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Absence of Innate MyD88 Signaling Promotes Inducible Allograft Acceptance

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Prior experimental strategies to induce transplantation tolerance have focused largely on modifying adaptive immunity. However, less is known concerning the role of innate immune signaling in the induction of transplantation tolerance. Using a highly immunogenic murine skin transplant model that resists transplantation tolerance induction when innate immunity is preserved, we show that absence of MyD88, a key innate Toll like receptor signal adaptor, abrogates this resistance and facilitates inducible allograft acceptance. In our model, absence of MyD88 impairs inflammatory dendritic cell responses that reduce T cell activation. This effect increases T cell susceptibility to suppression mediated by CD4⁺CD25⁺ regulatory T cells. Therefore, this study provides evidence that absence of MyD88 promotes inducible allograft acceptance and implies that inhibiting innate immunity may be a potential, clinically relevant strategy to facilitate transplantation tolerance. The Journal of Immunology, 2006, 177: 5307–5316.

Solid organ transplantation is the treatment of choice for end stage disease of various organs (1, 2). The success of transplantation has largely been due to improvements in both surgical techniques and immunosuppressive medications that impair the adaptive immune system (3). Despite this success, recipients of solid organ transplantation manifest excess mortality and morbidity due to the side effects of lifelong immunosuppression and graft loss from chronic rejection (4, 5). Hence, protocols that induce transplantation tolerance will be a great advance for transplantation as well as for the prevention and treatment of autoimmune disease.

Importantly, the majority of experimental studies examining tolerance induction have focused on adaptive immunity. However, the innate immune system, which provides the initial response to noxious stimuli (3), is essential for triggering the adaptive immune response. Less is known concerning the mechanisms by which the innate immune system is activated in allograft rejection. Elucidation of the role of innate immunity during allotransplantation could provide potential novel targets for promoting transplant tolerance (3).

Innate immunity represents the first line of defense in response to microbial invasion (6). TLRs are critical sentinel receptors of the innate system that sense the presence of conserved microbial motifs (6). Additionally, TLRs sense nonbacterial products such as RNA, DNA, and chromatin and may play a role in autoreactivity (7). TLRs are expressed on dendritic cells (DCs) and other cell types including epithelial and endothelial cells (6, 8), and to date at least 11 TLRs have been discovered. TLRs signal through specific adaptor proteins (e.g., MyD88), which leads to DC maturation and priming of naive T cells (8). Hence, innate TLR-driven immunity initiates adaptive immune function (9, 10).

Although the signals that promote innate immune system activation in allograft rejection are poorly understood, TLRs appear to be involved. For example, TLR-driven MyD88-dependent immunity is critical for the rejection of minor mismatched skin allografts by inducing DC maturation, priming of graft-reactive T cells, and inducing Th1 immunity (11). Additionally, innate immunity via TLR signaling plays an important role in ischemia reperfusion injury (12–14). In a fully allogeneic skin transplant model, MyD88 signaling was not required for rejection in untreated recipients, although Th1 cytokines were reduced and MyD88⁻/⁻ recipients manifest a delayed ability to reject vascularized allografts (15). Nonetheless, TLR signaling through MyD88 may still contribute to the rejection response in fully mismatched recipients and inhibition of such signals could promote induction of tolerance. Alternatively, Th1 cytokines may be required for tolerance (16, 17). Furthermore, TLR activation may have disparate effects on regulatory T cell (T regs) function (9, 10). Thus, in the current study, we add the role of MyD88 signaling in the induction of transplant tolerance. To examine this, we used a fully allogeneic skin allograft model because such allografts, in contrast to cardiac and islet allografts, are more resistant to tolerance induction (18–21).

We found that absence of MyD88 allows long term skin allograft survival under cover of costimulatory blocking agents that are normally ineffective in a highly resistant skin allograft model (20). Importantly, MyD88-deficient recipients exhibit impaired DC inflammatory responses that reduce T cell proliferation and increase T effector susceptibility to the suppressive effects of CD4⁺CD25⁺ T regs.

Materials and Methods

Mice

B6.129/SyJ-MyD88⁻/⁻ mice were generously provided by Dr. S. Akira (Osaka University, Osaka, Japan) and were backcrossed 10 times onto both the C57BL/6 (H2b) and BALB/c (H2d) backgrounds (designated as...
B6.MyD88^−/− and BALB/c:MyD88^−/− mice, respectively). Caspase 1-deficient mice (B6.129/SvJ-Caps-1tm1Flv/J, referred to as B6.IEC^−/−, mice) were provided by Dr. R. Flavell (Yale University, New Haven, CT). B6.129S7-RAG1^tm1Mom (designated as B6.RAG^−/−), B6, and BALB/c (H2^d) mice were purchased from The Jackson Laboratory. CBA mice (H2^K) were purchased from the National Cancer Institute. All mice were kept in pathogen-free conditions and prophylactically given sulfatrim antibiotics intermittently in the drinking water. The Yale University Institutional Animal Care and Use Committee approved use of animals in this study. 

Skin transplantation and Ab treatment

Full-thickness trunk skin was transplanted from 6- to 8-wk donor mice (BALB/c-H2^d) and were sutured on B6. recipients (H2^d) as previously described (15). Rejection was defined as graft necrosis of the entire graft area. Invariably, recipients that accepted their allografts for >150 days demonstrated preserved graft size, hair growth, and had no cellular infiltration or evidence of inflammation demonstrated by histology (data not shown). The experimental tolerance protocol consisted of anti-CD40L (anti-CD154, clone MR1) 500 μg/mouse on days +2, +4, +6, and +8 relative to transplantation and CTLA-4 Ig 500 μg/mouse on day +2 relative to transplantation both via i.p. injection (reagents generously provided by C. Larsen, Emory University, Atlanta, GA). Anti-CD25 (clone PC61) was generously provided by D. Rothstein (Yale University), and 150 μg/mouse was given on day −2 i.p. before transplantation. We determined that this treatment completely depleted the CD4^+ CD25^+ subpopulation by 96% and also specifically depleted Foxp3^+ cells (data not shown). Isotype control Ig 150 μg/mouse (IgG1) was obtained from Bioexpress. rIL-6, monoclonal IL-6 inhibiting Ab (2 μg/ml) and isotype control were purchased from R & D systems.

Adaptive transfer

B6.RAG^−/− mice that were transplanted with MyD88^+/+ BALB/c skin grafts 2 wk previously were adoptively transferred, via i.v. tail vein injection with 2 × 10^5 CD4^+ CD25^− Tregs harvested from B6. MyD88^−/− recipients that had accepted their BALB/c:MyD88^+/+ allografts for >150 days and cotransfused with 1 × 10^6 MyD88^+/+ B6. CD4^+ CD25^− T effectors from naive mice. Similar experiments were performed with CD4^+ CD25^− Tregs purified from MyD88-sufficient groups at >150 days after transplantation. This group invariably had rejected their allografts at this time point.

ELISPOT

ELISPOT was performed as per previously published work (22). To assess the T cell recall response after transplantation, recipient spleen cells (0.1–1 × 10^6) were harvested at day +14 posttransplantation (or other indicated time points), purified for CD4^+ and CD8^+ T cells as described below, and cultured with 0.3–3 × 10^5 irradiated (28 Gy) donor stimulator spleen cells per well. Third party donor spleen cells and naive responder cell controls were included. For B6.MyD88^−/− recipients, BALB/c:MyD88^+/+ stimulators were used, although similar results were obtained when MyD88^−/− stimulators were used for mutant responders and vice versa. For the DC ELISPOT assay, CD11c^+ splenic cells were purified as described below at 1 wk after transplantation. This time point was identified as the optimal time to assess cytokine production in preliminary experiments using MyD88^−/−-transplanted mice (data not shown). The DCs were plated (3 × 10^3/well) overnight in 96-well ELISPOT plates coated with the Ab of interest. No ex vivo stimulation was used for the DC experiments, and results were corrected for baseline levels of cytokine production by use of DCs from untransplanted control mice from the representative groups. All cells were cultured in RPMI, 10% FCS plus penicillin streptomycin during the ELISPOT procedure. Plates were read on a CTI automatic ELISPOT reader and analyzed using Immunospot 3.1 software (CTL). Results were expressed as spots per 1 × 10^6 cells or otherwise indicated.

Cell sorting and flow cytometric staining

After staining for cell surface markers, lymphoid cells were fixed and permeabilized overnight and then stained with Foxp3-specific PE-conjugated rat anti-mouse mAb (clone FJK-16S, IgG2a) or isotype control Ab at 4°C according to the manufacturer’s instructions (eBiosciences). To purify CD4^+ T effectors, spleen cells were harvested from mutant and MyD88^−/− mice, respectively, and purified via magnetic negative selection using the EasySep magnetic sorting system (StemCell Technologies). A similar protocol was used for purifying CD8^+ T cells. The CD4^+ cells were then purified for effectors or T regs (>95% purity) by staining with FITC-conjugated rat anti-mouse CD25, PE-conjugated rat anti-mouse CD45RB, and Cyochrome-conjugated rat anti-mouse CD4 Abs (eBiosciences) and sorting the CD4^+ CD25^− CD45RB^hi or CD4^+ CD25^+ CD45RB^lo cells via FACS (FACS Advantage; BD Biosciences). DCs (CD11c^+; purity >80% purity, assessed by flow cytometry) were purified via a positive selection magnetic protocol (EasySep; Stemcell), CD11c, markers of costimulatory molecules CD86 and CD80 and the chemokine receptor CCR7, were stained with relevant fluorescent mAbs or isotype controls (eBiosciences). Staining was assessed via flow cytometry. All flow cytometric data were acquired on a FACS-Calibur flow cytometer, and data were analyzed using FlowJo software (Treestar).

MLR, generation and activation of bone marrow-derived DCs (BMDCs), and proliferative assays

Allogeneic BMDCs were harvested as per our previously published work where bone marrow cells are harvested; depleted of T, B, and NK cells; and cultured in the presence of GM-CSF (15). To perform the MLR to examine the effect of MyD88 signaling within T cells, irradiated allogeneic (BALB/c) BMDCs (1 × 10^5 or 1 × 10^5) were added to 1 × 10^5 B6.MyD88^+/+ or MyD88^−/− CD4^+ CD25^+ T effectors or CD8^+ T cells for 2 days in 96-well plates in complete Bruf’s medium (Invitrogen Life Technologies). At this point, the cells were resuspended in RPMI 1640, 10% FCS with 1% penicillin and streptomycin, transferred to ELISPOT plates specific for IL-2, and cultured for another 20 h; the plates were then analyzed as described above. To assess the effect of MyD88-dependent cytokines on T cell proliferation in an MLR and on T reg-mediated allosuppression, B6.MyD88^+/+ and MyD88^−/− BMDCs were stimulated with LPS (50 ng/ml) in complete Bruf’s medium. After 12 h the supernatants were harvested (denoted as stimulated medium) and used to culture syngeneic MyD88^+/+ T effectors (1 × 10^5/well) that were allostimulated with irradiated BALB/c spleen cells (1:1 ratio). In certain experiments, 1 × 10^5 MyD88^−/− B6 Tregs (CD4^+ CD25^−) were then added to the wells. Proliferation was measured after 72 h of culture. During the last 12 h of culture, [3H]thymidine was added to the wells, and DNA was harvested and analyzed by a scintillation counter as an indicator of cell proliferation (PerkinElmer Life Science). Control wells included: stimulators or T effectors in rest medium (i.e., medium that was harvested from DCs that were not TLR activated where the same dose of LPS used to stimulate DCs was added after the culture supernatants were collected and before the MLR); T effectors cultured in stimulated medium without allogeneic spleen cells; and media only wells.

To perform the T reg-suppressive assays in response to CD3 activation, increasing numbers of CD4^+ CD25^− Tregs (1 × 10^5, 1 × 10^4, 1 × 10^3) were added to 96-well plates containing 5 × 10^5 CD4^+ CD25^− T effectors that were stimulated with anti-CD3, 0.3–3 μg/ml (Sigma-Aldrich) along with 5 × 10^5 irradiated, syngeneic, splenic feeder cells in complete medium for 3 days, [3H]thymidine was added to the wells to monitor cell proliferation as described above. Controls included Tregs stimulated with anti-CD3, anti-CD3-stimulated effectors only and T effectors and Tregs alone without stimulation, respectively.

Statistical analysis

Survival analysis between groups was calculated using the log rank method. Comparison of means was performed using a two-tailed t test. Repeated measures were assessed using a two-way ANOVA. All results were generated using GraphPad Prism software. Statistical significance was considered as p < 0.05.

Results

Absence of MyD88 synergizes with costimulatory receptor blockade and facilitates allograft acceptance

To test the hypothesis that an absence of MyD88 facilitates transplantation tolerance, we used a fully allogeneic murine skin allograft model that resists the tolerizing effects of costimulatory receptor blockade (specifically anti-CD154 and CTLA4-Ig, both of which inhibit signal 2 from APCs to T cells) (23) in wild-type recipients (24). Hence, we administered costimulatory receptor blockade to transplant groups that were either sufficient (wild type) or deficient in MyD88. In the absence of MyD88 (i.e., BALB/c MyD88^+/+ skin [H2^b]+→B6.MyD88^−/− recipients [H2^b^]) the vast majority of transplant recipients (10 of 13) accepted their skin allografts indefinitely (>200 days), and graft rejection was significantly delayed when recipients are only MyD88 deficient (i.e., BALB/c MyD88^+/+→B6.MyD88^−/−) (Fig. 1A). In contrast,
MyD88−/− recipients that received MyD88+/+ skin allografts and the tolerance protocol rejected their allografts with a median survival time (MST) of 27 days (p < 0.0001 vs MyD88-deficient group).

Consistent with a prior report, the absence of MyD88 did not delay graft rejection when the tolerance protocol was not administered (MST 12 days in MyD88-sufficient and -deficient groups, respectively) (15). Although untreated MyD88-deficient recipients mount an effective immune response similar to that exhibited by MyD88+/+ recipients (15), MyD88-deficient recipients are much more susceptible to tolerogenic effects of costimulatory blockade. Although MyD88 is downstream of TLRs, it is also downstream of IL-1 and IL-18 (25). Hence, to determine whether graft acceptance in the MyD88+/+ recipients was dependent on defective signaling via IL-1 or IL-18, we used caspase-1-deficient recipients that cannot signal via IL-1 and IL-18 (11, 26). The results show that these recipients remained resistant to the effects of costimulatory blockade (MST 36 days, p < 0.01 vs MyD88−/− recipient group) (Fig. 1). This suggests that graft acceptance in the MyD88−/− recipients was independent of the IL-1 and IL-18 signaling.

MyD88 signaling is important for DC inflammatory responses and migration after transplantation and treatment with costimulatory receptor blockade

Activation of DCs with TLR agonists causes them to produce proinflammatory cytokines, which are important for initiating adaptive immunity (26). This occurs principally via priming Th1 immune responses and inhibiting the effect of T reg cells (9, 26–28). Our prior studies have shown that MyD88 signaling within DCs is important for Th1 alloimmunity (11, 15); however, the impact of MyD88 signaling on DC production of proinflammatory cytokines during allotransplantation has not been previously documented (11, 15). We next examined whether splenic DCs produced proinflammatory cytokines after transplantation and whether these responses were altered in the absence of MyD88 and/or treatment with costimulatory blockade. Toward this end, we purified DCs from animals 7 days after transplantation with or without treatment with costimulatory blockade and tested their production of IL-6 and TNF-α via ELISPOT. DCs from MyD88+/+ transplanted recipients in the absence of costimulatory blockade demonstrated a substantial IL-6 and TNF-α production. Either administration of costimulatory blockade or absence of MyD88 alone significantly impaired DC proinflammatory cytokine production as compared with untreated MyD88+/+ transplant recipients (Fig. 2A). These responses were further impaired in MyD88−/− recipients treated with costimulatory blockade (Fig. 2A). This indicates that costimulatory blockade and an absence of MyD88 act synergistically to impair DC proinflammatory cytokine production after transplantation.

In our prior work, we found that in MyD88+/+ transplanted recipients that did not receive costimulatory blockade, mature DCs (defined as either CD11c+CD80(high) or -86(high)) accumulate in the draining lymph nodes of allografts, whereas this effect was compromised in MyD88−/− recipients (15). These effects persisted in the presence of costimulatory blockade and were associated with an impaired ability of MyD88−/− DCs to up-regulate the chemokine homing receptor CCR7 (Fig. 2, B and C).

Absence of MyD88 leads to reduced CD4+ and CD8+ T cell priming after transplantation and treatment with costimulatory receptor blockade

Because MyD88 signaling was important for DC inflammatory responses during transplantation and because DC inflammatory responses are important for T cell priming and generation of T effectors (6, 29), we next examined whether MyD88 signaling altered CD4+ and CD8+ T cell priming after transplantation. Hence, we measured the number of both CD4+ and CD8+ IL-2- and IFN-γ-producing T cells either in the absence of immune modulation or after administration of costimulatory blockade. Either administration of costimulatory blockade or MyD88 deficiency alone led to impaired allospecific CD4+ and CD8+ T cell responses (Fig. 3, A and B), with the possible exception of the IL-2 response of wild-type recipients treated with costimulatory blockade (Fig. 3B). The combination of administration of costimulatory blockade and MyD88 deficiency manifest the most defective response indicating synergy between these two factors (Fig. 3, A and B). Finally, we found that defective cytokine responses within unsorted spleen cells persisted in MyD88-deficient recipients that had accepted their allografts for >150 days post transplantation (denoted as long term acceptors) as measured via ELISPOT (Fig. 3C). However, spleen cells from these recipients were not completely anergic and were able to produce a response to nonspecific stimulation with Con A (Fig. 3C).

No evidence of impaired in vitro intrinsic T cell defects in the absence of MyD88

The results in the previous paragraph could be explained either by defective MyD88−/− DC responses leading to reduced T cell priming or an intrinsic defect in MyD88−/− T cells. To examine whether absence of MyD88 impaired intrinsic T cell function, MyD88+/+ or MyD88−/− CD4+ or CD8+ T cells were stimulated with allogeneic spleen cells or activated nonspecifically with CD3 stimulation. The results indicate that MyD88−/− T cells functioned equally as well as MyD88+/+ counterparts in these assays (Fig. 4, A and B). Hence, the data indicate that absence of MyD88 does not intrinsically impair T cell function.
Synergy between an absence of MyD88 and treatment with costimulatory blockade is donor specific

To determine whether the synergy between treatment with costimulatory receptor blockade and an absence of MyD88 leads to donor-specific graft acceptance, long term B6, MyD88$^{-/-}$ recipients that had accepted their BALB/c MyD88$^{-/-}$ allografts for >150 days (denoted as acceptors) were rechallenged with either donor-specific (MyD88$^{-/-}$) BALB/c skin allografts or MyD88$^{+/+}$, third party skin allografts (CBA, H2k). The results demonstrate that donor-specific rechallenge allografts exhibited markedly prolonged survival (MST >100 days), whereas the third party allografts were rapidly rejected (MST 14 days, $p = 0.003$; Fig. 5). Similar results were obtained when rechallenge grafts were from MyD88-sufficient donors (i.e., BALB/c MyD88$^{+/+}$ rechallenge grafts, MST >30 days, $p = 0.004$ vs CBA, $p = 0.1$ vs MyD88$^{-/-}$ donor rechallenge grafts; Fig. 5). These data...
MyD88

each cytokine appear above each histogram bar. (MyD88 donor third party (CBA) spleen cells (data not shown).

cells and responders were nonresponsive when stimulated with irradiated (15), naive responders did not elicit a response to donor-specific spleen cells and vice versa (data not shown). In agreement with our prior work (15), naive responders did not elicit a response to donor-specific spleen cells and responders were nonresponsive when stimulated with irradiated donor third party (CBA) spleen cells (data not shown). A. Treatment with costimulatory blockade or absence of MyD88 reduces the number of IFN-γ CD4+ and CD8+ producing T cells. This effect is reduced further in MyD88−/− recipients treated with costimulatory blockade. B. Similar results were found with IL-2. C. Unsorted spleen cells were harvested from long term (>150 days posttransplant) B6 MyD88−/− acceptors and analyzed for responsiveness to irradiated donor stimulator spleen cells via ELISPOT. The data demonstrate that long term acceptors were hyporesponsive for all cytokines tested (IL-2, IL-6, TNF-α, and IFN-γ; <10 spots/1 × 106 spleen cells) yet were able to respond to nonspecific T cell activation with Con A (>1500 spots/1 × 106 spleen cells). Spot counts for each cytokine appear above each histogram bar. (MyD88+/− recipients of MyD88−/− allografts that were treated with costimulatory receptor blockade demonstrated a detectable response at 150 days after transplantation; IFN-γ, 836 ± 33 spots/1 × 106 cells; IL-2, 546 ± 25 spots/1 × 106 cells.)

demonstrate that the acceptance of allografts mediated by costimulatory receptor blockade in the absence of MyD88 is donor specific.

Synergy between an absence of MyD88 and treatment with costimulatory blockade that leads to graft acceptance occurs via a CD25-dependent regulatory mechanism

Because long term MyD88−/− acceptors demonstrated delayed rejection of donor-specific challenge grafts at a point when the effects of costimulatory blockade have likely dissipated, this suggests that immune regulation is one of the mechanisms of graft acceptance in these recipients. Given that CD4+CD25+ T regs are important for tolerance induction (30), we asked whether long term allograft acceptance induced by costimulatory receptor blockade in the absence of MyD88 involves a regulatory mechanism (30). B6 MyD88−/− mice were treated with either an inhibiting anti-CD25 Ab, which has been recently shown to functionally inactivate CD4+CD25+ T regs (31), or with an isotype control before transplantation with BALB/c MyD88−/− skin and administration of costimulatory receptor blockade. The results demonstrate that anti-CD25 treatment abrogates graft acceptance in MyD88−/−-transplanted recipients and recovers allogeneurncy (Fig. 6, A and B), indicating that CD4+CD25+ T regs are important for graft acceptance in these recipients. Experiments in MyD88+/− recipients that received costimulatory blockade demonstrated that rejection kinetics was not altered by CD25 inhibition (anti-CD25-treated MyD88−/− group MST, 22 days, n = 4, p = 0.2 vs MyD88−/− isotype-treated group). Additionally, we were able to transfer graft acceptance by isolating purified CD4+CD25+ T regs from long term B6 MyD88−/− acceptors and adoptively cotransferring the T regs with MyD88+/−/CD4−CD25− T effectors into B6.RAG−/− recipients that were previously transplanted with a MyD88+/− BALB/c skin allograft (Fig. 6C).

Absence of MyD88 does not intrinsically augment T reg function

To examine whether MyD88 deficiency itself leads to augmented suppressive CD4+CD25+ T reg function, we measured the suppressive ability of either MyD88+/− or MyD88−/− T regs both in vitro and in vivo. Our results show that MyD88−/− T regs do not possess superior ability to suppress in vitro T cell activation (Fig. 7A) or in vivo alloimmunity (Fig. 7B) as compared with MyD88+/− counterparts. Furthermore, we did not find evidence that the absence of MyD88 leads to increased numbers of splenic Foxp3+ cells in naive mice (FoxP3 is a transcription factor found in naturally occurring CD4+CD25+ T regs (30)) (Fig. 7, C and D). Nor did we find evidence of increased numbers of CD4+CD25+ T cells within MyD88−/− host spleen cells either pre- or posttransplantation as compared with MyD88+/− counterparts (data not shown). These findings indicate that MyD88 deficiency does not intrinsically enhance CD4+CD25+ T reg-suppressive function or the generation of T regs.

MyD88-dependent proinflammatory cytokines from TLR-activated DCs augment T cell proliferation during allogeneic stimulation

Prior reports indicate that proinflammatory cytokines are important for recovery of T cell function in immunosenescent cells and promoting autoimmunity (32, 33). We next examined whether proinflammatory cytokines from TLR activated (via LPS) DCs augmented the ability of MyD88+/− T effectors to respond to allogeneic stimulation. To examine the role of MyD88 signaling within TLR-activated DCs, we harvested culture media from either B6 MyD88+/− or MyD88−/− BMDCs.
after LPS stimulation. In agreement with prior studies (34), proinflammatory cytokines IL-6 and TNF-α, present in MyD88+/+ supernatants, were abrogated in supernatants from MyD88−/− DCs (data not shown). These media were then used to culture syngeneic T effector cells that were stimulated with allogeneic BALB/c spleen cells. The results demonstrated that culture media harvested from LPS-stimulated MyD88+/+ DCs augmented proliferation as compared with T effectors allostimulated in the presence of media harvested from LPS-stimulated MyD88−/− BMDCs (Fig. 8A). Media from this latter group did not augment T cell proliferation above control levels generated from media harvested from representative non-TLR-activated DCs (Fig. 8A). We also found that media harvested from LPS-stimulated MyD88+/+ DCs augmented T cell proliferation in response to allostimulation in a dose-dependent manner and were significantly superior to media harvested from LPS-stimulated MyD88−/− BMDCs (Fig. 8B).

Given that prior studies have identified IL-6 as an important cytokine for T cell activation and the induction of autoimmunity (33, 35, 36), we wished to determine whether IL-6 was an active component of the media harvested from TLR-activated MyD88+/+ BMDCs, which augmented T cell proliferation during allostimulation. Hence, we performed experiments in which we supplemented media from MyD88+/+ TLR-activated DCs with rIL-6. The dose of IL-6 used was determined by the difference in IL-6 concentration (38 ng/ml) measured by ELISA in the culture media produced by TLR-activated MyD88+/+ and MyD88−/− DCs, respectively. The results show that the addition of IL-6 to the MyD88−/− media significantly augmented the ability of T effectors to proliferate in response to allostimulation (Fig. 8C).

Next, we incubated T effectors in media harvested from TLR-activated MyD88+/+ BMDCs and added an IL-6-inhibitory Ab during allostimulation. The results of this experiment demonstrate that IL-6 inhibition reduced T effector proliferation (Fig. 8D). Hence, the above data indicate factors produced by TLR-activated DCs augment T cell proliferation in vitro; this augmentation is MyD88-dependent and appears to involve IL-6 as one of the active components.

**Media harvested from TLR-activated MyD88+/+ DCs inhibit T reg-mediated suppression of alloimmunity**

A prior study has found that MyD88-dependent cytokines produced from DCs modify T effector cell susceptibility to suppression mediated by T regs during nonspecific T cell activation (9). Given that our data provide evidence that MyD88 signaling is important for DC production of inflammatory cytokines after transplantation and that these MyD88-dependent cytokines augment T cell proliferation during allostimulation, we next examined whether these cytokines altered the ability of T regs to suppress T effector proliferation in response to allogenic stimulation with BALB/c spleen cells. Hence, MyD88+/+ B6.CD4−/−CD25−/− T effector cells were added to cultures of B6.CD4+CD25+ Tregs, which were then allostimulated with BALB/c spleen cells in either culture supernatants derived from TLR-activated MyD88-sufficient or -deficient DCs. The results demonstrate that in the presence of media from MyD88+/+ TLR-activated DCs, wild-type Tregs manifest an inferior ability to inhibit proliferation of syngeneic MyD88+/+ T effector cells in response to allostimulation. In contrast, T regs manifest a superior ability to suppress T effector function when cultured in the presence of media harvested from...
with donor-specific MyD88
(H18554)
demonstrate a marked prolonged survival in MyD88
and 3). We also show that donor-specific rechallenge allografts
model (37). We found that lack of MyD88 signaling in our model
transplant acceptance in a highly immunogenic skin allograft
gizes with costimulatory receptor blockade and induces long term
In our study, we provide evidence that absence of MyD88 syner-
MyD88
long term acceptors of mutant BALB/c allografts were rechallenged with a
MyD88
(CBA, BALB/c. MyD88
also manifested extended allograft survival vs third-party grafts, (i.e.,
BALB/c, MyD88+/+ rechallenge grafts, MST >30 days, p = 0.004 vs
CBA, p = 0.1 vs MyD88−/− donor rechallenge grafts).

MyD88−/− DCs (Fig. 8E). These data demonstrate that MyD88-
dependent cytokines prevent T reg-mediated suppression of allo-
geneic stimulation in vitro.

Discussion
In our study, we provide evidence that absence of MyD88 syner-
gizes with costimulatory receptor blockade and induces long term
transplant acceptance in a highly immunogenic skin allograft
model (37). We found that lack of MyD88 signaling in our model
impairs DC-inflammmatory responses and T cell priming (Figs. 2
and 3). We also show that donor-specific rechallenge allografts
simulate a marked prolonged survival in MyD88−/− recipients
that had accepted their primary allograft for >150 days as com-
pared with third-party allografts (Fig. 5). Because immuno-
regulation is a possible explanation for this result, we examined the
role of T regs in our model. Our results indicate that graft acceptance
induced by costimulatory receptor blockade in the absence of
MyD88 was dependent on CD4+CD25+ T regs since graft
acceptance was transferable after adoptive transfer of MyD88-
deficient CD4+CD25+ Tregs (Fig. 6C). Furthermore, func-
tional CD25 inhibition (31) abrogated graft acceptance (Fig.
6A) and recovered alloimmunity (Fig. 6B) in the MyD88-defi-
cient recipients. This result implies that in our model, graft
acceptance mediated by costimulatory receptor blockade re-
quires the presence of T regs.

We found that an absence of MyD88 enhanced neither the gen-
eration nor the intrinsic function of T regs (Fig. 7). We provide a
possible explanation to this issue by demonstrating that absence of
MyD88 impairs DC inflammatory responses after transplantation
(Fig. 2). We present evidence that the functional consequences of
this impaired inflammatory response lead to reduced in vitro T
effector proliferation during allostimulation (Fig. 8, A and B). We
also show that MyD88-dependent cytokines produced by TLR-
activated DCs impair T reg mediated suppression of allostimulated
T effectors (Fig. 8E). This effect occurred without altering the
nature or number of T regs in this assay. We noted that a minority of
MyD88−/− recipients that had accepted their allografts for >150
days subsequently rejected rechallenge donor-specific grafts. A

possible explanation for this is that the effects of costimulatory
blockade may have dissipated at this point and therefore the isch-
emia reperfusion injury upon rechallenge may have perturbed the
cytokine environment and tipped the balance from tolerance to
immunity. Overall, our data suggest that absence of MyD88 alters
the inflammatory cytokine environment, allowing T effectors to
proliferate less and be more easily suppressed by T regs. This
occurs without changing the intrinsic function or generation of
T regs.

Our study is compatible with a previous in vitro report that
demonstrated that IL-6 produced by TLR-activated DCs allowed
effector T cells to become resistant to the suppressive effects of T
regs, although the authors of this study found that this phenomenon
could not be entirely explained by IL-6 alone (9). We show that in
an alloimmune setting IL-6 is an important factor in augmenting T
cell proliferation, although our data do not exclude that other in-
flammatory cytokines may also be involved in this effect. Future in
vivo studies are required to examine the role of specific proinflam-
matory cytokines in transplantation tolerance induction, although it
is possible that there may be a high degree of redundancy between
cytokines. This is supported by studies conducted several years

FIGURE 5. Graft acceptance after treatment with costimulatory block-
ade in the absence of MyD88 is donor specific. B6.MyD88−/− long term
acceptors of mutant BALB/c allografts were rechallenged with a
MyD88−/− donor-specific (i.e., BALB/c skin allograft or a third party,
MyD88+/+ (CBA) allograft. Results demonstrate that third-party allografts
(C, MST 14 days) were rejected in an accelerated fashion as compared
with donor-specific MyD88−/− rechallenge grafts (○, MST >100 days,
p = 0.003). Mice rechallenged with MyD88−/+ BALB/c skin grafts (▼)
also manifested extended allograft survival vs third-party grafts, (i.e.,
BALB/c, MyD88+/+ rechallenge grafts, MST >30 days, p = 0.004 vs
CBA, p = 0.1 vs MyD88−/− donor rechallenge grafts).

FIGURE 6. Synergy between costimulatory receptor blockade and ab-
sestance of MyD88 leading to allograft acceptance occurs via a CD25-regu-
latory mechanism. A. Anti-CD25 treatment (□) before transplantation ab-
rogates BALB/c, MyD88−/− graft acceptance in B6.MyD88−/− recipients
with costimulatory receptor blockade (p < 0.01 vs isotype control-
treated group ○). B. Anti-CD25 treatment before skin transplantation and
with costimulatory receptor blockade in B6.MyD88−/− recipients
(□) led to recovery of T cell alloimmune responses as compared with
B6.MyD88−/− recipients treated with isotype control (○) at 2
wk after transplantation as measured by ELISPOT. Data are representative
do the two independent experiments. C. CD4+CD25+ T cells purified from
B6.MyD88−/− (who were long term acceptors (i.e., 150 days after trans-
plantation) of BALB/c, MyD88−/− allografts induced graft acceptance
when adoptively cotransferred with CD4+CD25+ MyD88−/+ T effec-
tors (Teff) into B6.RAG−/− recipients that were transplanted previously with a
MyD88−/+ BALB/c allograft. CD4−CD25− T effector (2 × 105) were co-
transferring with 1 × 106 CD4−CD25− T effectors via i.v. tail vein injec-
tion. This impaired the ability of T effectors to induce graft rejection (▲,
MST >180 days) vs transplanted B6.RAG−/− recipients that were trans-
ferred with T effectors only (●, MST 22 days, p < 0.01). Confusion of 2 ×
105 CD4+CD25+ MyD88−/+ T effectors and 1 × 105 MyD88−/− T effec-
tors (●) did not delay the ability of MyD88−/+ T effector to induce graft
rejection (MST 19 days, p = 0.3 vs infusion of MyD88−/+ T effectors alone).
ABSENCE OF MyD88 AND ALLOGRAFT ACCEPTANCE

MyD88 deficiency does not enhance the intrinsic suppressive ability of T regs. A, CD4+CD25+ T regs were FACs sorted from naive B6.MyD88+/+ or B6.My88+/− mice, and their ability to suppress T effector proliferation was measured by thymidine incorporation assay. B6.MyD88+/− T regs (∆) manifest equal suppressive in vitro function vs MyD88+/+ CD4+CD25+ T effector. B, Survival curve showing that CD4+CD25+ T regs purified from similarly treated MyD88-sufficient transplant recipients. T regs (2 × 10^5) purified either MyD88+/+ (A) or MyD88+/− recipients (●) were adoptively cotransferred, via i.v. tail vein injection, with 1 × 10^6 MyD88+/−CD4+CD25+ T effectors into a B6.RAG−/− recipient previously transplanted with a BALB/c MyD88−/− allograft. Both p < 0.01 vs survival in recipients infused with T effectors alone (■). C, Flow cytometric dot plots showing equal expression of Foxp3 in MyD88−/− vs MyD88+/+ spleen cells. Proportions are shown in lower right quadrant. D, Histogram plot showing equal expression of Foxp3 within the CD4+CD25− subpopulation in naive MyD88+/+ and MyD88−/− hosts. Blue, MyD88+/+; red, MyD88+/−; green, isotype control. Histograms are gated on the CD4+CD25+ subpopulation. Flow cytometric dot plots of CD4+ and CD25+ cells within the Foxp3+ subpopulation in naive MyD88−/− and MyD88+/+ mice. (Spleen cells from naive MyD88−/− and MyD88+/+ mice were of similar size and cellularity; data not shown.). WT, wild type; FL2-H, fluorescence.

FIGURE 7.
The potential clinical implications of our study are that transiently inhibiting innate immune pathways may provide targets to promote the efficacy of transplant tolerance protocols. This is potentially important given the rising appreciation of innate immunity in ischemia reperfusion injury (12–14). The other implication of our study is that an acute inflammatory response, for example an infection, which can augment innate immune signaling, may interfere with the efficacy of transplant tolerance protocols, leading to a break of tolerance and initiation of immunity. Indeed, there are prior reports that heterologous immunity to viral Ags prevents transplantation tolerance (44). This is supported by a recent study that demonstrated that lymphocytic choriomeningitis viral infection, which likely activates many components of innate immunity, impaired the ability of an allograft prolonging treatment consisting of donor cell transfusion and anti-CD154 to extend allograft survival (43).

In conclusion, our study provides proof-of-principle evidence that inhibiting specific innate immune signaling pathways may synergize with therapies that have the potential to induce transplantation tolerance. This information may be relevant to future clinical strategies aimed at inducing transplantation tolerance.

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Disclosures
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