Published in final edited form as:

Am J Transplant. 2010 July ; 10(7): 1524–1533. doi:10.1111/j.1600-6143.2010.03066.x.

Infection with the Intracellular Bacterium, *Listeria monocytogenes*, Overrides Established Tolerance in a Mouse Cardiac Allograft Model

Tongmin Wang[#], Emily B. Ahmed[#], Luqiu Chen^{*}, Jing Xu[#], Jing Tao[#], Chyung-Ru Wang[^], Maria-Luisa Alegre^{*}, and Anita S. Chong[#]

[#]The Section of Transplantation, Department of Surgery, The University of Chicago, Chicago, Illinois, USA

^{*}Section of Rheumatology, Department of Medicine, The University of Chicago, Chicago, Illinois, USA

[^]Department of Microbiology/Immunology, Northwestern University, Chicago, Illinois, USA

Abstract

Infections and TLR signals at the time of transplantation have been shown to prevent the induction of tolerance, but their effect on allografts after tolerance has been established is unclear. We here report that infection with *Listeria monocytogenes* precipitated the loss of tolerance and the MyD88- and T cell-dependent rejection of accepted cardiac allografts in mice. This loss of tolerance was associated with increases in the numbers of graft-infiltrating macrophages and dendritic cells, as well as CD4⁺FoxP3[−] and CD8⁺ T cells. Rejection was also associated with increased numbers of graft-infiltrating alloreactive as well as *Listeria*-reactive IFN γ -producing T cells. Rejection of the established grafts required both IL-6 and IFN β , cytokines produced during acute *Listeria* infection. However, IL-6 and IFN β alone, even when present at higher concentrations than during *Listeria* infection, were insufficient to break tolerance, while the combination of IL-6 and IFN β was able to break tolerance. These and in vitro observations that IL-6 but not IFN β enhanced T cell proliferation while IFN β but not IL-6 enhanced IFN γ production support a hypothesis that these cytokines play non-redundant roles. In conclusion, these studies demonstrate that the pro-inflammatory effects of infections can induce the loss of tolerance and acute rejection of accepted allografts.

Introduction

Successful induction of transplantation tolerance for achieving long-term allograft survival has been reported in a number of preclinical models (1–10) and in limited clinical scenarios

Corresponding Author: Anita S. Chong Ph.D., Section of Transplantation, Department of Surgery, The University of Chicago, 5841 S. Maryland Ave, Chicago, IL60637, USA. Tel: (773) 702-5521; FAX: (773) 702-5517; achong@uchicago.edu.

Tongmin Wang designed and performed experiments, data analysis and contributed to the manuscript. Jing Xu performed the heart transplants, Jing Tao the hydrodynamic injections, Emily B. Ahmed assisted with the in vitro experiments while Luqiu Chen assisted in the analysis of the graft infiltrating cells. Chyung-Ru Wang provided the *Listeria* and assisted in the initial design of experiments, while Maria-Luisa Alegre contributed to the experimental design and manuscript. Anita S. Chong designed the studies, and wrote the manuscript.

(11–15). Critical issues that have emerged from these successes include the identification of barriers to tolerance induction and the circumstances under which the tolerant state can be breached. These issues must be resolved if clinicians are to be able to assure patients that transplantation tolerance will result in stable and long-lasting allograft survival that is superior to current outcomes with pharmacological immunosuppression. Indeed, it has been long been observed that different organ or tissue types have different susceptibility to rejection and tolerance induction, with the liver being the most susceptible and the skin, small bowel and lung being the most resistant (http://www.ustransplant.org/annual_reports/current/113_surv-new_dh.htm). The breadth of the alloreactive T cell repertoire (16), presence of memory and homeostatically expanded T cells (17–19) as well as memory B cells (20) have also been identified as barriers that can independently prevent the induction of transplantation tolerance. The stimulation of innate immunity, via toll-like receptor (TLR) engagement (21–24) or infections (25) can prevent the development of allograft tolerance, and may do so via mechanisms that can be dependent on the type I interferon receptor (IFN α R1), IL-6 and Th17 effectors, but independent of TNFR2 and IL-12 (21, 24) (26). Additionally, the combination of IL-6 and TNF α has recently been implicated synergistically in the prevention of transplantation tolerance induction (27).

The identification of multiple barriers to tolerance induction contrasts with the relative paucity of reports on the factors that can abrogate tolerance after it has been established. Pharmacological blockade of PD-1-PD-L1, but not PD-1-PD-L2, interactions can reverse tolerance (28). More recently, it has been reported that the local or systemic degranulation of mast cells can induce acute rejection of established tolerant skin grafts, suggesting a potential link between allergy and loss of tolerance (29). However, it is unclear whether mast cell degranulation affects other organs comparably or plays a role in more robust models of tolerance. Infections have been hypothesized to be also capable of abrogating tolerance via stimulation of innate and adaptive immunity. Although they have been shown to prevent the induction of tolerance when occurring around the time of transplantation, supporting experimental data that they can abolish tolerance after it is established are lacking (30). In this study we provide proof-of-principle evidence that infection with the intracellular bacterium, *Listeria monocytogenes*, to abrogate tolerance and induce the acute rejection of established allografts in an IL-6- and IFN β -dependent manner.

Materials and Methods

Mice

C57BL/6 (B6, H-2^b), BALB/c (B/c, H-2^d), and IL-6^{-/-} mice on the B6 background were purchased from the Jackson Laboratory. MyD88^{-/-} mice on B6 backgrounds were kindly provided by Dr. S. Akira (Osaka University, Japan) (31). IFN α R1^{-/-} mice originally obtained from Dr. M. Aguet (32) were backcrossed to B6 mice for 7 generations. *FoxP3^{gfp}* knock-in mice were obtained from Dr. A. Rudensky (33), and backcrossed to B6 mice for 10 generations. *Listeria*-infected mice were used in agreement with Institutional Animal Care and Use Committee and the National Institutes of Health guidelines for animal use.

Mouse Transplantation and infection

Mouse abdominal heterotopic cardiac transplantation was performed using a technique described by Corry and colleagues (34). Tolerance was induced as previously described (25) by administration of anti-CD154 and donor specific transfusion (DST). Cardiac grafts were checked by palpation and the day of rejection was defined as the day of undetectable heartbeat. *Listeria monocytogenes* (LM-OVA) was grown in brain-heart infusion broth (BD Biosciences). A dose of 1.5×10^6 colony forming unit (i.p) (CFU) was chosen for infection experiments because all tolerant mice survived this dose and 50% of mice died when infected with 3×10^6 CFU. Ampicillin (25mg/mouse/day) was administered i.p. for 5 days, starting on day 2 post-infection for MyD88^{-/-} recipients.

In vivo depletion of CD4⁺ or CD8⁺ T cells

Two days before *Listeria* infection, CD4⁺ or CD8⁺ T cells were depleted by monoclonal antibody GK1.5 (0.2mg/mouse, iv) or YTS-169 (1mg/mouse, iv), respectively, for two consecutive days. Depletion was confirmed by checking peripheral blood using flow cytometry.

Plasmid preparation and injection

Mouse IL-6- and IFN β -expressing and control plasmids were purchased from Invivogen (San Diego, CA). Stock plasmid were isolated and prepared using EndoFree Plasmid Mega Kit (Qiagen). Plasmid DNA (5 μ g/mouse) were diluted in 1.9ml sterile nonpyrogenic 0.9% sodium chloride solution, and injected through tail vein within 5–6 seconds using a 3ml syringe with 27G_{1/2} needle (BD Bioscience).

Serum IFN β and IL-6 concentration

B6 mice were infected with *Listeria* (1.5×10^6 CFU i.p.) or injected with cytokine-expressing or control plasmids and the serum concentrations of IFN β and IL-6 were measured by ELISA (PBL Interferon Source and eBioscience).

Isolation of graft infiltrating cells

Graft infiltrating cells (GICs) were isolated as previously described (23). Isolated total graft infiltrating cells were stained using antibodies specific for CD4, CD8, CD11c, CD11b, B220, and FoxP3 (BD Bioscience) and analyzed by flow cytometry. To isolate T cells, GICs from two or three grafts were pooled and T cells were isolated using a Miltenyi Pan T cell Isolation Kit (Miltenyi Biotec Inc.).

IFN γ ELISPOT Assays

Pan T cells, CD4, or CD8 T cells were purified by negative selection using Miltenyi Cell Isolation Kits (Miltenyi Biotec Inc.), and cell purity were confirmed by flow cytometry analysis. Whole splenocytes (10^6 /well, in triplicate) or purified T cells, CD4⁺ or CD8⁺ T cells in the presence of irradiated (3000 rads) syngeneic (B6) or allogeneic (B/c) splenocytes (5×10^5 /well) were co-cultured for 12 hours. In some experiments, purified graft-infiltrating T cells were cultured in 10%FBS/RPMI overnight before use in the ELISPOT assay. In indicated experiment, syngeneic (B6) splenocytes were pre-stimulated with heat-killed

Listeria (HKLM; 10^8 CFU HKLM/ 60×10^6 splenocytes) overnight before used as stimulator. Recombinant mouse IFN β (1000U/ml; PBL Interferon Source) and IL-6 (500ng/ml; R&D Systems) were added to the ELISPOT assay, and the numbers of spots per well were enumerated using the ImmunoSpot Analyzer (CTL Analyzers LLC).

In vitro T cell proliferation and mixed lymphocyte reaction (MLR) assay

CD4⁺*FoxP3gfp-negative* T cells (8×10^5 /well) were sorted by MoFlo High-Performance Cell Sorter (Beckman Coulter, Inc.) from naïve *FoxP3gfp* knock-in mice and cultured with different ratios of CD4⁺*FoxP3gfp-positive* T cells in the presence of anti-CD3 and IL-2 and T cell depleted syngeneic splenocytes (5×10^5 /well). Recombinant mouse IL-6 or/and mouse IFN β were added to the cultures and pulsed on day 5 of culture with [³H]-thymidine for 7 hours. Incorporated [³H]-thymidine was counted with a TopCount NXT v2.13 Instrument (Packard). For MLR, whole splenocytes (8×10^5 /well) from naïve wild type B6 or long term tolerant recipients were cultured with irradiated syngeneic (B6), allogeneic (B/c) or third-party (C3H) splenocytes (8×10^5 /well) and recombinant mouse IL-6 or mouse IFN β for 3 days before pulsing with [³H]-thymidine for 7 hours.

Immunohistochemistry

Grafts were removed on the day of rejection, embedded with O.C.T. (Tissue-Tek Miles Inc), and immediately frozen in liquid nitrogen. Cryostat sections were stained with anti-IgG, anti-IgM, anti-CD19, anti-CD4, and anti-CD8 antibodies following standard immunohistochemical techniques.

Statistical Methods

The two-tailed Student's t test or ANOVA and post-hoc Tukey's or Bonferroni test for multiple comparisons was performed to determine statistical differences between groups. Graft mean survival time (MST) and *p* values were calculated using the Kaplan-Meier/log rank test (Prism 4.00, GraphPad Software, Inc.).

Results

Listeria infection induces the loss of cardiac allograft tolerance

To test whether established tolerance can be abrogated by an acute bacterial infection, 1.5×10^6 /mouse of Listeria was administered (i.p) at 60–100 days after transplantation of a BALB/c (B/c) heart (H-2^d) into a C57BL/6 (B6) recipient (H-2^b) and establishment of allograft tolerance using anti-CD154/DST. The Listeria infection was able to precipitate acute rejection of approximately 70% of cardiac allografts, but had no effect on syngeneic grafts (Fig 1a). Signaling through MyD88 (Fig 1b) and T cells was necessary for rejection, as the depletion of either CD4⁺ or CD8⁺ cells or use of MyD88-deficient recipients abrogated Listeria-induced rejection (Fig 1c). In contrast to the prevention of tolerance by Listeria infection, the rejection of the established allograft was not accompanied by increased alloantibody production, (Fig 1d).

Listeria infection activates alloreactive T cells in tolerant recipients

Histological examination of the rejected allografts revealed features of acute cellular rejection, namely increased numbers of graft-infiltrating CD4⁺ and CD8⁺ cells compared to grafts from recipients with syngeneic grafts (data not shown), or uninfected recipients (Fig 2a). We quantified a 110-fold increase in the total numbers of graft-infiltrating macrophages (CD11b⁺CD11c⁻) and a 50-fold increase in dendritic cells (DCs; Fig 2b). Specifically, a 34-fold increase in CD8⁺ DCs and 23-fold increase in CD11b⁺CD11c⁺ DCs, but only a modest 1.6-increase in B220⁺CD11c⁺ DCs in the rejected grafts compared to the tolerant grafts were observed. We also observed a 10–12-fold increase in graft-infiltrating CD4⁺ and CD8⁺ T cells in rejected allografts compared to uninfected, tolerant allografts (Fig 2c). Because allograft tolerance has been associated with an enrichment of Tregs in the graft (23, 35, 36), and the prevention of this state with reduced intra-graft CD4⁺FoxP3⁺Tregs (23, 35, 36), we quantified these cells and determined that rejection of established allografts was associated with a 7–10-fold increase in CD4⁺FoxP3⁻ T cells and a 1.7-fold increase in CD4⁺FoxP3⁺Tregs compared to tolerant grafts (Fig 2c). These alterations in the numbers of T cells translated into an increased ratio of FoxP3⁻:FoxP3⁺ CD4⁺ T cells (7:1 versus 68:1) in the rejecting allografts. Thus the rejection of tolerant grafts was not associated with reduced numbers of graft-infiltrating CD4⁺FoxP3⁺ T cells *per se* but to increased CD4⁺FoxP3⁻ T cells, resulting in increased ratios of CD4⁺FoxP3⁻:CD4⁺FoxP3⁺T cells.

To determine the specificities of the graft-infiltrating cells, we harvested these cells at the time of rejection and tested them in an IFN γ -ELISPOT assay. However the high frequency of cells spontaneously producing IFN γ prevented the determination of their specificities (data not shown). We therefore purified graft-infiltrating T cells and rested them overnight before using them in an IFN γ -ELISPOT assay. Increased IFN γ -producing cells were detected in the presence of B/c stimulators (Fig 3a) confirming the presence of alloreactive T cells. Increased frequencies of IFN γ -producing cells were also observed in the presence of LM-infected stimulators, suggesting the presence of LM-specific T cells or T cells non-specifically activated by the LM-infected stimulators. Collectively our data suggest that alloreactive T cells are essential to the rejection, but do not eliminate the possibility that LM-activated T cells that are non-alloreactive and possibly LM-specific could have also contributed to the rejection of the established allograft.

To confirm that the B/c-reactive T cells were not actually cross-reactive T cells that had been stimulated by the Listeria infection, we performed an *in vivo* CTL assay using B/c or Ly5.1 congenic B6 spleen cells as targets. To normalize for the spontaneous killing of alloreactive targets in this assay, 10 $\times$ 10⁶ B/c and 5 $\times$ 10⁶ congenic Ly5.1 B6 spleen cells were co-injected by intravenous tail-vein injection into naïve B6 mice or B6 mice that had been infected with LM-OVA (1.5 $\times$ 10⁶) or primed with B/c skin transplantation and 30 $\times$ 10⁶ B/c splenocytes (IP) on day -7. Six hours after transfer of B/c or Ly5.1 B6 spleen cells, we harvested the spleens and quantified the total numbers of B/c and Ly5.1 B6 recovered. We observed a selective reduction in the total numbers of B/c cells, relative to congenic Ly5.1⁺ B6 cells, recovered from the spleen of B/c primed recipients, but not in the Listeria-infected recipients (Fig 3b). These experiments confirm and extend previous observations that

Listeria-infection in B6 mice does not generate a functionally significant B/c-cross reactive population of T cells.

IFN β produced during Listeria infection is necessary but not sufficient to induce the reversal of tolerance

Our previous observations that IFN β produced following a peri-transplant Listeria infection is necessary and sufficient to prevent allograft tolerance (25, 37, 38) led us to test the necessity of IFN β in the breaking of established tolerance. Using IFN α R1^{-/-} mice, we observed that the ability to respond to IFN β is required for the loss of tolerance following Listeria infection, but is not necessary for allograft rejection in untreated recipients (Fig 4a). To test whether IFN β is sufficient to break established tolerance, we induced high concentrations of IFN β *in vivo* by introducing naked DNA plasmids encoding the IFN β gene via hydrodynamic injection (37, 38). Peak IFN β production (17,000 pg/ml) at 1–3 days post-injection was followed by a more sustained low-level (3,000 pg/ml) production for up to 14 days (Fig 4b). Thus the time-course of IFN β production following hydrodynamic injection was similar to that observed following Listeria infection, but yielded 10–20-fold higher levels. Nonetheless, rejection of the established allograft was not observed (Fig 4c), demonstrating that response to IFN β is necessary but insufficient to abrogate tolerance.

IL-6 produced during Listeria infection is necessary but not sufficient to induce the reversal of tolerance

Early inflammatory responses to Listeria infection can be classified into an initial MyD88-independent phase resulting in IFN β production and a MyD88-dependent phase that induces pro-inflammatory cytokines, including IL-6, IL-12, and TNF α (39). Because the abrogation of tolerance by Listeria infection is MyD88-dependent (Fig 1b) and IL-6 has potent effects on adaptive immunity (40–43), we tested whether IL-6 was necessary for the abrogation of tolerance. Tolerant IL-6^{-/-} recipients did not reject their allografts following Listeria infection (Fig 5a), but were able to reject B/c hearts in the absence of immunosuppression with kinetics comparable to wild-type mice (~8 days). Circulating IL-6 induced by the hydrodynamic injection of IL-6-encoding plasmids was 10–1000 fold higher than observed after Listeria infection (Fig 5b). However these high levels of circulating IL-6 were unable to precipitate acute rejection in the majority of established allografts (Fig 5c), indicating that, similarly to IFN β , IL-6 is necessary but insufficient for abrogating established tolerance.

IFN β and IL-6 are sufficient to induce the loss of established tolerance

Because both IL-6 and IFN β are produced during Listeria infection, we tested whether IL-6 and IFN β together could precipitate the loss of established tolerance. Sequential hydrodynamic injections of IL-6-expressing plasmids followed by IFN β -expressing plasmids resulted in the sequential production of high levels of serum IL-6 and IFN β *in vivo* (Fig 6a & 6b), while the simultaneous injection of both plasmids resulted in the IFN β production but an unexpected inhibition of IL-6 production (data not shown). The sequential administration of the IL-6- followed by the IFN β -expressing plasmid precipitated the acute rejection of established allografts (Fig 6c), while the simultaneous injection of both plasmids

did not (data not shown). These experiments establish the necessity and sufficiency of the combination of IL-6 and IFN β in abrogating transplantation tolerance.

The observations that IL-6 and IFN β together are necessary and sufficient to abrogate tolerance, but not each cytokine alone, prompted a hypothesis that these two cytokines play non-redundant roles. We confirmed and extended the observations by Pasare and Medzhitov (41) that IL-6, but not IFN β , enhanced the proliferation of conventional T cells (CD4⁺FoxP3⁻) in the absence or presence of regulatory CD4⁺FoxP3⁺ T cells (Fig 7a). We also investigated the impact of IL-6 and IFN β on splenocytes from tolerant recipients. Splenocytes from naïve B6 mice responded to both B/c and C3H stimulators while splenocytes from tolerant recipients did not proliferate in response to B/c stimulators but proliferated to C3H stimulators (Fig 7b). Despite donor-specific hyporeactivity, IL-6 was still able to enhance the proliferation of B/c-reactive T cells from tolerant recipients to levels comparable to T cells from naïve B6 mice, while IFN β was not (Fig 7b). This indicates that there are donor-reactive T cells in tolerant mice that have not been depleted and are likely anergic or suppressed, and that IL-6, but not IFN β , can over-ride this hypo-responsiveness and enhance T cell proliferation in tolerant recipients.

Because Type I IFNs have been reported to promote Th1 polarization and IFN γ -production (44–47), we tested whether IFN γ -production is differentially enhanced by IFN β or IL-6. We observed that IFN β , but not IL-6, increased the frequency of IFN γ -producing cells detected in splenocytes from tolerant recipients 14 days after *Listeria* infection (Fig 7c). Further IFN β enhanced IFN γ -production by both CD4⁺ and CD8⁺ T cells (Fig 7c). No or minimal IFN γ -producing cells were observed from the splenocytes from uninfected tolerant recipients (Fig 7d). These observations collectively suggest that IFN β , but not IL-6, stimulated IFN γ -production in primed effector cells already present in recipients where tolerance was abrogated by infection with *Listeria*, and that these effector cells were not present in uninfected stably tolerant mice.

Discussion

Viral parasitic and bacterial infections at the time of transplantation have been previously reported to prevent the induction of transplantation tolerance by costimulation-blockade therapies, however, none have been reported to break established tolerance. Our current observations represent the first demonstration in an experimental model that bacterial infections can breach established tolerance. Our observations further suggest that the requirements for overturning tolerance are more difficult to achieve compared to the prevention of this state, an observation that bodes well for transplantation tolerance as a means of achieving long-term graft survival. Indeed, 15-fold higher infectious dose of *Listeria* was required for the reversal of tolerance compared to preventing the induction of tolerance. This observation supported an overall hypothesis that significantly more by-stander pro-inflammatory signals, generated by a more acute *Listeria* infection, is necessary to reverse compared to preventing the induction of tolerance.

Consistent with this hypothesis of *Listeria* infection inducing the by-stander activation of alloreactive T cells are the observations that the reversal of tolerance by *Listeria* infection

requires the presence of both CD4⁺ and CD8⁺ cells; in contrast the prevention of tolerance by *Listeria* infection is dependent only on the presence of either CD4⁺ or CD8⁺ cells (25). We confirmed that the cells infiltrating the allograft following *Listeria* infection had both donor- and *Listeria*-reactivity raising the possibility that either alloreactive or both alloreactive and *Listeria*-specific T cells contributed to the rejection. *Listeria*-reactive T cells, in the absence of alloreactive T cells, were not able to cause the rejection of the syngeneic hearts, suggesting that alloreactive T cells are necessary for the rejection of the established allograft. Further, we demonstrated that B/c-reactive T cells were unlikely to have been generated as a result of cross-reactivity from the anti-*Listeria* responses because B/c targets were not preferentially eliminated relative to syngeneic B6 grafts at the peak of the anti-*Listeria* response (7 days after infection). This lack of cross-reactivity complements and extends previous observations that *Listeria* infection does not generate a functionally significant B/c reactive memory population (25), and support the conclusion that *Listeria* infection releases alloreactive T cells from the mechanisms that suppress their activation and maintain allograft tolerance.

Also consistent with this hypothesis of *Listeria*-mediated by-stander activation of alloreactive T cells are the observations that the reversal of tolerance requires signaling via the MyD88 adaptor molecule, while the prevention of tolerance induction by *Listeria* infection does not (25). Innate immune responses to *Listeria* infection can be divided into two phases, an early MyD88-independent phase that results in IFN β production, and a later MyD88-dependent phase that regulates the production of pro-inflammatory cytokines including IL-6. Consistent with this schema are the observations that the reversal of established tolerance was dependent on both IFN α R1 and IL-6, whereas the prevention of tolerance required only IFN α R1. Using the approach of hydrodynamic transfection of plasmids expressing IFN β and/or IL-6 to induce the production of 30–50-fold higher concentrations of circulating IL-6 or IFN β than observed following *Listeria* infection, we observed that each cytokine alone was not capable of abrogating established tolerance. In contrast, these cytokines alone are capable of preventing tolerance induction in a skin allograft model ((25) and unpublished data, EBA). These observations together with previous reports that IL-6 is critical to the control of *Listeria* infection while Type I IFNs unexpectedly confer increased susceptibility (39, 48) suggest opportunities for the preservation of tolerance without significantly compromising protective immunity.

The observations that the combination of IL-6 and IFN β was able to reverse established tolerance are consistent with the notion that these cytokines played non-redundant roles. Indeed we demonstrated that the anti-donor proliferative responses by spleen cells from tolerant recipients were enhanced by IL-6, but not by IFN β , despite hyporesponsiveness, relative to naïve spleen cells, in its absence. Further, IFN β but not IL-6 was able to enhance the frequency of IFN γ -producing cells from tolerant mice that had rejected the established allograft following infection with *Listeria*. Notably, no increase in IFN γ -producing cells were observed in tolerant mice that were not-infected with *Listeria*, suggesting that effector cells capable of IFN γ production were generated during the *Listeria* infection of tolerant recipients. Collectively these observations support a conclusion that IL-6 and IFN β act in a non-redundant manner to induce the proliferation of effector alloreactive T cells despite established regulation in tolerant recipients, and to enhance IFN γ production of these

clonally expanded alloreactive T cells. Further studies are necessary to test whether these observations of *Listeria* infection on effector T cells are replicated *in vivo*, namely that IFN β is required for generation of IFN γ -producing alloreactive cells following infection and IL-6 is necessary for cell proliferation, and are the basis for the loss of tolerance. The question of whether IFN γ production by CD4 and/or CD8 alloreactive T cells are necessary and sufficient for the loss of tolerance also requires future resolution.

In contrast to its effects on CD4⁺FoxP3⁻ and CD8⁺ cells, the impact of *Listeria* infection on CD4⁺FoxP3⁺ Treg cell numbers in the allografts is minimal, and not significantly different in tolerant versus at the time of rejection induced by *Listeria* infection in tolerant recipients. These observations are in contrast to the loss of tolerance induced by mast cell degranulation, where a reduction in the numbers of Tregs was associated with the loss of tolerance rejection (29). In addition, the suppressive activity of Tregs and their expression of key immunomodulatory molecules were also transiently reduced as a result of mast cell degranulation (29). These observations with mast cells are complementary to recent reports that potentially autoreactive effector T cells can be generated as a consequence of Foxp3 instability under an autoimmune microenvironment (49). Thus additional experiments are necessary to more rigorously test whether *Listeria* infection and IL-6/IFN β can affect the function of CD4⁺FoxP3⁺ cells, in addition to their direct effects on effector T cells.

In conclusion, acute intracellular *Listeria* infection can elicit conditions that override established tolerance and precipitate T cell-dependent but alloantibody-independent allograft rejection. However, the conditions for the reversal of established tolerance are more stringent than for preventing the induction of tolerance. These observations bode well for the stability of transplantation tolerance and may explain why manipulations capable of preventing the induction of tolerance generally fail to abrogate established tolerance. Investigations into the mechanism by which *Listeria* infections reversed established tolerance demonstrated the requirement of both IL-6 and IFN β , which are produced following *Listeria* infection. Thus, select infectious agents that can induce high levels of circulating Type I IFNs and/or IL-6 (50–53) are predicted as capable of eroding the state of established tolerance, but not infections that induce low levels of both cytokines or that induce high levels of only one cytokine. Finally, our observations underscore the importance of controlling infections and innate immune reactivity, and suggest the targeting of inflammatory cytokines such as Type I IFNs and IL-6 as a means of preserving allograft tolerance.

Acknowledgments

We acknowledge Drs. Albert Bendelac, Alexander Rudensky and Shizuo Akira for the generous gifts of the IFN α R1^{-/-}, *FoxP3*^{gfp} knock-in and MyD88^{-/-} mice respectively, and thank Dr. James W. Williams for his insightful comments and discussion. This work was supported by grants AHA0920115G to Tongmin Wang, AHA 0620026Z to Luqiu Chen, NIAID RO1 AI071080 to Maria-Luisa Alegre and NIAID R01 AI072630 to Anita S. Chong.

References

1. Bushell A, Morris PJ, Wood KJ. Transplantation tolerance induced by antigen pretreatment and depleting anti-CD4 antibody depends on CD4+ T cell regulation during the induction phase of the response. *Eur J Immunol.* 1995; 25(9):2643–2649. [PubMed: 7589139]
2. Cobbold SP, Adams E, Marshall SE, Davies JD, Waldmann H. Mechanisms of peripheral tolerance and suppression induced by monoclonal antibodies to CD4 and CD8. *Immunol Rev.* 1996; 149:5–33. [PubMed: 9005219]
3. Elster EA, Xu H, Tadaki DK, Montgomery S, Burkly LC, Berning JD, et al. Treatment with the humanized CD154-specific monoclonal antibody, hu5C8, prevents acute rejection of primary skin allografts in nonhuman primates. *Transplantation.* 2001; 72(9):1473–1478. [PubMed: 11707732]
4. Ildstad ST, Wren SM, Bluestone JA, Barbieri SA, Sachs DH. Characterization of mixed allogeneic chimeras. Immunocompetence, in vitro reactivity, and genetic specificity of tolerance. *J Exp Med.* 1985; 162(1):231–244. [PubMed: 3159825]
5. Kawai T, Cosimi AB, Colvin RB, Powelson J, Eason J, Kozlowski T, et al. Mixed allogeneic chimerism and renal allograft tolerance in cynomolgus monkeys. *Transplantation.* 1995; 59(2):256–262. [PubMed: 7839449]
6. Kenyon NS, Chatzipetrou M, Masetti M, Ranuncoli A, Oliveira M, Wagner JL, et al. Long-term survival and function of intrahepatic islet allografts in rhesus monkeys treated with humanized anti-CD154. *Proc Natl Acad Sci U S A.* 1999; 96(14):8132–8137. [PubMed: 10393960]
7. Kenyon NS, Fernandez LA, Lehmann R, Masetti M, Ranuncoli A, Chatzipetrou M, et al. Long-term survival and function of intrahepatic islet allografts in baboons treated with humanized anti-CD154. *Diabetes.* 1999; 48(7):1473–1481. [PubMed: 10389857]
8. Kirk AD, Burkly LC, Batty DS, Baumgartner RE, Berning JD, Buchanan K, et al. Treatment with humanized monoclonal antibody against CD154 prevents acute renal allograft rejection in nonhuman primates [see comments]. *Nat Med.* 1999; 5(6):686–693. [PubMed: 10371508]
9. Lenschow DJ, Zeng Y, Thistlethwaite JR, Montag A, Brady W, Gibson MG, et al. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg [see comments]. *Science.* 1992; 257(5071):789–792. [PubMed: 1323143]
10. Larsen CP, Elwood ET, Alexander DZ, Ritchie SC, Hendrix R, Tucker-Burden C, et al. Long-term acceptance of skin and cardiac allografts after blocking CD40 and CD28 pathways. *Nature.* 1996; 381(6581):434–438. [PubMed: 8632801]
11. Braud C, Baeten D, Giral M, Pallier A, Ashton-Chess J, Braudeau C, et al. Immunosuppressive drug-free operational immune tolerance in human kidney transplant recipients: Part I. Blood gene expression statistical analysis. *J Cell Biochem.* 2008; 103(6):1681–1692. [PubMed: 17910029]
12. Kawai T, Cosimi AB, Spitzer TR, Tolckoff-Rubin N, Suthanthiran M, Saidman SL, et al. HLA-mismatched renal transplantation without maintenance immunosuppression. *N Engl J Med.* 2008; 358(4):353–361. [PubMed: 18216355]
13. Ashton-Chess J, Giral M, Brouard S, Souillou JP. Spontaneous operational tolerance after immunosuppressive drug withdrawal in clinical renal allotransplantation. *Transplantation.* 2007; 84(10):1215–1219. [PubMed: 18049104]
14. Martinez-Llordella M, Lozano JJ, Puig-Pey I, Orlando G, Tisone G, Lerut J, et al. Using transcriptional profiling to develop a diagnostic test of operational tolerance in liver transplant recipients. *J Clin Invest.* 2008; 118(8):2845–2857. [PubMed: 18654667]
15. Brouard S, Mansfield E, Braud C, Li L, Giral M, Hsieh SC, et al. Identification of a peripheral blood transcriptional biomarker panel associated with operational renal allograft tolerance. *Proc Natl Acad Sci U S A.* 2007; 104(39):15448–15453. [PubMed: 17873064]
16. Wells AD, Li XC, Li Y, Walsh MC, Zheng XX, Wu Z, et al. Requirement for T-cell apoptosis in the induction of peripheral transplantation tolerance. *Nat Med.* 1999; 5(11):1303–1307. [PubMed: 10545998]
17. Wu Z, Bensinger SJ, Zhang J, Chen C, Yuan X, Huang X, et al. Homeostatic proliferation is a barrier to transplantation tolerance. *Nat Med.* 2004; 10(1):87–92. [PubMed: 14647496]

18. Adams AB, Williams MA, Jones TR, Shirasugi N, Durham MM, Kaech SM, et al. Heterologous immunity provides a potent barrier to transplantation tolerance. *J Clin Invest.* 2003; 111(12):1887–1895. [PubMed: 12813024]
19. Valujskikh A, Pantenburg B, Heeger PS. Primed allospecific T cells prevent the effects of costimulatory blockade on prolonged cardiac allograft survival in mice. *Am J Transplant.* 2002; 2(6):501–509. [PubMed: 12118893]
20. Burns AM, Ma L, Li Y, Yin D, Shen J, Xu J, et al. Memory alloreactive B cells and alloantibodies prevent anti-CD154-mediated allograft acceptance. *J Immunol.* 2009; 182(3):1314–1324. [PubMed: 19155477]
21. Porrett PM, Yuan X, LaRosa DF, Walsh PT, Yang J, Gao W, et al. Mechanisms underlying blockade of allograft acceptance by TLR ligands. *J Immunol.* 2008; 181(3):1692–1699. [PubMed: 18641305]
22. Thornley TB, Brehm MA, Markees TG, Shultz LD, Mordes JP, Welsh RM, et al. TLR agonists abrogate costimulation blockade-induced prolongation of skin allografts. *J Immunol.* 2006; 176(3):1561–1570. [PubMed: 16424185]
23. Chen L, Wang T, Zhou P, Ma L, Yin D, Shen J, et al. TLR engagement prevents transplantation tolerance. *Am J Transplant.* 2006; 6(10):2282–2291. [PubMed: 16970798]
24. Thornley TB, Phillips NE, Beaudette-Zlatanova BC, Markees TG, Bahl K, Brehm MA, et al. Type 1 IFN mediates cross-talk between innate and adaptive immunity that abrogates transplantation tolerance. *J Immunol.* 2007; 179(10):6620–6629. [PubMed: 17982052]
25. Wang T, Chen L, Ahmed E, Ma L, Yin D, Zhou P, et al. Prevention of allograft tolerance by bacterial infection with *Listeria monocytogenes*. *J Immunol.* 2008; 180(9):5991–5999. [PubMed: 18424719]
26. Chen L, Ahmed E, Wang T, Wang Y, Ochando J, Chong AS, et al. TLR signals promote IL-6/IL-17-dependent transplant rejection. *J Immunol.* 2009; 182(10):6217–6225. [PubMed: 19414775]
27. Shen H, Goldstein DR. IL-6 and TNF- α synergistically inhibit allograft acceptance. *J Am Soc Nephrol.* 2009; 20(5):1032–1040. [PubMed: 19357252]
28. Tanaka K, Albin MJ, Yuan X, Yamaura K, Habicht A, Murayama T, et al. PDL1 is required for peripheral transplantation tolerance and protection from chronic allograft rejection. *J Immunol.* 2007; 179(8):5204–5210. [PubMed: 17911605]
29. de Vries VC, Wasiuk A, Bennett KA, Benson MJ, Elgueta R, Waldschmidt TJ, et al. Mast cell degranulation breaks peripheral tolerance. *Am J Transplant.* 2009; 9(10):2270–2280. [PubMed: 19681828]
30. Welsh RM, Markees TG, Woda BA, Daniels KA, Brehm MA, Mordes JP, et al. Virus-induced abrogation of transplantation tolerance induced by donor-specific transfusion and anti-CD154 antibody. *J Virol.* 2000; 74(5):2210–2218. [PubMed: 10666251]
31. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, et al. Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity.* 1998; 9(1):143–150. [PubMed: 9697844]
32. Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, et al. Functional role of type I and type II interferons in antiviral defense. *Science.* 1994; 264(5167):1918–1921. [PubMed: 8009221]
33. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity.* 2005; 22(3):329–341. [PubMed: 15780990]
34. Corry RJ, Winn HJ, Russell PS. Primarily vascularized allografts of hearts in mice. The role of H-2D, H-2K, and non-H-2 antigens in rejection. *Transplantation.* 1973; 16(4):343–350. [PubMed: 4583148]
35. Graca L, Cobbold SP, Waldmann H. Identification of regulatory T cells in tolerated allografts. *J Exp Med.* 2002; 195(12):1641–1646. [PubMed: 12070291]
36. Lee I, Wang L, Wells AD, Dorf ME, Ozkaynak E, Hancock WW. Recruitment of Foxp3+ T regulatory cells mediating allograft tolerance depends on the CCR4 chemokine receptor. *J Exp Med.* 2005; 201(7):1037–1044. [PubMed: 15809349]

37. Herweijer H, Wolff JA. Gene therapy progress and prospects: hydrodynamic gene delivery. *Gene Ther.* 2007; 14(2):99–107. [PubMed: 17167496]
38. Wolff JA, Budker V. The mechanism of naked DNA uptake and expression. *Adv Genet.* 2005; 54:3–20. [PubMed: 16096005]
39. Pamer EG. Immune responses to *Listeria monocytogenes*. *Nat Rev Immunol.* 2004; 4(10):812–823. [PubMed: 15459672]
40. Kishimoto T. Interleukin-6: discovery of a pleiotropic cytokine. *Arthritis Res Ther.* 2006; 8 (Suppl 2):S2. [PubMed: 16899106]
41. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science.* 2003; 299(5609):1033–1036. [PubMed: 12532024]
42. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature.* 2006; 441(7090):235–238. [PubMed: 16648838]
43. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity.* 2006; 24(2):179–189. [PubMed: 16473830]
44. Farrar JD, Murphy KM. Type I interferons and T helper development. *Immunol Today.* 2000; 21(10):484–489. [PubMed: 11071526]
45. Farrar JD, Smith JD, Murphy TL, Leung S, Stark GR, Murphy KM. Selective loss of type I interferon-induced STAT4 activation caused by a minisatellite insertion in mouse Stat2. *Nat Immunol.* 2000; 1(1):65–69. [PubMed: 10881177]
46. Rogge L, D'Ambrosio D, Biffi M, Penna G, Minetti LJ, Presky DH, et al. The role of Stat4 in species-specific regulation of Th cell development by type I IFNs. *J Immunol.* 1998; 161(12):6567–6574. [PubMed: 9862683]
47. Krug A, Veeraswamy R, Pekosz A, Kanagawa O, Unanue ER, Colonna M, et al. Interferon-producing cells fail to induce proliferation of naive T cells but can promote expansion and T helper 1 differentiation of antigen-experienced unpolarized T cells. *J Exp Med.* 2003; 197(7):899–906. [PubMed: 12668648]
48. Dalrymple SA, Lucian LA, Slattery R, McNeil T, Aud DM, Fuchino S, et al. Interleukin-6-deficient mice are highly susceptible to *Listeria monocytogenes* infection: correlation with inefficient neutrophilia. *Infect Immun.* 1995; 63(6):2262–2268. [PubMed: 7768607]
49. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol.* 2009; 10(9):1000–1007. [PubMed: 19633673]
50. Moro MR, Bonville CA, Suryadevara M, Cummings E, Faddoul D, Kobayaa H, et al. Clinical features, adenovirus types, and local production of inflammatory mediators in adenovirus infections. *Pediatr Infect Dis J.* 2009; 28(5):376–380. [PubMed: 19319023]
51. Julkunen I, Melen K, Nyqvist M, Pirhonen J, Sareneva T, Matikainen S. Inflammatory responses in influenza A virus infection. *Vaccine.* 2000; 19 (Suppl 1):S32–37. [PubMed: 11163460]
52. Bonjardim CA. Interferons (IFNs) are key cytokines in both innate and adaptive antiviral immune responses--and viruses counteract IFN action. *Microbes Infect.* 2005; 7(3):569–578. [PubMed: 15792636]
53. Feld R. Bloodstream infections in cancer patients with febrile neutropenia. *Int J Antimicrob Agents.* 2008; 32 (Suppl 1):S30–33. [PubMed: 18778919]

Figure 1. Listeria infection breaks anti-CD154/DST-mediated cardiac allograft tolerance
 (A) B6 recipients of B/c heart grafts were untreated (Rej; n=5) or were treated with anti-CD154/DST to induce long-term graft acceptance (Tolerant). On day 60 post-transplantation, tolerant recipients were infected i.p. with 1.5×10^6 Listeria (Tolerant+LM; n=41); this control cohort was cumulative over the project period. Controls were recipients with syngeneic cardiac grafts infected with Listeria on day 60 post-transplantation (n=5). $p=0.0118$ for Tolerant vs Tolerant+LM. (B) Tolerant MyD88^{-/-} or wild type B6 mice were infected with LM on day 60 post-transplantation (n=6). $p=0.006$ for Tolerant MyD88^{-/-}+LM vs Tolerant WT+LM. (C) Two days before Listeria infection, tolerant recipients were treated with GK1.5 (0.2mg/mouse) or YTS-169 (1mg/mouse) mAbs to deplete CD4⁺ or CD8⁺ T cells (n=5/group). (D) No increase in allo-IgG titers following the abrogation of tolerance by Listeria infection (Tolerant+D60-LM), but significantly increased allo-IgG titers were detected in anti-CD154/DST-treated recipients infected with Listeria on the day of transplantation (Tolerant+D0-LM), or in untreated recipients (No Rx). $p=0.0108$ for Tolerant+D0-LM vs Tolerant+D60-LM on day 7; $p=0.0153$ for Tolerant+D0-LM vs Tolerant+D60-LM on day 14; $p=0.0012$ for No Rx vs Tolerant+D60-LM on day 21; $p=0.0004$ for No Rx vs Tolerant+D60-LM on day 28. All results represent the mean and standard deviation of at least 3 independent experiments.

Figure 2. Analyses of intra-graft immune responses

(A) Histology and immunohistochemistry of rejecting cardiac allografts harvested on day 7 post-Listeria infection (representative of 3 allografts/group). (B & C) Graft infiltrating cells were isolated and analyzed by flow cytometry. Total CD11b⁺CD11c⁻ (macrophages), dendritic cells (CD11c⁺) and dendritic cell subsets (pDC: B220⁺CD11b⁻CD11c⁺; IDC:CD8⁺CD11b⁻CD11c⁺; mDC: CD11b⁺CD11c⁺) as well as T cell subsets (CD4⁺, CD8⁺, FoxP3⁺, and FoxP3⁻) were calculated for Tolerant and Tolerant+LM groups. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

Figure 3. Specificity of intra-graft immune responses

(A) The frequency of IFN γ -producing T cells isolated from the allograft on day 7 post-Listeria infection. The results represent the mean and standard deviation of 3 independent experiments. (B) 10×10^6 B/c and 5×10^6 B6.Ly5.1 spleen cells were injected into naïve B6 mice or on day 7-post Listeria (1.5×10^6) infection or 7 days after B/c skin transplantation plus 30×10^6 B/c splenocyte (i.p.). Six hours after cell transfer, spleen cells were harvested, and the total B/c cell and B6.Ly5.1 cells recovered enumerated. ** $p < 0.01$; * $p < 0.05$.

Figure 4. Type I IFN signaling is necessary but not sufficient for abrogating tolerance following Listeria infection

(A) IFN α R1^{-/-} mice received B/c cardiac grafts and were untreated (Rej; n=4) or treated with anti-CD154/DST followed by Listeria infection on day 60 post-transplantation (Tolerant+LM; n=41). The control cohort is the same as in Fig 1. $p=0.0005$ for Tolerant IFN α R1^{-/-}+LM vs Tolerant WT+LM. (B) Wild type B6 mice were infected with Listeria or with mouse IFN β -expressing plasmid and serum IFN β levels were determined by ELISA. (C) Graft survival after IFN β (n=5) or control plasmid (n=4) injection ($p>0.05$).

Figure 5. IL-6 is necessary but not sufficient for abrogating tolerance following Listeria infection (A) IL-6^{-/-} mice transplanted with B/c cardiac grafts and were untreated (Rej; n=3), or were treated with anti-CD154/DST followed by Listeria infection on day 60 post-transplantation (Tolerant+LM; n=5). The control cohort is the same as in Fig 1. $p=0.0118$ for Tolerant IL-6^{-/-}+LM vs Tolerant WT+LM. (B) Wild type B6 mice were infected with Listeria or with mouse IL-6-expressing plasmid, and serum IL-6 levels determined (n=3/group). (C) Graft survival after IL-6 (n=4) or control plasmid (n=4) injection ($p>0.05$).

Figure 6.

IFN β and IL-6 are sufficient for abrogating tolerance. Tolerant B6 recipients were injected with mouse IL-6- followed by IFN β -expressing plasmids 5 days later (n=3/group). Serum IFN β (A) and IL-6 (B) were quantified and (C) graft survival determined, following IL-6+IFN β (n=8) or control plasmid (n=4) injections ($p=0.0019$).

Figure 7. Listeria infection of tolerant recipients induces a transient enhancement of alloreactive T cell responses and abrogation of tolerance

(A) Sorted CD4⁺ T cells (CD25⁺GFP⁺ and CD25⁻GFP⁻) from naïve *FoxP3*^{gfp} knock-in mice were stimulated at the indicated ratios with immobilized anti-CD3 and IL-2 and irradiated T cell-depleted syngeneic splenocytes. In some cultures, rIL-6 (500 ng/ml) or rIFN β (500 U/ml) was added, and cell proliferation was determined by [³H]-thymidine incorporation. (B) Spleen cells from naïve or tolerant B6 recipients were stimulated with irradiated syngeneic (B6), allogeneic (B/c) or third-party (C3H) splenocytes in the presence of IL-6 (1000 ng/ml) or mouse IFN β (1000 U/ml). (C) Whole splenocytes, CD4⁺ or CD8⁺ cells from the spleens of Tolerant+LM recipients, or (D) The frequency of IFN γ -producing T cells from Tolerant+LM or Tolerant recipients, stimulated in rIL-6 (500ng/ml) or/and rIFN β (1000U/ml), was determined with an IFN γ ELISPOT assay. All results represent the mean and standard deviation of 3 independent experiments. ****p*<0.001; ***p*<0.01; **p*<0.05.