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ORIGINAL ARTICLE

Regulatory T cell expressed MyD88 is critical for prolongation of allograft survival

Christopher M. Borges[1,2], Dawn K. Reichenbach[3], Beom Seok Kim[4], Aditya Misra[5,6], Bruce R. Blazar[3] & Laurence A. Turka[1]

1 Center for Transplantation Science, Department of Surgery, Harvard Medical School, Massachusetts General Hospital, Boston, MA, USA
2 Program in Immunology, Harvard University Division of Medical Sciences, Harvard University, Boston, MA, USA
3 Department of Pediatrics, Division of Blood and Marrow Transplantation, University of Minnesota, Minneapolis, MN, USA
4 Department of Internal Medicine, Yonsei University College of Medicine, Seoul, Korea
5 Summer Immunology Research Program, Harvard University, Boston, MA, USA
6 School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY, USA

Correspondence
Laurence A. Turka, Center for Transplantation Sciences, 149 13th Street, Room 5101, Boston, MA 02129, USA.
Tel.: 617-724-7740;
fax: 617-726-6925;
e-mail: lturka@partners.org

SUMMARY

MyD88 signaling directly promotes T-cell survival and is required for optimal T-cell responses to pathogens. To examine the role of T-cell-intrinsic MyD88 signals in transplantation, we studied mice with targeted T-cell-specific MyD88 deletion. Contrary to expectations, we found that these mice were relatively resistant to prolongation of graft survival with anti-CD154 plus rapamycin in a class II-mismatched system. To specifically examine the role of MyD88 in Tregs, we created a Treg-specific MyD88-deficient mouse. Transplant studies in these animals replicated the findings observed with a global T-cell MyD88 knockout. Surprisingly, given the role of MyD88 in conventional T-cell survival, we found no defect in the survival of MyD88-deficient Tregs *in vitro* or in the transplant recipients and also observed intact cell homing and expression of Treg effector molecules. MyD88-deficient Tregs also fail to protect allogeneic bone marrow transplant recipients from chronic graft-versus-host disease, confirming the observations of defective regulation seen in a solid organ transplant system. Together, our data define MyD88 as having a divergent requirement for cell survival in non-Tregs and Tregs, and a yet-to-be defined survival-independent requirement for Treg function during the response to alloantigen.

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Introduction

The myeloid differentiation primary response gene 88 (MyD88) is a key adaptor molecule downstream of all toll-like receptors (TLRs) except TLR-3, as well as the IL-1 receptor (IL-1R) and family members (IL-18R and IL-33R) [1,2]. Although traditionally thought of as a key molecule for innate immune responses, MyD88 has a critical, T-cell-intrinsic role. Previously, we have demonstrated that in T cells, MyD88 activates both the NF-κB and phosphoinositide 3-kinase (PI3K) pathways, ultimately promoting proliferation, IL-2 production, and survival *in vitro* [3,4]. *In vivo*, T cell expressed MyD88 is required for resistance to the protozoan parasite *T. gondii* [5] and for optimal T-cell survival *in vivo*, during the early stages of acute lymphocytic

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choriomeningitis virus (LCMV) infection [6,7]. In the latter case, the requirement for MyD88 is linked to its role in IL-33 signaling [8].

The role of MyD88 signals during transplantation has been studied as well. Germ line MyD88 deficiency in both donor and recipient leads to long-term skin engraftment without immunosuppression in a minor antigen (H–Y) mismatch model [9] and facilitates induction of long-term survival by costimulatory blockade in MHC-mismatched skin transplantation [10]. Conversely, we and others have shown that treatment of mice with TLR ligands can abrogate the skin or cardiac allograft prolonging effects of costimulatory blockade (CoB) [11–13].

In most of the above models, $CD4^+Foxp3^+$ regulatory T cells (Tregs) are critical for the promotion of long-term allograft survival and tolerance [14]. Similar to naïve and effector T cells, Tregs express many TLRs and stimulation of these TLRs on mouse and human Tregs can both enhance or inhibit the suppressive function of Tregs *in vitro* and *in vivo* [15–17]. 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 [18]. In contrast, TLR-2 engagement with Pam3CSK4 did not decrease Treg function *in vitro* and *in vivo*, but rather promoted Treg survival via induction of Bcl-x_L [19]. While these data demonstrate a role for MyD88 signals in the alloimmune response, the use of germ line MyD88 knockout mice precluded determining its cell-specific roles.

To investigate this issue focusing on the role of MyD88 in T cells, we utilized conditional knockout mice whereby MyD88 was deleted specifically in all T cells or confined to Tregs. We found that mice that lacking MyD88 in their T cells or Tregs were relatively resistant to the induction of long-term survival of MHC class II-mismatched skin and cardiac grafts. MyD88-deficient Tregs also failed to protect allogeneic bone marrow transplant recipients from chronic graft-versus-host disease. Contrary to expectations, we found no survival defect in MyD88-deficient Tregs. These data uncover an important survival-independent role for MyD88 during the alloimmune response.

Materials and methods

Mice

Mice with a T-cell-specific deletion of MyD88, termed MyD88-ΔT mice, have been previously described [6]. Mice with a $Foxp3^+$ Treg-specific deletion of MyD88, termed MyD88-ΔTreg animals, were produced by crossing Foxp3-Cre mice [20] to $MyD88^{fl/fl}$ mice (Fig. S1). Foxp3-Cre, $MyD88^{fl/fl}$, MyD88-ΔT, and MyD88-ΔTreg mice were maintained as breeding colonies in our animal facility. C57Bl/6, B6(C)-H2-Ab1bm12/KhEgJ (bm12), B10.BR, and $Foxp3^{tm1Kuch}$ (FoxP3-GFP) mice [21] were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All colonies were maintained in accordance with the protocols approved by the Institutional Animal Care and Use Committees of the Massachusetts General Hospital and the University of Minnesota.

Skin transplantation and treatment

Skin transplantation was performed as described [22]. Animals were monitored daily for rejection (defined as >80% necrosis). Where indicated, mice received 0.25 mg anti-CD154 (clone: MR1; BioXCell, Lebanon, NH, USA) and 1 mg/kg rapamycin (LC Laboratories, Woburn, MA, USA) in a carboxymethyl cellulose and Tween 80 solution as described [11].

Cardiac transplantation and treatment

Heterotopic heart transplantation was performed as previously described [23]. Graft survival was assessed by daily palpation. Where indicated, mice received 0.5×10^6 Treg (defined as $CD4^+GFP^+$ cells, isolated by cell sorting from FoxP3-GFP mice) via retro-orbital injection 7 days prior to heart transplantation.

Induction of chronic GVHD

cGVHD was induced as previously described in B10.BR recipients exposed to 8.3 Gy X-ray irradiation prior to infusion of C57Bl/6 bone marrow and 0.07×10^6 splenic T cells from FoxP3-Cre or MyD88-ΔTreg mice [24]. Tests to measure pulmonary function were performed as previously described [24]. Briefly, mice were anesthetized and weighed, and lung function was assessed by whole body plethysmography using the Flexivent FX system (Scireq). Data were analyzed using the Flexivent software version 7.6.

Antibodies and flow cytometry

Single cell suspensions were prepared in PBS supplemented with 2% BSA and 0.1% sodium azide and stained with the mAbs to the following molecules

purchased from BioLegend (San Diego, CA, USA); CD4 (GK1.5), CD8 (53-6.7), CD25 (PC61), CD44 (IM7), CD62L (MEL-14), CD45 (30-F11) Thy1.2 (53-2.1), TCRβ (H57-597), annexin V, IFN-γ (XMG1.2), IL-17A (TC11-18H10.1), CD28 (37.51), CTLA-4 (UC10-4B9), PD-1 (29F.1A12), GITR (DTA-1), granzyme B (NGZB), CD39 (Duha59), CD73 (TY/11.8) and Lag 3 (C9B7W). mAb to Foxp3 (FJK-16s) was purchased from eBioscience (San Diego, CA, USA). Live/Dead Aqua was purchased from Life Technologies (Grand Island, NY, USA). 7-AAD was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Cell fixation and permeabilization was performed using the Intracellular Fix/Perm Buffer Set (eBioscience). For studies utilizing YFP expressing cells, cell fixation with 2% paraformaldehyde prior to above fix/perm procedure was performed to preserve YFP fluorescence. Flow cytometric analysis was performed on LSRII (BD Biosciences, San Jose, CA, USA) or Navios (Beckman Coulter, Brea, CA, USA) flow cytometers. Data analysis was performed using FlowJo (version 10.0.7 Tree Star, Ashland, OR, USA).

Histology

After harvesting, skin grafts were embedded in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA, USA), and submerged in liquid nitrogen for 20 s; 10-μm sections were fixed to slides in acetone for 5 min at −20°C and stained for 1 hour with αCD4 PE (GK1.5) (BioLegend) and DAPI (Life Technologies) at room temperature. Image analysis and merging were performed using ImageJ (Version 1.48).

Skin digestion

Skin grafts were harvested as described [25] and digested into a single cell suspension for 60–90 min. Where indicated, single cell suspensions were incubated with leukocyte activation cocktail (BD Biosciences) for 2 h at 37°C before staining for flow cytometry.

In vitro culture

Purified (StemCell Technologies, Vancouver, BC, USA) $CD4^+$ T cells from pooled spleens and peripheral lymph nodes were sorted for naïve cells ($CD4^+CD62L^{hi}$ $CD44^-CD25^-$Foxp3-), or Tregs ($CD4^+Foxp3^+$ or $CD4^+CD25^+$ cells on a SORP FACS ARIA II (BD Biosciences, San Jose). 2–5 × 10^5 cells were then resuspended in complete RPMI media and plated in a 48-well plate with 5 μg/ml plate bound anti-CD3 and anti-CD28 (BioLegend), a 96-well flat bottom plate with 5 μg/ml plate bound anti-CD3 and 1 μg/ml anti-CD28, or a 96-well round bottom plate with 3 × 10^5 APCs treated with mitomycin C (Sigma-Aldrich, St. Louis, MO, USA) and 2 μg/ml anti-CD3. Plates were incubated at 37°C for 72 h. When indicated, cells were incubated with leukocyte activation cocktail for 4 h after indicated culture time. *In vitro* suppression assays were performed as described in [26].

Western blotting

$CD4^+YFP^-$ and $CD4^+YFP^+$ cells were sorted using FACS to greater than 95% purity. Western blotting was performed as described in [26]. αMyD88 (1:200 dilution) was purchased from R&D Systems (Minneapolis, MN, USA), and α-β-actin (1:1000 dilution) was purchased from Cell Signaling (Danvers, MA, USA).

Results

T cell expressed MyD88 is critical for bm12 allograft survival

As a first step to determine the role of T cell expressed MyD88 during the alloimmune response, we transplanted bm12 skin onto wild-type (WT) or $MyD88^{fl/fl}$ × CD4-*Cre* (MyD88-ΔT) mice. Significantly delayed rejection in this model can be induced by anti-CD154 plus rapamycin (hereafter termed costimulatory blockade CoB) [11]. We observed that untreated MyD88-ΔT mice rejected bm12 skin allografts with similar kinetics (MST = 11 days) as untreated WT mice (MST = 14 days; Fig. 1a). As expected, CoB prolonged graft survival (>50 days) in the majority of wild-type recipients; however, we noted a nonstatistically significant trend toward more rapid rejection in the MyD88-ΔT recipient animals. In this same strain combination, bm12 → B6, significantly delayed rejection of cardiac allografts is reliably achieved without immunosuppression [27–29], and consistent with prior studies, untreated WT mice accepted bm12 cardiac allografts with a MST greater than 100 days (MST undefined). In contrast, we found that MyD88-ΔT mice rejected their grafts with a MST of 39 days (Fig. 1b, $P = 0.0012$). These data indicate that T-cell-intrinsic MyD88 is not only dispensable for rejection, but may be required for prolongation of graft survival.

We and others have shown that successful long-term engraftment of bm12 skin and heart transplants in B6 mice is Treg dependent [11,29], and thus, we

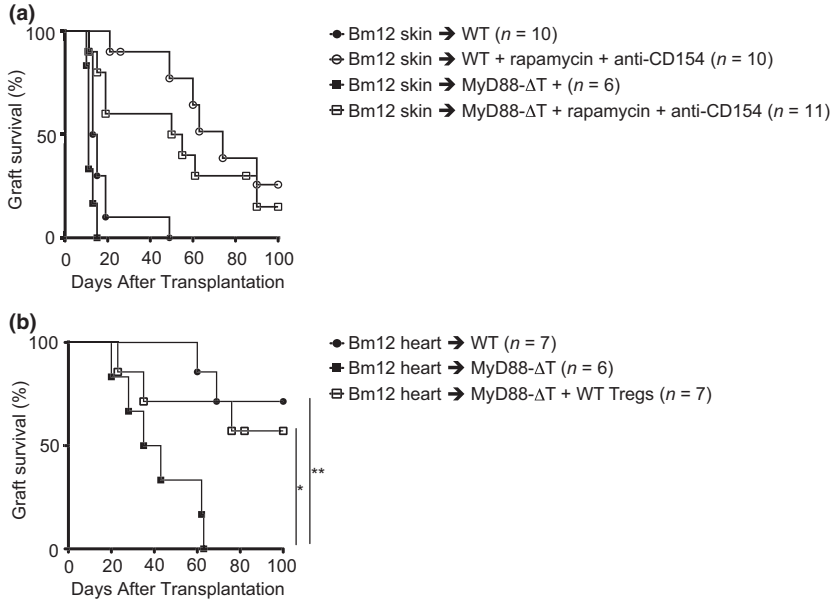

Figure 1 (a) bm12 skin allografts were transplanted onto WT or MyD88-ΔT recipients. Mice received either no treatment (● and ■, respectively), or i.p. injection of 0.25 mg anti-CD154 (Clone: MR1, administered on Days 0, 2, and 4) and i.p. injection of 1 mg/kg rapamycin (administered on Days 0, 2, 4, 6, 8, 10, and 12) (○ and □, respectively). Data are pooled from 2 independent experiments. MST: 13, 11, 90, 53 days (●, ■, ○, □), respectively. (b) bm12 cardiac allografts were transplanted into WT or MyD88-ΔT recipients. Mice received either no treatment (● and ■, respectively), or i.v. injection of 0.5×10^6 sorted WT Tregs (isolated from FoxP3^{GFP} mice 7 days prior to transplant (□). Data are pooled from 2 independent experiments. MST: undefined, 34 days, and undefined (●, ■, □), respectively. $*P < 0.05$. $**P < 0.01$.

hypothesized that a defective regulatory response in MyD88-ΔT mice could account for the findings above. To test this, we asked whether adoptive transfer of WT Tregs into MyD88-ΔT mice prior to bm12 cardiac transplant would rescue bm12 cardiac allograft survival. Indeed, infusion of 0.5×10^6 sorted WT Foxp3$^+$ cells 7 days prior to transplantation was sufficient to reconstitute "normal" graft survival (MST undefined; Fig. 1b, $P = 0.0213$). Taken together, these experiments demonstrate that T cell expressed MyD88 is critical for Treg-dependent prolongation of graft survival.

Treg expressed MyD88 is dispensable for Treg survival

Previously, we have shown a survival defect of MyD88-deficient T cells during the *in vivo* immune response to a pathogen [6,7]. We hypothesized given the large precursor frequency of alloreactive cells [30], that a survival defect might not have an impact on rejection kinetics but could affect the less numerous alloresponsive Treg population. To directly analyze whether MyD88 was critical for Treg survival, we cultured naïve T cells or Tregs (defined as CD4$^+$CD25$^+$) from WT or MyD88-ΔT mice with anti-CD3, anti-CD28 with or without IL-2. Consistent with our previously published findings *in vivo* findings [6,7], we found reduced survival (defined as annexinV$^+$7-AAD$^+$) among stimulated MyD88-deficient naïve T cells compared to WT cells (Fig. 2a and b). Surprisingly, however, we found that no significant difference in survival in the two the Treg populations (Fig. 2a and b).

To more directly address the role of MyD88 in Tregs, we crossed MyD88$^{fl/fl}$ mice with Foxp3$^{YFP-Cre}$ mice to generate animals with a Treg-specific MyD88 deficiency (MyD88-ΔTreg). We first bred female MyD88$^{fl/fl}$ mice to be heterozygous for Foxp3$^{YFP-Cre}$. As the Foxp3 locus is on the X chromosome, due to random X-chromosome inactivation, these mice should have a 50:50 ratio of Cre$^+$YFP$^+$ MyD88-deficient T regs to Cre$^-$YFP$^-$ MyD88-sufficient Tregs, thus allowing us to assess the survival of MyD88-deficient Tregs *in vivo* under competitive conditions. Using serial biweekly bleeds from age 6–36 weeks, we found no significant difference in the percentage of YFP$^+$ cells between control Foxp3$^{YFP-Cre/+}$ mice, Foxp3$^{YFP-Cre/+}$ mice with a single floxed-MyD88 allele, and Foxp3$^{YFP-Cre/+}$ mice with both alleles of MyD88 floxed (Fig. 2c). Moreover, in each of these groups of mice, the percentage of YFP$^+$ cells did not alter over time 7 months of observation. Due to the variation in YFP$^+$ cell frequency from mouse to mouse, the percentage of YFP$^+$ cells among the total Foxp3$^+$

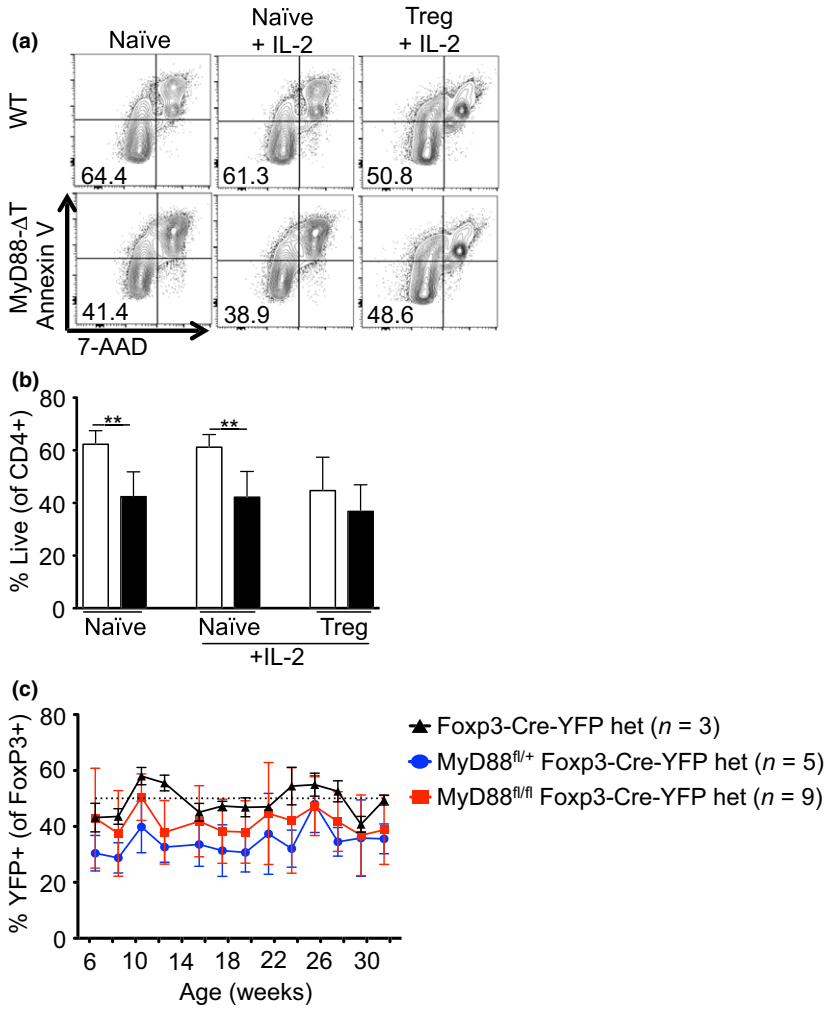

Figure 2 (a) Representative flow cytometry plots of sort-purified $CD4^+CD25^+$ Tregs or $CD4^+CD25^+CD62L^+CD44^-$ naïve, non-Tregs from WT (white bars) or MyD88-ΔT (black bars) mice stained with annexin V and 7-AAD after 72 h of *in vitro* culture with anti-CD3 and anti-CD28, with and without 10 ng/ml IL-2. Numbers in lower left quadrant indicate frequency of cells within that quadrant. (b) Quantification of data from 5 independent experiments of A. (c) Female $MyD88^{+/+}$ $Foxp3^{+/Cre-YFP}$ (Foxp3-Cre-YFP het) $MyD88^{fl/+}$ $FoxP3^{+/Cre-YFP}$ ($MyD88^{fl/+}$ Foxp3-Cre-YFP het) and $MyD88^{fl/fl}$x$Foxp3^{+/Cre-YFP}$ ($MyD88^{fl/fl}$ Foxp3-Cre-YFP het) mice were bled biweekly starting at 6 weeks of age for 26 weeks. Percent YFP positive cells were assessed among total $Foxp3^+CD4^+$ antibody stained cells. Data are pooled from a minimum of 2 independent experiments. Error bars display standard deviation. $^{**}P < 0.01$.

population was not significantly different between the three groups of mice. However, we do observe a trend toward lower percentages of YFP^+ cells within the $MyD88^{fl/+}$ $Foxp3^{YFP-Cre/+}$ het and $MyD88^{fl/fl}$ $Foxp3^{YFP-Cre/+}$ het groups. The fact that there is not a gene dosage effect, that is, levels of YFP+ cells in the single allele knockout mice ($MyD88^{fl/+}$ $Foxp3^{YFP-Cre/+}$ het) are lower than in either the wild-type mice (Foxp3-Cre het) or the double allele knockout mice ($MyD88^{fl/fl}$ $Foxp3^{YFP-Cre/+}$ het) argues against an actual MyD88-related effect. Thus, these data demonstrate equivalent *in vivo* survival of MyD88-deficient and wild-type Tregs under homeostatic conditions.

To further characterize MyD88-deficient Tregs, we next asked whether loss of MyD88 resulted in a difference in Treg expression of several hallmark Treg proteins, that is, CTLA-4, Lag-3, Granzyme B, CD39, and CD73, all of which have been implicated in Treg function [26,31–39]. We observed no difference in the expression of any of the above markers on MyD88-deficient Tregs relative to WT Tregs that were analyzed directly *ex vivo*, or activated *in vitro* with anti-CD3 plus anti-CD28 for 20 or 72 h (Fig. 3a–e). In addition, MyD88-deficient Tregs functioned equivalent to WT Tregs in a standard *in vitro* suppression assay (Fig. 3f).

Treg expressed MyD88 is critical for prolonged bm12 skin graft survival

We next examined graft survival in MyD88-ΔTreg mice. Similar to the observations in mice with MyD88-deleted in all T cells, we observed that MyD88-ΔTreg mice treated with CoB rejected bm12 skin grafts at a higher frequency than WT recipients (MST: 37 days and undefined, respectively; Fig. 4, $P = 0.0008$). To corroborate these observations with our finding above (Fig. 1) that adoptive transfer of WT Tregs could prevent rejection of bm12 cardiac allografts in MyD88-ΔT recipients, we utilized FoxP3$^{YFP-Cre/+}$ mice to assess whether a complement of WT Tregs could promote allograft survival in MyD88-ΔTreg mice. Indeed, we found that rejection of bm12 skin allografts was delayed in CoB-treated MyD88$^{fl/fl}$ FoxP3$^{YFP-Cre/+}$ recipients (MST: 59 days) compared with MyD88-ΔTreg recipients (MST: 37 days; Fig. 3). Together, these data demonstrate that Treg expressed MyD88 is critical for delay of bm12 skin graft rejection using CoB.

MyD88-deficient Tregs efficiently migrate to and survive within bm12 skin grafts

Tregs within the allograft (intragraft Tregs) are critical for graft survival, suggesting that observation of similar Treg frequencies in the periphery may not be indicative of the inability for MyD88-deficient Tregs to promote long-term allograft survival locally [40,41]. Having observed no defect in MyD88-ΔTregs either *in vitro* or in the steady state *in vivo*, it was therefore important to

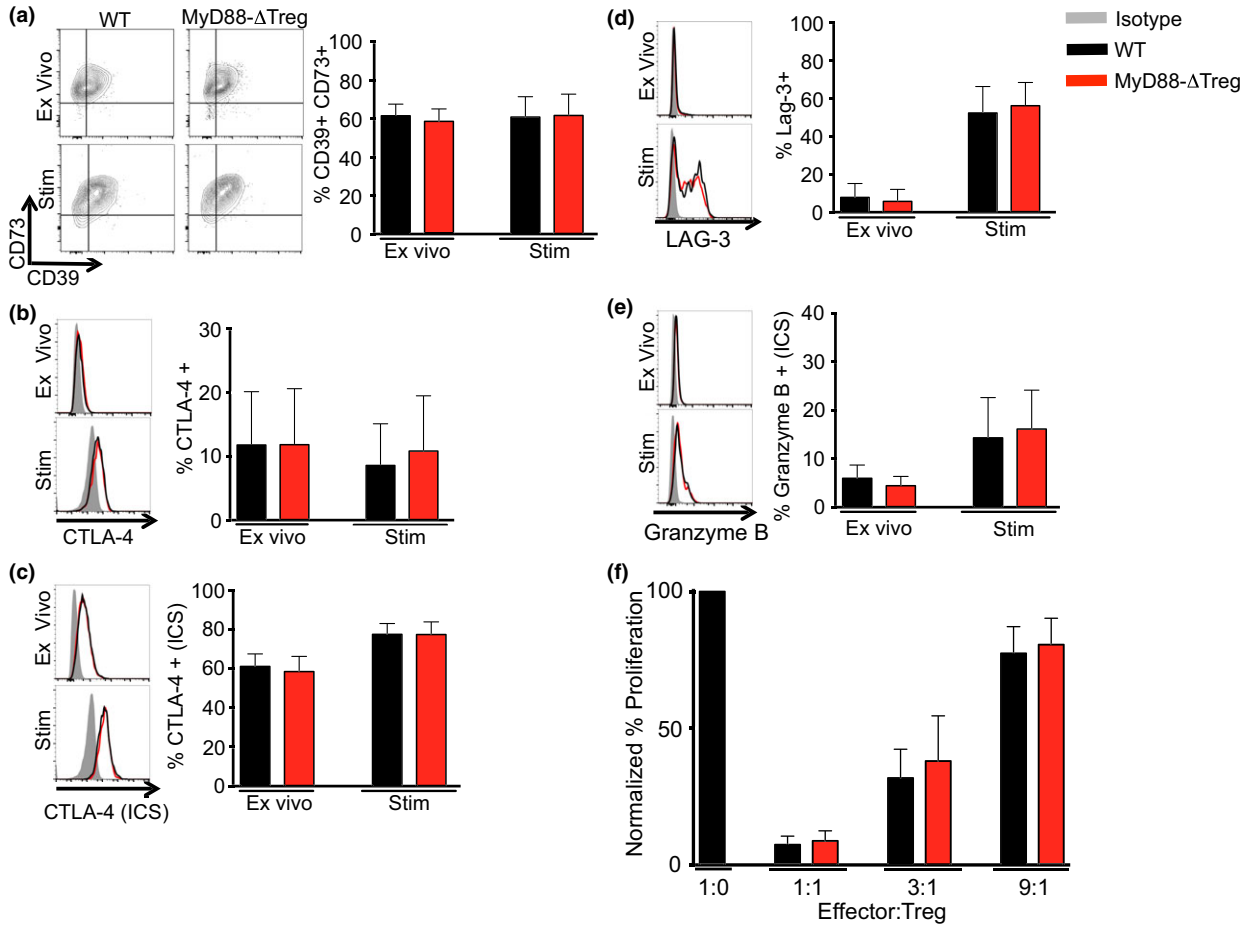

Figure 3 WT (black lines and black bars, minimum $n = 10$ mice) and MyD88-ΔTreg (red lines and red bars, minimum $n = 11$ mice) CD4$^+$Foxp3$^+$ cells were isolated from pooled spleen and peripheral lymph nodes. Cells were analyzed directly *ex vivo*, or stimulated with anti-CD3 and anti-CD28 for 20 h. At each time point, (a) CD39 and CD73, (b) surface CTLA-4, (c) intracellular CTLA-4, (d) Lag-3 and (e) Granzyme B were assessed. Shaded gray area indicates appropriate isotype control for indicated stain. Representative flow cytometry plots shown from a minimum of 3 independent experiments. (f) *In vitro* suppression assay. Quantification of percent proliferation of WT naïve T cells cocultured with irradiated APCs and indicated ratios of WT (black bars) or MyD88-ΔTreg (red bars) (normalized to proliferation of WT naïve cells without Treg). Data are pooled from 5 independent experiments. Error bars display standard deviation.

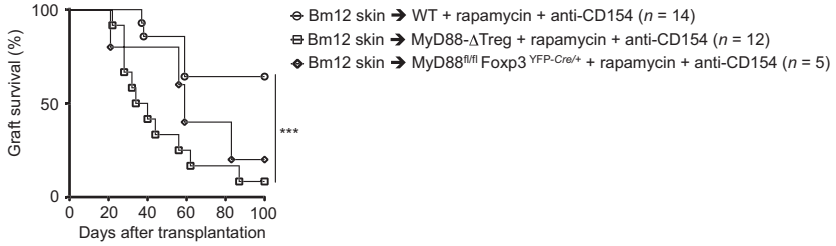

Figure 4 bm12 skin allografts were transplanted onto WT (n = 14 recipients, 7 male Cre-hemizygous mice, 7 female homozygous mice). MyD88$^{fl/fl}$Foxp3$^{+/Cre}$ heterozygous (n = 5 female Cre heterozygous recipients) or MyD88-ΔTreg (n = 12 recipients, 8 male Cre-hemizygous mice, 4 female Cre-homozygous mice) treated with CoB as in Fig. 1. (○, ◊, and □, respectively). Data are pooled from 3 independent experiments. MST: undefined, 37 and 59 days (○, ◊, □), respectively. ***$P < 0.001$.

examine their migrational ability and survival in transplanted animals. 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 [42–44]. There were no differences in the expression of CCR4, CCR6, or CD103 across time points within the spleen or pLN from MyD88-ΔTreg or WT recipients pretransplant, or 7, 14, and 21 days post-transplant (data not shown), suggesting that MyD88-deficient Tregs would be able to migrate to the skin efficiently.

We did not observe any difference in Foxp3$^+$ T cell frequency in blood, spleen, or peripheral lymph nodes from MyD88-ΔTreg or WT recipients pretransplant or 7, 14, and 21 days post-transplant (data not shown), and as well the frequencies of Tregs isolated from bm12 skin grafts 7 and 14 days post-transplant were similar. However, at day 21 post-transplant, we observed a significant decrease in Tregs harvested from MyD88-ΔTreg recipients in comparison with WT recipients (Fig. 5a). Nonetheless, based on a bead-based cell counting assay, we found that grafts harvested from MyD88-ΔTreg and WT recipients contained the same number of Tregs while the non-Treg number significantly increased in MyD88-ΔTreg recipients, indicating that the decreased Treg frequency was the result of an increase in non-Treg absolute number (Fig. 5b and c). Of note, there was no difference in the viability of intragraft Tregs harvested from WT vs. MyD88-ΔTreg recipients (Fig. 5d), nor in their localization with the graft as both types of cells homed to the hypodermal region (Fig. 5e). Together, the above data demonstrate that MyD88-deficient Tregs migrate and persist in bm12 skin grafts at similar numbers and in the same location within the graft as do WT Tregs.

Next, we asked whether a Treg survival defect was revealed following activation *in vitro* in the presence of CoB. To accomplish this, we activated sorted MyD88-deficient Tregs from MyD88-ΔTreg mice with anti-CD3 and anti-CD28 in the presence of 1 ng/ml, 10 ng/ml, and 100 ng/ml of rapamycin with or without 100 μg/ml anti-CD154. As seen in Fig. 5f, 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 in the presence of these drugs (data not shown). Importantly, at each concentration, no significant difference in the frequency of annexinV/7-AAD cells between WT or MyD88-deficient Tregs was observed (Fig. 5f), further confirming our findings that MyD88 was dispensable for Treg survival.

Impaired suppression by MyD88-deficient Tregs in GVHD

We considered that the impaired *in vivo* suppression we observed might be a situation specific to the skin transplant model. To test a distinct *in vivo* model of alloantigen responsiveness, we utilized a model of chronic GVHD (cGVHD) induced by infusion of bone marrow cells and splenic T cells. In this model, where disease pathology is manifest as immunoglobulin deposition in the lung and liver, the splenic T cells are critical for supporting B cells to become antibody secreting cells that are required for cGVHD generation and maintenance [45]. Similar to the results seen with skin transplantation, MyD88-deficient Tregs were unable to mediate optimal protection of B6 recipients of B10.BR bone marrow from cGVHD. We observed that recipients of MyD88-deficient splenocytes, despite having similar elastance to recipients of WT splenocytes, had significantly higher airway resistance and lower compliance, indicating that MyD88-deficient Tregs were not able to protect from cGVHD as efficiently as WT Tregs (Fig. 6a–d). Importantly, recipients of either WT or MyD88-deficient splenocytes had comparable survival and body weights, indicating that these mice did not experience acute GVHD.

Transplant International 2016; 29: 930–940

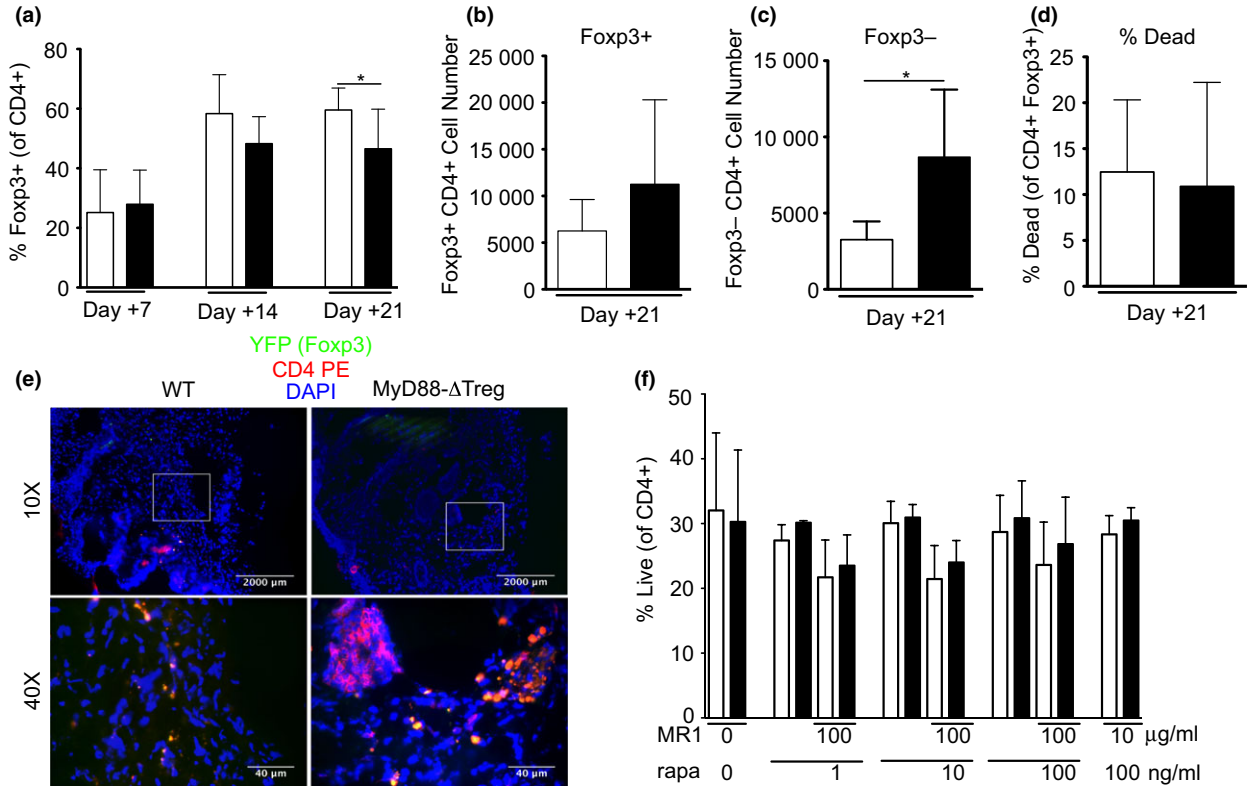

Figure 5 (a) bm12 skin grafts were harvested from WT (white bars, $n \geq 5$) or MyD88-ΔTreg (black bars, $n \geq 3$) recipient mice, and frequency of Foxp3$^+$ CD4+ cells was assessed by flow cytometry at each indicated time point post-transplant. (b,c) Intragraft Foxp3$^+$ (panel b) and Foxp3$^-$ (panel c) 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 with a bead-based flow cytometric counting assay; data are representative of 2 independent experiments. (d) Skin grafts were harvested from WT (white bars, $n = 5$) or MyD88-ΔTreg (black bars, $n = 6$) recipient mice, digested, and stained for Foxp3 and with Live/Dead Aqua. Frequency of Live/Dead Aqua-positive cells is indicated, data are representative of 2 independent experiments. (e) Fluorescent imaging of skin grafts from WT or MyD88-ΔTreg recipient mice. Sections were imaged at 10 × and 40 × magnification. White box on 10 × magnification images indicates area imaged with 40 × objective. Data are representative of grafts harvested from $n = 6$ WT mice and $n = 8$ MyD88-ΔTreg mice. (f) Frequency of annexin V/7-AAD- WT (white bars) or MyD88-ΔTreg (black bars) Treg after activation and culture with anti-CD3, anti-CD28, and IL-2 with indicated concentrations of anti-CD154 and/or rapamycin for 72 h. Quantification of data from a minimum of 3 independent experiments. Error bars display standard deviation. *$P < 0.05$.

Discussion

Our data demonstrate the unexpected finding of a regulatory T-cell-intrinsic role for MyD88 in the ability to modulate the response to alloantigens *in vivo*. Using models of skin and heart transplantation, as well as chronic GVHD, all of which have defined roles for Tregs in restraining the allo-effector response, MyD88-deficient T cells had diminished *in vivo* function. While an obvious mechanistic candidate for this finding is impaired cell survival, given the known defect in survival of naïve MyD88-deficient T cells responding to pathogens, we found no abnormalities in cell viability *in vivo* or *in vitro* between MyD88-deficient and wild-type Tregs. Therefore, the more rapid rejection of bm12 allografts by MyD88-ΔTreg recipients, as well as the inability of MyD88-ΔTreg splenocytes to protect from cGVHD, indicates a cell survival-independent defect.

In naïve T cells, the p85 subunit of PI3K associates with tyrosine 257 in the TIR domain of MyD88, which then phosphorylates 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 leads to NF-κB and Bcl-x_L expression and enhanced the survival of T cells [3,4]. Interestingly, in a standard model of adoptive transfer-induced colitis, MyD88-deficient effector T cells could not induce a wasting phenotype and colonic inflammation in Rag$^{-/-}$ recipients. This suggests that MyD88 may also promote T-cell function in addition to cell survival. Similarly, MyD88-deficient Tregs were unable to protect Rag$^{-/-}$ recipients of effector T cells as

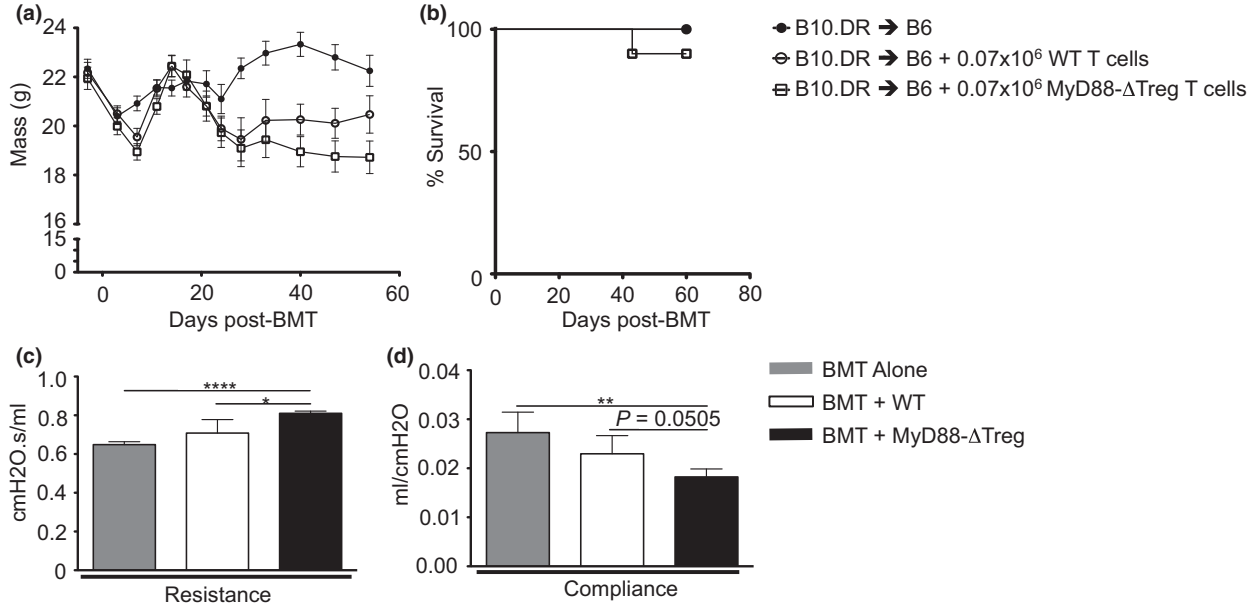

Figure 6 (a) Mouse weight and (b) survival curve following induction of cGVHD in B10.BR mice following B6 bone marrow transplant (●) and B6 bone marrow transplant plus adoptive transfer of either WT (○) or MyD88-ΔTreg (□) splenocytes. (c) Airway resistance and (d) compliance measured on day 28 post-BMT from recipients of B10.BR bone marrow alone (gray bars), B10.BR bone marrow plus WT splenocytes (white bars) or B10.BR bone marrow plus MyD88-ΔTreg splenocytes (black bars). Error bars display standard error of the mean. $*P < 0.05$. $**P < 0.01$. $***P < 0.001$.

well as WT Tregs from colitis like disease, suggesting that MyD88-deficient Tregs do not function equivalently to WT Tregs. [46] Furthermore, Treg-intrinsic MyD88 signaling has been shown to negatively regulate Foxp3 by promoting expression of IRF1, which binds to IRF1 response elements in the Foxp3 promoter [47]. Together, the above data demonstrate that Treg and T cell expressed MyD88 can modulate cell survival and function. Thus, it may appear paradoxical that in our transplant models, using mice in which MyD88 is lacking in the entire T cell compartment, the dominant effect observed is due to loss of MyD88 in Tregs. We believe that the likely explanation for this observation is the extremely large and vigorous nature of the alloreactive response, in which large numbers of cells are recruited very early and expand rapidly with the result that a defect in survival of effector T cells would be "subclinical".

During transplantation, MyD88 may be activated following ligation of TLRs and/or the IL-1R family of receptors. Recently, haptoglobin, a molecule released from necrotic cells, was described to be upregulated in skin grafts following transplantation. Furthermore, 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 [48]. In addition, Pam3Cys, PolyI:C, LPS, and CpG DNA, all microbial products abrogated the allograft prolonging effects of CoB further indicating that innate immune activation via the TLR signaling pathways promotes rejection and results in the inability to induce long-term allograft survival [11–13].

The IL-1R family member IL-33R, one of the upstream receptors of MyD88, prolongs cardiac allograft survival, despite promoting T_H2-associated cytokines [49,50]. Prolongation of graft survival in IL-33-treated recipients is dependent on Tregs and recipient expression of IL-33R and is associated with an increase in intragraft Tregs [51]. More recently, IL-33 was shown to stimulate IL-2 production by dendritic cells which selectively expanded suppressive IL-33R$^+$ Tregs, demonstrating a potential mechanism for its action [52]. Together, these data demonstrate that MyD88 signals can either promote or inhibit allograft survival, the net result likely being context dependent.

Previous work has shown that MyD88 expression in Tregs is important to promote mucosal tolerance [53]. In those studies, loss of Treg MyD88 resulted in a relative deficiency of intestinal Tregs and exacerbated IL-17-dependent inflammation in experimental colitis. Additionally, T follicular regulatory cells were decreased both in number and in function, leading to a dysbalance in IgA and alterations in intestinal flora. The potential mechanism by which loss of MyD88 might perturb Treg function remains under investigation, but may include alterations in cell

metabolism given the recently described critical role of TLR signaling in driving metabolic reprogramming in dendritic cells to enable optimal activation and function [54].

Our data highlight the importance of cell type-specific targeting of inflammatory pathways during the alloimmune response. Targeting of inflammatory pathways has been demonstrated to improve allograft survival, as inhibition of TNF-α has been shown to prolong cardiac allograft survival in rats and intestinal transplantation in patients [55,56]. As targeted therapeutics become more advanced, it may be possible to target MyD88, or other inflammatory pathways, specifically in dendritic cells and/or non-Tregs, resulting in enhanced long-term allograft survival and tolerance.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Western blot confirming MyD88 deletion in FoxP3-YFP$^+$ cells. CD4$^+$ YFP$^+$ and YFP$^-$ cells were sorted from 8 week old MyD88-ΔTreg mice and prepared for western blot as per methods section. Blots shown were exposed for 30 s.

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