment and prevention. Many researchers believe that targeting specific T cell surface molecules may lead to effective treatments. Our data support these beliefs although the elimination of CD62L was shown to not be critical for T_{reg} protection from aGvHD[28]. Previously it was thought that the difference between haploidentical mismatched and complete mismatched transplant models lied solely in the degree of genetic disparity, however our investigation of *RORC* demonstrated that cytokine production may also vary between these models altering the degree of disease severity[2].

While the success of individual drugs has been minimal, combination steroid therapy has been beneficial for some patients. Our data confirm the benefits of combinational therapy demonstrating that many cytokines and signaling pathways are important for disease manifestations. The identification of novel targets for drug development and the growing knowledge about GvHD promises more effective treatment options for patients.

Figure 5.1

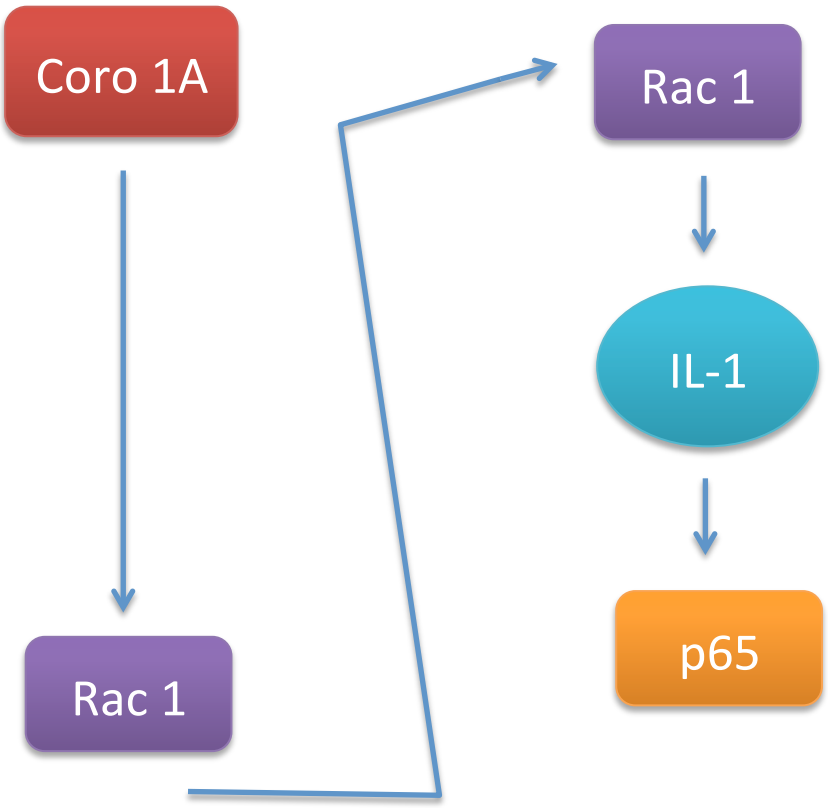


Figure 5.1. Model of Coro 1A Regulation of p65.

Figure 5.2

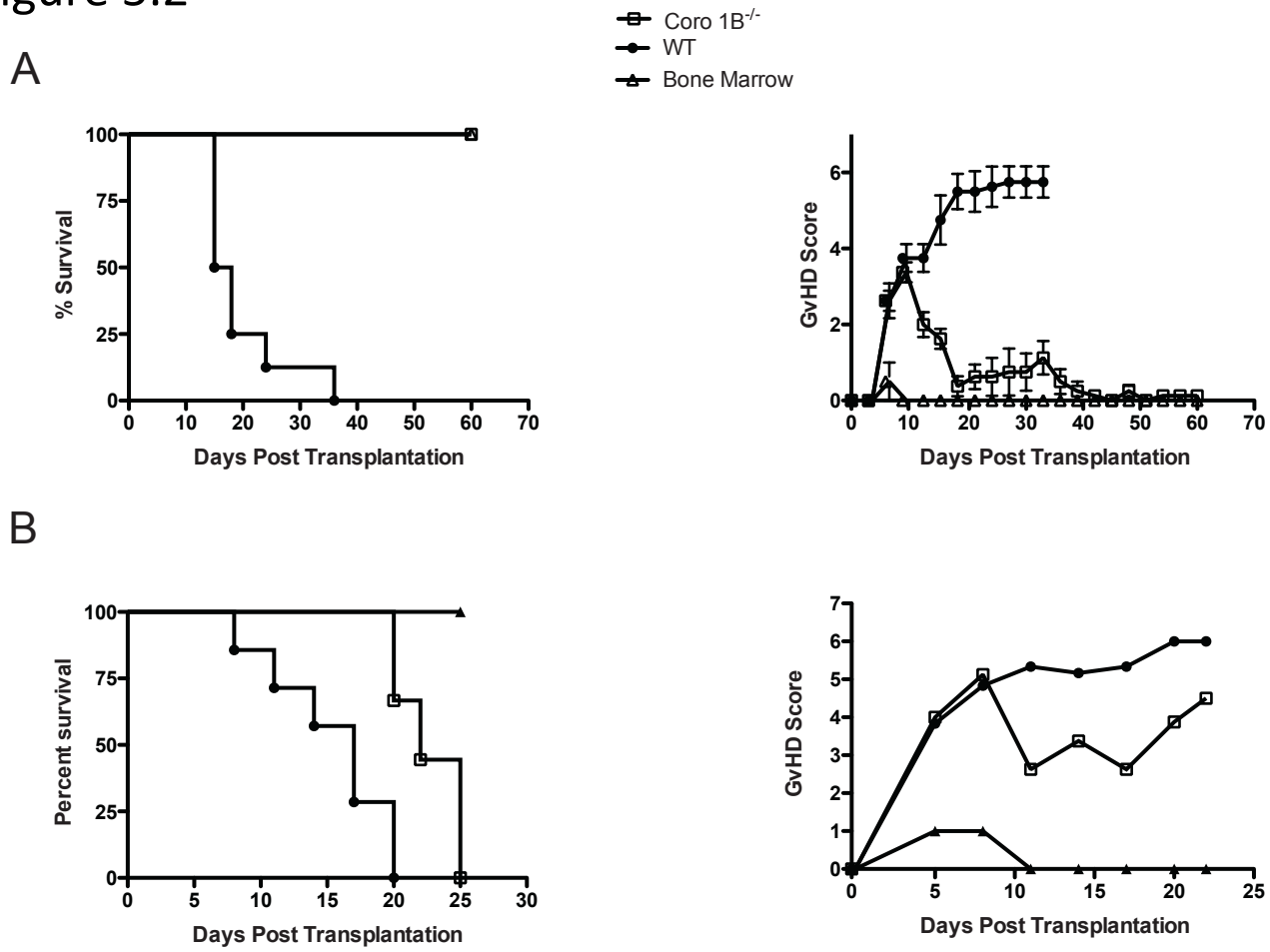


Figure 5.2 GvHD Pathogenesis using Coro 1B$^{-/-}$ T Cells. (A) 4 x 10^6 Coro 1B$^{-/-}$ or WT T cells with 3 x 10^6 TCD bone marrow cells were injected into lethally irradiated B6D2 recipients. n=8 for Coro 1B$^{-/-}$ and WT recipients, n=3 for bone marrow only. (B) 5 x 10^5 Coro 1B-/- or WT T cells with 5 x 10^6 TCD BM cells were infused into lethally irradiated BALB/c recipients. n=8 for Coro 1B$^{-/-}$, n=6 for WT, and n=2 for bone marrow only

Figure 5.3

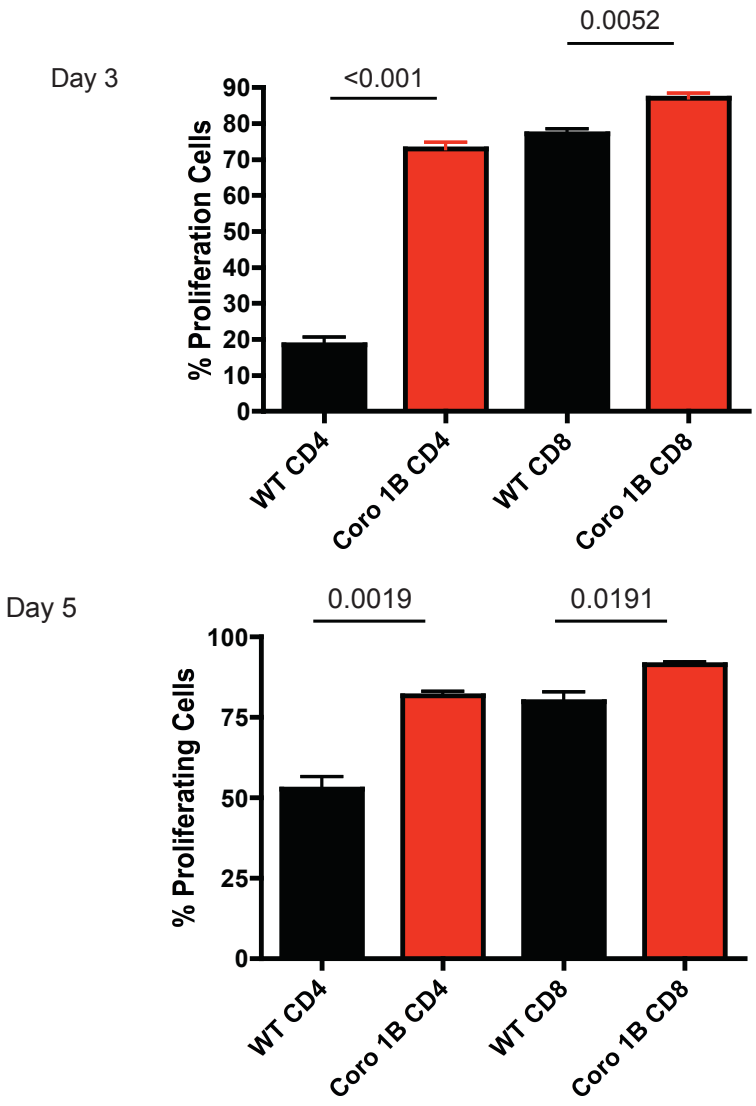


Figure 5.3 *In Vitro* Proliferation of Coro 1B$^{-/-}$ T Cells. Coro 1B$^{-/-}$ and WT T cells were isolated from naïve mice. T cells were labeled with CFSE. Equal amounts of T cells and irradiated B6D2 splenocytes were cultured for 3 or 5 days before harvesting. Cells were stained for CD4 and CD8 and analyzed by flow cytometry.

References

1. Carlson MJ, West ML, Coghill JM, Panoskaltsis-Mortari A, Blazar BR, et al. (2009) In vitro-differentiated TH17 cells mediate lethal acute graft-versus-host disease with severe cutaneous and pulmonary pathologic manifestations. Blood 113: 1365–1374. doi:10.1182/blood-2008-06-162420.

2. Fulton LM, Carlson MJ, Coghill JM, Ott LE, West ML, et al. (2012) Attenuation of Acute Graft-versus-Host Disease in the Absence of the Transcription Factor RORγt. J Immunol. doi:10.4049/jimmunol.1200858.

3. Kobbe G, Schneider P, Rohr U, Fenk R, Neumann F, et al. (2001) Treatment of severe steroid refractory acute graft-versus-host disease with infliximab, a chimeric human/mouse antiTNFalpha antibody. Bone Marrow Transplant 28: 47–49. doi:10.1038/sj.bmt.1703094.

4. Hervé P, Flesch M, Tiberghien P, Wijdenes J, Racadot E, et al. (1992) Phase I-II trial of a monoclonal anti-tumor necrosis factor alpha antibody for the treatment of refractory severe acute graft-versus-host disease. Blood 79: 3362–3368.

5. Yu Y, Wang D, Liu C, Kaosaard K, Semple K, et al. (2011) Prevention of GVHD while sparing GVL effect by targeting Th1 and Th17 transcription factor T-bet and RORγt in mice. Blood 118: 5011–5020. doi:10.1182/blood-2011-03-340315.

6. Yi T, Chen Y, Wang L, Du G, Huang D, et al. (2009) Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. Blood 114: 3101–3112. doi:10.1182/blood-2009-05-219402.

7. Nistala K, Adams S, Cambrook H, Ursu S, Olivito B, et al. (2010) Th17 plasticity in human autoimmune arthritis is driven by the inflammatory environment. Proc Natl Acad Sci USA 107: 14751–14756. doi:10.1073/pnas.1003852107.

8. Wagner JE, Thompson JS, Carter SL, Kernan NA, Unrelated Donor Marrow Transplantation Trial (2005) Effect of graft-versus-host disease prophylaxis on 3-year disease-free survival in recipients of unrelated donor bone marrow (T-cell Depletion Trial): a multi-centre, randomised phase II-III trial. Lancet 366: 733–741. doi:10.1016/S0140-6736(05)66996-6.

9. Antin JH (2011) T-cell depletion in GVHD: less is more? Blood 117: 6061–6062. doi:10.1182/blood-2011-04-348409.

10. Shimabukuro-Vornhagen A, Hallek MJ, Storb RF, Bergwelt-Baildon von MS (2009) The role of B cells in the pathogenesis of graft-versus-host disease. Blood 114: 4919–4927. doi:10.1182/blood-2008-10-161638.

11. Socié G, Mary J-Y, Lemann M, Daneshpouy M, Guardiola P, et al. (2004) Prognostic

value of apoptotic cells and infiltrating neutrophils in graft-versus-host disease of the gastrointestinal tract in humans: TNF and Fas expression. Blood 103: 50–57. doi:10.1182/blood-2003-03-0909.

12. Saad AG, Alyea EP, Wen PY, Degirolami U, Kesari S (2009) Graft-versus-host disease of the CNS after allogeneic bone marrow transplantation. J Clin Oncol 27: e147–e149. doi:10.1200/JCO.2009.21.7919.

13. Hall A (1998) Rho GTPases and the actin cytoskeleton. Science 279: 509–514.

14. Sit S-T, Manser E (2011) Rho GTPases and their role in organizing the actin cytoskeleton. J Cell Sci 124: 679–683. doi:10.1242/jcs.064964.

15. Castro-Castro A, Ojeda V, Barreira MIA, Sauzeau V, rida IN-LE, et al. (2011) Coronin 1A promotes a cytoskeletal-based feedback loop that facilitates Rac1 translocation and activation. EMBO J 30: 3913–3927. doi:10.1038/emboj.2011.310.

16. Jefferies CA, O'Neill LA (2000) Rac1 regulates interleukin 1-induced nuclear factor kappaB activation in an inhibitory protein kappaBalpha-independent manner by enhancing the ability of the p65 subunit to transactivate gene expression. J Biol Chem 275: 3114–3120.

17. Dannemann M, Prüfer K, Lizano E, Nickel B, Burbano HA, et al. (2012) Transcription factors are targeted by differentially expressed miRNAs in primates. Genome Biol Evol 4: 552–564. doi:10.1093/gbe/evs033.

18. Choo A, Palladinetti P, Holmes T, Basu S, Shen S, et al. (2008) siRNA targeting the IRF2 transcription factor inhibits leukaemic cell growth. Int J Oncol 33: 175–183.

19. Wysocki CA, Burkett SB, Panoskaltsis-Mortari A, Kirby SL, Luster AD, et al. (2004) Differential roles for CCR5 expression on donor T cells during graft-versus-host disease based on pretransplant conditioning. J Immunol 173: 845–854.

20. Brunstein CG, Miller JS, Cao Q, McKenna DH, Hippen KL, et al. (2011) Infusion of ex vivo expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. Blood 117: 1061–1070. doi:10.1182/blood-2010-07-293795.

21. Edinger M, Hoffmann P, Ermann J, Drago K, Fathman CG, et al. (2003) CD4+CD25+ regulatory T cells preserve graft-versus-tumor activity while inhibiting graft-versus-host disease after bone marrow transplantation. Nat Med 9: 1144–1150. doi:10.1038/nm915.

22. Sallusto F, Lenig D, Förster R, Lipp M, Lanzavecchia A (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 401: 708–712. doi:10.1038/44385.

23. Anderson BE, McNiff J, Yan J, Doyle H, Mamula M, et al. (2003) Memory CD4+ T

cells do not induce graft-versus-host disease. J Clin Invest 112: 101–108. doi:10.1172/JCI200317601.

24. Pavletic SZ, Fowler DH (2012) Are we making progress in GVHD prophylaxis and treatment? Hematology Am Soc Hematol Educ Program 2012: 251–264. doi:10.1182/asheducation-2012.1.251.

25. Alousi AM, Weisdorf DJ, Logan BR, Bolaños-Meade J, Carter S, et al. (2009) Etanercept, mycophenolate, denileukin, or pentostatin plus corticosteroids for acute graft-versus-host disease: a randomized phase 2 trial from the Blood and Marrow Transplant Clinical Trials Network. Blood 114: 511–517. doi:10.1182/blood-2009-03-212290.

26. Levine JE, Paczesny S, Mineishi S, Braun T, Choi SW, et al. (2008) Etanercept plus methylprednisolone as initial therapy for acute graft-versus-host disease. Blood 111: 2470–2475. doi:10.1182/blood-2007-09-112987.

27. Zheng J, Liu Y, Liu Y, Liu M, Xiang Z, et al. (2013) Human CD8+ regulatory T cells inhibit GVHD and preserve general immunity in humanized mice. Sci Transl Med 5: 168ra9. doi:10.1126/scitranslmed.3004943.

28. Carlson MJ, Fulton LM, Coghill JM, West ML, Burgents JE, et al. (2010) L-selectin is dispensable for T regulatory cell function postallogeneic bone marrow transplantation. Am J Transplant 10: 2596–2603. doi:10.1111/j.1600-6143.2010.03319.x.

29. Alyea E, Weller E, Schlossman R, Canning C, Webb I, et al. (2001) T-cell--depleted allogeneic bone marrow transplantation followed by donor lymphocyte infusion in patients with multiple myeloma: induction of graft-versus-myeloma effect. Blood 98: 934–939.