Defining a Role for T Regulatory Cell Expressed MyD88 During the Response to Allografts

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Defining a role for T regulatory cell expressed MyD88 during the response to allografts

A dissertation presented

by

Christopher Michael Borges

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

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Defining a role for T regulatory cell expressed MyD88 during the response to allografts

Abstract

The myeloid differentiation primary response gene 88 (MyD88) is an adaptor protein proximally downstream of the Toll-like receptor (TLRs) and the IL-1 receptor family (IL-1R, IL-18R and IL-33R). The TLR-MyD88 signaling pathway enables the innate immune system to sense inflammation and promote adaptive immune responses. TLRs are also expressed on T cells, and we have previously demonstrated that T cell intrinsic MyD88 signaling promotes T cell survival and is required for optimal responses to pathogens such as *T. gondii* and LCMV. Surprisingly however, we observed that mice with a targeted T cell deletion of MyD88 (MyD88^{fl/fl} x CD4-Cre, termed MyD88-ΔT) reject bm12 cardiac allografts, and bm12 skin allografts when treated with αCD154 and rapamycin, at a higher frequency than wild type (WT) mice. As Tregs are critical for graft prolongation following costimulatory blockade in this model, we hypothesized a “defect” in MyD88-ΔT Tregs in the above model. As MyD88-ΔT mice do not allow us to differentiate the effect of MyD88 deletion specifically on Tregs versus the T cell compartment as a whole, we created mice with a targeted deletion of MyD88 specific to Tregs (MyD88^{fl/fl} x FoxP3-Cre-YFP, MyD88-ΔTreg). MyD88-ΔTreg mice, similar to MyD88-ΔT mice have a similar Treg frequency when compared to WT mice and have no observable signs of autoimmunity. We found that MyD88-ΔTreg mice failed to accept bm12 skin allografts at the same frequency as WT control mice when given CoB, suggesting that the phenotype we observed in the MyD88-ΔT mice was likely caused by MyD88 deficient Tregs. As T cell intrinsic MyD88 is required for T cell survival during the response to pathogens, we thus hypothesized that the inability for MyD88 deficient Tregs to promote long-term allograft survival was due to an
inability of these cells to survive. Surprisingly, through a series of in vivo and in vitro experiments, we observed that MyD88 deficient Tregs survive as well as WT Tregs under competitive conditions with WT Tregs, during activation with \( \alpha \) CD3 and \( \alpha \) CD28, as well as in the allograft 21 days post transplantation. In addition, MyD88 deficient Tregs survived as well as WT Tregs when activated and cultured with rapamycin and \( \alpha \) CD154. In an attempt to reconcile the inability of MyD88-deficient Tregs to promote graft survival with their surprising ability to survive as well as WT Tregs, we next assessed the function of MyD88-deficient Tregs. In an in vitro suppression assay, MyD88-deficient Tregs suppressed effector T cell proliferation as well as WT Tregs at multiple Treg:T effector ratios. In addition, MyD88-deficient Tregs expressed similar levels of the Treg functional proteins CTLA-4, PD-1, Granzyme B, Lag-3, GITR, CD39, CD73 and CD28 as WT Tregs, suggesting that the function of MyD88-deficient Tregs may only be impaired during the response to alloantigen. Together, these data define MyD88 as having a divergent requirement for cell survival in non-Tregs and Tregs, and a yet-to-be defined functional requirement in Tregs during the response to alloantigen.
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Here's to the crazy ones,
the misfits, the rebels,
the troublemakers,
the round pegs in the square holes...
the ones who see things differently.
They're not fond of rules
and they have no respect for the status quo.
You can quote them, disagree with them,
glorify, or vilify them.
But the only thing you can't do is ignore them,
because they change things...
they push the human race forward.
And while some may see them as the crazy ones,
we see genius.

**Because the people who are crazy enough to think they can change the world, are the ones who do.**

Rob Siltanen, Ken Segall &
Steve Jobs
Chapter 1: Introduction

Overview and History of Transplantation

Solid organ transplantation has improved the well being of thousands of patients with chronic organ failure since the first kidney was transplanted by Dr. Joseph Murray between two identical twins in 1954 at The Peter Bent Brigham Hospital in Boston.(Harrison et al., 1956; Merrill et al., 1956) As most patients requiring a solid organ transplant do not have an identical twin for use as a donor, transplantation between two genetically disparate individuals became necessary. However, even before Dr. Murray’s pioneering surgery, it was clear that that tissue rejection would be a barrier to organ transplantation between two genetically disparate individuals. Despite previous descriptions of successful skin grafting in humans, Drs. Peter Medawar and Thomas Gibson were the first investigators to detail that while autografts were not destroyed following transplantation, grafts transplanted from a patient’s brother were eventually destroyed.(Gibson and Medawar, 1943) This study and others by Medawar in a large number of rabbits were the first to describe in detail an adaptive immune response against tissue transplanted from the same species with “ordinary genetic diversity”.(Medawar, 1944) Following these studies, Billingham, Brent and Medawar demonstrated that leukocytes are the key mediators of graft rejection by placing A strain skin onto CBA strain recipients. Cells from the recipient lymph nodes were transferred into a different cohort of naïve CBA mice, and accelerated graft rejection in the secondary hosts was observed, clearly due to the infused leukocytes from the primary host lymph nodes.(Billingham et al., 1954)

We now appreciate that graft rejection occurs predominantly as a result of a highly polymorphic region of the genome called the major histocompatibility complex (MHC).(Gorer, 1948; Snell, 1948) Genetic differences between individuals were first appreciated when a tumor derived from an albino mouse grew when transplanted to any other albino mouse and F1 hybrids with black mice, but was rejected when transplanted into black mice.(Gorer, 1937)
Alloreactive is the term used to describe the recognition of genetic disparities among individuals of the same species. There are two well-studied pathways of allorecognition; direct and indirect. (Lechler and Batchelor, 1982) Direct alloreactivity is defined as the response of T cells specific for any peptide in association with self MHC that are reactive with an allogeneic MHC loaded with an endogenous peptide. (Lombardi et al., 1989; Morris et al., 2011) While this idea was first appreciated in mixed lymphocyte cultures/reactions (MLC/MLR) with irradiated F1 lymphocytes triggering the proliferation of parental lymphocytes, only recently have crystal structures of allogeneic TCRs complexed with MHC molecules demonstrated that alloreactive TCRs directly recognized peptide+MHC via. 2 mechanisms; either the TCR contact points (CDRs) were concentrated along the peptide, or rotated to be in greater contact with the differing peptides of the MHC itself. (Colf et al., 2007; Macdonald et al., 2009; Wilson, 1967) Of note, Matzinger and Bevan previously proposed both of these non-mutually exclusive binding patterns of the TCR to allo-MHC as an explanation for the vigorous nature of the response to alloantigen (discussed further below). (Bevan, 1984; Matzinger and Bevan, 1977) In addition, evidence for direct alloreactivity came from experiments indicating that in vitro culture of thyroid grafts prior to transplantation resulted in prolonged graft survival. Interestingly, infusion of donor splenocytes after transplantation of the cultured graft resulted in rejection indicating that a supply of donor antigen, presumably via APCs, egressed from the graft during in vitro culture. (Lafferty et al., 1976) Furthermore, (ASxAUG)F1 renal allografts were not rejected when transplanted into AS strain rats treated with DST and AS anti-AUG antiserum. (Batchelor et al., 1979) When the grafts were re-transplanted into naïve AS strain rats (secondary recipients), they were rejected only if donor dendritic cells collected from the thoracic duct were transferred at the time of transplantation, suggesting that host T cells were directly primed by donor dendritic cells, presumably expressing MHC class II, and eventually rejected the graft. (Lechler and Batchelor, 1982)
Indirect alloreactivity is defined as the response of T cells to an allogeneic MHC peptide in the context of self MHC. Groups studying the effect of pre-transplant organ culture on graft survival originally proposed the idea of an indirect pathway as a means to explain graft survival in some strain combinations but not others. (La Rosa and Talmage, 1983; Lechler and Batchelor, 1982) Formal evidence for indirect alloreactivity was demonstrated when skin grafts from MHC Class II deficient mice were rejected with similar kinetics as WT skin grafts, demonstrating that direct recognition of donor MHC Class II was not required for graft rejection and CD4 cells (in this model) were capable of responding to donor MHC Class I in the context of host MHC Class II. (Auchincloss et al., 1993) Furthermore, dendritic cells from C57BL/6 (I-Eα-/I-Aβ+) recipients stained positive for an antibody specific for I-Eα peptides presented by I-Aβ MHC Class II molecules (Y-Ae) following adoptive transfer of I-Eα+ dendritic cells into the footpad, demonstrating that recipient cells could process and present allogeneic MHC derived from cellular products. (Inaba et al., 1998) Together, the above discussion demonstrates that alloantigen recognition occurs via two pathways, direct and indirect, and ultimately leads to vigorous rejection of transplanted organs.

**Immunosuppression for Long-Term Allograft Survival**

Irrespective of whether alloantigen is presented via the direct or indirect pathways, the response to alloantigen is robust due to a high alloreactive T cell precursor frequency (i.e. the frequency of cells in the periphery capable of responding to alloantigen). While the precursor frequency to T cells specific for foreign peptide antigens is estimated to be 1 in $10^4$ to 1 in $10^6$, the alloreactive precursor frequency was demonstrated to be 1 in $10^2$ or 1 in 10. (Blattman et al., 2002; Butz and Bevan, 1998; Moon et al., 2007; Suchin et al., 2001) This high precursor frequency results in vigorous immune mediated rejection of allografts and necessitated the development of strategies that could be used to prevent the unwanted response to a
transplanted organ. Initially, total body irradiation (TBI) resulted in increased skin allograft survival in rabbits,(Dempster et al., 1950) but effectiveness in patients was low.(Murray, 1992; Murray et al., 1960) The administration of 6-mercaptopurine inhibited rejection of kidney allografts in dogs,(Calne, 1960) and the imidazole derivative of it, azathioprine or Imuran, proved to be highly effective in prolonging allograft survival in patients.(Murray et al., 1963)

Beginning in the late 1970s, a new class of immunosuppresants became available with the discovery of a metabolite from the fungus *Tolypocladium inflatum*. Cyclosporine A, now understood to prevent calcium flux following TCR signaling by binding to cyclophilin and inhibiting calcineurin,(Clipstone and Crabtree, 1992; Handschumacher et al., 1984) prevented allograft rejection in a high frequency of animals and patients.(Borel et al., 1976; Calne et al., 1978) Isolated from *Streptomyces tsukubaensis* about a decade after cyclosporine A,(Kino et al., 1987a; Kino et al., 1987b) FK506 (tacrolimus) was demonstrated to inhibit T cell activation by binding to FK506-binding protein 12 (FKB12), a protein similar in function to cyclophilin.(Bierer et al., 1990b; Siekierka et al., 1989) Clinically, FK506 was demonstrated to be as effective, if not more effective than cyclosporine A in a variety of transplant cases.(Group, 1994; Starzl et al., 1989) Finally, in the mid 1970s rapamycin (sirolimus) was isolated from *Streptomyces hygroscopicus* found on The Easter Island Rapa Nui, and was found to have potent anti-fungal properties.(Sehgal et al., 1975; Vezina et al., 1975) However, shortly after its discovery, rapamycin was indicated to have immunosuppressive properties in a model of autoimmune encephalomyelitis and arthritis,(Martel et al., 1977) and was later confirmed to be effective in promoting long term allograft survival in animals and patients.(Brattstrom et al., 1997; Calne et al., 1989; Ferron et al., 1997) Like FK506, rapamycin binds to FKB12, however inhibition of cell proliferation is due to interference with mTOR function instead of inhibition of calcineurin.(Bierer et al., 1990a; Dumont and Su, 1996; Sabers et al., 1995)

Another approach used to prolong allograft survival took advantage of biologics. Élie Metchnikoff made “anti-lymph gland serum” by exposing either rat or guinea pig cellular
suspensions to serum he made from injecting rabbits with either guinea pig or rat lymph node and splenic cellular suspensions. He demonstrated specificity of the serum, as it was only toxic to the strain of cells from which the immunizing cells were derived. (Metchnikoff, 1899)

Metchnikoff’s studies were furthered by serum developed against lymphocytes (and more specifically T cells by immunizing rabbits or horses with thymocytes, termed ATG) which was proven highly effective in vitro as well as in the clinic, and is still in use today. (Birtch et al., 1971; Cosimi et al., 1976; Pappenheimer, 1917) While the use of ATG was powerful clinically, the use of monoclonal antibodies allowed for greater specificity. Once developed, monoclonal antibodies to CD4, CD8 and all T cells (CD3) were used as a diagnostic tool (through flow cytometry) and anti-CD3 was used as a highly specific depleting antibody in transplant patients. (Cosimi et al., 1981)

Costimulatory Blockade for Long-Term Allograft Survival

Prior to the formal description of T cells, (Miller and Mitchell, 1968; Mitchell and Miller, 1968) the finding that thymectomized neonatal mice transplanted with allogeneic skin grafts later in life were not rejected demonstrated that T cells were required for allograft rejection. (Miller, 1962) We now appreciate that in order to respond to alloantigen, T cells must be activated through their TCR (triggered by binding peptide+MHC) (Dembic et al., 1986; Katz et al., 1973; Kindred and Shreffler, 1972; Zinkernagel and Doherty, 1974) and receive costimulatory signals, through CD28-B7.1 on a cognate antigen presenting cell (APC) resulting in the transcription and stability of IL-2 mRNA and expression of anti-apoptotic proteins such as Bcl-xL (Boise et al., 1995; Fraser et al., 1991; Jenkins et al., 1991; June et al., 1989; Lindstein et al., 1989) TCR stimulation in the absence of costimulation resulted in the failure of T cells to proliferate, suggesting that blockade of the CD28 costimulatory pathway blocked antigen specific T cell activation. (Jenkins et al., 1990; Schwartz, 1990) Indeed, infusion of a high affinity fusion protein containing the extracellular domain of human CTLA-4 fused to a human common
γ-immunoglobulin chain (termed CTLA4Ig) (Linsley et al., 1991) resulted in the blockade of acute rejection of fully mismatched cardiac allografts in rats, demonstrating that the CD28-B7 interaction was required for the response to alloantigen. (Turka et al., 1992) This study, and a study improving on the prior results by co-administering donor splenocytes (DST) with CTLA4Ig were the first reports of costimulatory blockade improving fully mismatched allograft survival in rodents (Lin et al., 1993) and held the promise of specifically targeting alloreactive T cells, but sparing pathogen reactive T cells, resulting in patients that exhibited donor-specific non-responsiveness but were otherwise immunocompetent.

Many studies have now used CTLA4Ig and other costimulatory blocking agents to prolong allograft survival in rodents; one other example being the blockade of the CD40-CD40L/CD154 costimulatory pathway. CD40 is a member of the TNF receptor family that is constitutively expressed on B cells, dendritic cells and other APCs and when engaged by activated T cells expressing its ligand CD40L/CD154/gp39, accounts for T cell dependent B cell activation (proliferation and class switching) as well as the upregulation of B7, among other costimulatory molecules that are critical for robust T cell activation. (Armitage et al., 1992; Noelle et al., 1992; Ranheim and Kipps, 1993) This costimulatory pathway was targeted with an anti-CD154 monoclonal antibody (mAb) in combination with DST resulting in the long-term survival of fully mismatched pancreatic islet cell as well as fully mismatched skin grafts. (Markees et al., 1997; Parker et al., 1995) Despite promising results using costimulatory blockade for long-term allograft survival in rodent models, translation to primate models and the clinic was unfortunately not as successful. (Kanmaz et al., 2004; Kawai et al., 2000; Kirk et al., 1997; Levisetti et al., 1997)

**T regulatory cells**

In parallel to the costimulatory blockade studies discussed above, many groups were beginning to investigate a T cell subset that could suppress immune responses *in situ*. Two
groups are credited with the initial discovery and description of this suppressor population of T cells. In 1969, Nishizuka and Sakakura demonstrated that neonatally thymectomized mice experienced autoimmunity of the ovary when their thymus was removed 3 days, but not 1 or 7 days, after birth. Interestingly, autoimmunity could be rescued by grafting newborn thymus 4 days after day 3 thymectomy (on day 7 of life), demonstrating for the first time that a population of thymocytes was required to prevent autoimmunity. (Nishizuka and Sakakura, 1969) In 1972, Gershon and colleagues adoptively transferred syngeneic thymocytes into irradiated hosts that were then immunized with sheep red blood cells. After 7 days, splenocytes were harvested from these mice and injected into newly irradiated recipients with or without naïve thymocytes, and after 3,4 or 5 days incorporation of $^{125}$I 5-iodo-2-deoxyuridine ($^{125}$I UDR) was measured in the spleens from the secondary recipients. Interestingly, less $^{125}$I UDR was incorporated in splenocytes from the mice that were immunized with the antigen experienced splenocytes in addition to naïve thymocytes than antigen experienced splenocytes alone, suggesting that a population of thymocytes was suppressing the proliferation of splenocytes to antigen rechallenge. (Gershon et al., 1972) After a hiatus in the study of T suppressor cells, while performing adoptive transfer experiments in a rat model of long-term allograft survival induced by cyclosporine, Hall and colleagues identified a CD4+ IL-2 receptor (CD25)+ T cell that prevented the rejection of graft reactive T cells present after transplantation. Adoptive transfer of CD4+CD25+ cells transfered the tolerance induced to cardiac allografts with cyclosporine to naïve, secondary recipients. (Hall et al., 1990) Accordingly, adoptive transfer of CD4+CD25- T cells alone into Balb/c nude mice led to wide-spread autoimmune disease that could be subsequently rescued by co- transfer with CD4+CD25+ T cells, demonstrating that the CD4+CD25+ cell population was critical for maintaining peripheral tolerance. (Sakaguchi et al., 1995)

The scurfy mouse was initially developed out of a spontaneous mutation in 1950 and was later characterized to have broad autoimmune pathology associated with lymphoid and
myeloid multi-organ infiltrates. (Godfrey et al., 1991; Russell et al., 1959) The mutation causing the scurfy phenotype in mice was identified as FoxP3, a transcriptional regulator with an ortholog identified in the human disease immunodysregulation, polyendocrinopathy, enteropathy, X-linked, or IPEX. (Bennett et al., 2001; Brunkow et al., 2001) Expression of FoxP3 was shown to promote suppressive activity in T cells, demonstrating that FoxP3 was the principle gene for T regulatory cell (Treg) function. (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003)

**Treg Suppressive Mechanisms**

Tregs suppress via a number of likely non-mutually exclusive mechanisms. *In vitro*, Treg suppression was dependent on TCR stimulation and cell contact with the Treg target. (Thornton and Shevach, 2000) It is now appreciated that Tregs depend on TCR signaling for their development and function *in vivo*. For example, Tregs were undetectable in Rag2−/− DO.11.10 TCR transgenic mice, wherein CD4 T cells were specific for a foreign peptide derived from chicken ovalbumin. (Itoh et al., 1999) In contrast, TCR transgenic mice specific for a self-antigen developed Tregs only when the self-peptide was expressed using a second transgene. (Jordan et al., 2001) While these studies made clear that the TCR was critical for Treg development, the role of the TCR on Treg following Treg development has remained elusive. By using mice with an inducible Treg specific deletion of the TCR, it is now appreciated that the TCR is required on Tregs for their suppressive function through induction of a number of target genes utilized by Tregs to suppress effector T cells. (Levine et al., 2014; Vahl et al., 2014)

Unlike their effector T cell counter parts, Tregs do not produce IL-2 in response to antigen or TCR stimulation resulting in the lack of an autocrine proliferative stimulus. Instead, Tregs relied on exogenous sources of IL-2 for their proliferation, survival and function, predominantly produced by CD25low T cells. (D'Cruz and Klein, 2005; Fontenot et al., 2005; Setoguchi et al., 2005) Because Tregs expressed the high affinity IL-2 receptor but failed to produce IL-2, one proposed mechanism of suppression was that Tregs consume IL-2, depleting
it from the microenvironment. In support of this, naïve T cells from IL-2-GFP knock-in mice, which expressed CD25 but did not produce IL-2 (similar to Tregs), were able to suppress the proliferation of WT naïve T cells in vitro. Furthermore, less IL-2 was detected in Tregs and naïve T cell co-culture supernatants than naïve T cell culture supernatants, and Tregs were found to bind more IL-2 than their naïve counterparts. (Pandiyan et al., 2007) Despite this in vitro evidence for Treg consumption of IL-2, IL-2 or IL-2 receptor knockout mice have autoimmune rather than immunosuppressive phenotypes, suggesting that naïve T cells can efficiently proliferate and cause disease even in the absence of IL-2. (Caudy et al., 2007; Horak, 1995; Kramer et al., 1995)

Signaling through CD28 in developing thymocytes resulted in apoptosis and negative selection, despite being critical for mature T cell activation. (Punt et al., 1997; Punt et al., 1994) This paradox is further complicated as mice deficient in CD28, or its ligand B7, have reduced numbers of Tregs in the thymus and the periphery, suggesting that CD28 was critical for Treg development. (Salomon et al., 2000; Tai et al., 2005; Tang et al., 2003) Because the above studies used germ line CD28 knockout mice, it was difficult to elucidate a specific role for Treg development in the thymus, function and maintenance in the periphery. Using conditional deletion of CD28 in cells expressing FoxP3 (termed CD28ΔTreg mice), CD28 was demonstrated to be essential for Treg survival and function, as CD28ΔTreg mice develop lymphadenopathy and splenomegaly as well as autoimmune skin and lung lesions. (Zhang et al., 2013a) CD28 signaling in Tregs is required for CCR6 expression and efficient homing of Tregs to the skin, as well as CTLA-4 and PD-1 expression, surface proteins critical for Treg function (discussed below). (Zhang et al., 2013a)

CTLA-4 is an inhibitory molecule expressed by activated T cells that opposes CD28 costimulatory signaling; binding of CTLA-4 to B7.1 and B7.2 results in decreased T cell activation through a blockade in cell cycle progression and inhibition of IL-2.
transcription. (Brunner et al., 1999; Krummel and Allison, 1996) Mice deficient in CTLA-4 succumbed to fatal multi-organ autoimmunity shortly after birth, suggesting that CTLA-4 signaling was critical in preventing aberrant immune activation. (Tivol et al., 1995; Waterhouse et al., 1995) Furthermore, CTLA-4 deficiency specifically in the Treg compartment of mice resulted in a fatal T cell mediated autoimmune disease similar to the phenotype observed in CTLA-4 germ line knockout mice, demonstrating that Treg expressed CTLA-4 is critical for Treg function. (Wing et al., 2008) While Treg expressed CTLA-4 has long thought to block CD80 and CD86 interactions with T effector cell CD28 through direct binding, CTLA-4 has recently been demonstrated to trans-endocytose CD80 and CD86 from APCs, resulting in an APC lacking the necessary ligands for effector cells to be costimulated through CD28 following TCR activation. (Gu et al., 2012; Qureshi et al., 2011)

PD-1 is another inhibitory molecule expressed on activated T cells, and signaling via its ligands PD-L1 (expressed on APCs, B cells, T cells and endothelial cells) and PD-L2 (expressed exclusively on DCs and macrophages) were important in restraining T cell activation that would otherwise result in autoimmunity. (Brown et al., 2003; Freeman et al., 2000; Latchman et al., 2001) PD-1 is also expressed on Tregs, and blockade of the interaction between PD-1 and PD-L1 resulted in decreased suppressive function. (Baecher-Allan et al., 2003) In addition, PD-L1 signaling through PD-1 on naïve T cells resulted in the conversion of naïve T cells to Tregs by attenuating AKT and mTOR phosphorylation, suggesting that this pathway was critical for de novo Treg development. (Francisco et al., 2009; Krupnick et al., 2005)

Cytokines are important positive and negative signals used by immune cells to communicate. TGF-β1 is one of three structurally similar cytokines in the TGF-β super family with broad tissue expression that generally results in cell growth inhibition. TGF-β1 was identified to be critical for immune regulation when germline TGF-β1 knockout mice were found to experience multi-organ inflammatory infiltrates and a wasting phenotype at 3 weeks of
Furthermore, mice lacking the TGF-β receptor TGF-BRII specifically on T cells also developed autoimmunity, suggesting that signaling by TGF-β through the TGF-β1 receptor was critical to prevent aberrant immune activation. (Gorelik and Flavell, 2000; Lucas et al., 2000) As TGF-β is produced by a wide variety of cell types, it is not surprising that Tregs also produce secreted and membrane-bound TGF-β that was critical for the suppression of T cell proliferation in vitro and in vivo. (Chen et al., 2005; Nakamura et al., 2001) Treg produced membrane bound TGF-β provides further evidence for a cell-contact dependent mechanism of suppression as discussed above. Initially, membrane bound TGF-β is associated with the latency associated peptide (LAP), rendering TGF-β inactive. (Gentry and Nash, 1990; Lawrence et al., 1984) Multiple mechanisms have been proposed to explain the activation of membrane bound TGF-β; LAP can be stripped off of the inactive TGF-β molecule by plasmin, or binding to the αvβ6 integrin, resulting in a conformational change rendering TGF-β active. (Munger et al., 1999; Yehualaeeshet et al., 1999) Despite the apparent importance of TGF-β in regulating immune homeostasis as discussed above, it is not a mutually exclusive mechanism of suppression as FoxP3 negative T cells exposed to TGF-β were capable of causing skin graft rejection. (Regateiro et al., 2012)

The cytokine IL-10 is produced by APCs as well as T cells, including Tregs, and is critical for immune regulation, as mice lacking IL-10 developed colitis associated with immune infiltrates and increased IgA deposits in intestinal tissues. (de Waal Malefyt et al., 1991; Fiorentino et al., 1989; Iwasaki and Kelsall, 1999; Kuhn et al., 1993) Furthermore, IL-10 production specifically by Tregs was critical to prevent adoptive transfer colitis in Rag-2−/− or SCID mice. (Asseman et al., 1999; Groux et al., 1997) IL-10 administration prior to cardiac transplantation prevented graft rejection while IL-10 administration at the time of, or after cardiac transplantation in mice resulted in enhanced rejection and resulted in resistance to immunosuppression. (Li et al., 1999a, b; Li et al., 1997; Qian et al., 1996) These data suggest
that IL-10 signaling requires temporal regulation in order to promote immune regulation, especially in the context of organ transplantation.

IL-27, a dimer of IL-27β paired with IL-27α is produced by activated APCs and synergizes with IL-12 to trigger proliferation of naïve T cells and production of IFN-γ. (Pflanz et al., 2002) While there was no apparent lymphoproliferation or autoimmunity in mice lacking the IL-27β gene (Ebi3), Ebi3 was upregulated in Tregs relative to non-Tregs. (Gavin et al., 2007; Nieuwenhuis et al., 2002) Interestingly, a novel Ebi3-IL-12α heterodimer termed IL-35 was secreted by Tregs. Recombinant IL35 suppressed naïve T cell proliferation in vitro, and Tregs from either Ebi3−/− mice or IL-12a−/− mice failed to suppress Treg proliferation in vitro and could not prevent adoptive transfer colitis in Rag-1−/− mice, demonstrating that IL-35 production by Tregs is another suppressive mechanism mediated by Treg produced cytokines. (Collison et al., 2007)

Perforins and granzymes are molecules classically implicated in CD8 cytotoxic T lymphocyte killing of virally infected cells. (Heusel et al., 1994; Kagi et al., 1994) Interestingly, Tregs also express granzyme A and/or granzyme B, and use these molecules to kill effector cells through perforin dependent and independent mechanisms in vitro. (Gondek et al., 2005; Grossman et al., 2004a; Grossman et al., 2004b) While Tregs did not express granzyme B in naïve mice, tumor infiltrating Tregs upregulated granzyme B. Interestingly, Tregs lacking granzyme B and perforin were unable to efficiently kill effector cells resulting in decreased tumor growth, suggesting that Tregs utilized granzyme B and perforin in vivo to suppress effector cells. (Cao et al., 2007)

The purine nucleoside adenosine accumulates in inflammatory microenvironments and signaling through the high affinity surface receptor, A2A, results in an accumulation of immunosuppressive intracellular cyclic adenosine mono-phosphate (cAMP). (Bodin and Burnstock, 1998; Cronstein et al., 1983; Huang et al., 1997; Ohta and Sitkovsky, 2001; Richman
and Wyborny, 1964) In T cells, accumulation of cAMP via A2A signaling blocked cell proliferation, IL-2 production, the ability to lyse target cells and IFN-γ secretion. (Henney and Lichtenstein, 1971; Huang et al., 1997; Lappas et al., 2005; Novak and Rothenberg, 1990) Interestingly, Tregs contained high basal levels of cAMP in comparison to naïve CD4+ T cells, and transferred cAMP into target cells via gap junctions as a suppressive mechanism. (Bopp et al., 2007)

The cell-surface proteins CD39, an ecto-nucleoside triphosphate diphosphohydrolase that catalyzes the reaction of extracellular ATP into AMP, and CD73, an ecto-5'-nucleotidase that then dephosphorylates adenosine 5'-monophosphate into adenosine, are expressed by many immune cell types such as neutrophils, macrophages and T cells. (Barankiewicz et al., 1994; Kansas et al., 1991; Maliszewski et al., 1994; Misumi et al., 1990; Steinberg and Silverstein, 1987; van Waeg and Van den Berghe, 1991; Wang and Guidotti, 1996) Unique from other T cells however, Tregs co-express CD39 and CD73, and adenosine generated from Treg expressed CD39 and CD73 has been shown to suppress T cell proliferation in vitro. Importantly Tregs from WT, but not CD39−/− mice, were unable to promote Balb/c skin graft survival when co-transferred with CD4+CD25+ cells into Rag1−/− mice, demonstrating that CD39 expression by Tregs was also critical for in vivo function. (Deaglio et al., 2007) Furthermore, CD73 expression was induced independent of FoxP3 expression on CD4 cells, and in CD8 cells, dendritic cells and macrophages by TGF-β, linking the excretion of TGF-β to the parallel suppressive mechanism of adenosine production. (Regateiro et al., 2011)

**Tregs and Long-Term Allograft Survival**

Despite demonstrating that a lymphocyte population was important for promoting long-term allograft survival in the 1970s, (Kilshaw et al., 1975) no clear evidence for a role of suppressor cells became apparent until the work of Hall and colleagues in the early
1990s. (Batchelor et al., 1977) Many groups began intense study of the role of Tregs in transplant tolerance, predominantly utilizing adoptive transfer models to demonstrate that T cells from tolerant recipients could both transfer tolerance to T cell depleted naïve animals, but also prevent rejection by non-tolerant T cells. (Davies et al., 1996) In the early 2000s, many groups began to apply the newly appreciated Treg effector mechanisms (discussed above) to determine whether Tregs utilized similar mechanisms during the response to alloantigen. Tregs from CBA mice with long-term surviving B10 cardiac allografts were co-transferred with naïve CD45RB\textsuperscript{high} T cells into T cell depleted CBA recipients and were able to promote survival of a B10 skin graft in an IL-10 dependent manner. (Hara et al., 2001) In a follow up study, donor-specific Tregs generated from treatment of CBA mice with αCD4 and DST could prevent rejection dependent on IL-10 receptor and CTLA-4 when adoptively transferred to T cell depleted CBA recipients before receiving a B10 skin graft. (Kingsley et al., 2002) While the above studies demonstrated that Tregs isolated from secondary lymphoid organs were necessary and sufficient to transfer tolerance and prevent rejection in naïve recipients, it has also been demonstrated that graft infiltrating Tregs are necessary to promote long-term allograft survival. (Graca et al., 2002; Kendal et al., 2011) To summarize the above data, Tregs present in both the secondary lymphoid organs as well as in the allograft are a critical cell type in promoting long-term allograft survival.

**Barriers to Transplantation Tolerance**

Despite extensive study on immunosuppression and Tregs, translating allograft tolerance from rodents to patients has not been as successful as initially hoped. (Kanmaz et al., 2004; Kawai et al., 2000; Kirk et al., 1997; Levisetti et al., 1997) Many differences between rodents and primates account for the disparity in these results; two well-studied differences for the resistance to costimulatory blockade in large animals and the clinic are immune memory and infection.
The capacity of the adaptive immune system to retain memory to previously encountered antigen was first realized when Sir Edward Jenner vaccinated children with cowpox and found that they were protected from smallpox. (Riedel, 2005) Centuries later, it is now appreciated that antigen specific T cells persist at a higher frequency after infection than they do in naïve animals. (Hou et al., 1994; Whitmire et al., 1998) These memory T cells express higher constitutive levels of transcripts for effector proteins such as IFN-γ, perforins and granzymes that are translated rapidly following antigen recognition. (Bachmann et al., 1999; Grayson et al., 2001; Veiga-Fernandes et al., 2000) While this process is essential for effective immunity against pathogens, we now realize that that immune memory is a major barrier to long-term allograft survival.

Each unique T cell receptor is cross-reactive, as in one TCR can recognize multiple peptide-MHC complexes. (Bhardwaj et al., 1993; Mason, 1998; Wucherpfennig and Strominger, 1995) Memory T cells with TCRs specific for viral antigens can also react to alloantigen. (Braciale et al., 1981; Sheil et al., 1987; Yang and Welsh, 1986) In contrast to the low or undetectable levels of memory T cells in naïve mice, about 50% of the human T cell repertoire is composed of memory T cells, of which a significant portion is alloreactive. (Cossarizza et al., 1996; Lombardi et al., 1990; Macedo et al., 2009) As memory T cells have been shown to be resistant to the interventions (i.e. costimulatory blockade, antibody depletion and regulation via Tregs) employed to promote long-term allograft survival, it is thus not surprising that many of these methods fail in primates and/or humans. (Adams et al., 2003; Pearl et al., 2005; Yang et al., 2007)

In addition to pathogen specific memory cells serving as a barrier to long-term allograft survival, the pathogens that lead to immune memory can also prevent long-term graft survival even before memory cells are formed. As most transplant recipients are immunosuppressed, infection with environmental pathogens occurs often and is associated with increased frequency of graft rejection. (May et al., 1978; Simmons et al., 1974) In mice, viral infection (namely LCMV)
before, at the time of, or soon after transplantation prevented long-term allograft survival induced by co-stimulatory blockade. (Brehm et al., 2003; Turgeon et al., 2000; Welsh et al., 2000; Williams et al., 2002) Interestingly, infection with vaccinia virus or mouse cytomegalovirus did not affect the survival of skin allografts, suggesting that activation of the immune system by certain viruses was sufficient to trigger graft rejection following costimulatory blockade. (Welsh et al., 2000) Together, the above data demonstrated that activation of the immune system by a pathogen and/or their products was detrimental to long-term allograft survival.

The Innate Immune Response to Pathogens

Despite the critical nature of the adaptive immune response to pathogens, the innate immune system is essential for the initial recognition of a pathogen and the initial activation of T cells and B cells. The innate immune system is composed of sentinel cells which express germ-line encoded, non-rearranged, evolutionarily conserved receptors to pathogen associated molecular patterns (PAMPS). (Hoffmann et al., 1999; Janeway and Medzhitov, 2002) Toll-like receptors (TLRs) are a class of pattern recognition receptors (PRRs) that enable cells of the innate immune system to detect moieties unique to pathogens. Thirteen TLRs have been identified in mice and ten TLRs have been identified in humans; some examples of TLRs and TLR ligands are TLR 4 (binds to lipopolysaccharide (LPS)), (Hoshino et al., 1999; Poltorak et al., 1998; Qureshi et al., 1999) TLR 2 (binds to peptidoglycan), (Schwandner et al., 1999; Takeuchi et al., 1999) TLR 5 (binds to flagellin), (Hayashi et al., 2001) TLR 3 (binds to dsRNA), (Alexopoulou et al., 2001) TLR 7 (binds to ssRNA), (Diebold et al., 2004) and TLR 9 (binds to methylated bacterial CpG DNA). (Hemmi et al., 2000)

The TLR signaling pathway from ligand binding the leucine-rich repeat extracellular domain, to gene expression is well characterized, and begins with the recruitment of myeloid differentiation primary response gene 88 (MyD88) to the N-terminal intracellular signaling Toll/interleukin receptor (TIR) domain of all TLRs except TLR3 (which utilizes the adaptor
Toll/IL-1R domain-containing adaptor-inducing IFN-β, termed TRIF). (Alexopoulou et al., 2001; Medzhitov et al., 1998; Oshiumi et al., 2003; Yamamoto et al., 2002) The TIR-TIR interaction between MyD88 and a TLR’s intracellular domain allows for the recruitment of IL-1 receptor-associated kinase (IRAK) family members via a hemophilic death domain interaction. (Wesche et al., 1997) IRAK-4 is the MyD88 proximal IRAK which recruited and phosphorylated IRAK-1 and IRAK-2. (Li et al., 2002; Lin et al., 2010) Phosphorylated IRAK-1 and 2 recruited and activated TNF-receptor-associated factor 6 (TRAF-6), an E3 ubiquitin ligase that ubiquitylated and activated the kinase TAK1. (Xia et al., 2009; Ye et al., 2002) Activated TAK1 phosphorylated IκB kinase (IKK), which in turn phosphorylated IκB. (DiDonato et al., 1997; Li et al., 2005; Mercurio et al., 1997; Wang et al., 2001) While IκB sequesters the transcription factor NF-κB in the cytoplasm of quiescent cells, phosphorylated IκB is marked for proteosomal degradation by ubiquitin, allowing NF-kB to enter the nucleus and promote the transcription of a number of inflammatory genes including IL-6, IL-12 and TNF-α. (Baeuerle and Baltimore, 1988; Beg et al., 1993; Beg et al., 1992; Chen et al., 1995; Collart et al., 1990; Ganchi et al., 1992; Libermann and Baltimore, 1990; Murphy et al., 1995; Shakhov et al., 1990) It is also worth noting that TLR 4 can signal via the TRIF pathway (also utilized by TLR 3) through the TRIF-related adaptor molecule (TRAM), which led to the production of type-I interferons and dendritic cell maturation. (Doyle et al., 2002; Hoshino et al., 2002; Kawai et al., 2001; Yamamoto et al., 2003a; Yamamoto et al., 2003b) As would be expected, germ line MyD88 knockout mice were unable to mount responses efficiently to a variety of pathogens such as L. monocytogenes, M. tuberculosis, L. major and T. gondii, indicating broad defects in innate immune responses and thus, adaptive immunity. (Fremond et al., 2004; Muraille et al., 2003; Scanga et al., 2002; Seki et al., 2002) Accordingly, primary and secondary immunization to ovalbumin was unable to induce a memory Th1 response, however, because these studies were performed in germ line MyD88

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knockout mice, the cell type specific requirement for MyD88 in this, and the above infection models is unclear. (Pasare and Medzhitov, 2004)

The Innate Immune Response to Allografts

The innate immune system is not only activated in response to pathogens, however. It has long been appreciated that the innate immune system, and specifically TLRs, can become activated after tissue injury. The “danger model” initially proposed by Matzinger, explained the innate immune response to tissue injury and because surgery results in tissue injury, it is not surprising that the innate immune system would be activated in response to a surgical procedure. (Matzinger, 1994) When tissues were injured, especially during reperfusion of an organ, the tissue contained a high concentration of oxygen free radicals. (McCord, 1985) Patients that received renal allografts treated with a highly specific free-radical scavenger experienced improved graft survival, demonstrating that eliminating one of the products of tissue injury could prolong allograft survival. (Land et al., 1994) Furthermore, ischemia/reperfusion liver injury was ameliorated in TLR4−/− mice, demonstrating that activation of the innate immune system, specifically through a TLR, led to immune-mediated tissue damage. (Zhai et al., 2004)

Much work has focused on the stimulation of the TLR signaling pathway, particularly TLRs-2 and 4, through the release of endogenous danger molecules by necrotic cells such as low-molecular weight hyaluronan (a byproduct of the break down of high-molecular weight hyaluronan upon disruption of the extracellular matrix), heparin sulfate, fibronectin extra domain A (an alternative splice product produced in response to tissue injury) and biglycan (an extracellular matrix component). (Johnson et al., 2002; Okamura et al., 2001; Schaefer et al., 2005; Scheibner et al., 2006; Tesar et al., 2006) More recently, haptoglobin, another molecule released from necrotic cells, increased at the graft site following skin transplantation. Expression of haptoglobin accelerated graft rejection while genetic deletion of haptoglobin in the donor delayed rejection kinetics in a MyD88 dependent, but TLR-2 and 4 independent manner,
demonstrating that a molecule released following tissue injury directly enhanced skin graft rejection. (Shen et al., 2012) In addition, Pam3Cys (a TLR-2 agonist), PolyI:C (a TLR-3 agonist), LPS (a TLR-4 agonist) and CpG DNA (a TLR-9 agonist) abrogated the skin allograft prolonging effect of αCD154 and rapamycin or αCD154 and DST, or the cardiac allograft prolonging effect of αCD154 in mice, further indicating that innate immune activation via the TLR signaling pathways results in the inability to promote long-term allograft survival. (Chen et al., 2006; Porrett et al., 2008; Thornley et al., 2006)

To further solidify a role for MyD88 signaling during the response to alloantigen, minor or major mismatched skin were transplanted onto germ-line MyD88 knockout recipients. In a minor-mismatch (HY) model of skin transplantation, male skin grafts were accepted by female germ line MyD88 knockout mice while they were otherwise rejected by wild type female mice. Importantly, graft rejection in this model was independent of TLR-2, TLR-4 and the IL-1R family, indicating that MyD88 may be signaling downstream of another pathway. (Goldstein et al., 2003)

In addition, MyD88-deficient B6 mice accepted Balb/c skin grafts in a model where they were otherwise rejected by WT B6 mice when both groups were treated with costimulatory blockade. (Walker et al., 2006) While the above data implicates MyD88 signaling in accelerated graft rejection, the cell type specific requirement for MyD88 signaling during the response to alloantigen remains unclear as the above models utilized germ line MyD88 knockout mice.

**T cell expressed MyD88**

Despite being classically studied in the context of innate immune signaling, TLRs and MyD88 are expressed and utilized by T cells. (Gelman et al., 2004; Hornung et al., 2002; Zarember and Godowski, 2002) Work from the Turka Lab demonstrated that the p85 subunit of PI3K associated with tyrosine 257 in the TIR domain of MyD88, which then phosphorylated AKT and GS3K, synergizing with CD28 signaling leading to a proliferative response and IL-2
production. Additionally, in the absence of CD28 signaling, MyD88-dependent TLR-9 signaling led to NF-κB and Bcl-xL expression and enhanced the survival of T cells.\textcite{Gelman et al., 2006} T cell expressed MyD88 was critical for T cell survival and function in a variety of in vivo models. Utilizing bone marrow chimeras to isolate MyD88 deletion to T cells, T cell expressed MyD88 was shown to be critical to mount an effective immune response to, and protect mice against \textit{T. gondii} infection independent of IL-1R and IL-18R signaling.\textcite{LaRosa et al., 2008}

While splenocytes were shown to produce less IFN-γ in response to \textit{T. gondii} antigens in vitro, T cell expressed MyD88 was critical for IFN-γ production that resulted in paneth cell death and intestinal dysbiosis during \textit{T. gondii} infection in vivo.\textcite{LaRosa et al., 2008; Raetz et al., 2013} During ehrlicial infection (via the tick transmitted, obligate intracellular pathogen \textit{E. muris}), T cell expressed MyD88 was critical for T cell production of IFN-γ, which resulted in activation of hematopoietic stem cells in the bone marrow in response to the infection.\textcite{Zhang et al., 2013b}

Through models whereby T cells were adoptively transferred from MyD88 germline knockout mice, and conditional deletion of MyD88 systems, T cell expressed MyD88 was demonstrated to promote T cell survival in a model of LCMV infection, demonstrating that T cell expressed MyD88 was critical for T cell survival in vivo.\textcite{Rahman et al., 2008; Rahman et al., 2011} Lastly, in a model of adoptive transfer-induced colitis, Rag\textsuperscript{−/−} recipients of MyD88-deficient effector T cells did not experience a wasting phenotype and colonic inflammation whereby Rag\textsuperscript{−/−} recipients of WT effector T cells lost a significant amount of weight associated with inflamed colon tissue.\textcite{Fukata et al., 2008} Together, these data demonstrate that T cell expressed MyD88 is critical for T cell function and survival during responses to pathogens and in inflammatory bowel disease. It is worth noting that in all of these systems there were TLR ligands present from either pathogen or commensals, suggesting that T cells may directly sense TLR ligands in addition to innate immune recognition of TLR ligands.
Furthermore, TLRs and MyD88 are also expressed and utilized by Tregs. (Caramalho et al., 2003; Crellin et al., 2005; Peng et al., 2005) For example, treatment of Tregs with flagellin during an *in vitro* suppression assay resulted in decreased proliferation of effector T cells, suggesting that TLR-5 engagement resulted in increased Treg suppressive function. (Crellin et al., 2005) TLR-2 signaling on Tregs via the synthetic ligand Pam3Cys or the fungus *C. albicans* increased the proliferation but decreased the suppressive capacity of Tregs *in vitro* and *in vivo*. (Sutmuller et al., 2006) However, in a different model system, TLR-2 engagement with Pam3CSK4 did not decrease Treg function *in vitro* and *in vivo*, but rather promoted Treg survival via induction of Bcl-xL. (Chen et al., 2009) Exposure of Tregs to CpG DNA resulted in decreased suppression, either through direct inhibition of Treg function and/or costimulation of responding effector cells resulting in effector T cell resistance to suppression by Tregs. (LaRosa et al., 2007; Peng et al., 2005) Together, these disparate findings suggest that consequences of TLR signaling on Treg may be TLR context and/or model dependent.

Addressing the role of MyD88 in Tregs provides a more general understanding of the result of TLR signaling on Tregs. In a model of adoptive transfer-induced colitis, Rag−/− recipients of MyD88-deficient Tregs and WT effector T cells lost a significant amount of weight associated with inflamed colon tissue whereby Rag−/− recipients of WT Tregs and WT effector T cells did not experience this wasting phenotype, suggesting that MyD88 signaling, in general, was important for Treg function. (Fukata et al., 2008) While these data suggest that MyD88 signaling is important for cell function, the use of cells that developed in MyD88/TLR germ line knockout mice complicate interpretations based on the known defects of MyD88/TLR germ line knockout mice as discussed above. Additionally, whether the inability of MyD88−/− Tregs to protect against colonic inflammation was due to a direct functional defect, or a functional defect due to the inability of MyD88−/− Tregs to survive was not addressed. To analyze the role of MyD88 signaling in Tregs with more precision, we utilized conditional knockout mice in which MyD88 deletion was restricted to all T cells and/or only Tregs. The goal of my work over the past five years has
been to characterize the role of T cell, and more specifically Treg expressed MyD88 during the response to alloantigen. In the following chapters, I will present compelling data that demonstrate T cell and Treg expressed MyD88 are critical for long-term allograft survival.
References


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Chapter 2: Defining a role for T regulatory cell expressed MyD88 during the response to allografts

Attributions

Christopher Borges conceptualized the study, wrote the chapter, designed, performed and analyzed the majority of experiments. Laurence Turka conceptualized the study and provided significant advice in regards to experimental design and data analysis. JiHoon Chang provided conceptual advice and performed experiments (western blotting). Martin Fan performed experiments (Parent into F1 adoptive transfer). Weihua Gong performed experiments (cardiac transplantation). Aditya Misra performed experiments and analyzed data (Treg surface molecule expression).

Introduction

As discussed in Chapter 1, MyD88 is a critical adaptor molecule proximally downstream of all of the TLRs except TLR-3, as well as the IL-1 receptor (IL-1R) family members (IL-1R, IL18R and IL-33R). (Casanova et al., 2011; Medzhitov et al., 1998) Although traditionally studied as a key molecule for innate immune responses, MyD88 has an essential T cell intrinsic role. Our group has demonstrated that MyD88 associates with the p85 subunit of PI3K, leading to the activation of the AKT and GSK-3 pathways resulting in proliferation and IL-2 production in vitro. (Gelman et al., 2006) Bone marrow chimeras lacking MyD88 specifically in T cells succumbed to infection with the protozoan parasite T. gondii at a dose which was non-lethal in bone marrow chimeras sufficient for MyD88 in the T cell compartment. (LaRosa et al., 2008) More recently, T cell expressed MyD88 was shown to be critical for T cell production of IFN-γ and T_{H1} polarization during infection with T. gondii. (Raetz et al., 2013) Furthermore, MyD88-deficient and sufficient P14 TCR transgenic CD8 T cells were co-adoptively transferred into LCMV infected B6 hosts. After 3 days in vivo, a decrease in MyD88-deficient P14 TCR
transgenic T cells relative to WT P14 TCR transgenic T cells was observed, suggesting that T cell intrinsic MyD88 was required for T cell persistence during the response to a viral pathogen. (Rahman et al., 2008) In addition, tetramer positive MyD88-deficient CD8 T cells decreased in frequency following LCMV infection in mice that lack MyD88 specifically in T cells (MyD88-ΔT mice). (Rahman et al., 2011) Together, these data demonstrated that effector T cell expressed MyD88 was critical for cell function and survival during the response to an array of pathogens.

CD4+FoxP3+ T regulatory (Treg) cells are critical for maintenance of peripheral tolerance to self antigens and preventing autoimmune lesions in rodents and humans. (Bennett et al., 2001; Brunkow et al., 2001; Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003) In addition, Tregs are critical for long-term allograft survival in many rodent models. (Wood and Sakaguchi, 2003) Tregs suppress effector cells through a variety of surface molecules including CTLA-4 and cytokines including IL-10 and TGF-β. (Asseman et al., 1999; Chen et al., 2005; Groux et al., 1997; Nakamura et al., 2001; Wing et al., 2008) Similar to their naïve and effector counterparts, Tregs also express TLRs and stimulation of these TLRs, in particular TLR-2, on mouse and human Tregs reduces the suppressive function of Tregs in vitro and in vivo. (Caramalho et al., 2003; Nyirenda et al., 2015; Sutmuller et al., 2006) However, in a different model system, TLR-2 engagement did not decrease Treg function, but rather promoted Treg survival, suggesting that consequences of TLR signaling on Treg may be context or model dependent. (Chen et al., 2009)

Our group and others have shown that treatment of mice with TLR agonists opposed the effects of costimulatory blockade on long-term allograft survival. (Chen et al., 2006; Porrett et al., 2008; Thornley et al., 2006) In a minor (HY)-mismatch model of skin transplantation, male skin grafts were accepted by female germ line MyD88 knockout mice while they were otherwise rejected by wild type female mice. (Goldstein et al., 2003) In addition, MyD88-deficient B6 mice
accepted Balb/c skin grafts in a model where they were otherwise rejected by WT B6 mice when both groups were treated with a costimulatory blockade regimen. (Walker et al., 2006) These data suggest that MyD88 signaling can prevent long-term allograft survival and negate the graft-prolonging effects of costimulatory blockade. While the above data implicated MyD88 signaling in accelerated graft rejection, the cell specific requirement for MyD88 signaling remained unclear as the above models utilized germ line MyD88 knockout mice.

Here, we wish to investigate the requirement for T cell expressed MyD88 during the response to alloantigen. We hypothesize that alloreactive T cells require MyD88 for cell survival, similar to pathogen reactive T cells. This hypothesis is attractive because pharmacologic inhibition of MyD88 could lead to the death of alloreactive T cells following transplantation, while sparing non-responding (i.e. non-alloreactive) T cells, as implicated by previous and unpublished studies indicating that MyD88 is required for cell survival during proliferation. Furthermore, as Tregs are a subset of T cells, we wish to confirm that Treg expressed MyD88 is required for Treg survival during the response to alloantigen as long-term allograft survival is dependent on Tregs in our model. To test these hypotheses, we utilized mice with cell-specific deletion of MyD88 from T cells and Tregs. These mice not only permit us to define an unappreciated role for T cell and Treg expressed MyD88 during the response to alloantigen, but also reconcile the inconsistent results on the effect of TLR signaling on Treg function by using a more precise model.

**T cell expressed MyD88 is critical for long-term bm12 allograft survival**

Previous studies of allograft tolerance and rejection by our group and others utilized the Bm12 mouse which is genetically identical to the C57BL/6 mouse except for a three amino acid substitution in the I-Aβ1 chain of the MHC Class II locus. (McIntyre and Seidman, 1984) C57BL/6 mice reject bm12 skin allografts while cardiac allografts are spontaneously accepted long-term. (McKenzie et al., 1979; Wang et al., 1990) CD25+ Tregs were later shown to be
necessary for graft survival in C57BL/6 recipients, as the long-term survival of Bm12 cardiac allografts was abrogated when a regimen of αCD25 mAb was administered. (Schenk et al., 2005) Furthermore, bm12 skin graft survival can be prolonged with a regimen of αCD154 and rapamycin (CoB). (Porrett et al., 2008) Thus, utilizing the bm12 strain as donor mice is advantageous as the response to alloantigen is limited to CD4+ T cells, is dependent on Tregs and permits the study of long-term allograft survival with and without costimulatory blockade.

As a first step to determine the role of T cell expressed MyD88 during the response to allografts, we transplanted bm12 skin onto WT mice or mice with conditional deletion of MyD88 in all T cells (MyD88-ΔT mice). We found that untreated MyD88-ΔT mice rejected bm12 skin allografts with similar kinetics (MST= 11 days) as compared to untreated WT mice (MST=14 days; Figure 2.1).

These data were surprising to us as MyD88-deficient effector cells were unable to survive as well as WT effector cells during infection, and thus unable to efficiently mount a response to pathogens. (LaRosa et al., 2008; Rahman et al., 2008; Rahman et al., 2011) Upon
treatment of MyD88-ΔT and WT bm12 skin graft recipients with αCD154 and rapamycin, we observed that a higher proportion of MyD88-ΔT mice rejected their grafts earlier (MST=52.5 days) than WT mice (MST=74 days)(Figure 2.1). While WT mice accepted bm12 cardiac allografts with a MST greater than 100 days (MST undefined, Figure 2.2), we found that MyD88-ΔT recipient mice rejected their grafts with a MST of 39 days. Together, these data suggest that T cell intrinsic MyD88 is dispensable for rejection, but critical for long-term bm12 graft survival. From the above data, two non-mutually exclusive hypotheses can be formed to explain our observations; 1) unlike pathogen specific MyD88-deficient T cells, alloreactive MyD88-deficient T cells do not require MyD88 for their survival and thus persist and respond to alloantigen and 2) because both of these models require Tregs for induction of long-term allograft survival, MyD88-deficient Tregs may not survive during the response to alloantigen, similar to MyD88-deficient effector T cells during infection.

Figure 2.2:

Bm12 cardiac allografts were transplanted into WT or MyD88-DT recipients. Mice received either no treatment (● and ■, respectively), or i.v. injection of 0.5x10^6 sorted WT FoxP3-GFP + (WT Treg) cells 7 days prior to transplant (□). Data are pooled from 2 independent experiments. MST: Undefined, 34, and undefined days (●, ■, □) respectively. *, p<0.05. **, p<0.01.
**T cell expressed MyD88 is required for effector T cell survival during the response to alloantigen**

To address the hypothesis that alloreactive MyD88-deficient T cells were able to survive as well as WT T cells during the response to alloantigen, unlike their pathogen reactive counterparts, we utilized a parent into F₁ adoptive transfer model whereby the transferred T cells recognized alloantigen on the host, but the host T cells were tolerant of the transferred T cells. Through co-adoptive transfer of WT and MyD88-ΔT T cells, we not only assessed the relative proportion of WT to MyD88-ΔT cells, but also assessed the frequency of Annexin V+ Live/Dead+ (i.e. dead or dying) WT or MyD88-ΔT cells. B6 (donor) cells could be differentiated from CB6F1 (host) cells via staining of MHC class I molecules, as B6 and CB6F1 mice share H-2Kᵇ, H-2Dᵇ and I-Aᵇ, but do not share H-2Kᵈ, H-2Dᵈ, H-2Lᵈ, I-Aᵈ or I-Eᵈ. Therefore staining with H-2Kᵇ and H-2Kᵈ specific antibodies could differentiate host cells (H-2Kᵇ and H-2Kᵈ positive) from donor cells (only H-2Kᵇ positive). Differentiation of WT donor T cells from MyD88-ΔT donor T cells was possible because WT B6 T cells were CD45.1 positive while B6 WT mice MyD88-ΔT donor T cells were CD45.2 positive (Figure 2.3A).

Following differentiation of host and donor cells, we found a significantly lower frequency of MyD88-ΔT CD4 cells relative to WT CD4 cells 3 and 7 days post transfer (Figure 2.3B). In contrast, MyD88-ΔT CD8 cells were significantly higher in frequency than WT CD8 T cells at Day 3 post transfer but significantly lower in frequency than WT CD8 T cells at Day 7 post transfer (Figure 2.3B). One possibility was that a slightly higher frequency of MyD88-ΔT CD8+ cells were adoptively transferred relative to WT CD8+ cells resulting in the significantly higher proportion of MyD88-ΔT CD8+ cells relative to WT CD8+ cells observed on Day +3. While proportions of adoptively transferred MyD88-ΔT T cells to WT T cells can be suggestive of an increase or decrease in cell survival capacity, we next assessed cell survival directly by staining
with Annexin V and Live/Dead Aqua. We observed a higher proportion of Annexin V+ Live/Dead+ MyD88-ΔT CD4 and CD8 cells relative to WT T cells 3 and 7 days post adoptive (Figure 2.3C).

Together, these data demonstrate that MyD88 is required for alloreactive T cell survival.

While these data are similar to the previously defined role for T cell expressed MyD88 during infection, the data are in contrast to our more recent data that demonstrated MyD88-ΔT mice were able to reject bm12 allografts. One important difference between the response to
pathogens and the response to alloantigen is that the precursor frequency of alloreactive cells is much higher (1 in $10^2$ or 1 in 10) than the precursor frequency of pathogen reactive cells (1 in $10^4$ or 1 in $10^6$) (Blattman et al., 2002; Butz and Bevan, 1998; Moon et al., 2007; Suchin et al., 2001). Therefore, despite the failure of MyD88-$\Delta$T T cells to survive during the response to alloantigen, rejection potentially could still occur due to the higher starting frequency of potential alloreactive responders relative to potential pathogen-reactive responders.

**T regulatory cell expressed MyD88 is critical for long-term allograft survival**

Together, the above data show that mice lacking MyD88 exclusively in the T cell compartment reject bm12 skin and cardiac grafts and are resistant to the allograft prolonging effects of $\alpha$CD154 and rapamycin despite the inability of MyD88 deficient T cells to survive during the response to alloantigen. Because long-term allograft survival of both skin and cardiac grafts was dependent on Tregs in these models, we next hypothesized that MyD88-deficient Tregs were not functioning and/or surviving as well as WT Tregs. To address this hypothesis, we first asked whether we could rescue bm12 cardiac allograft survival by administering WT Tregs prior to transplantation of MyD88-$\Delta$T mice with bm12 cardiac allografts. Indeed, infusion of 0.5x10^6 sorted WT FoxP3+ cells 7 days prior to transplantation rescued bm12 cardiac allograft survival in MyD88-$\Delta$T recipients. (MST undefined; Figure 2.2). While these data, coupled with the requirement for Tregs to promote long-term bm12 skin graft survival, were suggestive of a functional and/or survival defect in Tregs from MyD88-$\Delta$T mice, we wanted to more precisely address the requirement for Treg expressed MyD88.

To accomplish this, we crossed MyD88^{fl/fl} mice to FoxP3-Cre mice resulting a mouse that specifically lacked MyD88 in FoxP3+ cells, termed MyD88-$\Delta$Treg mice. In addition to expressing Cre, FoxP3+ cells also expressed YFP, allowing us to conveniently assay the Treg compartment by flow cytometry or immunofluorescent imaging without the need for intracellular
staining. (Rubtsov et al., 2008) Western blotting for MyD88 from cell lysates derived from sort purified YFP+ and YFP- cells from MyD88-ΔTreg mice confirmed that YFP+ cells indeed expressed low, to undetectable levels of MyD88 (Figure 2.4).

MyD88-ΔTreg mice developed normally and did not present any phenotypic abnormalities with age. In support of the above findings, we observe that MyD88-ΔTreg mice were similarly resistant to the allograft prolonging effects of αCD154 and rapamycin, as CoB treated MyD88-ΔTreg recipients rejected bm12 skin grafts at a higher frequency than WT recipients (MST: 37 days and undefined respectively; Figure 2.5) To corroborate these findings with our above finding that WT Tregs could prevent rejection of bm12 cardiac allografts in MyD88-ΔT recipients, we utilized female FoxP3-Cre heterozygous mice. In theory, half of the FoxP3+ cells in these mice express Cre while the other half do not due to the random X-chromosome inactivation that occurs in female cells. When crossed to MyD88fl/fl mice, in theory 50% of the Tregs in these animals would lack MyD88 while the remaining 50% expressed a WT
copy of Cre, permitting us to study MyD88-deficient Tregs with a complement of WT Tregs without the need for an adoptive transfer or bone marrow chimera. Interestingly, we found that rejection of bm12 skin allografts was delayed in MyD88fl/fl FoxP3-Cre het recipients in comparison to MyD88-ΔTreg recipients (MST: 59 days; Figure 2.5), however the frequency of grafts surviving at 100 days post transplant was not significantly different than MyD88-ΔTreg recipients. Together, these data demonstrate that T cell, and more specifically Treg, expressed MyD88 is critical for long-term bm12 allograft survival.

Characterization of MyD88-deficient Tregs during the response to alloantigen

As a first step to explore the resistance of MyD88-ΔTreg mice to the allograft-prolonging effects of costimulatory blockade, we first assessed Treg frequency in secondary lymphoid organs of recipient mice following transplantation with bm12 skin allografts and treatment with CoB. As our group has demonstrated that T cell expressed MyD88 was critical for cell survival during the response to pathogens, we hypothesized that MyD88 would also be critical for Treg survival, and Treg cell death during the response to alloantigen would result in the observed
resistance to the effects of CoB. We observed no difference in the frequency of FoxP3+ cells in WT or MyD88-ΔTreg recipients before transplant, or on day 14 or day 21 after transplant in the blood, spleen of peripheral lymph nodes (Figure 2.6A-C). We observed a significant increase in Tregs from the spleens, but not blood or pLNs of MyD88-ΔTreg recipients (relative to Tregs from WT recipients) on day 7 post transplant, however this increase in frequency was isolated to the spleen at this time point (Figure 2.6A-C). Although there was largely no difference in Treg frequency between WT or MyD88-ΔTreg recipients in their peripheral lymphoid organs, the possibility remained that MyD88 deficient Tregs were unable to traffic to the skin graft, and/or survive within the skin graft and locally suppress the effector response.

Figure 2.6:

Percent FoxP3+ cells of CD4+ cells in blood, spleen and peripheral lymph nodes from WT (white bars) or MyD88-ΔTreg (black bars) recipients pre, or at indicated time post transplant.
To assess the ability of MyD88-deficient Tregs to migrate to the skin graft, we first analyzed the expression of CCR4, CCR6 and CD103, three receptors important for skin homing. (Siegmund et al., 2005; Wei et al., 2006; Yamazaki et al., 2008) In addition, CCR4 knockout mice treated with DST and αCD154 fail to accept cardiac allografts, demonstrating that CCR4, presumably facilitating homing to the allograft, was important in preventing cardiac allograft rejection. (Lee et al., 2005) While we observed significant decreases in MyD88-deficient Tregs expressing CCR4 from the spleen on Day 7 post transplant, CCR6 from the spleen and pLNs on Day 7 post transplant, CD103 from the spleen on Day 14 post transplant and from the pLNs on Day 21 post transplant (all relative to WT Tregs), there was no consistent trend across time points within the spleen (Figure 2.7A) or pLN (Figure 2.7B).

Figure 2.7:

Expression of the skin homing receptors CCR4, CCR6 and CD103 on FoxP3+CD4+ cells in A. spleen or B. pLN from WT (white bars) or MyD88-ΔTreg (black bars) mice at indicated time post transplant. Each bar is representative of a minimum of n=3 mice from each group at each time point. *, p<0.05. **, p<0.01.
CD4+FoxP3+ cells within the allograft (intragraft Tregs) are critical for graft survival, suggesting that observation of similar Treg frequencies in the secondary lymphoid organs may not be indicative of the inability for MyD88-deficient Tregs to promote long-term allograft survival due to a survival defect that specifically occurs within the graft. (Gondek et al., 2008; Graca et al., 2002) In addition, as graft survival in all of the models we utilized was dependent on Tregs, and based on the known requirement for MyD88 during the effector T cell response during infection, we hypothesized that MyD88 signaling specifically might be important for Treg survival within the allograft, a highly inflammatory site similar to conditions during infection. Interestingly, we observed no difference in the frequency of Tregs isolated from bm12 skin grafts 7 and 14 days post transplant (Figure 2.8). However, at day 21 post transplant, we observed a significant decrease in Tregs harvested from MyD88-ΔTreg recipients relative to WT recipients (Figure 2.8).

This observed decrease in frequency could have been due to a decrease in the absolute number of Tregs, or a similar absolute number of Tregs accompanied by an increase in the
absolute number of non-Tregs. To test these possibilities, we utilized a bead-based cell counting assay to quantify the absolute number of intragraft Tregs and non-Tregs from WT or MyD88-ΔTreg recipients harvested on day 21 post transplant. Absolute cell counting revealed that grafts harvested from MyD88-ΔTreg recipients contained the same absolute number of Tregs while the absolute number of non-Tregs significantly increased, indicating that the decreased Treg frequency was not due to a decrease in absolute Treg cell number, but rather an increase in absolute non-Treg cell number (Figure 2.9A and B).

Figure 2.9:

![Bar chart](image)

Intragraft FoxP3+ and FoxP3- cellularity was calculated using the number of CD45+ cells isolated from WT (white bars, n=5) or MyD88-ΔTreg (black bars, n=6) recipient mice using a bead based flow cytometric counting assay 21 days after transplantation. Data are representative of 2 independent experiments. *, p<0.05.

These data are corroborated by our survival kinetics which showed that a significant proportion of MyD88-ΔTreg recipients rejected their grafts soon after the day 21 post transplant time point, which would be associated with an increase in non-Treg numbers within the skin graft. In support of these data, we also observed similar frequencies of live intragraft Tregs harvested from WT or MyD88-ΔTreg recipients indicated by negative staining of the Live/Dead Aqua dead cell dye (Figure 2.10A and B).
Although Tregs from MyD88-ΔTreg recipients populated bm12 skin grafts with similar numbers, the possibility that MyD88-deficient Tregs were not trafficking to the same region of the graft as Tregs from WT recipients remained, despite similar expression of the canonical skin homing markers as demonstrated above (Figure 2.7). To test this, we imaged grafts harvested from WT or MyD88-ΔTreg mice 21 days post transplant co-stained with CD4. Not only did we confirm our absolute cell counting data by observing similar numbers of Tregs from skin grafts transplanted on WT or MyD88-ΔTreg recipients, but we found that the Tregs from both recipients home to the hypodermis region of the skin graft (Figure 2.11). These data were in
contrast to previously published data that showed skin resident Tregs home to hair follicles, but were consistent with the vascularized nature of the hypodermis. (Gratz et al., 2014; Sanchez Rodriguez et al., 2014) Together, the above data demonstrated that despite resistance to the effects of CoB, MyD88-deficient Tregs migrate and persist in bm12 skin grafts to the same degree, and in a similar location as WT Tregs.

**MyD88 is dispensable for Treg survival**

While the above data suggest that MyD88-deficient Tregs survive as well as WT Tregs in vivo during the response to alloantigen, we aimed to directly test this as it was surprising to us that MyD88 might be dispensable for Treg survival while being required for non-Treg T cell survival. As cell survival is difficult to assess in vivo, and even more difficult to assess directly within a tissue, we again utilized our breeding scheme to produce mice heterozygous for FoxP3-Cre. As discussed above, when crossed to MyD88<sup>fl/fl</sup> mice, in theory 50% of the Tregs would
delete MyD88 while the other 50% of Tregs would express MyD88. While the frequency of Cre+ cells among total FoxP3+ cells (detected by expression of YFP) fluctuated bi-weekly, we observed no significant difference in the frequency of Cre+ cells among total FoxP3+ cells between FoxP3-Cre het and MyD88fl/+ or MyD88fl/fl Cre het mice (Figure 2.12).

Next, to directly analyze whether MyD88 was dispensable for Treg survival, we cultured sorted naïve or Treg (defined by CD4+ CD25+) cells from WT or MyD88-ΔT mice with αCD3, αCD28 and IL-2. Utilizing MyD88-ΔT mice instead of MyD88-ΔTreg mice for this assay enabled us to culture MyD88-deficient naïve cells, which do not survive as well as WT T cells after activation, with MyD88-deficient Tregs from the same animal, removing any potential variables between MyD88-ΔT mice and MyD88-ΔTreg mice. As expected, fewer MyD88-deficient naïve cells were Annexin V- 7-AAD- (live) following activation with and without IL-2 than WT naïve cells (Figure 2.13A and B). In contrast, WT and MyD88-deficient Tregs have similar frequencies
of Annexin V- 7-AAD- cells (Figure 2.13A and B), demonstrating that MyD88 was dispensable for Treg survival following activation in vitro.

Next, we wanted to ask whether a Treg survival defect in vitro was only revealed following activation in the presence of rapamycin and αCD154. To accomplish this, we activated sorted MyD88-deficient Tregs with αCD3 and αCD28 in the presence of 1ng/mL, 10ng/mL and 100ng/mL of rapamycin with 100µg/mL αCD154. As seen in Figure 2.14B, these drug concentrations did not result in a lower frequency of Annexin V- 7-AAD- cells, indicating that Treg survival did not decrease despite blocked proliferation (Figure 2.14A) in the presence of these drugs, in agreement to previous studies on the effects of αCD154 or rapamycin on Tregs.
in vitro. (Putnam et al., 2009; Rigby 2013) Importantly, at each concentration, no significant difference in the frequency of Annexin V-7-AAD- between WT or MyD88-deficient Tregs was observed (Figure 2.14B), further confirming our in vivo findings that MyD88 was dispensable for Treg survival. Despite our efforts to explain the resistance of MyD88-ΔTreg mice to long-term allograft survival through Treg survival, it was now clear that MyD88-deficient Tregs survive just as well as WT Tregs, in general and specifically during the response to alloantigen.

Figure 2.14:

A. Percent dividing CD4+ cells (as determined by CFSE dilution) and B. frequency of Annexin V-7-AAD- WT (white bars) or MyD88-ΔTreg (black bars) Treg after activation and culture with αCD3, αCD28 and IL-2 with indicated concentrations of αCD154 and/or rapamycin for 72 hours. Quantification of data from a minimum of 3 independent experiments.
MyD88-deficient Tregs do not have reduced expression of FoxP3 or aberrantly produce inflammatory cytokines.

We next aimed to study whether Treg expressed MyD88 was required for Treg function during the response to alloantigen, potentially resulting in the resistance of MyD88-ΔTreg mice to the effects of costimulatory blockade. While we did not observe any difference in frequency or FoxP3 expression of FoxP3+ cells in vivo, we asked whether FoxP3 expression might differ between WT and MyD88-deficient Tregs following activation with αCD3, αCD28 and IL-2. While we observed that a portion of WT or MyD88-ΔTregs lost FoxP3 expression as previously described, we observed no difference in FoxP3 expression, as measured by frequency of YFP+ cells after 72 hours in culture, between WT and MyD88-deficient Tregs (Figure 2.15A). (Feng et al., 2014)

Furthermore, when we culture WT or MyD88-deficient Tregs with Th1 skewing conditions (IL-12), there was no difference in FoxP3 expression after 72 hours of culture, suggesting that even under conditions favoring the differentiation of an effector cell type, FoxP3 expression was unaffected in MyD88-ΔTregs (Figure 2.15B).

Figure 2.15:

A. WT

B. WT

C. Quantification of data from A. and B. pooled from a minimum of 3 independent experiments.

WT or MyD88-ΔTreg Treg were sort purified and activated for 72 hours with A. Th0 conditions (αCD3, αCD28 and IL-2) or with B. Th1 conditions (αCD3, αCD28, IL-2, IL-12 and αIL-4). Representative flow cytometry plots of YFP (FoxP3) expression. C. Quantification of FoxP3 expression data from A. and B.
While we did not observe any difference between WT Treg and MyD88-deficient Treg expression of FoxP3, another possibility was that MyD88-deficient Tregs were aberrantly producing inflammatory cytokines, such as IFN-γ, that could lead to graft rejection. Following in vitro activation for 72 hours, we observed no difference in FoxP3+ cell production of IFN-γ between WT or MyD88-deficient Tregs after culture with αCD3, αCD28, IL-2 with or without IL-12 (Figure 2.16A and B).

Furthermore, intragraft Tregs from WT or MyD88-ΔTreg recipients produce similar amounts of IFN-γ and IL-17 21 days after transplantation and treatment with CoB. (Figure 2.17A and B) Notably, we observed a significant decrease in the frequency of IL-17+FoxP3+ Tregs isolated from MyD88-ΔTreg ears relative to those isolated from WT ears, further solidifying the notion that MyD88 deficient Tregs do not aberrantly produce inflammatory cytokines that could result in the resistance of MyD88-ΔTreg mice to the effects of CoB (Figure 2.17B).
MyD88-deficient Tregs efficiently regulate effector cell proliferation and express hallmark functional molecules.

In an attempt to further investigate the mechanism by which MyD88-ΔTreg were resistant to the effects of CoB in the context of Bm12 skin transplantation, we next characterized the basic function of MyD88-deficient Tregs. Despite not always correlating well to in vivo suppressive function, an in vitro suppression assay has previously been shown to demonstrate whether Tregs can effectively prevent effector T cells proliferation. (Thornton and Shevach, 1998; Zhang et al., 2013) As shown in Figure 2.18, MyD88-deficient Tregs prevented the proliferation of WT effector cells as efficiently as WT Tregs, suggesting that a defect in suppression in our model may occur only during the response to alloantigen in vivo.
We next asked whether a difference in Treg expression of any hallmark Treg functional proteins could result in the resistance of MyD88-ΔTreg mice to the effects of CoB that may not necessarily be revealed in the above in vitro suppression assay. To accomplish this, we assessed surface and/or intracellular expression of CTLA-4, PD-1, CD28, GITR, Lag-3, Granzyme B, CD39 and CD73 on MyD88-deficient Tregs, all of which have been implicated in Treg function.(Baecher-Allan et al., 2003; Deaglio et al., 2007; Gondek et al., 2005; Grossman et al., 2004a; Grossman et al., 2004b; Huang et al., 2004; Krupnick et al., 2005; Le Moine et al., 1998; McHugh et al., 2002; Wing et al., 2008; Zhang et al., 2013) We observed no difference in any of the above markers on MyD88-deficient Tregs relative to WT Tregs that were analyzed directly ex vivo, or activated in vitro with αCD3 and αCD28 for 20 hours (Figure 2.19). These data suggest that even though MyD88-deficient Tregs were unable to promote long-term bm12 skin graft survival, their functional marker phenotype and in vitro function appears to be the same as WT Tregs, indicating a more complex role for Treg expressed MyD88 during the response to alloantigen than can currently be defined.
Figure 2.19:

A: Representative flow cytometry plots of A. CD39 and CD73, B. surface CTLA-4, C. intracellular CTLA-4, D. Lag-3, E. Granzyme B and F. GITR on WT (black lines, minimum n=10 mice) and MyD88–Treg (red lines, minimum n=11 mice) Treg from pooled spleen and pLN directly ex vivo or after 20 hours stimulation with αCD3 and αCD28. Shaded gray area indicates appropriate isotype control for indicated stain. Data are pooled from a minimum of 3 independent experiments.
Discussion

Together, the above data demonstrate that T cell, and Treg expressed MyD88 is critical for rapamycin and αCD154 to prolong bm12 allograft survival. Bm12 skin and cardiac long-term allograft survival is dependent on the presence of Tregs, and we thus hypothesized from these transplantation studies that MyD88 deficient Tregs were defective either in their ability to survive or directly inhibit effector T cell proliferation in response to alloantigen. Interestingly, in contrast to our hypothesis that Treg expressed MyD88 would be critical for Treg survival, similar to the pathogen reactive non-Treg requirement for survival, we found that Treg expressed MyD88 was dispensable for Treg survival. MyD88-deficient Treg frequency was similar to WT Treg frequency in the blood, spleen and pLN following transplantation with bm12 skin and treatment with αCD154 and rapamycin, suggesting that MyD88-deficient Tregs had no survival defect in the peripheral lymphoid organs. As the lab’s previous findings that T cell expressed MyD88 was critical for T cell survival during infection, we next hypothesized that there might not be systemic inflammation during the response to a skin allograft, potentially explaining the similar Treg frequencies we observed in the secondary lymphoid organs.

Studies from other groups demonstrated that Tregs within the allograft were critical in the promotion of long-term allograft survival, and we next wanted to address the survival capacity within the allograft, as the allograft is highly inflammatory, similar to the environment that cells experience during an infection.(Gondek et al., 2008; Graca et al., 2002) While we did not observe a difference in FoxP3+ cell frequency among graft-resident CD4+ T cells at 7 and 14 days post transplant, we observed a significant decrease in MyD88-deficient FoxP3+ cells relative to WT cells 21 days post transplant (Figure 2.8). While this decrease in frequency could have been due to a decrease in the absolute number of Tregs, it could also have been due to an increase in non-Treg cells relative to Tregs. To address these possibilities, we used a bead-based cell counting assay to quantify the absolute number of Tregs and non-Tregs isolated from
transplanted bm12 skin. We found that the absolute number of WT and MyD88-deficient Tregs was similar, while the absolute number of non-Tregs was significantly higher in grafts harvested from MyD88-ΔTreg recipients compared to WT recipients (Figure 2.9), consistent with our graft-survival kinetics indicating that the majority of grafts are rejected by MyD88-ΔTreg recipients between 20 and 40 days post transplant (Figure 2.5). In addition, we demonstrated that similar frequencies of intragraft Tregs from MyD88-ΔTreg recipients stained positive for Live/Dead Aqua as intragraft Tregs from WT mice, indicating that there was not a higher frequency of dead Tregs in grafts harvested from MyD88-ΔTreg recipients 21 days after transplant, in agreement with our cell counting data (Figure 2.10).

While these data indicated that MyD88-deficient Tregs were surviving as well as WT Tregs in the allograft, further study was required in order to prove that MyD88-deficient Tregs did not have a survival defect, as complicating factors including the long enzymatic tissue digestion and low cell numbers made precise assessment of cell survival difficult. First, we utilized our FoxP3-Cre heterozygous system to assess MyD88-deficient Treg survival in vivo under competitive conditions with WT Tregs over 32 weeks of age, in lieu of a bone-marrow chimera adoptive transfer models. We found that FoxP3+ cells that lacked 1 (MyD88^{fl/+} FoxP3-Cre het) or 2 (MyD88^{fl/fl} FoxP3-Cre het) copies of MyD88 did not significantly differ in frequency among total FoxP3+ cells as compared to FoxP3+ cells with both copies of MyD88 (Figure 2.12). If MyD88 deficient Tregs failed to survive when in competition with WT Tregs, we would expect the frequency of MyD88-deficient Tregs to decline among the total FoxP3+ cell frequency (Figure 2.12, red line), however, we observed that there was no significant difference in the frequency of MyD88 deficient Tregs among total Tregs over 32 weeks. These data suggest that MyD88-deficient Tregs do not exhibit a survival defect in naïve, unmanipulated mice as they can compete with WT Tregs for space regardless of age.
The survival defect in MyD88-deficient non-Tregs initially observed in the infection models discussed above was extended to an in vitro model where MyD88-deficient non-Tregs fail to survive as well as WT non-Tregs following activation. To extend our in vivo findings, we next aimed to culture MyD88-deficient Tregs and WT Tregs side by side with MyD88-deficient non-Tregs, cells that have a well-defined survival defect. To accomplish this, we cultured non-Tregs (CD4+, CD25-, CD62L+, CD44-) and Tregs (CD4+, CD25+) sorted from WT or MyD88-ΔT mice. As expected, we observed a significantly lower frequency of live MyD88-deficient non-Tregs as compared to live WT non-Tregs after activation with or without 10ng/mL IL-2 (Figure 2.13). Interestingly, we observed no significant difference between the frequencies of live MyD88-deficient and WT Tregs after activation with αCD3, αCD28 and IL-2 (Figure 2.13). The possibility remained however that MyD88-deficient Tregs failed to survive beyond our ability to detect in MyD88-ΔTreg transplant recipients treated with αCD154 and rapamycin, so we next determined whether MyD88-deficient Treg survival differed from WT Treg survival in vitro during activation with αCD154 and rapamycin. To accomplish this, we sorted FoxP3- and FoxP3+ cells from WT or MyD88-ΔTreg mice and activated them with αCD3 and CD3-depleted splenocytes (APC source) with increasing concentrations of rapamycin and/or rapamycin with 100µg/mL αCD154. CD3-depleted splenocytes were used as opposed to αCD28 because the effect of αCD154 blockade on T cells is dependent on APCs. Despite observing a block in proliferation when cultured with all combinations of rapamycin and αCD154 (Figure 2.14A), we observed no difference between the frequency of live WT or MyD88-deficient Tregs (Figure 2.14B).

These data indicate that MyD88-deficient Tregs survived as well as WT Tregs following activation in vitro with or without αCD154 and rapamycin, demonstrating that MyD88 was paradoxically dispensable for Treg survival. Many differences could account for the divergent requirement for MyD88 in Tregs and non-Tregs. One difference is that FoxP3+ cells have a higher turnover rate than non-Tregs under homeostatic conditions as measured by BRDU.
incorporation. (Fisson et al., 2003; Pierson et al., 2013; Setoguchi et al., 2005; Walsh et al., 2006) To maintain constant Treg frequencies in the context of increased proliferation, a constitutively expressed, Treg intrinsic apoptosis program must be active. Knockout of the pro-apoptotic genes Bak and Bax resulted in an increase in Treg numbers in vivo, however knockout of the anti-apoptotic genes Bcl-2 and Bcl-xL, two anti-apoptotic proteins that have been shown to be important (but not required) for non-Treg survival, were also dispensable for Treg survival. (Boise et al., 1995; Dahl et al., 2000; Pierson et al., 2013; Wojciechowski et al., 2007) Similar to Bcl-xL, the anti-apoptotic protein Mcl-1 is upregulated upon T cell activation and is also expressed in Tregs, however unlike Bcl-xL, the role for Mcl-1 is non-redundant in Tregs in that Mcl-1 deletion results in decreased Treg survival capacity. (Dzhagalov et al., 2008; Pierson et al., 2013) Higher constitutive expression of anti-apoptotic proteins, such as Mcl-1, could account for the resistance of MyD88-deficient Tregs to cell death. Another possibility, discussed further in Chapter 3 of this thesis, is that differences in the metabolic programs of Tregs and non-Tregs could account for the divergent survival observed in MyD88-deficient Tregs and non-Tregs.

Because we surprisingly demonstrated that MyD88 was not required for Treg survival in general or during the response to alloantigen, the Treg-specific requirement for MyD88 to promote long-term allograft survival required further study. We next hypothesized that MyD88-deficient Tregs were unable to promote long-term allograft survival due to a functional defect rather than a survival defect. However, MyD88-deficient Tregs suppress non-Treg proliferation as efficiently as WT Tregs, and express hallmark Treg functional proteins at similar levels as WT Tregs (Figures 2.18 and 2.19). In addition, MyD88-deficient Tregs do not aberrantly produce inflammatory cytokines in the allograft or in vitro following activation (Figures 2.16 and 2.17). These data, compounded with our observation that MyD88-ΔTreg mice do not develop autoimmunity or a lymphoproliferative disease at any age may indicate that MyD88-deficient Tregs may specifically lose functional capacity during the response to alloantigen.
Although unlikely, it remains possible that MyD88-deficient Tregs have decreased expression of any of the Treg hallmark functional proteins we assayed for specifically in the allograft. Furthermore, it is possible that a functional protein or pathway beyond our ability to detect is down-regulated in MyD88-deficient Tregs generally or specifically in the allograft. To address these possibilities, we have sorted FoxP3+ cells from bm12 skin grafts 21 days post transplantation from either WT or MyD88-ΔTreg recipients with the goal of performing comparative gene expression analysis by RNA sequencing in an attempt to determine why MyD88-ΔTreg mice are resistant to the effects of rapamycin and αCD154.

Together, our data importantly define a differential requirement for MyD88 in T cells. Previously defined T cell specific survival factors, such as Bcl-xL and Mcl-1, were consistently dispensable or required for survival in non-Tregs and Tregs (of note, while Bcl-xL increased effector T cell survival during T cell activation, it was surprisingly dispensable for effector T cell survival, suggesting other pro-survival proteins can compensate for the loss of Bcl-xL in both Tregs and non-Tregs). (Boise et al., 1995; Dahl et al., 2000; Dzhagalov et al., 2008; Pierson et al., 2013; Zhang and He, 2005) MyD88 is the first T cell intrinsic survival molecule to our knowledge that is required for non-Treg survival but dispensable for Treg survival. This observation would support the exciting idea that pharmacologic targeting of MyD88 could induce death of effector T cells while sparing Tregs, a potential therapeutic angle for autoimmune disorders where depletion of effector T cells but sparing of Tregs is essential.
Materials and Methods

Mice

Mice with a T cell specific deletion of MyD88, termed MyD88-ΔT animals, have been previously described. (Rahman et al., 2011) Mice with a FoxP3+ cell specific deletion of MyD88, termed MyD88-ΔTreg animals, were produced by crossing FoxP3-Cre mice (Rubtsov et al., 2008) to MyD88fl/fl mice. FoxP3-Cre, MyD88fl/fl, MyD88-ΔT and MyD88-ΔTreg mice were maintained as breeding colonies in our animal facility. C57Bl/6, B6(C)-H2-Ab1bm12/KhEgJ (bm12), CB6F1 and Foxp3tm1Kuch (FoxP3-GFP) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). (Bettelli et al., 2006) All colonies were maintained in accordance with the protocols approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital.

Skin transplantation and treatment of recipients

As previously described, tail skin was removed from donor mice and trimmed to a 1 cm x 1 cm piece. (Billingham, 1954) Recipient animals (8-10 weeks old) were prepared by shaving and aseptic application of betadine and 70% ethanol. A 1 cm x 1 cm incision was made on the left or right trunk, and donor skin was placed on the open area. 4 sutures were applied at each corner of the graft. Triple antibiotic ointment was applied to graft area, and vaseline coated gauze was placed over the graft, followed by a bandage. Bandages and gauze were removed 7 days following surgery and grafts were visually monitored daily for rejection (defined as >80% necrosis). Where indicated, mice were administered (by i.p. injection) 0.25mg αCD154 (clone: MR1, BioXcell, Lebanon, NH) on days 0, +2 and +4 and 1mg/kg rapamycin (LC Laboratories, Woburn, MA) in a carboxy methyl cellulose and Tween 80 solution on days 0, +2, +4, +6, +8, +10 and +12.
Cardiac transplantation and treatment

Heterotopic heart transplantation was performed as previously described (Corry et al., 1973) Graft survival was assessed by daily palpation, and upon absence of palpable graft function, recipient mice were anesthetized before sacrifice so that cessation of beating could be visually confirmed. Grafts were then removed and prepared for histological confirmation of rejection (see below). Where indicated, mice received 0.5x10^6 Treg (defined as CD4+GFP+ cells, isolated by cell sorting from FoxP3-GFP mice) via retro-orbital injection 7 days prior to heart transplantation.

Antibodies and flow cytometry

Single cell suspensions were prepared from blood, spleen, peripheral lymph nodes (inguinal, axillary, brachial and cervical), ear or skin graft in FACS Media (PBS, 2% BSA, 0.1% Sodium Azide) and stained with the following mAbs purchased from BioLegend (San Diego, CA); αCD4 (GK1.5), αCD8 (53-6.7), αCD25 (PC61), αCD44 (IM7), αCD62L (MEL-14), αCCR4 (2G12), αCCR6 (29-2L17), αCD103 (2E7), αCD45 (30-F11) αThy1.2 (53-.1), αTCRβ (H57-597), Annexin V, αIFN-γ (XMG1.2), αIL-17A (TC11-18H10.1), αCD28 (37.510), αCTLA4 (UC10-4B9), αPD-1 (29F.1A12), αGITR (DTA-1), αGranzyme B (NGZB), αCD39 (Duha59), αCD73 (TY/11.8) and αLag 3 (C9B7W). αFoxP3 (FJK-16s) was purchased from eBioscience (San Diego, CA). Live/Dead Aqua was purchased from Life Technologies (Grand Island, NY). 7-AAD was purchased from BD Biosciences (Franklin Lakes, NJ). Cell fixation and permeabilization (fix/perm) was performed using the Intracellular Fix/Perm Buffer Set (eBioscience). For FoxP3-Cre het longitudinal studies, cell fixation with 2% paraformaldehyde prior to above fix/perm procedure was performed to preserve YFP fluorescence. Flow cytometric analysis was performed on a LSRII (BD Biosciences, San Jose, CA) or Navios (Beckman Coulter, Brea, CA)
flow cytometer. Data analysis was performed using Flow Jo (version 10.0.7 Tree Star, Ashland, OR).

**Histology**

Donor hearts were harvested from recipient animals following cessation of beating and fixed overnight in 10% buffered formalin. Tissues were then processed as previously described and imbedded in paraffin. (Zhang et al., 2013) Transverse sections of the left and right ventricles were prepared and stained with hemotoxin and eosin. Slides were examined by an observer without knowledge of the treatment protocol of the recipients.

Skin grafts were harvested from recipient animals at indicated time points, embedded in OCT Compound from Tissue-Tek, Sakura Finetek USA (Torrance, CA), and submerged in liquid nitrogen for 20 seconds. Samples were stored at -80 degrees Celsius until cryo-sectioning. 10µm sections were fixed to slides in acetone for 5 minutes at -20 degrees Celsius, washed 2 times with TBST from Boston Bio-Products (Ashland, MA) and stained for at least 1 hour with αCD4 PE (GK1.5) from BioLegend and DAPI from Life Technologies (diluted in TBST) at room temperature or 4 degrees Celsius. Slides were washed 2 times with TBST, and imaged. Image analysis and merging was performed using ImageJ (Version 1.48).

**Skin Digestion**

As previously described, (Riol-Blanco et al., 2014) ear skin or skin grafts were harvested from indicated mice and digested into a single cell suspension for 60-90 minutes using Liberase TM from Roche (Indianapolis, ID) and Hyaluronidase (Life Technologies). Before digestion of skin grafts, any remaining sutures were removed. Where indicated, single cell suspensions were incubated with Leukocyte Activation Cocktail (BD Biosciences) for 2 hours at 37 degrees Celsius before being stained for analysis by flow cytometry.
In vitro Culture

Purified (StemCell Technologies, Vancouver, BC) CD4+ T cells from pooled spleens and peripheral lymph nodes of MyD88-ΔTreg, FoxP3-Cre, MyD88-ΔT or MyD88-fl/fl mice were sorted for naïve (CD4+, FoxP3-, CD62L+, CD44-, CD25-), FoxP3+ (CD4+, FoxP3+) or CD25+ (CD4+, CD25+) cells on a SORP FACS ARIA II (BD Biosciences, San Jose, CA). Alternatively, where indicated, CD4+ T cells were isolated from spleens and peripheral lymph nodes from WT or MyD88-ΔTreg using eBioscience MagniSort negative selection. 2x10^5 (when using 96 well plate) or 5x10^5 (when using 48 well plate) cells were then resuspended in RPMI media supplemented with 10% FBS, 1% Penicillin/Streptomycin, 1% L-glutamate, 1% non-essential amino acids and 0.1% 2-mercaptoethanol (Complete RPMI Media) and plated in one of the following conditions:

1) In a 48-well plate with 5µg/mL plate bound αCD3 and αCD28 (BioLegend)
2) In a 96-well flat bottom plate with 5µg/mL plate bound αCD3 and 1µg/mL αCD28
3) In a 96-well flat bottom plate with 5µg/mL plate bound αCD3 and 2µg/mL αCD28
4) In a 96-well round bottom plate with 3x10^5 APCs treated with mitomycin C from Sigma-Aldrich (St. Louis, MO) and 2µg/mL αCD3

Where indicated, cytokines from Peprotech (Rocky Hill, NJ) and/or R&D Systems (Minneapolis, MN) and antibodies (BioLegend) for T_H1 (10 ng/ml IL-2, 5 ng/ml IL-12, 5 µg/mL αL-4) and T_H17 (50 ng/ml IL-6, 1 ng/ml TGF-β, 50 µg/well αIL-2, 5 µg/mL αIL-4, 5 µg/mL αIFNγ) conversion, rapamycin (0.1µg/mL, 0.01µg/mL and 0.001µg/mL) and/or αCD154 (Clone: MR1, 100µg/mL or 10µg/mL), DMOG (10µM) or DMSO were added to the culture. Where indicated, plates were incubated at 37 degrees Celsius for 20 or 72 hours. When indicated, cells were incubated with Leukocyte Activation Cocktail (BD Biosciences) for 4 hours at 37 degrees Celsius after indicated culture time. Cells were then prepared for flow cytometry as indicated above.
In vitro suppression assay

Purified (StemCell Technologies, Vancouver, BC or eBioscience, San Diego, CA) CD4+ T cells from pooled spleens and peripheral lymph nodes of MyD88-ΔTreg or FoxP3-Cre mice were sorted for naïve (CD4+, FoxP3-, CD62L+, CD44-, CD25-) and FoxP3+ (CD4+, FoxP3+) cells. Naïve cells from FoxP3-Cre mice were labeled with cell trace violet (Invitrogen), and cultured with 3x10^5 APCs irradiated with 2500 rads from FoxP3-Cre mice and 0.5mg/mL soluble αCD3. Tregs from FoxP3-Cre or MyD88-ΔTreg were added to wells at 1:1, 3:1 and 9:1 (naïve:Treg) ratios, and cells were cultured for 3 days at 37 degrees Celsius and then prepared for flow cytometry as indicated above.

Adoptive Transfer

Purified (StemCell Technologies or eBioscience) T cells (CD4+ and CD8+) from pooled spleens and peripheral lymph nodes of MyD88-ΔT B6 CD45.2 or WT B6 CD45.1 were prepared and stained with CFSE or Cell Trace Violet (Invitrogen). Cells were suspended at a 1:1 ratio (MyD88-ΔT:WT B6), and 10x10^6 total T cells were injected retro-orbitally to CB6F1 recipients. Spleens and peripheral lymph nodes were harvested from CB6F1 recipients 3 and 7 days post adoptive transfer. Donor B6 cells were differentiated from recipient CB6F1 through positive staining with H-2K^b but not H-2K^d during flow cytometry analysis.

Western Blot

Purified samples were obtained by FACs or magnetic bead isolation and boiled for 4-10 minutes in sample buffer normalized to cell pellet size. A polyacrylamide gel (4-15% gradient) was prepared, and 15μL of sample was loaded into appropriate wells. Gel was run at 80 volts for a minimum of 30 minutes, then 100 volts until dye front reached the bottom of the gel. Gel was released from cast, and stored in transfer buffer while membrane was prepared by washing.
for 10 seconds in methanol. Proteins were transferred to membrane at 90 volts for 1 hour on ice. Membrane was blocked for 1 hour in 5% milk in TBST, washed in 1x TBST for 5 minutes, and incubated with indicated antibodies overnight. α-β-actin (1:1000 dilution) was purchased from Cell Signaling (Danvers, MA), αMyD88 (1:200 dilution) was purchased from R&D Systems and αHif-1α (1:500 dilution) was purchased from Caymen Chemical (Ann Arbor, MI). Membrane was then washed for 3 times for 15 minutes in 1x TBST before being incubated with secondary antibody conjugated to HRP (Bio-Rad, Hercules, CA) for 1 hour at room temperature. Membrane was washed 4 times for 15 minutes each, quickly dried and exposed to appropriate developing solution. Membrane was developed in dark room for indicated time periods. Developed film was scanned at 300DPI.

**Imiquimod Treatment**

5mg Imiquimod cream (5%) was applied to the dorsal and ventral aspects of WT and MyD88-ΔTreg ear skin for 6 consecutive days. Each day, mice were anesthetized while treated and non-treated ears were measured in identical locations for 17 consecutive days using a digital micrometer (Mitutoyo).

**Data analysis and Statistics**

Data were tabulated and graphed in Graph Pad Prism (version 5.0). For all experiments, a two-tailed students T-test was used to determine statistical significance, with the exception of the data represented in Figure 2.13 where a one-tailed students T-test was used to determine statistical significance. For survival curves, (Figures 2.1, 2.2 and 2.5) a log-rank (Mantel-Cox) test was used to determine statistical significance.
References:


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Chapter 3: Discussion, conclusions and future directions

T cell expressed MyD88 is critical for long-term allograft survival

Inflammation, in the form of cytokine release and immune cell recruitment, serves as the primary mechanism for the immune response to a barrier breach. One way to think about inflammation is as a gradient. High levels of inflammation, such as those present during the response to a pathogen are associated with an effector T cell response. In the context of organ transplantation, high levels of inflammation contribute to graft rejection mediated by CD4 T helper cells and CD8 cytotoxic T cells. In contrast, low levels of inflammation are associated with immune tolerance. In the context of organ transplantation, immune tolerance leads to long-term allograft survival, promoted at least in part by Tregs. (Wood and Sakaguchi, 2003) Because the level of inflammation is critical in ensuring long-term allograft survival versus graft rejection, further understanding of inflammatory pathways is essential.

One inflammatory pathway is the TLR signaling pathway. Engagement of any of the TLRs except TLR-3 by PAMPs leads to MyD88 signaling, activation of NF-κB and the eventual transcription of inflammatory cytokines such as IL-6 and TNF-α. Furthermore, signaling through the IL-1R family members IL-1R, IL-18R and IL-33R leads to similar cytokine production. Despite much effort in characterizing this pathway in cells of the innate immune system, our group and now others have demonstrated a T cell intrinsic role for MyD88 signaling. During the response to a pathogen, T cells that lack MyD88 are unable to survive and efficiently respond to the pathogen, demonstrating that MyD88 signaling is critical for T cell survival. (LaRosa et al., 2008; Rahman et al., 2008; Rahman et al., 2011) Based on these data, we next hypothesized that MyD88 deficient T cells would fail to respond to alloantigen due to their inability to survive during an antigen specific response. Surprisingly, bm12 skin and cardiac allografts were rejected from MyD88-ΔT mice at a higher frequency as compared to WT mice (Figures 2.1 and...
2.2). In addition, a regimen of αCD154 and rapamycin used to prolong bm12 skin allograft survival was not as effective in MyD88-ΔT mice as compared to WT mice (Figure 2.1). These data suggest two non-mutually exclusive possibilities: 1) the MyD88-deficient T cell response to alloantigen did not result in cell death as the response to a pathogen did, and/or 2) as long-term allograft survival in these models is dependent on Tregs, and because Tregs presumably utilize the same survival factors as non-Tregs, MyD88-deficient Tregs fail to survive during the response to alloantigen and thus fail to promote graft survival. Interestingly, adoptive transfer of MyD88-ΔT T cells into an allogeneic mouse (parent into F1 hybrid model) resulted in a higher frequency of cell death relative to WT T cells, demonstrating that alloreactive MyD88-deficient T cells failed to survive similar to their pathogen reactive counterparts (Figure 2.3).

**Treg expressed MyD88 is dispensable for Treg survival**

While the above data suggested that MyD88-deficient non-Tregs failed to survive following the response to alloantigen, it was essential to demonstrate that MyD88-deficient Tregs also were unable to survive during the response to alloantigen and thus failed to promote long-term allograft survival. To accomplish this, we restricted MyD88 deletion specifically to Tregs by crossing MyD88<sup>fl/fl</sup> mice to FoxP3-Cre mice (termed MyD88-ΔTreg mice, Figure 2.4). MyD88-ΔTreg mice treated with CoB failed to accept bm12 skin grafts at similar frequencies as WT mice (Figure 2.5). Interestingly, complement of MyD88-ΔT mice with WT Tregs via adoptive transfer prolonged bm12 cardiac graft survival and complement of MyD88-ΔTreg mice with WT Tregs via female mice possessing one copy of FoxP3-Cre and one copy of WT FoxP3 (FoxP3-Cre heterozygous mice) resulted in improved allograft survival in each system, demonstrating that the survival and or functional defect of MyD88-deficient Tregs could be rescued by WT Tregs (Figures 2.2 and 2.5).
We next hypothesized that Treg expressed MyD88 was critical for Treg survival based on our previous findings that T cell expressed MyD88 was critical for T cell survival. This hypothesis had precedence, as the requirement for the survival molecules Bcl-xL and Mcl-1 are similar between T cells and Tregs. (Boise et al., 1995; Dahl et al., 2000; Dzhagalov et al., 2008; Pierson et al., 2013; Zhang and He, 2005) Interestingly however, through a series of in vitro and in vivo experiments we have now demonstrated that MyD88 is dispensable for Treg survival. Of note, we cultured Tregs and non-Tregs from MyD88-ΔT mice and as expected, MyD88 deficient non-Tregs failed to survive while MyD88-deficient Tregs survived as well as WT in the same culture conditions (Figure 2.13). These data suggest the exciting and novel possibility that MyD88 has a divergent requirement for cell survival in Tregs and non-Tregs.

Except for promotion of long-term allograft survival, MyD88-deficient Tregs have similar functional capacity

The observation that MyD88-ΔT and MyD88-ΔTreg mice were not amenable to the skin graft-prolonging effects of CoB served as a deep mystery after our demonstration that MyD88-deficient Tregs could survive as well as WT Tregs during the response to alloantigen. Because Tregs are critical for long-term allograft survival in our system, we next began to analyze Treg function, as the possibility remained that MyD88 signaling was contributing to Treg function during the response to alloantigen. (Porrett et al., 2008) Interestingly, MyD88-deficient Tregs suppressed effector T cell proliferation as well as WT Tregs in an in vitro suppression assay (Figure 2.18). Furthermore, MyD88-deficient Tregs had similar expression patterns of hallmark Treg functional markers such as CTLA-4, GITR, granzyme B, Lag-3, CD39 and CD73 directly ex vivo or after in vitro activation as WT Tregs (Figure 2.19). Together, these data suggest that MyD88-deficient Tregs have a functional defect beyond our current ability to detect
Contribution of MyD88 to Treg function specifically during inflammatory responses could explain why MyD88-ΔTreg mice do not spontaneously develop lymphoproliferative disorders or autoimmunity. In order to better understand the Treg intrinsic requirement for MyD88 during a non-specific inflammatory response, we induced inflammation in the skin using imiquimod, a TLR-7 agonist. This reaction is dependent on production of IL-23 that stimulates production of IL-17 and IL-22 by resident γδ T cells. These cytokines induce the recruitment of monocytes and neutrophils into the skin resulting in swelling of the ear. Aside from the recruitment of mononuclear cells, this reaction is limited to skin resident cells. (Riol-Blanco et al., 2014) While it is not clear whether Tregs are required for resolution of this specific inflammatory response, Tregs are critical in the resolution of other, similar skin inflammatory responses. (Kish et al., 2005; Ring et al., 2006) We applied Imiquimod to the ears of MyD88-ΔTreg and WT mice, and measured ear swelling over 17 days. There was no difference in the ear thickness between WT and MyD88-ΔTreg mice during the initial swelling phase, or the resolution phase (Figure 3.1). These data suggest that the presence of an alloantigen, not solely inflammation, may be a factor in the failure of MyD88 deficient Tregs to promote long-term skin allograft survival.

Because of the apparent specific requirement for MyD88 during the response to alloantigen but our inability to detect any differences in hallmark Treg functional proteins, we are now turning to next-generation sequencing to probe for any functional differences between WT and MyD88-deficient Tregs. To accomplish this, we sorted Tregs and non-Tregs from spleen, ears (non-inflamed skin) and bm12 skin grafts from WT and MyD88-ΔTreg recipients 21 days after transplantation and treatment with CoB. These cells are now being prepared for RNA-sequencing to determine any differences in gene expression in WT and MyD88-deficient Tregs and may potentially yield an avenue of future investigation as to why MyD88 is required for Treg function during the response to alloantigen.
Future directions

The above studies present many exciting avenues of study. As eluded to in the discussion section of Chapter 2, these studies will focus on 1) the requirement for MyD88 in non-Treg, but not Treg survival and 2) the role for MyD88 in promoting Treg function.

Upstream signals to MyD88 that are critical for Treg function during the response to alloantigen.

While our ongoing study of functional differences between WT and MyD88-deficient Tregs through gene expression analysis will inform us of why MyD88-deficient Tregs do not
function as well as WT Tregs in the context of the response to alloantigen, this study will not elucidate the upstream signals from MyD88 that are important in promoting Treg function. All of the TLRs except for TLR-3 and the IL-1R family members IL-1R, IL-18R and IL-33R signal through MyD88. Previous work has implicated TLRs in Treg function. For example, as previously discussed, treatment of Tregs with flagellin during an in vitro suppression assay resulted in decreased proliferation of effector T cells, suggesting that TLR-5 engagement resulted in increased Treg function.(Crellin et al., 2005) TLR-2 signaling on Tregs via the synthetic ligand Pam3Cys or the fungus C. albidans increased the proliferation but decreased the suppressive capacity of Tregs in vitro and in vivo.(Sutmuller et al., 2006) However, in a different model system, TLR-2 engagement with Pam3CSK4 did not decrease Treg function in vitro and in vivo. (Chen et al., 2009) Exposure of Tregs to CpG DNA resulted in decreased suppression, either through direct inhibition of Treg function and/or costimulation of responding effector cells resulting in effector T cell resistance to suppression by Tregs.(LaRosa et al., 2007; Peng et al., 2005)

The IL-1R family member IL-33R (ST2) promotes Treg function during the response to alloantigen as well as in the intestine. Despite increasing Th2 effector T cells, IL-33 significantly prolongs cardiac allograft survival. Prolongation of graft survival in IL-33 treated recipients is dependent on Tregs and recipient expression of ST2, and is associated with an increase in intragraft Tregs.(Turnquist et al., 2011) More recently, IL-33 was shown to stimulate IL-2 production by dendritic cells which selectively expanded suppressive ST2+ Tregs, demonstrating a potential mechanism by which IL-33 could prolong Treg dependent allograft survival.(Matta et al., 2014) Furthermore, ST2 has been shown to be selectively expressed on colonic Tregs, and colonic Treg expression of ST2 is critical for Treg suppression of aberrant immune activation in the gut.(Schiering et al., 2014) Together, these data demonstrated that signaling through IL-33R, and thus MyD88, was critical for Treg function in this model.
In the above models, the investigators utilized germ-line knockouts, bone marrow chimeras and/or adoptive transfers to investigate the role of TLR or IL-1R family signaling on Tregs. While these models provide a system to address the requirement of a single molecule on Tregs, T cell development and/or immune homeostasis is often disrupted in these mice as all cells lack the molecule under investigation. Thus, results may not be indicative of the actual biological requirement for the molecule, or differ between experimental systems as noted above. Advances in conditional knockouts have made it possible to reconcile these findings, and one potential avenue of future study is to conditionally knock out receptors upstream of MyD88 to determine their role on Tregs in a more precise system.

Of all the upstream molecules of MyD88 discussed above, currently only TLR-4^{fl/fl} mice are commercially available solidifying our choice of MyD88 as a broad adapter molecule for many inflammatory pathways. However, with the advent of CRISPR-Cas9 genome editing technology, inserting a cassette with lox-p sites flanking a gene of interest, such as TLR-2 or ST2/IL-33R, can now be done efficiently and at low cost, and may be of interest for future studies that wish to specify upstream signals through MyD88 that promote Treg function.

Furthermore, experiments using well defined model systems to test Treg function could be utilized to determine whether MyD88 is indeed required for Treg function specifically during the alloresponse. First, we wish to expand our bm12 skin and heart transplant studies to a chronic GVHD (cGVHD) model whereby protection from disease is dependent on a functional Treg compartment. This study would allow us to determine Treg function during the response to an alloantigen in a non-solid organ transplant model, and also across a full MHC mismatch. Preliminary data suggest that MyD88-deficient Tregs are unable to protect B10.BR recipients as well as WT Tregs from chronic GVHD induced following a B6 bone marrow transplant and adoptive transfer of either WT or MyD88^{-Δ}Treg following treatment with cyclophosphamide and TBI (this study is being done in collaboration with the Blazer Lab at The University of Minnesota). These data suggest that MyD88 is critical for Treg function during the response to alloantigen
more broadly than the isolated MHC Class II mismatch solid organ transplant models as discussed above.

In addition to solidifying the notion that MyD88 is critical for Treg function during the alloresponse, we need to determine whether MyD88 is dispensable for Treg function during other immune reactions. One model that has classically been used to test Treg function is a model of experimental autoimmune encephalomyelitis (EAE), as disease severity and resolution is dependent on Tregs. (Kohm et al., 2002; McGeachy et al., 2005) If MyD88 deficient Tregs are defective in suppression specifically during the response to alloantigen, we would expect the disease severity in MyD88-ΔTreg mice to be similar to WT mice. However, if we observed that MyD88-ΔTreg mice developed worse disease, or failed to resolve disease, then it would suggest that the defect in MyD88-deficient Treg function was more broad. Another model to test Treg function is a model of skin infection by L. major, whereby immunity to recurrent infections is dependent on a functional Treg compartment. Without functional Tregs, L. major is effectively cleared and pathogen loads during secondary challenge are greater in mice that contain a functional Treg compartment. (Belkaid et al., 2002) If MyD88-deficient Tregs were not functional during the response to L. major, we would expect that MyD88-ΔTreg mice would have a higher pathogen load upon secondary challenge with L. major than WT mice. However, if MyD88-deficient Tregs functioned as well as WT mice during this response to a pathogen, we would observe that pathogen loads were similar upon secondary challenge with L. major. These studies, in addition to our imiquimod experiment above, would solidify whether MyD88 is required for Treg function specifically during the response to alloantigen, or whether MyD88 is required for Treg function during all immune responses.

**Metabolism**

Another paradigm arising from the above studies is that MyD88 is required for effector non-Treg cell survival but not for Treg survival. Based on the consistent requirement for the survival molecules Bcl-xL and Mcl-1 in Tregs and non-Tregs, we were surprised to discover that
the requirement for MyD88 was inconsistent between these two subsets of CD4 T cells. (Boise et al., 1995; Dahl et al., 2000; Dzhagalov et al., 2008; Pierson et al., 2013; Zhang and He, 2005)

To determine the direction of future study, our efforts focused on known differences between Tregs and non-Tregs. One key difference between non-Tregs and Tregs appreciated more recently are the metabolic programs each cell type uses. Tregs have been shown to depend more on lipid oxidation while effector non-Tregs have been shown to depend more on glycolysis. (Gerriets et al., 2015; Michalek et al., 2011)

One factor that regulates this metabolic difference between oxidative Treg metabolism and glycolytic non-Treg metabolism is pyruvate dehydrogenase (PDH), which promotes oxidative phosphorylation through the conversion of cytosolic pyruvate into acetyl-CoA which can be utilized by the mitochondria. Accordingly, effector T_{H17} cells express high levels of the PDH inhibitor pyruvate dehydrogenase kinase (PDHK) while Tregs express lower levels of PDHK, providing a potential mechanism for the difference in metabolic programs between effector T cells and Tregs. (Gerriets et al., 2015)

Another factor that controls Treg and non-Treg fate is hypoxia inducible factor 1α (Hif-1α). Hif-1α is stabilized under conditions of low O_2, and transcribes a myriad of genes including many that enhance proliferation and cell survival by decreasing O_2 consumption through a shift towards glycolysis. (Palazon et al., 2014; Semenza, 2014)

Interestingly, T_{H17} but not Treg inducing conditions in vitro induced a glycolytic program as well as high levels of Hif-1α. Germ-line deletion of Hif-1α resulted in skewing towards Treg differentiation in vitro and protection from the progression of EAE. (Shi et al., 2011)

Together, these data show that the glycolytic program differs from predominantly oxidative phosphorylation in Tregs and predominantly glycolysis in non-Tregs, and thus it is possible that MyD88-deficient Tregs do not undergo apoptosis because they rely on oxidative phosphorylation more than glycolysis to serve their energetic needs (Figure 3.2).
To more formally address this, one possibility would be to force Tregs to depend more on glycolysis by inhibiting oxidative phosphorylation during activation in vitro with αCD3 and αCD28 in the presence of the known oxidative phosphorylation inhibitor oligomycin. (Huijing and Slater, 1961) If this hypothesis were correct, we would expect that inhibition of oxidative phosphorylation in WT Tregs would have no effect on Treg survival despite increasing the dependence on a glycolytic program while MyD88-deficient Tregs would fail to survive when the dependence on a glycolytic program was induced by the inhibition of oxidative phosphorylation.

Figure 3.2:

Model depicting potential mechanism for the differential requirement for MyD88 in non-Tregs and Tregs. One fundamental difference between non-Tregs and Tregs is their metabolic program, with non-Tregs depending more on glycolysis and Tregs depending more on oxidative phosphorylation (ox phos). We hypothesize that forcing MyD88-deficient Tregs into a glycolytic program either by inhibiting ox phos (oligomycin) or promoting glycolysis (DMOG) will result in MyD88-deficient Treg cell death.
While a more direct approach to this question would be to pharmacologically enhance glycolysis, there are currently no defined drugs that directly enhance glycolysis. However, many transcription factors, such as Hif-1α (as mentioned above), enforce a glycolytic program after activation. Hif-1α can be enforced by inhibiting the Hif-1α inhibitor PHD using dimethyloxalyl glycine (DMOG), and presumably one of the consequences of constant Hif-1α activation is enforcement of a glycolytic program. While there are likely many consequences of treating Tregs with DMOG, preliminary data indicated that a higher frequency of MyD88-deficient Tregs experience cell death as compared to WT Tregs treated with DMOG, suggesting that MyD88-deficient Tregs that are forced into a glycolytic program are unable to survive as well as WT Tregs (Figure 3.3). This preliminary data warrants deeper study of whether MyD88 is required for any cell with an active glycolytic program.

![Figure 3.3:](image)

Preliminary data demonstrating that MyD88-deficient Tregs treated with DMOG during activation conditions are more susceptible to cell death than control treated MyD88-deficient Tregs. *, p<0.05.

**Clinical Implications**

While our initial goal was to potentially target alloreactive T cells by inhibition of MyD88 following transplantation, the data in this thesis demonstrate that T cell expressed MyD88 is
required for long-term allograft survival and thus MyD88 inhibition would be undesirable. Although MyD88 deletion spared Tregs from cell death, MyD88-deficient Tregs appear to have a functional defect during the response to alloantigen. Our data would suggest that MyD88 might specifically be an ideal therapeutic target of alloreactive T cells, but not non-alloreactive T cells and Tregs as the vast majority of non-alloreactive T cells do not proliferate (and thus do not depend on MyD88 for survival) and as we have demonstrated here, MyD88 is dispensable for Treg survival. However, because Treg expressed MyD88 appears to be critical for Treg function during the response to alloantigen, MyD88 inhibition could not be used to promote long-term graft survival in the clinic.

In addition to long-term allograft survival, there are other clinical scenarios whereby specific deletion of effector T cells but not Tregs is desired, and transient inhibition of Treg function could potentially be tolerated. One of these clinical scenarios is autoimmune disease. One could imagine that a short dose of a MyD88 inhibitor could result in robust deletion of autoreactive T cells while sparing Tregs. Although it has not been formally demonstrated, inhibition of MyD88 may also lead to a decrease in Treg function in scenarios other than the response to alloantigen, and thus drug dose would need to be carefully balanced with a potential loss of Treg function. In addition, inhibition of MyD88 would prevent innate immune activation to pathogens via the MyD88 pathway, and thus patients would be at risk for opportunistic infections. However, because non-deleted cells (i.e. any non-proliferating non-Treg T cell or Tregs) would remain present and only the Tregs potentially transiently impaired, once the drug is withdrawn, cells should regain function and the patient should be minimally immunosuppressed. Furthermore, cells of the innate immune system could still be activated in response to a pathogen via the TLR-3, TLR-4, Rig-I-like receptors, Nod-like receptors and any other MyD88-independent pattern recognition pathways, reducing the risk of transient MyD88 inhibition on the innate immune response to pathogens. (Takeuchi and Akira, 2010) This strategy would result in deletion of any proliferating autoreactive T cells and the potential for
transient functional inhibition of Tregs and cells of the innate immune system that would likely be reversed once treatment stopped.

**Concluding Remarks**

Together, our data clearly demonstrate that MyD88 is dispensable for Treg survival despite being required for non-Treg survival. Furthermore, we show that even though we detected no difference in MyD88-deficient Treg function as compared to WT Treg function, MyD88-deficient Tregs clearly do not function as well as WT Tregs during the response to alloantigen. Deeper investigation into what the functional difference between MyD88-deficient and WT Tregs might be is currently ongoing via gene expression analysis. Also, we are focused on defining metabolic differences as one potential mechanism by which MyD88-deficient Tregs survive just as well as WT Tregs while their effector T cell counterparts do not.
References


