

**IFN- $\alpha/\beta$  INDUCTION BY dsRNA AND TOLL-LIKE RECEPTORS SHORTENS  
ALLOGRAFT SURVIVAL INDUCED BY COSTIMULATION BLOCKADE**

**A Dissertation Presented**

**By**

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Thomas B. Thornley

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## ABSTRACT

Costimulation blockade protocols are promising alternatives to the use of chronic immunosuppression for promoting long-term allograft survival. However, the efficacy of costimulation blockade-based protocols is decreased by environmental insults such as viral infections. For example, lymphocytic choriomeningitis virus (LCMV) infection at the time of costimulation blockade treatment abrogates skin allograft survival in mice. In this dissertation, we test the hypothesis that viruses shorten allograft survival by activating the innate immune system through pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs).

To investigate the role of innate immunity in shortening allograft survival, costimulation blockade-treated mice were co-injected with TLR2 (Pam<sub>3</sub>Cys), TLR3 (polyinosinic:polycytidylic acid, poly(I:C)), TLR4 (lipopolysaccharide, LPS), or TLR9 (CpG DNA) agonists, followed by transplantation with skin allografts 7 days later. Costimulation blockade prolonged skin allograft survival that was shortened in mice co-injected with TLR agonists. To investigate the underlying mechanisms of this observation, we used synchimeric mice, which circulate trace populations of anti-H2<sup>b</sup> transgenic alloreactive CD8<sup>+</sup> T cells. In synchimeric mice treated with costimulation blockade, co-administration of all four TLR agonists prevented deletion of alloreactive CD8<sup>+</sup> T cells. These alloreactive CD8<sup>+</sup> T cells 1) expressed the proliferation marker Ki-67, 2) upregulated CD44, and 3) failed to undergo apoptosis. We also demonstrate that costimulation blockade-treated CD8 $\alpha$ -deficient mice exhibit prolonged allograft survival when co-injected with LPS. These data suggest that TLR agonists shorten allograft survival by impairing the apoptosis of alloreactive CD8<sup>+</sup> T cells.

We further delineate the mechanism by which TLR agonists shorten allograft survival by demonstrating that LPS and poly(I:C) fail to shorten allograft survival in IFN-RI-deficient mice. Interestingly, the ability of poly(I:C) to more potently induce IFN- $\alpha/\beta$  than LPS correlates with its superior abilities to shorten islet allograft survival and induce allo-specific CTL activity as measured by an *in vivo* cytotoxicity assay. The ability to shorten allograft survival and induce IFN- $\alpha/\beta$  is a TLR-dependent process for LPS, but is a TLR-independent process for poly(I:C). Strikingly, the injection of IFN- $\beta$  impairs alloreactive CD8<sup>+</sup> T cell deletion and shortens allograft survival, similar to LPS and poly(I:C). These data suggest that LPS and poly(I:C) shorten allograft survival by inducing IFN- $\alpha/\beta$  through two different mechanisms.

Finally, we present data showing that viruses (LCMV, Pichinde virus, murine cytomegalovirus and vaccinia virus) impair alloreactive CD8<sup>+</sup> T cell deletion and shorten allograft survival, in a manner comparable to LPS and poly(I:C). Similar to LPS, LCMV and MCMV exhibit an impaired ability to shorten allograft survival in MyD88-deficient mice. These data suggest that the MyD88 pathway is required for certain viruses and TLR-agonists to shorten allograft survival.

In this dissertation, we present data supporting an important role for TLRs and IFN- $\alpha/\beta$  in shortening allograft induced by costimulation blockade. Our findings suggest that targeting these pathways during the peri-transplant period may enhance the efficacy of costimulation blockade protocols in the clinic.

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## ABBREVIATIONS

APCs, antigen-presenting cells

cDC, conventional dendritic cell

CTL, cytotoxic T lymphocyte

DC, dendritic cell

dsRNA, double-stranded RNA

DST, donor-specific transfusion

GP, glycoprotein

GVHD, graft versus host disease

H2, histocompatibility-2 locus

HSP, heat shock protein

IFN- $\alpha/\beta$ , interferon-alpha/beta

IFN-RI, interferon receptor I

IKK, I $\kappa$  Kinase

IL, interleukin

i.p., intraperitoneally

IPC, interferon-producing cell

IRAK, IL-1 receptor-associated kinase

IRF, interferon regulatory factor

ISGF, interferon stimulated growth factor

ISRE, interferon-stimulated response element

i.v., intravenous

JAK, Janus-associated kinase

KU, kilounit

LCMV, lymphocytic choriomeningitis virus

LPS, lipopolysaccharide

mAb, monoclonal antibody

MCMV, murine cytomegalovirus

MDA, melanoma differentiation-associated gene

MFI, mean fluorescence intensity

MHC, the major histocompatibility complex

MST, median survival time

MyD88, myeloid differentiation factor 88

NP, nucleoprotein

PAMP, pathogen-associated molecular pattern

PBL, peripheral blood lymphocyte

pDC, plasmacytoid dendritic cell, interferon-producing cell

PKR, protein kinase RNA

poly(I:C), poly(inosinic acid):poly(cytidylic acid)

PRR, pattern recognition receptor

PV, Pichinde virus

PFU, plaque-forming units

RIG, retinoic acid inducible gene

RIP, receptor interacting protein

s.d., standard deviation

ssRNA, single-stranded RNA

STAT, signal transducer and activator of transcription

TANK, TNFR-associated factor family member-associated NF- $\kappa$ B activator

TCR, T cell receptor

TLR, Toll-like receptor

TNF $\alpha$ , tumor necrosis factor alpha

TNFR, tumor necrosis factor receptor

TRAF, TNFR-associated factor

TGF, transforming growth factor

TRIF, TIR-domain-containing adaptor protein-inducing IFN- $\beta$

Tyk, Tyrosine Kinase

VSV, vesicular stomatitis virus

VV, vaccinia virus

WBI, Whole-body irradiation

## CHAPTER I: INTRODUCTION TO TRANSPLANTATION

### A Brief History of Transplantation and Alloreactivity

#### *Transplantation: The Early Years*

There is considerable historical evidence that successful reconstructive rhinoplasty was performed using a patient's autologous skin more than 2 millennia ago by Indian physicians (1). Anthropologic findings substantiate that physicians performed these surgeries in order to reverse superficial damage inflicted as punishment for a criminal offense or by the sword in battle. Undoubtedly, any attempt to transplant tissue from unrelated donors failed due to a lack of understanding about the immunologic basis for organ rejection and the inability to contravene the immune system. In fact, a latter-day Italian reconstructive surgeon, Gaspare Tagliacozzi, wrote the following about similar surgeries that he performed during the 16<sup>th</sup> century:

*"The singular character of the individual entirely dissuades us from attempting this work on another person. For such is the force and power of individuality, that if any one should believe that he could achieve even the least part of the operation, we consider him plainly superstitious and badly grounded in physical science." (2,3)*

Tagliacozzi recognized the immune system's power long before the discovery of the molecular and cellular basis for immune reactions, astutely observing the nature of self/non-self discrimination as "the force and power of individuality." He believed that force to be so strong that he deemed anyone naïve enough to battle it as "plainly

superstitious and badly grounded in physical science.” The centuries to follow would yield less contempt for those who attempted to prevent organ rejection; however, despite numerous advances in transplantation techniques during the early twentieth century (4-6), the failed outcome of organ rejection continues to frustrate surgeons to the present day.

### *Discovery of the MHC*

It would be centuries before George Snell would genetically identify “the force and power of individuality” described by Tagliacozzi as the histocompatibility-2 (H2) locus in mice (7,8). Snell identified the H2 locus as the genetic determinant of susceptibility or resistance to transplantable tumors by screening congenic mice, which only differ from the background strain at a small and defined genetic interval. He discovered four different alleles (H2<sup>a</sup>, H2<sup>b</sup>, H2<sup>d</sup>, and H2<sup>p</sup>) of the H2 locus in four different strains of mice (characteristic strain, C57BL/6, BALB/c and P) that are so-named today (8). The discovery of the H2 locus, later known as the Major Histocompatibility Complex (MHC) when homologs were identified in other animal species, dawned a new era of transplantation that would unearth the biological basis for organ rejections observed in centuries past.

Sir Peter Medawar, working independently of Snell, was the first to report that organ rejection was an immunologic process (9). He made this landmark discovery through his pioneering work with allografts, grafts transplanted between two genetically different members of the same species, in humans (10) and rabbits (11). Medawar established the immunologic basis for rejection by observing that the first allograft was rejected after an initial delay (first-set rejection) while a second allograft was rejected much more rapidly (second-set rejection), a hallmark of immunologic memory. This

observation would later transition into his work on immunosuppression as a means of prolonging graft survival, a topic discussed later in this chapter.

In 1975, Peter Doherty and Rolf Zinkernagel discovered that cytotoxic T cell activity only occurred optimally in an H2 compatible system (12,13). The observation that a T cell only recognizes antigen presented on self-MHC, known as MHC-restriction, forever changed the landscape of immunology by linking the MHC locus to the immune response. In subsequent years, scientists extended this seminal observation to the current understanding that the clonal specificity of a T cell is conferred by the ability of a T cell receptor (TCR) to interact with a foreign peptide presented in an MHC molecule on an antigen-presenting cell (APC). For the transplant community, these findings would unite Snell's self/non-self determinant, the MHC, with Medawar's early immunology.

Zinkernagel's observation that the TCR recognizes antigen presented in the context of the MHC eventually defined a mechanism for a confounding phenomenon known as alloreactivity. Alloreactivity is the response observed when T cells from one host are activated by cells derived from a genetically distinct host of the same species (allogeneic), but not cells derived from a genetically identical host (syngeneic) (14-16). Scientists hypothesize that alloreactive T cells are generated through a combination of polymorphism within the MHC locus of a species and the degeneracy of the TCR.

#### *Alloreactivity*

The MHC locus exhibits a great deal of structural homology, but a high degree of polymorphism at the peptide-binding region. It follows that one allelic variant presents different peptides than another allelic variant. Consequently, self-MHC/peptide complexes will not clonally delete T cells with an affinity for allogeneic MHC/peptide

complexes during thymic education. Additionally, T cells are predisposed to interact with the generic structure of the MHC because positive thymic education selects TCRs that possess a low affinity for the MHC. It is therefore expected that a subset of mature T cells will recognize allogeneic MHC/peptide complexes with high affinity (17).

The observation that a single TCR recognizes multiple antigens has been confirmed in several experimental systems (18), and demonstrates that the TCR is degenerate. This characteristic of the TCR partially explains the observation that there is a disproportionately high frequency of T cells that recognize alloantigens, estimated at 0.1-10% (19-21), when compared to the frequency of T cells that recognize a nominal peptide presented by self MHC, estimated at 0.001% (22,23). Further investigation has led to the identification of two distinct types of allorecognition: direct and indirect.

#### *Direct and Indirect Presentation*

One subset of alloreactive T cells recognizes the allogeneic MHC/peptide complex present on donor APCs. This type of recognition is referred to as direct recognition or direct presentation. The direct recognition process is initiated when passenger leukocytes from the donor graft migrate to the secondary lymphoid tissues, where they encounter alloreactive T cells (17). The donor APCs provide both the antigen and the costimulatory signals necessary for T cell activation. Since donor APCs express MHC class I and MHC class II, they activate both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Fully activated alloreactive effector cells then precipitate allograft rejection by producing pro-inflammatory cytokines, providing T cell help for alloantibody production, and killing donor cells directly after migrating into the periphery (17).

A second subset of alloreactive T cells recognizes allogeneic peptides presented by self-MHC. This process is analogous to the one that occurs during the immune recognition of pathogens, where host APCs present foreign peptides on self-MHC. This process is referred to as indirect recognition or indirect presentation. Cross-priming, the ability of host APCs to present foreign peptides on both MHC class I and MHC class II, allows the APC to activate CD4<sup>+</sup> and CD8<sup>+</sup> T cells that recognize alloantigen indirectly. The manner in which the indirect pathway contributes to allograft rejection remains unclear; however, it is known that CD4<sup>+</sup> and CD8<sup>+</sup> T cells that recognize alloantigens by the indirect pathway can cause injury to the allograft (17). Scientists hypothesize that this occurs when effector T cells recognize and subsequently destroy recipient cells in the vasculature of the graft bed that are presenting donor-derived peptides. The destruction of the vasculature may terminate blood supply to the allograft, causing its death (17).

## **Immunosuppression**

### *Early Immunosuppression*

Sir Peter Medawar's discovery that organ rejection is an immunologic process led to a groundswell of attempts to prolong allograft survival by contravening the immune system. Medawar first used cortisone, a glucocorticoid known to have immunosuppressive properties. Unfortunately, the immunosuppressive effects of cortisone were too mild to impede one of the most robust immune reactions discovered to date, and had a modest effect on allograft survival in rabbits (24). In contrast, whole body irradiation (WBI) was reported to prolong kidney allograft survival in related and unrelated donors (9). However, WBI had a remarkably high rate of morbidity in the

absence of bone marrow co-transplantation, and its use in conjunction with glucocorticoids was soon abandoned (9).

#### *Azathioprine to Sirolimus: Modern Immunosuppressants*

The lethality of WBI led scientists to pursue pharmacologic methods of ablating the immune system (25). Azathioprine, its analog 6-mercaptopurine, and its eventual successor mycophenolate mofetil, would gain popularity as early, lymphocyte-specific immunosuppressants. These drugs are purine analogs that are incorporated into RNA and DNA through the *de novo* purine synthesis pathway. They are relatively lymphocyte-specific because lymphocytes primarily use the *de novo* pathway for purine biosynthesis, while other cell types use both the salvage and *de novo* pathways (25). These drugs were effective in prolonging allograft survival, but also proved to be fairly toxic (9).

Cyclosporine A, and its successor tacrolimus (FK506), achieved wide-spread usage for their greater selectivity and lower toxicity relative to cytoablative drugs (26). Cyclosporine A and tacrolimus inhibit the activation of calcineurin, a critical T cell signaling molecule that is required for the activation of the transcription factor NFAT. This inhibition impairs the production of NFAT dependent proteins, including interleukin-2 (IL-2) and CD40 (25), and impairs the maturation of dendritic cells (25,27). Great clinical successes have been achieved with cyclosporine A (9,28-30) and tacrolimus (9,31,32). The outcomes are similar in allograft acceptance and, unfortunately, adverse events (9,33). The side effects include toxicity to the kidney and pancreatic islets, and an increase in the incidence of B cell lymphomas (9,34-38).

A final class of pharmacologic immunosuppressants contains the target of rapamycin inhibitors, consisting of rapamycin, sirolimus, and everolimus. Each of these

drugs inhibits a protein named the target of rapamycin, which is responsible for the entry into cell cycle following IL-2R signaling. The target of rapamycin inhibitors impair T cell proliferation, quenching the T cell response (25). Unfortunately, these drugs also carry substantial side effects, including impaired proliferation in non-lymphoid cells that leads to defects in other organ systems, such as the lung (25,39).

#### *Anti-lymphocyte Serum: An Early Immunotherapeutic*

The discovery that organ rejection was an immunologic process identified a new use for anti-lymphocyte serum, a pool of polyclonal antibodies directed at various lymphocyte markers. Anti-lymphocyte serum was originally developed as an anti-inflammatory several decades before Medawar demonstrated that it had the capacity to prolong allograft survival (40,41). However, the fact that anti-lymphocyte serum non-specifically obliterated the adaptive immune system and failed to prolong allograft survival beyond several weeks stymied its widespread use. However, rather than abandoning the use of antibodies in transplantation, scientists have tailored their use through the advent of monoclonal antibodies (mAbs) with more specific targets and a deeper understanding of the immune system. This strategy has yielded a wide range of mAbs that target cytokine receptors and costimulatory pathways, have the capacity to suppress the immune system and, potentially, induce a state of donor-specific tolerance that we discuss later in this chapter.

#### *Immunosuppression as a Cure and a Pathology*

Despite several decades worth of monumental advances in the field, successful transplantation still requires the long-term administration of immunosuppressive drugs. Over time, these drugs have increased in safety and efficacy; however, they still carry

increased risks of infection, neoplasia and organ toxicity (26). These side effects, while preferable when the alternative outcome is death, limit the use of organ transplantation to cure diseases such as diabetes. Only the most critical diabetic patients will meet the threshold at which the benefits of transplantation will surmount the consequences of life-long immunosuppression, even with cutting-edge islet transplantation procedures embodied by the Edmonton protocol (42).

The immunosuppressive regimen for the Edmonton protocol consists of two doses of dacluzimab (anti-IL-2R mAb) during the peri-transplant period in addition to continuous post-operative treatment with tacrolimus and sirolimus (43). This combination is highly effective at achieving insulin-independence in patients shortly after transplantation, at a rate of 94% at 1 month after transplantation. However, insulin independence wanes over time, to as low as 10% at 5 years after transplantation (44). The loss of insulin-independence may be a direct result of tacrolimus treatment, which exhibits islet-specific toxicity. Additionally, patients have encountered other adverse events related to immunosuppressive therapy, including increased incidences in infection and neoplasia (44).

Unfortunately, the serious and sometimes lethal consequences of immunosuppression therapy are inherent to the non-specific nature of immunosuppressants. Therefore, it is the goal of transplantation scientists to devise a method of prolonging allograft survival in a donor-specific manner that obviates the need for immunosuppression. This goal, also referred to as transplantation tolerance, will ideally become the final frontier in transplantation research.

## **Transplantation Tolerance**

### *The Goal of Transplantation Tolerance*

The concept of transplantation tolerance, the survival of a donor allograft in the absence of immunosuppression, arose to circumvent concerns about the side effects arising from immunosuppression therapy. The idea is to take advantage of a host's natural mechanisms for inducing tolerance to self-antigens in order to induce tolerance to donor antigens without altering the recipient's ability to respond to subsequent immunologic challenges such as infection. This approach eliminates the need for long-term immunosuppression, and broadens the appeal of transplantation as a cure for diseases such as diabetes. Using a combination of improved islet isolation techniques and less toxic immunosuppressive drugs, the Edmonton protocol has recently enjoyed great clinical success in treating critically ill diabetic patients; however, this protocol could be vastly improved by the introduction of tolerance and the elimination of immunosuppression.

### *Peripheral vs. Central Transplantation Tolerance*

The immune system has developed two forms of tolerance for preventing reactivity with self-antigens (autoreactivity), central tolerance and peripheral tolerance. Central tolerance involves the deletion of autoreactive cells in the primary lymphoid organs during development, the thymus for T cells and the bone marrow for B cells. Autoreactive cells that escape deletion during the induction of central tolerance undergo peripheral tolerance in the periphery. These cells fail to receive full activation signals when they first encounter antigen and undergo apoptosis or become non-responsive.

Immunologists have developed ways to achieve both central and peripheral tolerance in a transplant setting.

In 1953, Medawar and colleagues obtained the first experimental evidence that central tolerance leads to the acceptance of skin allografts in freemartin cattle (9,45). During fetal development, freemartin calves – which are fraternal, dizygotic twins – share a common uterine blood supply that leads to the cross-circulation of leukocytes between the offspring (9,46). Consequently, lymphocytes are negatively selected against both self and fraternal antigens. Medawar and colleagues demonstrated that this in-turn leads to the survival of skin allografts obtained from fraternal littermates (9,45). This observation led Main and Prehn to experimentally induce hematopoietic chimerism by treating mice with WBI and allogeneic bone marrow cells, followed by transplantation with skin allografts obtained from the same strain as the donor bone marrow (9,47). They successfully induced tolerance to skin allografts using this experimental model; however, animals developed lethal graft-versus-host disease (GVHD), a reaction where passenger leukocytes in the donor bone marrow or graft mount an immune response against the host. GVHD is a common side effect of bone marrow transplantation when the recipient and donor are not substantially matched for histocompatibility.

In 1975, Kevin Lafferty hypothesized a mechanism for the induction of T cell tolerance in the periphery. He theorized that a T cell requires two signals to become activated where: 1) signal 1 is the interaction between the TCR and the MHC/peptide complex on the APC, and 2) signal 2 is a costimulatory signal provided by the APC (48,49). Subsequently, it was experimentally demonstrated *in vitro* that T cells which received signal 1 in the absence of signal 2 became non-responsive (49,50), a state

referred to as anergy. The existence of a comparable *in vivo* state was debated for years, until it was described independently by Hengartner (51) and Oldstone (52) in 1991 using two similarly elegant experimental systems. These investigators generated double-transgenic mice that express: 1) lymphocytic choriomeningitis virus (LCMV) glycoprotein (GP) (51) or nucleoprotein (NP) (52) under the control of the rat insulin promoter, making the expression islet specific, and 2) a transgenic TCR that recognizes a peptide from the transgenic LCMV protein. In unmanipulated mice, the transgenic T cells emigrate from the thymus into the periphery, but remain tolerant to islets expressing GP or NP (51,52). Infection with LCMV reverses this state of tolerance, leading to a diabetic phenotype resulting from the destruction of pancreatic islets expressing the transgenic protein.

These data support a mechanism where the autoreactive T cells in naïve mice encounter antigen in the absence of costimulation and become tolerant. In contrast, the autoreactive T cells in LCMV-infected mice encounter antigen in the context of an active infection, receive the proper costimulatory signals, and become functionally active. Scientists now understand that the relationship between T cell activation and T cell tolerance is substantially more complicated; however, the underlying model still serves as the conceptual basis for the induction of peripheral transplantation tolerance. It has been demonstrated in numerous systems that the *in vivo* interruption of the costimulatory process leads to the induction of tolerance in an antigen-specific manner (49).

#### *Costimulation Blockade*

In the decades since Lafferty proposed the 2-signal model of T cell activation, scientists have established that a minimum of 3 signaling events are necessary for a T cell

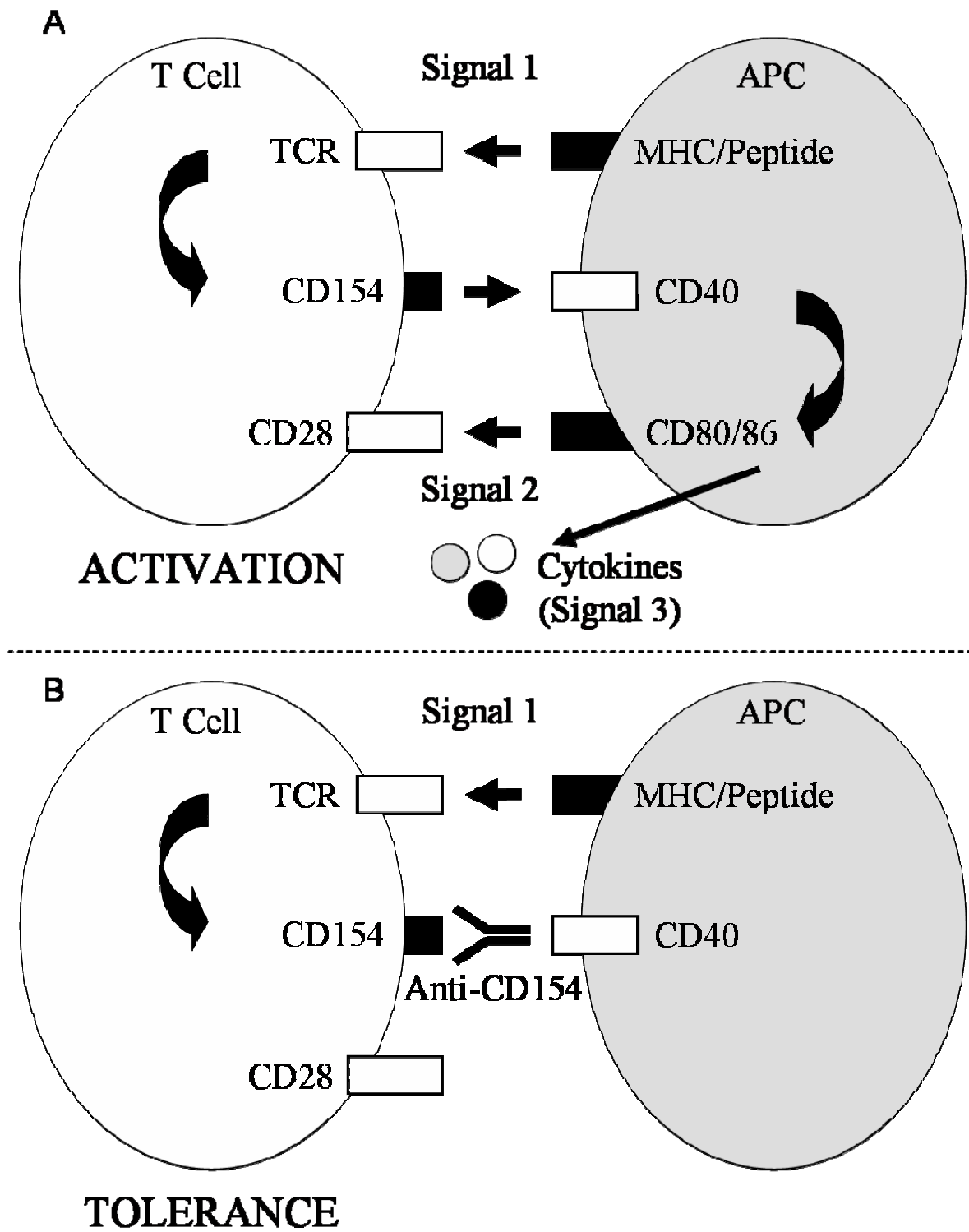
to become completely activated. 1) The T cell receives the primary signal by engaging antigen through the TCR (signal 1); 2) the T cell upregulates CD154, which matures the APC by engaging CD40; and 3) the mature APC upregulates costimulatory molecules (signal 2) and secretes cytokines (signal 3) that provide the T cell with required costimulatory signals (**Fig 1A**). A growing body of work suggests that a T cell must receive a cytokine signal (signal 3) in order to become completely activated (53-56).

Early research demonstrated that the induction of T cell tolerance requires uninterrupted signal 1, leaving subsequent steps as available targets for therapeutic intervention. Our lab has successfully developed a two-step costimulation blockade protocol that interferes with the interaction between CD154 on the T cell and CD40 on the APC (**Fig 1B**). This protocol (57) consists of antigen, in the form of a donor-specific transfusion (DST) of donor splenocytes, and four peri-transplant injections of anti-CD154 mAb, which blocks the interaction between CD154 and CD40 (**Fig 2A**) (58,59). Our lab has shown that this protocol prevents the upregulation of costimulatory molecules on APCs in the donor DST (60), supporting our model that T cells receive the primary signal in the absence of costimulation. Consequently, our protocol leads to the deletion of alloreactive CD8<sup>+</sup> T cells (61) and the generation of a regulatory cell population, which are critical steps for the induction of peripheral transplantation tolerance (62-64). This protocol successfully prolongs allograft survival in mice (57,65) and non-human primates (66-69).

As costimulation blockade protocols move closer to clinical reality, there is growing concern that environmental insults such as viral infections may compromise their efficacy. Infection with LCMV at the time of DST and anti-CD154 mAb treatment

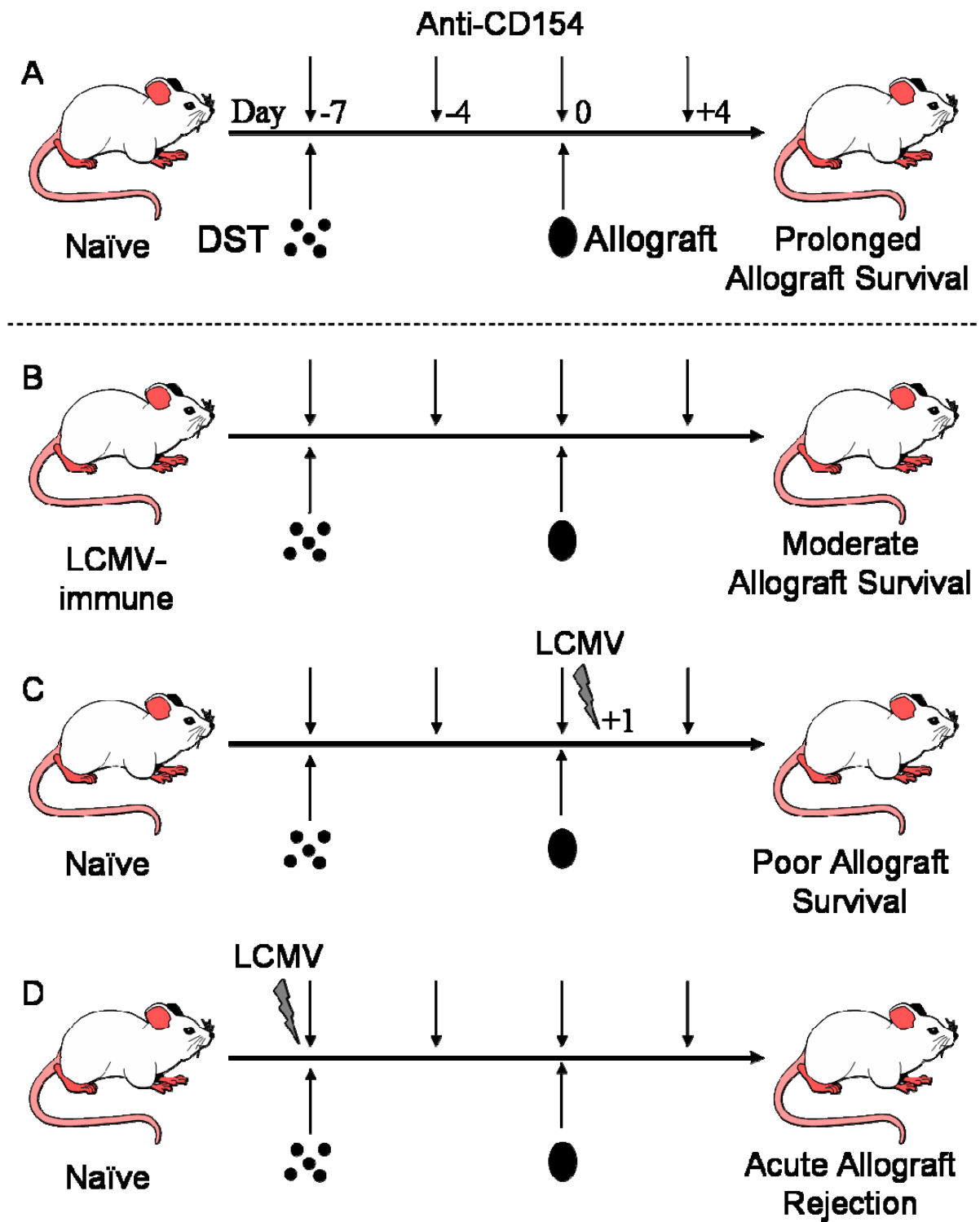
(70,71) or shortly after transplantation (72) shortens allograft survival induced by costimulation blockade. Additionally, Toll-like receptors (TLRs) and their pro-inflammatory role in responding to infection and ischemia are being increasingly seen as a serious obstacle to solid organ transplantation (73-75).

**FIGURE 1: COSTIMULATION BLOCKADE RENDERS T CELLS TOLERANT**



**Figure 1 Legend:** (A) The 3-step model for T cell activation. The TCR recognizes antigen in the form of an MHC/peptide complex on the APC (signal 1), leading to the upregulation of CD154 on the T cell. CD154 then interacts with CD40 on the APC, resulting in the upregulation of costimulatory molecules, including CD80 and CD86, and the expression of pro-inflammatory cytokines. The costimulatory molecules (signal 2) and the pro-inflammatory cytokines (signal 3) provide the additional stimuli needed for complete T cell activation. (B) The anti-CD154 mAb blocks the interaction between CD154 and CD40, preventing the maturation of the APC. The T cell receives signal 1 in the absence of signal 2 or signal 3, and is rendered tolerant.

**FIGURE 2: LCMV ALTERS THE OUTCOME OF COSTIMULATION BLOCKADE**



**Figure 2 Legend:** (A) Our standard costimulation blockade protocol is depicted. Mice are injected with DST, consisting of 10 million donor splenocytes, and anti-CD154 mAb on day -7 relative to transplantation with a fully allogeneic skin or islet graft on day 0. Additional injections of anti-CD154 mAb are given on days -4, 0, and +4 relative to transplantation. This protocol leads to prolonged allograft survival. (B) Treatment of LCMV-immune mice with our costimulation blockade protocol results in shortened skin allograft survival compared to naïve mice (76). (C) LCMV infection one day after transplantation results in shortened skin allograft survival compared to uninfected mice (72). (D) LCMV infection at the time of costimulation blockade results in acute allograft rejection (70,77).

## **Viral Abrogation of Transplantation Tolerance**

### *Heterologous Immunity Alters the Outcome of Allograft Survival*

The classic view of clonal T cell activation is that one TCR interacts with one cognate antigen. However, we now understand that the TCR is degenerate, and can recognize multiple related and unrelated antigens. The ability of an antigen-specific T-cell to cross-react with multiple antigens, known as heterologous immunity, can influence immunodominance, protective immunity, and immunopathology during subsequent viral infections (18,78,79). Of particular interest to transplant scientists, however, are the observations that: 1) virus-specific T cells cross-react with allo-antigens (80-82), and 2) LCMV-immune mice are resistant to tolerance induction by costimulation blockade (71,76). Scientists have hypothesized that cross-reactive memory cells are not deleted by costimulation blockade, leading to a resistance to tolerance induction that culminates in shortened allograft survival in LCMV-immune mice (**Fig 2B**).

### *Viral Infection After Transplantation*

Surprisingly, mice infected with LCMV one day after transplantation also exhibit shortened allograft survival (**Fig 2C**) (72). Interestingly, the longer after transplantation, the less impact LCMV infection has on allograft survival. The deletion of alloreactive CD8<sup>+</sup> T cells is complete at this time, making it improbable that LCMV is interfering with deletion. However, it is possible that LCMV activates and expands new cross-reactive thymic emigrants that have entered the circulation since costimulation blockade treatment. Further research is necessary to elucidate the mechanisms by which LCMV shortens allograft survival during the post-transplantation timeframe.

### *Viral Infection Concurrent to Costimulation Blockade Treatment*

Interestingly, mice infected with LCMV at the time of costimulation blockade treatment exhibit acute allograft rejection (**Fig 2D**) (70,77). The shortened allograft survival correlates with an impaired deletion of alloreactive CD8<sup>+</sup> T cells (70,77); however, the mechanisms underlying these observations remain unclear and will be the focus of this thesis. We hypothesize that LCMV shortens allograft survival by activating Toll-like Receptors (TLRs), which activate APCs independently of the interaction between CD40 and CD154, thereby circumventing the costimulation blockade.

## CHAPTER II: IMMUNOBIOLOGY OF TOLL-LIKE RECEPTORS

### Toll-like Receptors

#### *The Danger Signal Hypothesis*

The demonstration that mature T cells become tolerant when they receive signal 1 in the absence of signal 2 and signal 3 naturally led scientists to question how the immune system is able to determine the appropriate circumstances under which to provide the second and third signals that decide between functional activation and tolerance. This decision-making process is paramount to immunologic function; its outcome means the difference between protective immunity and pathogenesis, and autoimmunity and self-tolerance.

On March 22<sup>nd</sup>, 1996 – some two decades after Lafferty proposed the 2 signal model for T cell activation – three separate research teams published three related articles that shed light on the topic (83-85). These groups demonstrated that neonatal T cells could be activated when they encountered the appropriate APC (84), dose of viral antigen (85), or adjuvant (83). Collectively, these authors modified Medawar's model of neonatal tolerance, and showed that it was the manner under which antigen was presented, not the stage of life, which determined activation versus tolerance (86).

The March 22<sup>nd</sup>, 1996 issue of *Science* reinvigorated the pursuit of the 'danger signal' model, which was postulated by Charles Janeway, Jr. several years earlier (87). The model dictated that complete T cell activation was only achieved with antigen (signal 1) in combination with an environmental insult, a danger signal, which resulted in costimulation (signal 2). Eventually, the search for the molecular basis of the danger

signal focused on pattern recognition receptors (PRRs). PRRs are germ-line encoded receptors that recognize conserved microbial components, known as pathogen-associated molecular patterns (PAMPs), as well as host-derived molecules that are only released during cellular damage. Through evolution, these invariable receptors have gained the ability to distinguish unequivocally self from non-self or altered self.

A diverse array of PRRs have been identified, each of which translates a physical (88) or biological (89,90) insult into a pro-inflammatory response that signals a dangerous presence. During the past decade, the search has centered around Toll-like receptors (TLRs), which have become synonymous with the molecular embodiment of the danger signal (91).

#### *A Conserved Family of Receptors*

Members of the TLR family exhibit structural homology to the Toll receptor in *Drosophila melanogaster*, which is responsible for dorsal-ventral patterning during embryonic development and the immune response to fungal pathogens (89). The first human homolog of Toll was identified in 1997 (92), shortly after Polly Matzinger and colleagues reignited interest in the ‘danger signal’ hypothesis (84). Strikingly, Janeway and colleagues demonstrated that a constitutively active form of human Toll, later known as TLR4, leads to a pro-inflammatory response that favors T cell activation. The response includes the activation of NF- $\kappa$ B, the induction of pro-inflammatory cytokines, and the upregulation of costimulatory molecules in human cells (92). This discovery marked the beginning of a movement that would uncover 12 mammalian Toll-like homologues and their role in bridging the innate and adaptive immune systems.

Twelve members of the TLR family have been identified in mammals. The PAMP-specificities, sub cellular localization, and primary signaling pathways of the first nine members are summarized in **Table I**. Some of these receptors have limited intrinsic signaling capabilities and function only in concert with other receptors. For example, TLR1 and TLR6 become functional when they form heterodimers with TLR2 (91). Additionally, scientists have not ascribed a specific role to some members. TLR11 is non-functional due to a premature stop-codon found in humans, and TLR10 and TLR12 have no confirmed ligands (91). Despite the diversity of patterns recognized by TLRs, they utilize a limited number of signaling pathways to execute their functions (91).

#### *The MyD88 Pathway*

The adaptor molecule myeloid differentiation factor-88 (MyD88) is an essential signaling component for every TLR, except TLR3, and the cytokine receptors IL-1R and IL-18R (91). This includes TLRs that are located extracellularly (TLR1,2,4-6) and endosomally (TLR7-9). Active MyD88 recruits and activates IL-1R associated kinase-4 (IRAK4), IRAK1 and the tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6). Activated TRAF6 catalyzes the activation of the I $\kappa$ B Kinase (IKK), which facilitates the phosphorylation and release of I $\kappa$ B from the transcription factor NF- $\kappa$ B. NF- $\kappa$ B then translocates to the nucleus and activates the expression of pro-inflammatory cytokines, including IL-6, IL-12 and TNF $\alpha$  (**Fig 3**).

The induction of IFN- $\alpha/\beta$  is MyD88-independent in all cells except plasmacytoid dendritic cells (pDCs). pDCs, also known as interferon producing cells (IPCs), express TLR7 and TLR9 with exclusivity. In pDCs, the MyD88-dependent activation of IRAK-1 activates interferon response factor-7 (IRF-7), a transcription factor that rapidly and

efficiently activates the expression of IFN- $\alpha/\beta$  (91). The recognition of viral ssRNA by TLR7 and viral DNA by TLR9 induces copious amounts of IFN- $\alpha/\beta$  in pDCs. It is therefore believed that pDCs play a critical role in driving IFN- $\alpha/\beta$  production in response to certain viruses (**Fig 3**).

#### *The TRIF Pathway*

The induction of IFN- $\alpha/\beta$  in conventional dendritic cells (cDCs), which express TLR3 and TLR4, is dependent on the TIR-domain-containing adaptor protein-inducing IFN- $\beta$  (TRIF) (91). Active TRIF recruits and activates receptor-interacting protein 1 (RIP1) and TRAF-family-member associated NF- $\kappa$ B activator (TANK) binding kinase 1 (TBK1). TBK1 phosphorylates IRF3, which translocates to the nucleus and activates the transcription of IFN- $\alpha/\beta$ . RIP1 also mediates the activation of NF- $\kappa$ B, which activates the transcription of pro-inflammatory cytokines as discussed for the MyD88-dependent pathway (**Fig 3**).

#### *Maturation of APCs by Toll-like Receptors*

In addition to pro-inflammatory cytokine production, TLR-activation upregulates the expression of costimulatory molecules on APCs. Interestingly, the upregulation of costimulatory molecules by the TLR4-agonist LPS requires the adaptor molecule TRIF (93). This observation suggests that the induction of IFN- $\alpha/\beta$  is responsible for the TLR-dependent upregulation of costimulatory molecules. This hypothesis is supported by the findings that IFN- $\alpha/\beta$  promotes the maturation of DCs (94,95). Therefore, TLR-activation has both direct and indirect consequences for the activation of the adaptive immune system.

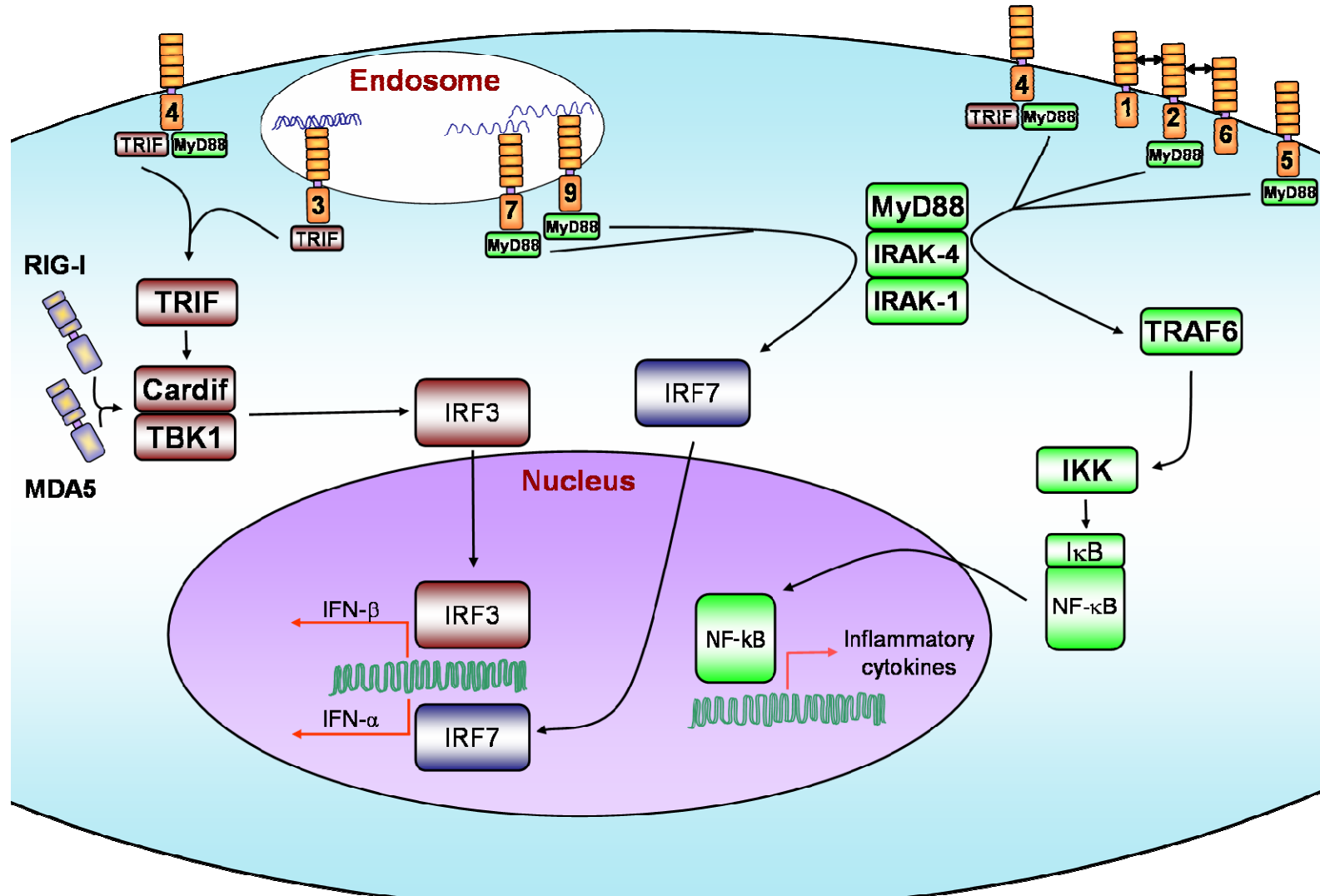
**TABLE I: SUMMARY OF THE TLR FAMILY**

TLR	Major Ligand(s) (Origin)	Localization	Adaptor(s)	Transcription
1 & 2	Tri-acylated Lipopeptides (B)	Extracellular, Heterodimer	MyD88	NF- $\kappa$ B
2	Zymosan (F) CMV, HSV-1, MV, VV (V)	Extracellular Forms heterodimers with surface receptors	MyD88	NF- $\kappa$ B
3	dsRNA (V)	Endosomal	TRIF	IRF3
4	Lipopolysaccharide (B) HSP60, HSP70 (H), Taxol (P) RSV, MMTV (V)	Extracellular	MyD88 TRIF	NF- $\kappa$ B IRF3
5	Flagellin (B)	Extracellular	MyD88	NF- $\kappa$ B
6 & 2	Di-acylated Lipopeptides (B) Peptidoglycan (B)	Extracellular, Heterodimer	MyD88	NF- $\kappa$ B
7	ssRNA (V)	Endosomal	MyD88	NF- $\kappa$ B IRF7
8	ssRNA (V)	Endosomal, Non-functional in mice	MyD88	NF- $\kappa$ B IRF7
9	CpG DNA (B), Viral DNA (V) MCMV (V)	Endosomal	MyD88	NF- $\kappa$ B IRF7

**Table I Legend:** Summary of the major ligands, sub cellular localization and signaling pathways for TLR1 through TLR9 (91)

Key: (B)acterial, (F)ungal, (H)ost, (P)lant, (V)iral (91,96).

**FIGURE 3: TLR SIGNALING**



**Figure 3 Legend:** The intracellular signaling pathways are depicted for TLR family members 1-9. TLR1, TLR2, TLR4, TLR5 and TLR6 signal through the classical MyD88 pathway in conventional DCs. MyD88 recruits IRAK4 and IRAK1, which recruit and activate TRAF6. TRAF6 facilitates the nuclear translocation of the transcription factor NF- $\kappa$ B by activating the kinase IKK. NF- $\kappa$ B activates the transcription of pro-inflammatory cytokines. TLR3 and TLR4 recruit the Cardif/TBK1 complex through the TRIF adaptor molecule, whereas the RNA helicase receptors RIG-I and MDA5 recruit the Cardif/TBK1 complex directly. Active TBK1 induces the nuclear translocation of IRF3, a transcription factor that induces IFN- $\beta$  expression. TLR7, TLR8 (not shown) and TLR9 activate the non-classical MyD88 pathway in pDCs. The MyD88-dependent recruitment of IRAK4 and IRAK1 leads directly to the activation and nuclear translocation of IRF7, a transcription factor that induces large quantities of IFN- $\alpha/\beta$  (91,96).

## **The Innate Response to Viruses and Transplantation**

### *The Innate Anti-viral Response*

The innate immune system has evolved specialized mechanisms that recognize viruses. We hypothesize that these specialized components account for the ability of LCMV to shorten allograft survival (70,77). The innate response to viruses includes a variety of PRRs, including TLRs and cytoplasmic RNA helicase receptors. Collectively, these PRRs play a critical role in initiating a strong anti-viral immune response, and may provide a potent barrier to the induction of transplantation tolerance.

### *TLR-dependent Recognition of Viral Components*

The synthesis of double-stranded RNA (dsRNA) does not occur naturally in mammalian cells, but it is a required step in the replication cycle of many viruses. This combination makes it the perfect candidate for fail-safe recognition by the innate immune system. In 2001, TLR3 was identified as a PRR that recognizes dsRNA (97). As discussed in the previous section, TLR3 is expressed in the endosomal compartment of APCs and generates a strong IFN- $\alpha/\beta$  response through recruitment of the adaptor molecule TRIF (91).

Several years later, it was discovered that viral single-stranded RNA (ssRNA) could be recognized in the endosomal compartment by TLR7 in murine cells (98,99) and its homologue TLR8 (99) in human cells. The recognition of ssRNA by TLR7 and TLR8 is sequence-specific, targeting guanosine- and uridine-rich sequences. This specificity prevents these receptors from recognizing endogenous ssRNA (91). The expression of TLR7 and TLR8 is restricted to pDCs, which are specialized interferon-producing cells. As discussed in the previous section, TLR7 and TLR8 utilize an alternative MyD88

pathway that leads to the activation of IRF7, a transcription factor that leads to high levels of IFN- $\alpha/\beta$  expression.

In addition to viral RNA, TLRs also recognize viral DNA. TLR9 recognizes CpG-rich DNA sequences found in certain herpes viruses, including murine cytomegalovirus (MCMV) (100). Similar to TLR7 and TLR8, TLR9 is located in the endosome of pDCs and activates the MyD88/IRF7 pathway to induce high levels of IFN- $\alpha/\beta$  in response to viral infection (91).

In addition to viral nucleic acids, TLRs also have the capacity to recognize viral glycoproteins. TLR4 is responsible for innate recognition of respiratory syncytial virus fusion protein (101) and murine mammary tumor virus envelope protein (100,102). TLR2 recognizes glycoproteins found on measles virus (103), human cytomegalovirus (104), herpes simplex virus (105) and vaccinia virus (106). The TLR-mediated recognition of viral glycoproteins is virus-specific, and it remains unclear whether the recognition is an evolved response or merely coincidental. The only certainty is that the list will expand in the near future.

#### *TLR-independent Recognition of Viral Components*

The excitement surrounding the 2001 discovery of TLR3 nearly eclipsed the original dsRNA PRR, protein kinase RNA (PKR), which was discovered several decades earlier (107). PKR is a cytoplasmic serine-threonine kinase that binds to dsRNA and activates the transcription factor NF- $\kappa$ B (108). The anti-viral role that PKR plays *in vivo* remains unclear due to the presence of additional PRRs that recognize dsRNA, some with overlapping functions. The PRRs identified to bind to dsRNA include PKR, TLR3, retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5

(MDA5). RIG-I (109) and MDA5 (110) are newly identified RNA helicase receptors localized in the cytoplasm. These receptors recruit the Cardif/TBK1 complex directly, which leads to the activation and nuclear translocation of the transcription factor IRF3. IRF3 induces the transcription of IFN- $\alpha/\beta$ , an important anti-viral cytokine (91).

The cytoplasmic recognition of dsRNA by helicase receptors is postulated to play an important sentinel role in virus-infected cells, where the dsRNA is found in the cytoplasm. In contrast, it is hypothesized that endosomally located TLRs are involved in viral recognition of phagocytosed material, which is trafficked to the endosome. The complex and intricate nature of the dsRNA-recognition machinery has made it difficult to ascribe a particular role to a particular receptor within the network overall (111).

#### *IFN- $\alpha/\beta$ : The Common Antiviral Player*

Despite the diversity of mechanisms that the innate immune system has evolved to sense viral invaders, there are common mediators of anti-viral immunity. The quintessential anti-viral player is, of course, IFN- $\alpha/\beta$ . IFN- $\alpha/\beta$  is a family of cytokines that includes 13 types of IFN- $\alpha$  and a single form of IFN- $\beta$ . As discussed in previous sections, the transcription of IFN- $\alpha/\beta$  is induced upon activation of the transcription factors IRF3 and IRF7 (94,95). All members of the IFN- $\alpha/\beta$  family activate a common dimeric receptor, IFN-RI.

Generally, the production of IFN- $\alpha/\beta$  is biphasic. Most PRRs activate IRF3, which induces a weak response consisting mostly of IFN- $\beta$ . This initial bolus of IFN- $\beta$  signals through IFN-RI, activating the transcription factor IRF7. IRF7 then potently activates the transcription of IFN- $\alpha$  and IFN- $\beta$ . This positive-feedback loop allows the production of large amounts of IFN- $\alpha/\beta$  in response to the activation of IRF3 by PRRs

that recognize viral RNA (112). Interestingly, TLR7, TLR8 and TLR9 directly activate IRF7 in pDCs, which are known to rapidly produce large quantities of IFN- $\alpha/\beta$ . The ability of these receptors to bypass the weak activator IRF3 and activate directly the potent activator IRF7 may explain this important observation (94,95).

IFN- $\alpha/\beta$  produces a diverse set of responses that aids the adaptive immune system in clearing virus and protects uninfected cells from infection. The activation of IFN-RI leads to the phosphorylation of the Janus-associated kinase-1 (Jak1) and the tyrosine kinase-2 (Tyk2), which in-turn activate signal transducer and activator of transcription-1 (STAT-1) and STAT-2. Upon translocation to the nucleus, STAT-1 and STAT-2 form a heterotrimer with IRF9, called the interferon-stimulated growth factor-3 (ISGF3). ISGF3 binds upstream of IFN-inducible genes at sites known as IFN-stimulated response elements (ISRE) and activates their transcription.

IFN-RI is ubiquitously expressed, but transcriptional activation is often cell-type specific. For example, IFN- $\alpha/\beta$  upregulates the expression of MHC and costimulatory molecules on DCs and enhances nitric oxide production in macrophages (113). Biologically, this leads to efficient antigen presentation and T cell priming by DCs and efficient intracellular killing by macrophages. IFN- $\alpha/\beta$  also acts on T cells directly, inducing apoptotic death and inhibiting proliferation of CD8<sup>+</sup> T cells. The apoptotic attrition is antigen-independent and particularly striking in the memory compartment (114). This stands in contrast to impaired proliferation, which is restricted to T cells that have not engaged antigen (115). Recently, a positive role for IFN- $\alpha/\beta$  has been described in T cell activation. It provides a costimulatory signal 3 to CD8<sup>+</sup> T cells, lowering the threshold of activation (54). The mechanisms by which these opposing roles are

regulated in CD8<sup>+</sup> T cells remain unclear, although mounting evidence suggests that the biological outcome is regulated by STAT family members (54,115,116).

### *Response of TLRs to Transplantation and Ischemia*

The tissue injury resulting from surgical procedures induces an inflammatory response known as ischemia-reperfusion injury. Surgeons have long recognized the inflammatory response, but scientists are only beginning to uncover its molecular basis. In recent years, several endogenous ligands have been identified, including heat-shock proteins (HSPs) (117,118), uric acid (119,120), polymeric hyaluronic acid (121) and chromatin (122). It is hypothesized that extensive tissue damage, such as that caused by surgical manipulation, results in the large-scale release of these intracellular ligands into the extracellular space. It is unclear how some of these ligands initiate an inflammatory response, although TLRs have emerged as candidate receptors for several ligands (73).

### *Activation of TLRs May Shorten Allograft Survival Induced by Costimulation Blockade*

Our lab has previously demonstrated that LCMV-infected mice treated with costimulation blockade exhibit shortened allograft survival compared to uninfected mice (70,77). In this dissertation, we test the hypothesis that LCMV shortens allograft survival by activating the innate immune system in a CD154/CD40-independent manner, thereby bypassing the costimulation blockade and its tolerogenic effects. In support of this hypothesis, we present convincing data that TLR agonists shorten allograft survival by impairing the deletion of alloreactive CD8<sup>+</sup> T cells in an IFN- $\alpha/\beta$ -dependent manner.

## CHAPTER III: MATERIALS & METHODS

### Animals

C57BL/6 ( $H2^b$ ), CBA/J ( $H2^k$ ), and BALB/c ( $H2^d$ ) mice were obtained from the National Cancer Institute (Frederick, MD) or The Jackson Laboratory (Bar Harbor, ME). C57BL/10ScSnJ ( $H2^b$ ), C57BL/10ScNJ (TLR4<sup>-/-</sup>,  $H2^b$ , *Tlr4*<sup>lps-del</sup>), C.C3-*Tlr4*<sup>Lps-d</sup>/J (TLR4<sup>-/-</sup>,  $H2^d$ ), B6.129S1-*Il12rb2*<sup>tm1Jm/J</sup> (B6.IL-12R<sup>-/-</sup>,  $H2^b$ ), B6.129S1-*Tlr3*<sup>tm1Flv/J</sup> (B6.TLR3<sup>-/-</sup>,  $H2^b$ ), B6.129S2-*Cd8a*<sup>tm1Mak/J</sup> (B6.CD8α<sup>-/-</sup>,  $H2^b$ ), B6.129S7-*Il1r1*<sup>Tm1Imx/J</sup> (B6.IL-1R<sup>-/-</sup>,  $H2^b$ ), and B6.129P2-*Il18*<sup>Tm1Aki/J</sup> (B6.IL-18<sup>-/-</sup>,  $H2^b$ ) mice were obtained from The Jackson Laboratory and bred at the animal facility at the University of Massachusetts Medical School (Worcester, MA). C57BL/10ScNJ mice carry a null mutation of TLR4 and fail to express either TLR4 RNA or protein (123). C.C3-*Tlr4*<sup>Lps-d</sup>/J mice are derived from the original C3H/HeJ TLR4<sup>-/-</sup> strain (123) in which the TLR4 mutation has been backcrossed onto the BALB/c background at The Jackson Laboratory (<http://jaxmice.jax.org/info/>). C57BL/10-*Rag2*<sup>-/-</sup> mice (RAG2<sup>-/-</sup>,  $H2^b$ ) were obtained from Taconic (Hudson, NY) and bred at the animal facility at the University of Massachusetts Medical School. B6.129S2-*Tnfrsf1b*<sup>tm1Mwm/J</sup> (B6.TNFR2<sup>-/-</sup>,  $H2^b$ ) and B6.129S2-*Tnfrsf1a*<sup>tm1Mac/J</sup> (B6.TNFR1<sup>-/-</sup>,  $H2^b$ ) mice were the gift of Dr. Francis Chan (University of Massachusetts Medical School), who originally obtained them from The Jackson Laboratory. B6.MyD88<sup>-/-</sup> N6 ( $H2^b$ ) (124,125) and B6.IFN-IR<sup>-/-</sup> N12 ( $H2^b$ ) (126,127) mice were the gift of Dr. Egil Lien (University of Massachusetts Medical School), who originally obtained the B6.MyD88<sup>-/-</sup> mice from Dr. Douglas Golenbock (University of Massachusetts Medical School) and the B6.IFN-IR<sup>-/-</sup> mice from Dr.

Jonathan Sprent (Scripps Research Institute, La Jolla, CA). B6.TLR2<sup>-/-</sup> (*H2<sup>b</sup>*) mice were the gift of Dr. Evelyn Kurt-Jones (University of Massachusetts Medical School). (CBA/J × KB5.CBA)F<sub>1</sub> CD8<sup>+</sup> T cell TCR-transgenic mice were developed by Dr. A. Mellor (Medical College of Georgia, Augusta, GA) and bred in our animal facility (128). The TCR transgene is expressed in CBA (*H2<sup>k</sup>*) mice by CD8<sup>+</sup> cells, and the transgenic TCR has specificity for native H2-K<sup>b</sup> (128).

All animals were certified to be free of Sendai virus, pneumonia virus of mice, murine hepatitis virus, minute virus of mice, ectromelia, LDH elevating virus, mouse poliovirus, Reo-3 virus, mouse adenovirus, LCMV, polyoma, *Mycoplasma pulmonis*, and *Encephalitozoon cuniculi*. They were housed in a specific pathogen-free facility in microisolator cages, given autoclaved food and acidified water, and maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School and the recommendations in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996).

#### **Generation of CD8<sup>+</sup> KB5 TCR Transgenic Synchronic CBA/J Mice**

To study the fate of host alloreactive CD8<sup>+</sup> T cells in mice treated with costimulation blockade, we used KB5 TCR-transgenic hematopoietic synchronic mice generated as described previously (129). Briefly, CBA/J non-transgenic mice were treated with 200 cGy of whole-body gamma irradiation from a <sup>137</sup>Cs source (Gammacell 40; Atomic Energy of Canada, Ottawa, Ontario, Canada or Mark I-30 Series 2000 Ci; JL Shepherd & Associates, San Fernando, CA) and given a single i.v. injection of 0.5×10<sup>6</sup> KB5 bone marrow cells. In order to allow sufficient time for hematopoietic chimerism to

develop, the KB5 synchimeric mice received skin allografts at 12–17 weeks of age, 8–12 weeks after irradiation and injection of KB5 transgenic bone marrow. We refer to these mice as KB5 synchimeric mice (129).

### **Skin Transplantation Procedures**

Recipient mice of the specified strain were treated with a donor-specific transfusion (DST), anti-CD154 mAb, and given skin allografts, as described previously (65,130). Briefly, the DST, consisting of  $10^7$  splenocytes from female BALB/c or C57BL/6 mice, was injected intravenously in a volume of 0.5 ml. DST was given on day –7 relative to the day of skin transplantation. Purified hamster anti-mouse CD154 mAb (clone MR1) was obtained from the National Cell Culture Center (Minneapolis, MN). The concentration of contaminating endotoxin was determined by a commercial firm (Charles River Endosafe, Charleston, SC) and was uniformly <10 units EU per mg of mAb. Mice were injected i.p. with anti-CD154 mAb (0.5 mg per dose) on days –7, –4, 0, and +4 relative to the day of skin transplantation. Full-thickness skin grafts 1-2 cm in diameter were obtained from the flanks of donor mice and transplanted onto the dorsal flanks of recipients (65). Graft rejection was defined as the first day on which the entire graft was necrotic (65,130).

### **Islet Transplantation Procedures**

Recipient mice 6-12 wks of age were rendered diabetic by a single i.p. injection of streptozotocin (150 mg/kg). Diabetes was defined as a plasma glucose concentration of >250 mg/dl on at least two successive tests on two different days. Plasma glucose concentration was measured using a Beckman II glucose analyzer (Beckman, Fullerton, CA). All diabetic animals were treated with s.c. timed release insulin pellets (Linbits,

Linshin, Ontario, Canada) that were removed at the time of islet transplantation. Pancreatic islets were isolated by collagenase digestion (57) and were transplanted at a dose of 20 per g body weight into the renal subcapsular space of chemically diabetic recipients. Grafts that did not reduce plasma glucose concentration to <250 mg/dl within 48 h were deemed technical failures and were excluded from analysis. Mice were monitored every 2-3 days using Clinistix® reagent strips (Bayer, Elkhart, IN) for glycosuria. Plasma glucose concentration was measured in all animals that tested positive for glucose in their urine. Graft rejection was defined as recurrence of a plasma glucose concentration >250 mg/dl on two successive days. In the case of all islet recipients that were normoglycemic at the end of the period of experimental observation, graft function was confirmed by unilateral nephrectomy of the kidney bearing the transplant and documentation of the reappearance of diabetes.

### **Viruses and Cell Lines**

LCMV, strain Armstrong, and Pichinde virus (PV), strain AN3739, were prepared in baby hamster kidney cells as described previously (131). Vaccinia virus (VV) was propagated on L-929 cells (131). MCMV stocks, strain Smith, were obtained from the salivary glands of infected BALB/c mice (131). Mice were inoculated i.p. with  $5 \times 10^4$  PFU of LCMV,  $2 \times 10^7$  PFU of PV,  $10^5$  PFU of MCMV, or  $5 \times 10^4$  PFU of VV within 4 hours after DST treatment. The adherent L-929 cell line (NCTC clone 929), the RMA cell line ( $H2^b$ ), and the P815 cell line ( $H2^d$ ) were obtained from the ATCC (Manassas, VA) and maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 2 mM L-glutamine (Sigma, St Louis, MO).

### **Preparation and Injection of Toll-like Receptor Agonists**

Poly(inosinic acid):poly(cytidylic acid) (poly (I:C), Sigma and GE Healthcare, Piscataway, NJ) was dissolved in Dulbecco's PBS (D-PBS) at a concentration of 1 mg/ml. Stock was filtered through 0.45  $\mu$ m sterile nylon mesh (Becton Dickinson, Franklin Lakes, NJ) and stored at  $-20^{\circ}\text{C}$  until needed. The CpG oligonucleotide (5'-CT CCC AGC GTG CGC CAT-3') was generated on a phosphothioate backbone by Trilink BioTechnologies (San Diego, CA). Upon receipt, lyophilized CpG was suspended in D-PBS and stored at  $-20^{\circ}\text{C}$  until needed. LPS from *Escherichia coli* 0111:B4 (Sigma) was repurified as described previously (132), except that phenol-PBS phase separation was conducted at  $2,000\times g$  for 30 min in order to accommodate larger volumes. Repurified LPS was suspended in D-PBS with an assumed 10% loss during repurification (132). Purified LPS was stored at  $4^{\circ}\text{C}$  until used. Pam<sub>3</sub>Cys-Ser-(Lys)<sub>4</sub> (EMC Microcollections, Tuebingen, Germany) was dissolved in D-PBS and stored at  $-20^{\circ}\text{C}$  until needed. Recombinant mouse IFN- $\beta$  was obtained from R&D Systems (Minneapolis, MN). Mice were injected i.p. with the indicated ligand and dose in a volume of 0.5 ml of D-PBS within 1 h of DST treatment using ligand doses determined to be active in preliminary dose-titration experiments.

### **Flow Microfluorometry and Antibodies**

A mouse hybridoma cell line secreting the KB5-specific clonotypic Desiré (DES) mAb (133) was a gift from Dr. J. Iacomini (Harvard Medical School). FITC-conjugated anti-mouse IgG2a developing reagent (clone R19-15) for DES, anti-mouse CD8 $\alpha$ -PerCP (clone 53-6.7), anti-mouse CD44-APC (clone IM7), anti-human/mouse Ki-67-PE (clone

B56), anti-mouse IFN- $\gamma$ -PE (clone XMG1.2), anti-human/mouse active caspase-3-PE (clone C92-605), and purified anti-mouse CD16/32 (clone 2.4G2) mAbs were obtained from BD PharMingen (San Diego, CA). Isotype control mAbs and the Annexin V-PE Apoptosis Detection Kit I, which contains annexin V-PE and 7-AAD, were also obtained from BD PharMingen.

Single-cell suspensions from spleen or heparinized whole blood were made in RPMI 1640 and washed in D-PBS containing 1% fetal clone serum (HyClone, Logan, Utah) and 0.1% sodium azide (Sigma). Samples were incubated in anti-CD16/32 for 5 minutes at 4°C prior to incubation for 20 minutes with the clonotypic DES mAb. Samples were washed and incubated for 20 minutes with fluorescently labeled antibodies to cell surface markers and the secondary development antibody for DES. Samples were processed with FACS lysing solution (BD Biosciences, San Diego, CA) in accordance with the manufacturer's protocol. Labeled cells were washed, fixed with 1% paraformaldehyde (Polysciences, Warrington, PA) in D-PBS, and analyzed with a FACSCalibur, FACScan, or LSRII instrument (BD Biosciences) and FlowJo Software (Tree Star, Ashland, OR). Lymphoid cells were gated according to their light-scattering properties.

### **Annexin V Staining**

Apoptosis was quantified using the Annexin V-PE Apoptosis Detection Kit I (BD PharMingen) according to the manufacturer's instructions. Briefly, red blood cells were lysed with 0.84% NH<sub>4</sub>Cl solution. The samples were stained with the clonotypic antibody DES in D-PBS supplemented with 1% fetal clone serum and 0.1% sodium azide. The samples were then washed with Annexin V Binding Buffer (BD PharMingen)

and stained with anti-mouse IgG2a-FITC, anti-mouse CD8 $\alpha$ -PE-Cy7, 7-AAD, and annexin V-PE diluted in binding buffer. Samples were analyzed within 2 h of staining with a Becton Dickinson LSRII (BD Biosciences).

### **Ki-67 Staining**

Intracellular Ki-67 expression was determined in splenocytes directly *ex vivo*. Red blood cells were removed from samples with 0.84% NH<sub>4</sub>Cl solution and stained with the clonotypic antibody DES. The samples were washed and stained with anti-mouse IgG2a-FITC, anti-mouse CD8 $\alpha$ -PerCP, and anti-mouse CD44-APC. The samples were then fixed and permeabilized with Cytofix/Cytoperm<sup>TM</sup> solution and stained with either anti-human/mouse Ki-67-PE or a mouse IgG<sub>1k</sub>-PE isotype control antibody (clone MOPC-21, BD PharMingen) diluted in Perm/Wash Buffer<sup>TM</sup> (BD PharMingen). The samples were analyzed with a Becton Dickinson FACSCalibur (BD Biosciences).

### **Intracellular IFN- $\gamma$ Assay**

IFN- $\gamma$  production was assessed in spleen cells using the BD Cytofix/Cytoperm<sup>TM</sup> Kit with GolgiPlug<sup>TM</sup> (BD Pharmingen) as previously described (76,134). Briefly, single-cell suspensions were prepared from spleens and red blood cells were lysed using 0.84% NH<sub>4</sub>Cl. Splenocytes (2x10<sup>6</sup> cells) were incubated for 5 hours in GolgiPlug<sup>TM</sup> and 10 U/mL rIL-2 (R&D Systems, Minneapolis, MN) at 37°C in the presence of a syngeneic (RMA, *H2<sup>b</sup>*) or allogeneic (P815, *H2<sup>d</sup>*) cell line (0.5x10<sup>6</sup> cells per stimulation). Samples were stained with anti-CD8 $\alpha$ -PerCP, followed by fixation and permeabilization with BD Cytofix/Cytoperm<sup>TM</sup> and staining with anti-IFN- $\gamma$ -PE.

### **IFN- $\alpha$ / $\beta$ Bioassay**

IFN- $\alpha$ / $\beta$  was measured using a standard virus-inhibition bioassay (135,136). Unheparinized whole blood was obtained from mice 14 to 16 h after costimulation blockade treatment and centrifuged to obtain serum, which was diluted two-fold across a 96-well plate. Each well was seeded with  $3 \times 10^4$  mouse L-929 cells and incubated overnight.  $2 \times 10^5$  PFUs of vesicular stomatitis virus (VSV), strain Indiana, was then added to each well except for the uninfected control wells. Cultures were observed by microscopy for cytopathic effects (CPE) 2 days later. The IFN- $\alpha$ / $\beta$  titer was determined as the reciprocal of the dilution that provided 50% protection from CPE (135,136). In blocking experiments, sera were incubated in one KU of neutralizing anti-mouse IFN- $\alpha$  and one KU of neutralizing anti-mouse IFN- $\beta$  (EMD Biosciences, San Diego, CA) prior to serial dilution.

### ***In Vivo* Cytotoxicity Assay**

The *in vivo* cytotoxicity assay was performed as previously described (137-139). Briefly, single-cell suspensions were prepared from spleens that were harvested from C57BL/6 ( $H2^b$ , syngeneic) or BALB/c ( $H2^d$ , allogeneic) mice. Cells were washed with HBSS (Invitrogen Life Technologies), and each population was incubated in either 2.5 or 0.625  $\mu$ M CFSE (Invitrogen Life Technologies) for 15 min at 37°C. Splenocytes were washed with HBSS and combined at equal ratios.  $3 \times 10^7$  total cells were adoptively transferred i.v. into recipient mice that had been treated with 0.025mg of a depleting anti-NK1.1 antibody 24 to 48 hours earlier. Spleens from recipient mice were harvested 4 h later and lysed with FACS lysing solution (BD Biosciences) according to the manufacturer's instructions. Samples were analyzed with a FACSCalibur instrument

(BD Biosciences) and FlowJo software (Tree Star). Specific lysis was calculated by comparing the relative survival of each target population to the survival in NK cell-depleted naive mice, using the following equation as described previously (140):  $100 - (((\text{percentage of target population in experimental} / \text{percentage of syngeneic population in experimental}) / (\text{percentage of target population in NK1.1-depleted naive} / \text{percentage of syngeneic population in NK1.1-depleted naive})) \times 100)$ .

### **Statistics**

Statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA). Three or more means were compared by one-way ANOVA. Two means were compared using a two-tailed student's t-test. Allograft survival curves were generated by the Kaplan and Meier method and compared by the log-rank test. Duration of allograft survival is presented as the median. P values <0.05 are considered to indicate statistical significance.

# **CHAPTER IV: TLR AGONISTS IMPAIR COSTIMULATION BLOCKADE- INDUCED SKIN ALLOGRAFT SURVIVAL THROUGH A CD8-DEPENDENT MECHANISM**

## **Introduction**

Costimulation blockade protocols have been studied extensively as potential alternatives to the use of immunosuppressive drugs in transplantation procedures. Unfortunately, infection with LCMV at the time of DST and anti-CD154 mAb treatment abrogates the prolonged allograft survival induced by this protocol, presenting a potent barrier to its eventual use in the clinic. We are hopeful that a more thorough understanding of the mechanisms by which viruses shorten allograft survival will help to generate a clinically viable protocol that is both safe and effective in the presence of environmental stressors.

We hypothesized that infection with LCMV at the time of costimulation blockade treatment impairs allograft survival and the deletion of alloreactive CD8<sup>+</sup> T cells in part by activating TLRs. In order to obtain proof of principle, we first tested whether purified TLR agonists share LCMV's ability to shorten allograft survival. We also explored the role that alloreactive CD8<sup>+</sup> T cells play in this shortened allograft survival, and the means by which the deletion of these cells is impaired.

## Results

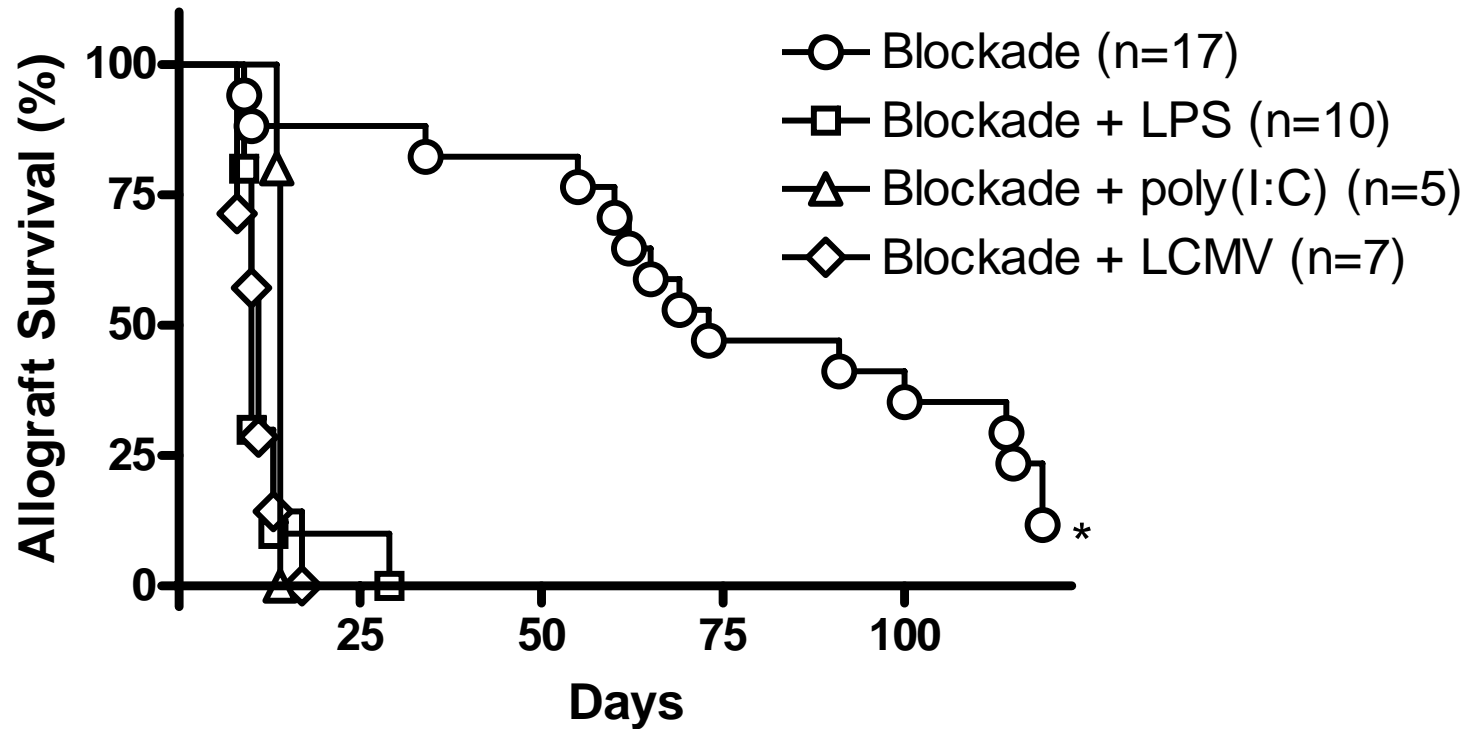
### 1. *TLR agonists shorten skin allograft survival induced by costimulation blockade*

We first confirmed the observation that infection with LCMV at the time of DST in our costimulation blockade protocol shortens skin allograft survival (70,72,141). C57BL/6 mice were treated with DST and anti-CD154 mAb on day -7, and then randomly divided into four groups. Group 1 received no additional pre-conditioning, whereas groups 2, 3 and 4 were injected with  $5 \times 10^4$  PFU of LCMV, 50  $\mu$ g of poly(I:C) or 100  $\mu$ g of re-purified LPS on day -7, respectively. All mice received additional injections of anti-CD154 mAb on days -4, 0 and +4, and a fully allogeneic BALB/c skin allograft on day 0. A schematic for our treatment protocol is depicted in **Fig 2A**.

In group 1, which received only the costimulation blockade treatment, mice exhibited prolonged allograft survival with a median survival time (MST) of 73 days (n=17; **Fig 4**). This stood in contrast to Group 2, which exhibited shortened allograft survival after LCMV infection (MST=11 days, n=7), as reported previously (70,72,141). Similarly, the administration of either the TLR3 agonist poly(I:C) (MST=14 days, n=5) or the re-purified TLR4 agonist LPS (MST=10 days, n=10) both led to acute allograft rejection (**Fig 4**). Skin allograft survival in mice treated with TLR agonists was similar to that observed in LCMV-infected mice (p=N.S.).

These data indicate that TLR activation is as effective as LCMV infection in abrogating skin allograft survival induced by costimulation blockade.

**FIGURE 4: TLR AGONISTS SHORTEN BLOCKADE-INDUCED ALLOGRAFT SURVIVAL IN C57BL/6 MICE**



**Figure 4 Legend:** C57BL/6 mice were treated with a BALB/c DST on day -7 and four doses of anti-CD154 mAb on days -7, -4, 0, and +4 relative to skin allografting on day 0 (standard costimulation blockade protocol). Additional indicated treatments were performed on day -7 as described in *Materials and Methods*. All groups received BALB/c skin allografts on day 0.

\*Significantly different from all other groups,  $p < 0.0001$ .

## *2. TLR agonists impair the deletion of alloreactive CD8<sup>+</sup> T cells*

We hypothesized that TLR agonists abrogate skin allograft survival by impairing the deletion of alloreactive CD8<sup>+</sup> T cells. To determine the fate of alloreactive CD8<sup>+</sup> T cells in a normal microenvironment, we used KB5 TCR transgenic hematopoietic synchimeric mice. The CD8<sup>+</sup> TCR transgene is expressed on a CBA (*H2<sup>k</sup>*) background and has specificity for H2-K<sup>b</sup>. Synchimeric mice were generated by injecting lightly irradiated CBA/J mice with KB5 transgenic bone marrow 8 weeks prior to commencing the experimental protocol. In this system, synchimeric mice circulate a self-renewing population of alloreactive anti-H2-K<sup>b</sup> CD8<sup>+</sup> T cells that mature in a normal microenvironment and can be identified by microfluorometry using the clonotypic antibody DES (129). To test our hypothesis that TLR agonists impair alloreactive CD8<sup>+</sup> T cell deletion, we co-injected costimulation blockade-treated synchimeric mice with TLR2 (Pam<sub>3</sub>Cys), TLR3 (poly(I:C)), TLR4 (LPS), or TLR9 (CpG) agonists and monitored the level of KB5 transgenic T cells in the peripheral blood.

As expected (129,141-143), KB5 synchimeric mice treated with C57BL/6 DST on day -7 and anti-CD154 mAb on days -7 and -4 exhibited a marked depletion of alloreactive CD8<sup>+</sup> T cells when analyzed on day -1, the day before transplantation in this protocol (**Table II**). The circulating level of CD8<sup>+</sup> DES<sup>+</sup> T cells fell from an average of  $4.69 \pm 1.20\%$  of peripheral blood lymphocytes (PBLs) prior to treatment to  $0.55 \pm 0.50\%$  (n=25) on day -1. Deletion of alloreactive CD8<sup>+</sup> T cells was impaired by all TLR agonists tested ( $p < 0.0001$  vs. DST and anti-CD154 mAb group; **Table II**).

To confirm that the failure to delete alloreactive CD8<sup>+</sup> T cells was associated with short skin allograft survival, these mice were then transplanted with C57BL/6 skin

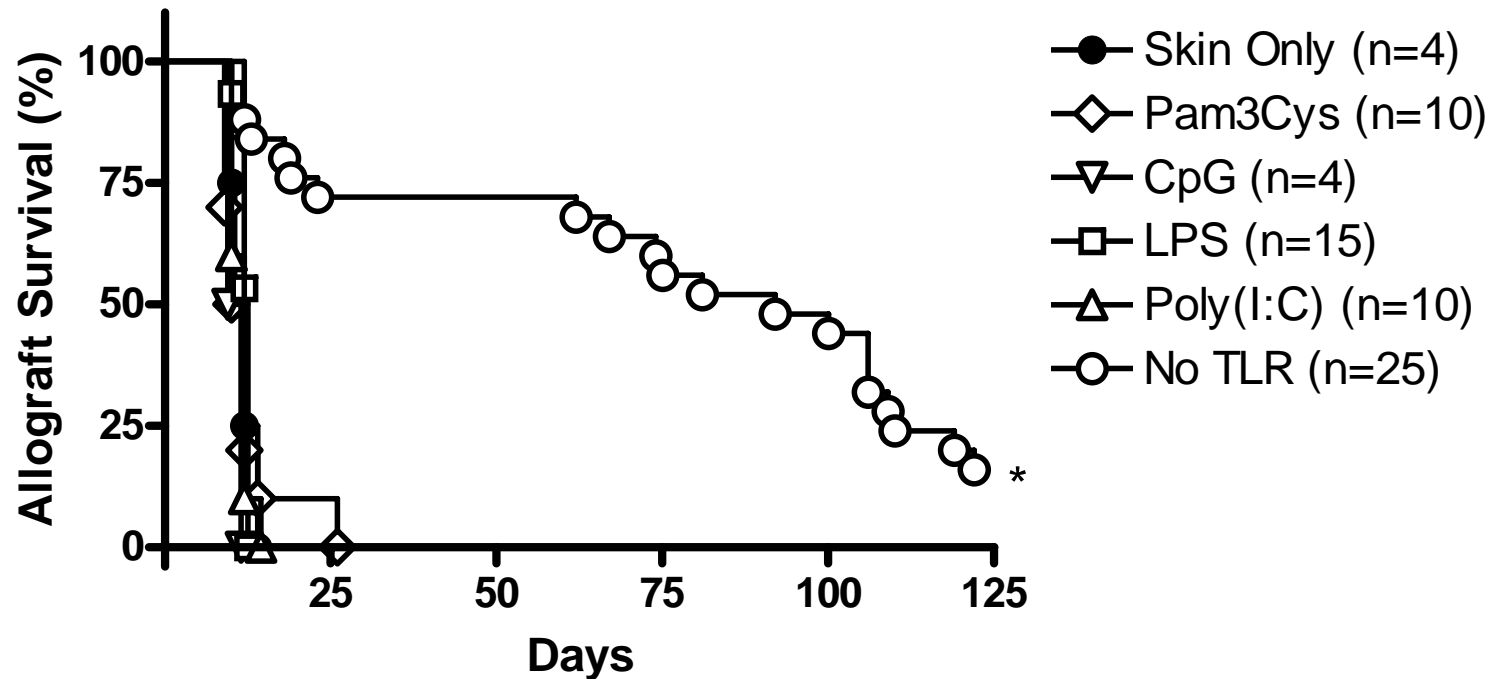
allografts on day 0 and given additional injections of anti-CD154 mAb on days 0 and +4. Similar to C57BL/6 mice, we observed that synchimeric mice treated with DST and anti-CD154 mAb exhibited prolonged skin allograft survival (MST=92 days, n=25; **Fig 5**). Corresponding to their failure to delete alloreactive CD8<sup>+</sup> T cells, all KB5 synchimeric mice treated with costimulation blockade plus a TLR agonist exhibited short skin allograft survival (MSTs=11 to 13 days,  $p<0.0001$  vs. DST and anti-CD154 mAb treated mice; **Fig 5**)

**TABLE II: TLR AGONISTS IMPAIR THE DELETION OF ALLOREACTIVE CD8<sup>+</sup> T CELLS IN BLOOD**

Treatment	TLR agonist (dose)	N	Chimerism Pre-treatment (%)	Chimerism 6d post-treatment (%)	Deletion (%)
<i>DST + Anti-CD154</i>	<i>None</i>	20	4.69 ± 1.20	0.55 ± 0.50*	88.3*
<i>DST + Anti-CD154</i>	<i>50µg Pam<sub>3</sub>Cys</i>	10	5.33 ± 0.79	4.37 ± 0.78	18.0
<i>DST + Anti-CD154</i>	<i>500µg Poly(I:C)</i>	10	5.38 ± 1.05	5.72 ± 1.24	-6.3
<i>DST + Anti-CD154</i>	<i>100µg LPS</i>	10	4.35 ± 0.93	3.14 ± 1.12	27.9
<i>DST + Anti-CD154</i>	<i>50µg CpG</i>	5	5.45 ± 0.53	3.59 ± 0.54	34.1
<i>No Treatment</i>	<i>None</i>	8	3.48 ± 1.08	4.05 ± 0.67	-16.5

**Table II Legend:** KB5 synchimeric mice were untreated or treated with C57BL/6 DST, anti-CD154 mAb, and the indicated TLR agonist on day 0 and anti-CD154 mAb on day +3 relative to recovery of blood for analysis on day 6. Peripheral blood mononuclear cells were stained with the anti-DES mAb, followed by the DES detection mAb (anti-mouse IgG2a-FITC) and anti-CD8α-PerCP mAb as described in *Materials and Methods*. \*p<0.0001 vs. all other groups.

**FIGURE 5: TLR AGONISTS SHORTEN ALLOGRAFT SURVIVAL IN SYNCHIMERIC CBA/J MICE**



**Figure 5 Legend:** KB5 syngeneic mice were given DST and anti-CD154 mAb according to our standard costimulation blockade protocol, with or without injection of the indicated agent on day -7 relative to skin grafting on day 0. The skin only group received no pre-conditioning prior to transplantation. All groups received C57BL/6 skin allografts on day 0.

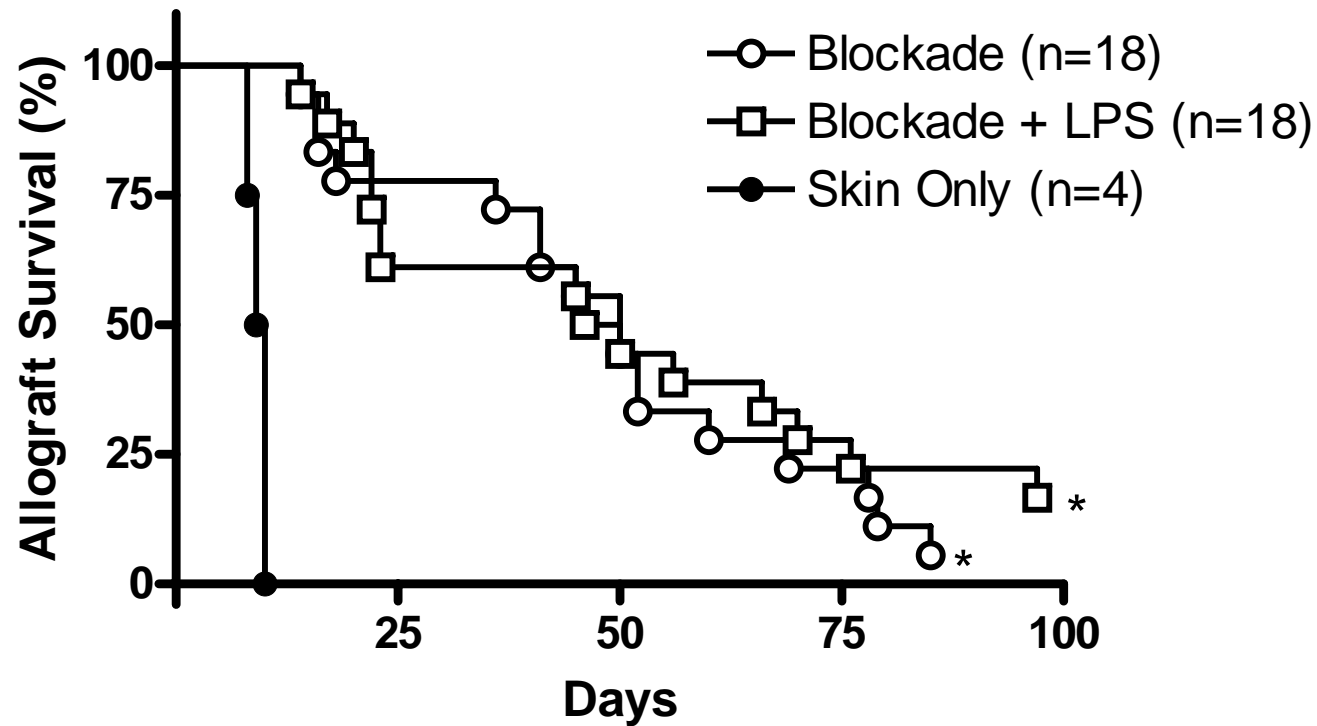
\*Significantly different from all others,  $p < 0.0001$ .

### 3. *CD8<sup>+</sup> cells are required for LPS to shorten allograft survival*

We hypothesized that the ability of TLR agonists to impair the deletion of alloreactive CD8<sup>+</sup> T cells was responsible for the shortened skin allograft survival. To test this, B6.CD8 $\alpha^{-/-}$  mice were treated with costimulation blockade with or without co-injection with LPS. Allograft survival was comparable in blockade-treated B6.CD8 $\alpha^{-/-}$  mice in the absence (MST=50 days, n=18) or presence (MST=48 days, n=18, p=N.S.) of LPS (**Fig 6**). As expected (129), untreated B6.CD8 $\alpha^{-/-}$  mice rapidly rejected BALB/c skin allografts (MST=10 days, n=4; **Fig 6**).

These data demonstrate a critical role for CD8<sup>+</sup> cells in impairing skin allograft survival induced by costimulation blockade.

**FIGURE 6: CD8<sup>+</sup> CELLS ARE REQUIRED FOR LPS TO SHORTEN SKIN ALLOGRAFT SURVIVAL**



**Figure 6 Legend:** B6.CD8 $\alpha^{-/-}$  mice were given DST and anti-CD154 mAb according to our standard protocol with or without injection of LPS on day -7 relative to skin grafting on day 0. All groups received BALB/c skin allografts on day 0. The difference in skin allograft survival between groups receiving costimulation blockade or costimulation blockade and LPS was not significantly different (p=N.S.).

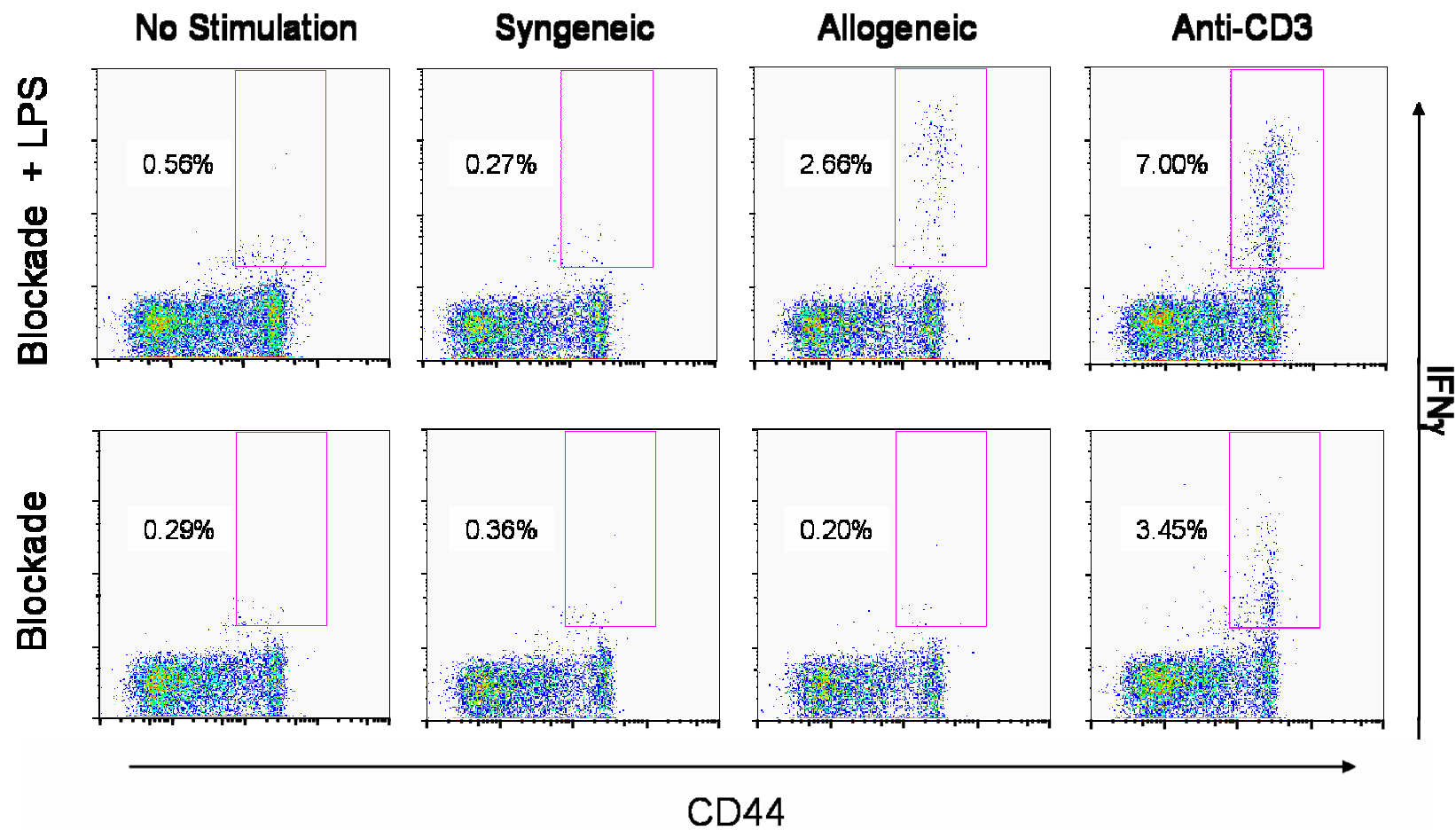
#### 4. Primed CD8<sup>+</sup> T cells are present after LPS-induced skin allograft rejection

To confirm that LPS prevented the deletion of functionally active alloreactive CD8<sup>+</sup> T cells, we recovered spleen cells from C57BL/6 mice 22 days after initiation of treatment with costimulation blockade, which was 15 days after receiving a BALB/c skin allograft. These cells were tested for their ability to produce IFN- $\gamma$  upon *in vitro* stimulation with alloantigen. Rapid production of IFN- $\gamma$  is a sensitive marker of functional effector/memory alloreactive CD8<sup>+</sup> T cells (76).

In C57BL/6 mice treated with costimulation blockade, the percentage of CD8<sup>+</sup> T cells producing IFN- $\gamma$  in response to *H2<sup>d</sup>* donor-specific allogeneic stimulation was low (0.20% and 0.11%, n=2) and similar to that observed after stimulation with syngeneic cells (0.36% and 0.29%, n=2; **Fig 7**). In contrast, the percentage of CD8<sup>+</sup> T cells producing IFN- $\gamma$  in mice treated with costimulation blockade and LPS was markedly higher after allogeneic stimulation (2.68% and 3.45%, n=2) than after syngeneic stimulation (0.27% and 0.45%, n=2; **Fig7**)

These data indicate that TLR agonists impair the deletion of functional effector CD8<sup>+</sup> T cells, permitting the generation of memory CD8<sup>+</sup> T cells in mice given skin allografts, supporting our hypothesis that CD8<sup>+</sup> T cells are responsible for the shortened skin allograft survival.

FIGURE 7: PRIMED CD8<sup>+</sup> T CELLS ARE PRESENT AFTER LPS-INDUCED SKIN ALLOGRAFT REJECTION



**Figure 7 Legend:** C57BL/6 mice ( $H2^b$ ) were given DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of LPS on day -7 relative to skin grafting on day 0. All groups received BALB/c ( $H2^d$ ) skin allografts on day 0. Animals were sacrificed 15 days after skin allograft transplantation. At this time point, mice treated with costimulation blockade had intact grafts, whereas mice treated with costimulation blockade plus LPS had rejected their grafts. Splenocytes received no stimulation, syngeneic stimulation (RMA,  $H2^b$ ), allogeneic stimulation (P815,  $H2^d$ ), or polyclonal anti-CD3 stimulation for 5 hours in the presence of brefeldin-A. Cells were stained for anti-CD44-FITC and anti-CD8 $\alpha$ -PerCP, followed by permeabilization and staining with anti-IFN- $\gamma$ -PE. Representative dot plots showing CD44 and IFN- $\gamma$  expression on gated CD8 $^+$  lymphocytes are shown.

### *5. LPS prevents the deletion and promotes the expansion of alloreactive CD8<sup>+</sup> cells*

We next wanted to determine whether LPS prevents the deletion of alloreactive T cells by promoting the survival of these cells or by enhancing the proliferation of a surviving subset of these cells. To address this, we quantified the absolute number of transgenic alloreactive CD8<sup>+</sup> T cells in the spleens of KB5 synchimeric mice at 24, 48, 72, and 96 h after treatment with DST and anti-CD154 mAb in the presence or absence of LPS co-administration.

LPS prevented the disappearance of the transgenic population from the spleen as early as 24 h after treatment with costimulation blockade (**Table III**). In mice treated with costimulation blockade,  $6.17 \pm 0.89 \times 10^5$  splenic KB5 CD8<sup>+</sup> T cells were recovered as compared to  $21.9 \pm 2.61 \times 10^5$  cells in mice receiving costimulation blockade and LPS (**Table III**;  $p < 0.01$ ). A small but significant decrease in cell number was observed in mice treated with costimulation blockade and LPS compared to untreated mice ( $p < 0.05$ ), but this change was statistically similar to a decrease caused by treatment with LPS alone ( $p = \text{N.S.}$ ). The transgenic CD8<sup>+</sup> T cell population then expanded dramatically in animals treated with costimulation blockade and LPS at 72 h. This trend was not observed in mice treated with LPS alone (**Table III**).

These data show that LPS prevents the loss of alloreactive CD8<sup>+</sup> T cells as early as 24 h after costimulation blockade treatment, and supports their antigen-specific expansion and accumulation by 72 h after treatment.

**TABLE III: LPS IMPAIRS THE DELETION OF ALLOREACTIVE CD8<sup>+</sup> T CELLS IN THE SPLEEN**

	<i>24 h</i>			<i>48 h</i>			<i>72 h</i>			<i>96 h</i>		
Treatment	N	KB5 (x10 <sup>5</sup> )	KB5 (%)	N	KB5 (x10 <sup>5</sup> )	KB5 (%)	N	KB5 (x10 <sup>5</sup> )	KB5 (%)	N	KB5 (x10 <sup>5</sup> )	KB5 (%)
<i>Blockade</i>	4	6.17 ± 0.89*	0.99 ± 0.21*	4	7.25 ± 3.92*	0.97 ± 0.42*	4	18.1 ± 4.90 <sup>†</sup>	2.56 ± 0.82 <sup>†</sup>	3	7.65 ± 3.41 <sup>‡</sup>	1.09 ± 0.51 <sup>‡</sup>
<i>Blockade + LPS</i>	4	21.9 ± 2.61	3.71 ± 0.47	4	45.8 ± 11.3	6.23 ± 1.03	4	64.0 ± 9.73	7.00 ± 0.97	3	46.2 ± 14.0	5.35 ± 1.16
<i>LPS Only</i>	3	22.4 ± 1.15	3.83 ± 0.10	3	21.8 ± 4.82	2.53 ± 0.25	3	26.2 ± 9.93	2.61 ± 0.50		ND	ND
<i>Untreated</i>	3	27.9 ± 1.85	5.27 ± 0.42	3	34.3 ± 5.33	5.72 ± 0.20	3	36.2 ± 1.67	5.48 ± 1.46	3	33.9 ± 15.4	5.79 ± 1.42

**Table III Legend:** KB5 synchimeric mice were untreated; treated with LPS on day 0; treated with C57BL/6 DST and anti-CD154 mAb on day 0 and anti-CD154 mAb on day +3; or treated with C57BL/6 DST, anti-CD154 mAb and LPS on day 0 and anti-CD154 mAb on day +3 relative to recovery of splenocytes for analysis on day 6. Splenocytes were stained as described in Table II. \*p<0.01 vs. all other groups. <sup>†</sup>p=N.S. vs. LPS only group and p<0.01 vs. all other groups. <sup>‡</sup>p<0.05 vs. untreated group and p<0.01 vs. costimulation blockade and LPS group.

#### 6. *LPS protects alloreactive CD8<sup>+</sup> cells from blockade-induced apoptosis*

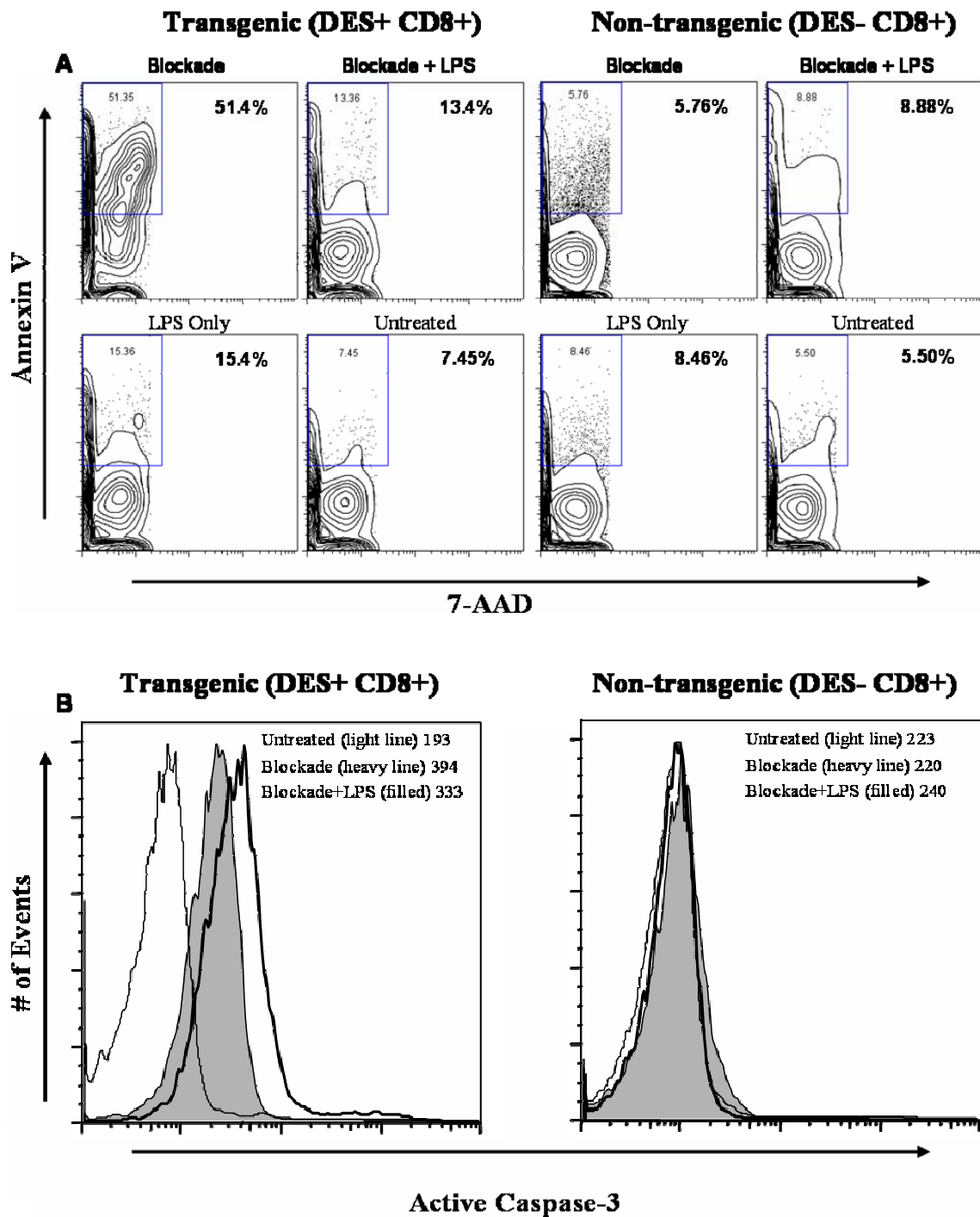
The observation that there was no dramatic deletion of alloreactive CD8<sup>+</sup> T cells following treatment with costimulation blockade and LPS led us to hypothesize that LPS protects these cells from costimulation blockade-induced apoptosis. To test this, apoptosis was measured in KB5 transgenic CD8<sup>+</sup> T cells 72 h after costimulation blockade treatment using the apoptotic marker annexin V and the viability dye 7-AAD.

Although few cells remained at 72 h after costimulation blockade treatment (**Table III**), a high percentage of these remaining cells stained with the apoptosis marker annexin V ( $42.3\% \pm 12.9\%$ ,  $n=8$ ; **Fig 8A, 9**). This finding suggests that the alloreactive T cells in mice treated with costimulation blockade were driven into apoptotic cell death. Strikingly, not only were more transgenic CD8<sup>+</sup> T cells present in mice treated with costimulation blockade and LPS, but a significantly lower percentage of these cells was undergoing apoptosis ( $13.4\% \pm 4.4\%$ ,  $n=8$ ,  $p<0.0001$  vs. DST and anti-CD154 mAb; **Fig 8A, 9**). This low level of apoptosis was similar to that observed in untreated syngeneic mice ( $8.8\% \pm 4.3\%$ ,  $n=6$ ) and syngeneic mice treated with LPS alone ( $10.8\% \pm 4.5\%$ ,  $n=6$ ). These data demonstrate that apoptosis induced by costimulation blockade is prevented by LPS by 72 h after treatment.

In contrast, the level of apoptosis in the CD8<sup>+</sup> DES<sup>-</sup> non-transgenic population was similar among all treatment groups ( $p=N.S.$ ; **Fig 9B**). These data demonstrate that costimulation blockade-induced apoptosis is restricted to the alloreactive T cell population and is a specific effect of LPS treatment in combination with antigen (DST) and anti-CD154mAb.

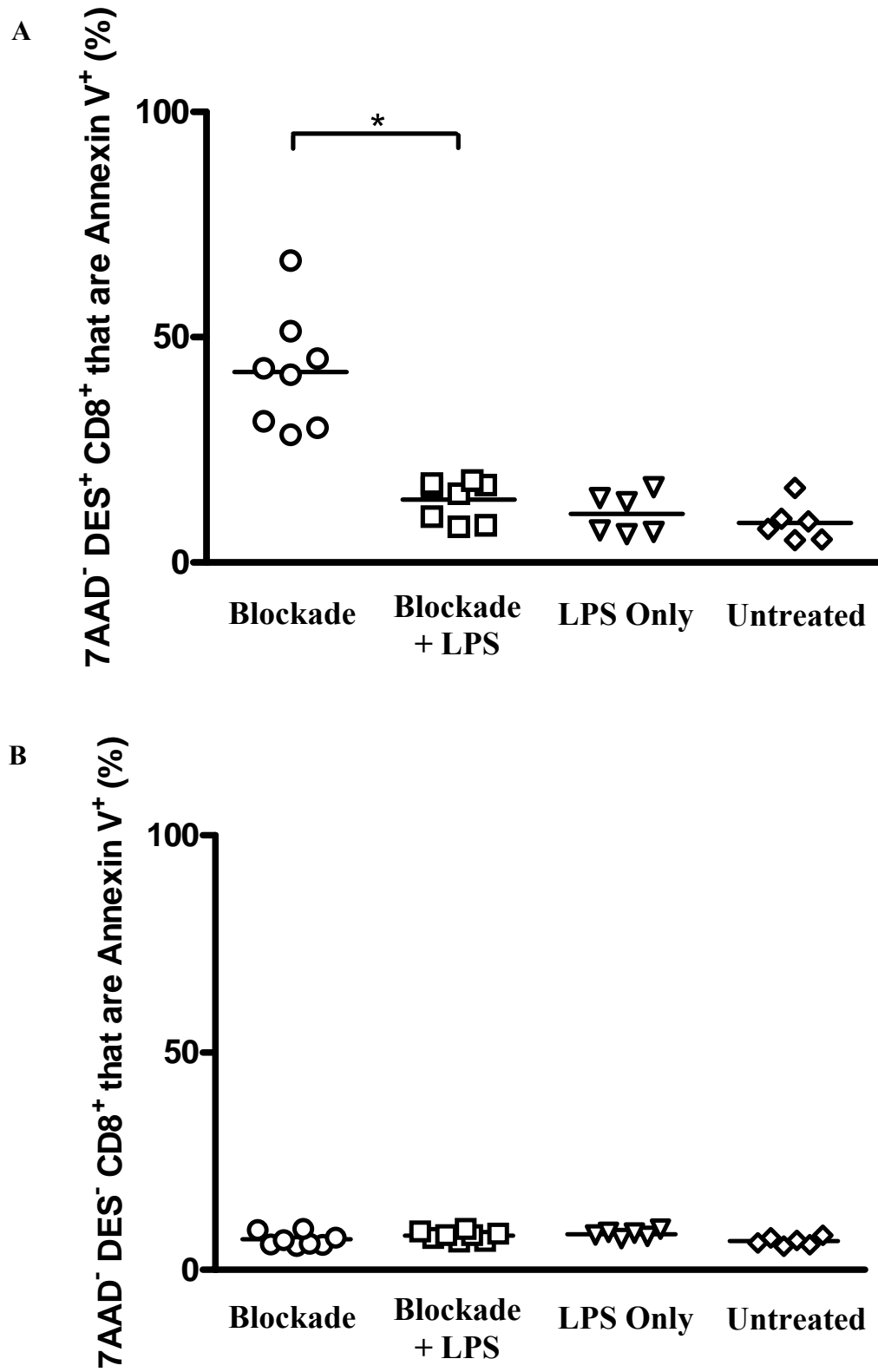
To confirm our annexin V data, we stained cells for the expression of active caspase-3, an effector caspase that is activated during the induction of apoptosis. We found that transgenic alloreactive CD8<sup>+</sup> T cells expressed lower levels of the pro-apoptotic marker after treatment with costimulation blockade and LPS (MFI=333, n=3) than costimulation blockade alone (MFI=394, n=3, p<0.01; **Fig 8B**). In contrast, non-transgenic CD8<sup>+</sup> T cells showed no detectable change in caspase-3 expression in any treatment group. These data confirm our finding that LPS impairs costimulation blockade-induced apoptosis 72 h after treatment.

**FIGURE 8: LPS PREVENTS BLOCKADE-INDUCED APOPTOSIS**



**Figure 8 Legend:** KB5 synchimeric mice were given DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of LPS on the day of DST and anti-CD154 mAb treatment. Splenocytes were recovered 72 h later and stained with anti-DES mAb followed by: (A) staining with the DES detection antibody (anti-mouse IgG2a-FITC), anti-CD8 $\alpha$ -APC, annexin V-PE and the viability dye 7-AAD, or (B) staining with the DES detection antibody (anti-mouse IgG2a-FITC) and anti-CD8 $\alpha$ -PerCP, followed by permeabilization and staining with anti-active caspase 3-PE. Representative contour plots (A) and histograms (B) are shown.

**FIGURE 9: LPS PREVENTS BLOCKADE-INDUCED APOPTOSIS**



**Figure 9 Legend:** KB5 synchimeric mice were untreated; treated with LPS; treated with DST and anti-CD154 mAb; or treated with DST, anti-CD154 mAb plus LPS according to our standard protocol. Splenocytes were recovered 72 h later and stained as described in Figure 8A. Graphs show the percent of cells binding annexin V after gating on live (7AAD<sup>-</sup>) transgenic (CD8<sup>+</sup> DES<sup>+</sup>, Panel A) or non-transgenic (CD8<sup>+</sup> DES<sup>-</sup>, Panel B) T cells. Data are pooled from two independent experiments. Each symbol represents an individual determination. \*p<0.001.

### *7. Costimulation blockade does not prevent the activation of alloreactive CD8<sup>+</sup> T cells*

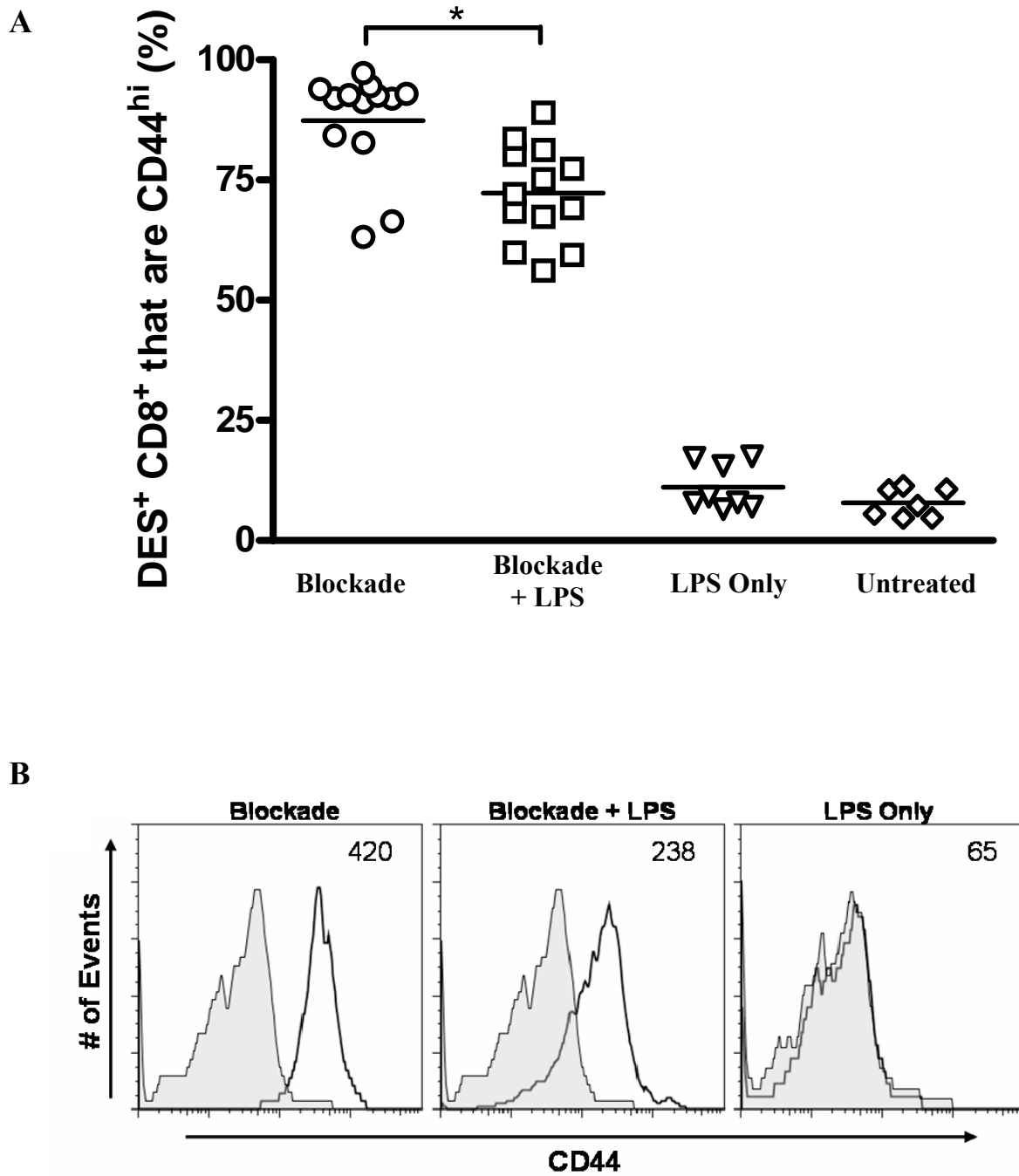
The observation that LPS promotes the expansion of alloreactive CD8<sup>+</sup> T cells 72 h after treatment with costimulation blockade prompted us to evaluate the effects of LPS on the activation and proliferation of these cells. We first quantified the expression of the activation marker CD44 on the transgenic KB5 (DES<sup>+</sup>) CD8<sup>+</sup> T cell population in the spleen 72 h after costimulation blockade treatment. Naïve transgenic CD8<sup>+</sup> T cells are CD44<sup>low</sup> (61). In contrast, the majority of CD8<sup>+</sup> T cells were CD44<sup>hi</sup> in costimulation blockade-treated animals without ( $87.4\% \pm 10.8\%$ , n=13) or with LPS treatment ( $72.3\% \pm 10.0\%$ , n=13; **Fig 10**). The median sample was then chosen as the representative histogram. The mean fluorescence intensity (MFI) of CD44 expression in the costimulation blockade-treated group (MFI=542, n=3) was slightly higher than in the group co-injected with LPS (MFI=337, n=3,  $p<0.01$ ).

In the absence of DST administration, LPS did not lead to a significant increase in the level of CD8<sup>+</sup>CD44<sup>hi</sup> T cells ( $11.1\% \pm 4.77\%$ , MFI=138, n=8) above the background level that was observed in untreated mice ( $7.84\% \pm 2.97\%$ , MFI=73, n=7,  $p=N.S.$ ; **Fig 10**). These results document that LPS does not activate alloreactive CD8<sup>+</sup> T cells in the absence of antigen (DST).

Costimulation blockade did not prevent alloreactive CD8<sup>+</sup> T cells from acquiring an activated (CD44<sup>hi</sup>) phenotype, but it was possible that these cells lacked the capacity to proliferate. To address this possibility, the expression of the nuclear proliferation antigen Ki-67 was quantified in the transgenic alloreactive CD8<sup>+</sup> T cell population in the spleens of KB5 synchimeric mice 72 h after costimulation blockade treatment. At this time point, the absolute number of transgenic alloreactive CD8<sup>+</sup> T cells was greatly

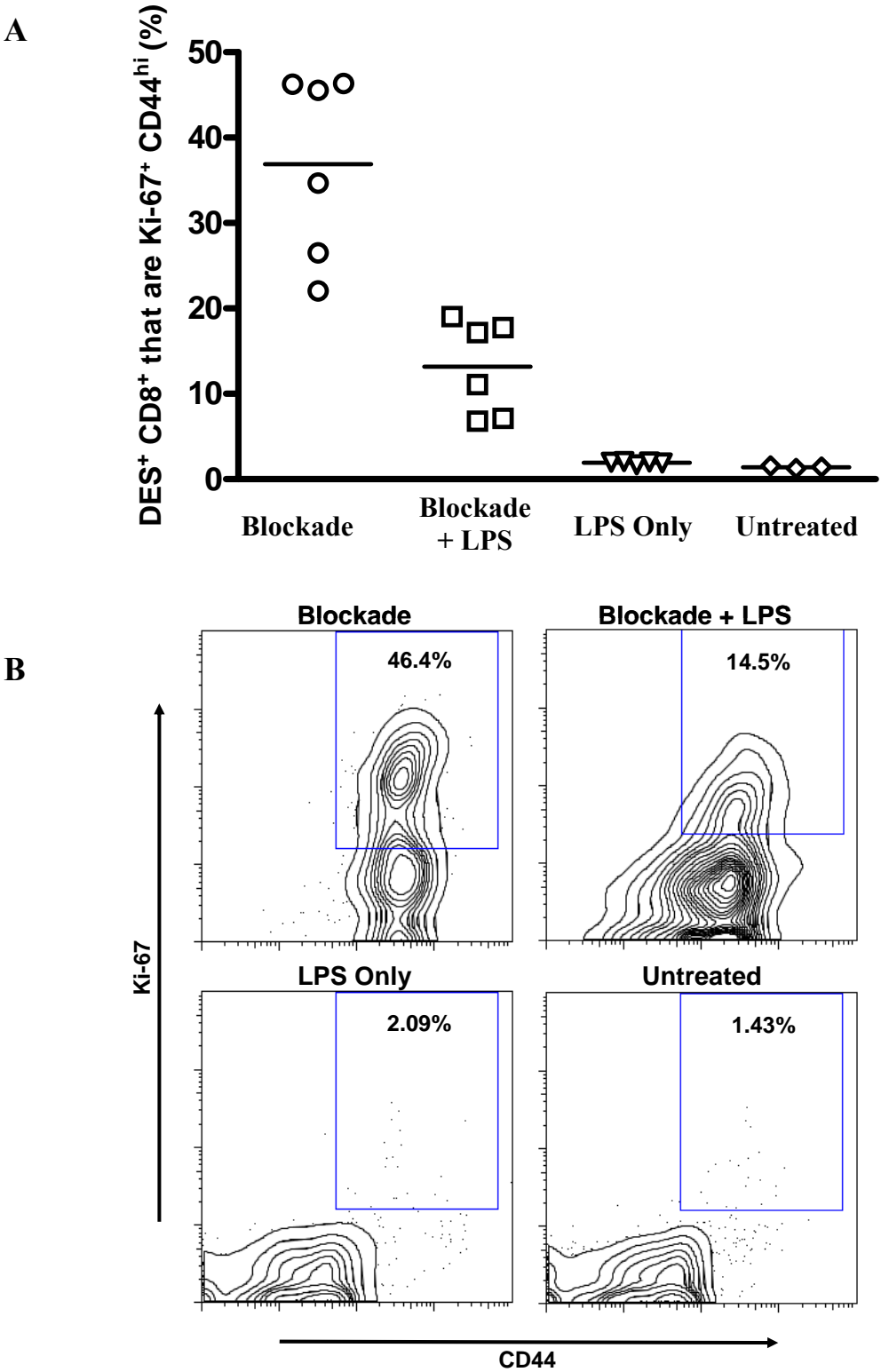
reduced in mice treated with costimulation blockade as compared to that observed in mice also treated with LPS (**Table III**). We observed a higher proportion of cells expressing the Ki-67 antigen in mice treated with costimulation blockade ( $36.9\% \pm 10.8\%$ ,  $n=6$ ) than in mice treated with costimulation blockade and LPS ( $13.3\% \pm 5.5\%$ ,  $n=6$ ,  $p<0.01$ ; **Fig 11**). This stands in contrast to Ki-67 expression in untreated mice ( $1.4\% \pm 0.1\%$ ,  $n=3$ ) and in mice treated with LPS alone ( $1.9\% \pm 0.2\%$ ,  $n=5$ ), which was low. These results show that LPS does not increase the activation or proliferative phenotype of alloreactive CD8<sup>+</sup> T cells in mice treated with costimulation blockade; the few cells still present in mice treated with costimulation blockade at 72 h were highly activated and proliferating.

**FIGURE 10: ALLOREACTIVE CD8<sup>+</sup> T CELLS HAVE AN ACTIVATED PHENOTYPE AFTER COSTIMULATION BLOCKADE**



**Figure 10 Legend:** Splenocytes were stained with anti-DES mAb, followed by staining with the DES detection mAb (anti-mIgG2a-FITC), anti-CD8-PerCP mAb and anti-CD44-APC mAb. Samples were fixed and analyzed by flow cytometry. Samples were gated on DES<sup>+</sup> CD8<sup>+</sup> lymphocytes and analyzed for CD44 expression. (A) A summary of experimental mice in four independent trials with means indicated by the solid bars (n=7 to 13/group). (B) The median sample for each group was selected as the representative flow histogram showing CD44 expression on gated DES<sup>+</sup> CD8<sup>+</sup> lymphocytes. Shaded histograms represent untreated control mice. The MFI of a representative sample from each experimental group is indicated in the upper-right-hand corner. \* p<0.001.

**FIGURE 11: ALLOREACTIVE CD8<sup>+</sup> T CELLS ARE REPLICATING AFTER COSTIMULATION BLOCKADE TREATMENT**



**Figure 11 Legend:** Splenocytes from KB5 synchimeric mice were analyzed 72 h after treatment with costimulation blockade in the presence or absence of co-injection with LPS. Splenocytes were stained with the clonotypic antibody DES, followed by staining with the DES detection mAb (anti-mIgG2a-FITC), anti-CD8-PerCP mAb and anti-CD44-APC mAb. Cells were then fixed, permeabilized and stained for the nuclear proliferation antigen Ki-67 with an anti-Ki-67-PE mAb. Samples were gated on DES<sup>+</sup> CD8<sup>+</sup> lymphocytes and analyzed for Ki-67 expression. (A) A summary of experimental mice from two independent experiments with the mean indicated by the solid bars (n=3 to 6 mice/group, each symbol represents an individual determination). (B) Representative contour plots showing CD44 and Ki-67 expression in gated DES<sup>+</sup> CD8<sup>+</sup> lymphocytes.

## Summary

The administration of an individual TLR agonist at the time of costimulation treatment is as effective at impairing allograft survival as infection with LCMV. The ability of the TLR4 agonist LPS to shorten allograft survival depends on a CD8<sup>+</sup> population. Importantly, LPS prevents the apoptosis-driven deletion of alloreactive CD8<sup>+</sup> T cells, but does not increase the activation phenotype of these cells. We conclude that the ability of LPS to prevent the deletion of alloreactive CD8<sup>+</sup> T cells plays a critical role in impairing skin allograft survival prolonged by costimulation blockade.

## **CHAPTER V: LPS SHORTENS ALLOGRAFT SURVIVAL THROUGH A MECHANISM THAT DEPENDS ON HOST TLR4 AND MyD88**

### **Introduction**

We have demonstrated that LPS shortens skin allograft survival induced by costimulation blockade; however, the mechanism by which LPS mediates this effect is not known. In this chapter, we address two important questions: 1) what cell population does the TLR4 agonist LPS act on, and 2) which TLR4 signaling pathways are required to shorten allograft survival?

TLR4 is expressed on donor cells in the DST and graft, as well as the host. Either source of TLR4 could account for the observation that LPS shortens allograft survival. LPS might be activating APCs in the donor DST directly, leading to their CD40-CD154-independent maturation. This maturation would provide the costimulatory signals required for the complete activation of alloreactive CD8<sup>+</sup> T cells, thus impairing their deletion and shortening allograft survival. Alternatively, it is possible that LPS acts on host cells to induce the expression of pro-inflammatory cytokines that are important for the survival of alloreactive CD8<sup>+</sup> T cells and allograft rejection. We therefore determined whether TLR4 is required on the donor, the host, or both.

TLR4 also has multiple downstream signaling pathways, mediated by key adaptor molecules such as MyD88 and TRIF (96). We therefore determined whether MyD88, a key regulator of pro-inflammatory cytokines, is required for LPS to shorten allograft survival induced by costimulation blockade.

## Results

### 1. *TLR4 is required on the host, but not on the donor*

It is possible that LPS abrogates skin allograft survival by engaging TLR4 on cells of host origin or, conversely, by engaging TLR4 directly on cells in the DST or skin allograft. To address the influence of host TLR4 expression on the effects of costimulation blockade, C57BL/10ScSnJ (TLR4<sup>+/+</sup>) and C57BL/10ScSnJ (TLR4<sup>-/-</sup>) mice were treated with costimulation blockade in the presence or absence of co-injection with LPS.

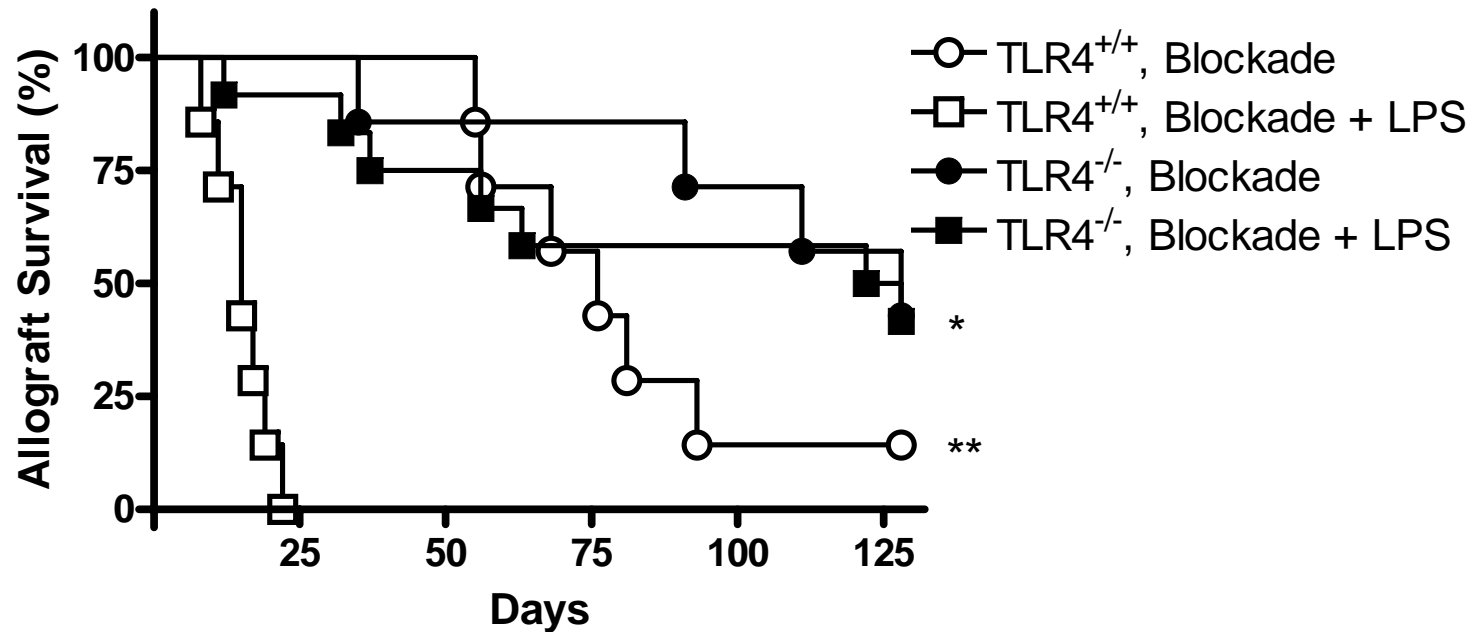
Treatment of TLR4<sup>+/+</sup> mice with costimulation blockade led to prolonged skin allograft survival (MST=76 days), which was shortened by LPS treatment (MST=15 days,  $p<0.0001$ ; **Fig 12**). In contrast, TLR4<sup>-/-</sup> mice treated with costimulation blockade in the presence (MST>128 days) or absence (MST>128 days) of LPS exhibited comparable skin allograft survival ( $p=N.S.$ ; **Fig 12**). Interestingly, skin allograft survival was significantly prolonged by costimulation blockade in TLR4<sup>-/-</sup> mice as compared to TLR4<sup>+/+</sup> mice ( $p<0.05$ ). These data suggest that TLR4 expression is required on host cells for LPS to shorten skin allograft survival induced by costimulation blockade.

To study the role of TLR4 expression on the DST and graft, we performed the converse experiment. C57BL/6 (TLR4<sup>+/+</sup>) mice were treated with BALB/c (TLR4<sup>+/+</sup>) or C.C3-*Tlr4*<sup>Lps-d</sup>/J (TLR4<sup>-/-</sup>) DST and anti-CD154 mAb in the presence or absence of LPS administration, and transplanted with a DST-matched skin allograft. As expected, allograft survival induced by costimulation blockade treatment (MST=76 days,  $n=7$ ) was shortened by LPS treatment (MST=15 days,  $n=7$ ) in C57BL/6 mice treated with TLR4<sup>+/+</sup> DST (**Fig 13**). Interestingly, allograft survival induced by costimulation blockade

(MST=48 days, n=9) was also shortened following LPS treatment (MST=11 days, n=8,  $p<0.0001$ ) in mice treated with TLR4<sup>-/-</sup> DST (**Fig 13**). These data indicate that LPS does not shorten allograft survival by activating TLR4 on the donor DST or allograft.

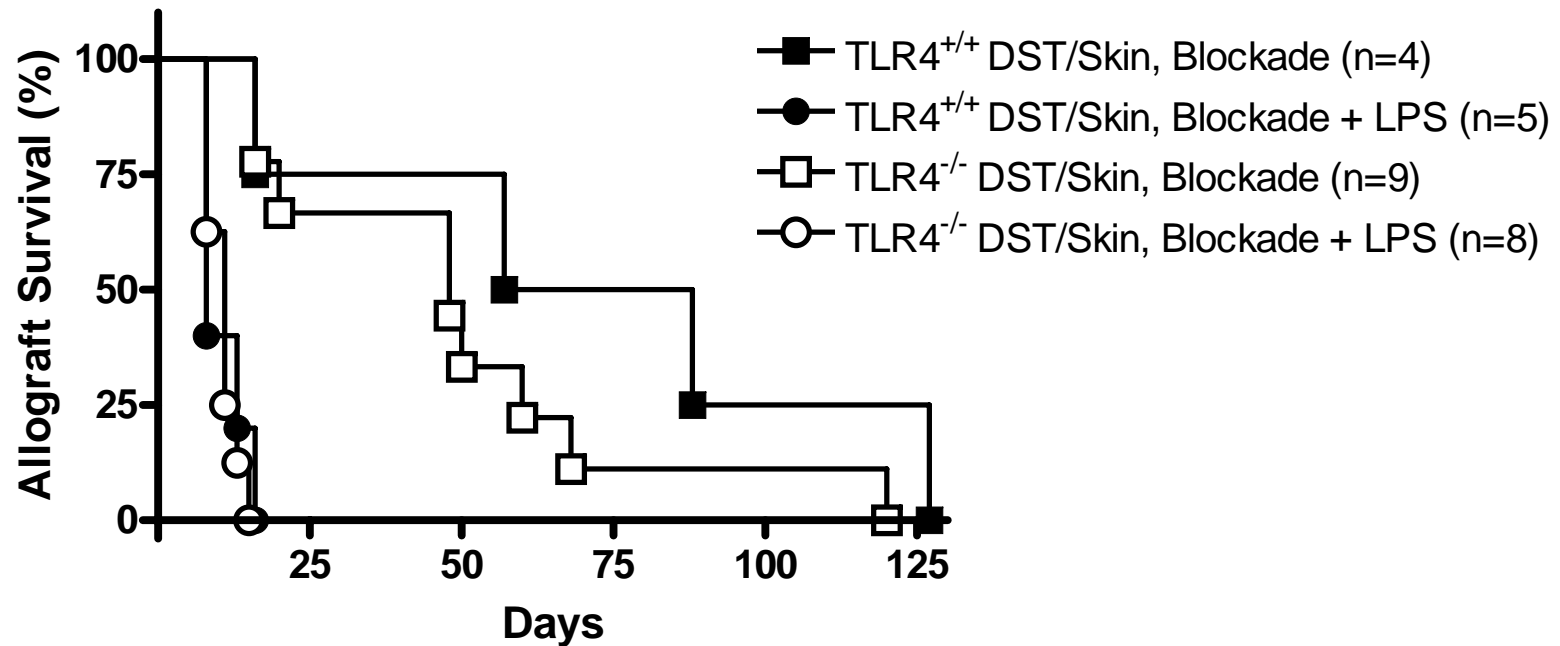
Together, these data demonstrate that TLR4 is essential on the host, but dispensable on the donor, for LPS to shorten prolonged allograft survival induced by costimulation blockade.

**FIGURE 12: TLR4 IS REQUIRED ON CELLS OF HOST ORIGIN**



**Figure 12 Legend:** C57BL/10ScSn (TLR4<sup>+/+</sup>) and C57BL/10ScN (TLR4<sup>-/-</sup>) mice were given BALB/c DST (TLR4<sup>+/+</sup>) and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of LPS on day -7 relative to skin grafting on day 0. All groups received BALB/c (TLR4<sup>+/+</sup>) skin allografts on day 0. \*Skin allograft survival in costimulation blockade-treated TLR4<sup>-/-</sup> mice was similar in the presence (MST>128 days, n=7) or absence (MST>128 days, n=12, p=N.S.) of LPS. \*\*Skin allograft survival in costimulation blockade-treated TLR4<sup>+/+</sup> mice (MST=76 days, n=11) was significantly prolonged compared to allograft survival in mice co-injected with LPS (MST=15 days, n=7, p<0.0001).

**FIGURE 13: TLR4 IS NOT REQUIRED ON THE DONOR DST OR ALLOGRAFT**



**Figure 13 Legend:** C57BL/6 mice were given BALB/c (TLR4<sup>+/+</sup>) or C.C3H-LPS<sup>d</sup> (TLR4<sup>-/-</sup>) DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of LPS on day -7 relative to skin grafting on day 0. All groups received DST-matched skin allografts on day 0. Skin allograft survival in costimulation blockade-treated C57BL/6 mice was significantly longer than the allograft survival observed in mice co-injected with LPS and DST derived from TLR4<sup>+/+</sup> (MST = 75 vs. 8 days, p=0.01) or TLR4<sup>-/-</sup> (MST = 48 vs. 11 days, p<0.0001) mice.

## *2. LPS does not impair deletion by activating cells in the DST*

We found that LPS does not require TLR4 on donor DST to impair allograft survival; however, it remained possible that treatment with TLR4-deficient DST, anti-CD154 mAb and LPS led to the deletion of alloreactive CD8<sup>+</sup> T cells that recognize antigen directly, and that the rejection observed was occurring through the indirect pathway. To assess this possibility, we treated KB5 synchimeric mice with C57BL/10ScSn (TLR4<sup>+/+</sup>) or C57BL/10ScN (TLR4<sup>-/-</sup>) DST and anti-CD154 mAb with or without a co-injection of LPS.

We found that treatment with costimulation blockade led to a deletion in the transgenic alloreactive CD8<sup>+</sup> T cell population, regardless of whether the DST was TLR4<sup>+/+</sup> (93.6 ± 3.80% , n=4) or TLR4<sup>-/-</sup> (Mean of 93.3 ± 3.01%, n=4; **Table IV**). Interestingly, LPS treatment led to a similar impairment of this deletion between groups treated with TLR4<sup>+/+</sup> (13.0 ± 14.0%, n=4, p<0.0001 vs. TLR4<sup>+/+</sup> DST and anti-CD154 group) or TLR4<sup>-/-</sup> (-6.19 ± 26.8%, n=4, p<0.001 vs. TLR4<sup>-/-</sup> DST and anti-CD154 group, p=N.S. vs. TLR4<sup>+/+</sup> DST, anti-CD154 and LPS group; **Table IV**) DST and anti-CD154 mAb.

These data indicate that TLR4 is not required on the donor for LPS to impair the deletion of alloreactive CD8<sup>+</sup> T cells that recognize antigen directly.

**TABLE IV: LPS DOES NOT IMPAIR DELETION BY ACTIVATING CELLS IN THE DST**

DST Donor	LPS	Chimerism Pre-treatment (%)	Chimerism 6d post-treatment (%)	N	Deletion (%)
<i>TLR4</i> <sup>+/+</sup>	-	2.85 ± 0.78	0.20 ± 0.14	4	93.6
<i>TLR4</i> <sup>+/+</sup>	+	2.56 ± 0.59	2.26 ± 0.80	4	13.0 <sup>*</sup>
<i>TLR4</i> <sup>-/-</sup>	-	2.38 ± 0.29	0.17 ± 0.09	4	93.3
<i>TLR4</i> <sup>-/-</sup>	+	1.68 ± 0.28	1.82 ± 0.70	4	-0.06 <sup>**</sup>

**Table IV Legend:** KB5 synchimeric mice were treated with C57BL/10ScSn (*TLR4*<sup>+/+</sup>) or C57BL/10ScN (*TLR4*<sup>-/-</sup>) DST and anti-CD154 mAb on day 0 and anti-CD154 mAb on day +3, with or without injection of LPS on day 0 relative to recovery of blood for analysis on day 6. Peripheral blood mononuclear cells were stained and analyzed as described in Table II. ANOVA analysis showed an overall significant difference among groups (p<0.0001). <sup>\*</sup>p<0.0001 vs. *TLR4*<sup>+/+</sup> DST and anti-CD154 group. <sup>\*\*</sup>p<0.001 vs. *TLR4*<sup>-/-</sup> DST and anti-CD154 group; p=N.S. vs. *TLR4*<sup>+/+</sup> DST, anti-CD154 and LPS group.

### 3. *LPS does not require TLR4 expression on host T cells*

Several recent reports have described the expression and function of TLRs on a subset of human (144,145) and murine (146,147) T cells. Combined with our data that TLR4 is required on the host, this presents the possibility that TLR agonists act directly on T cells. To address this possibility, we reconstituted irradiated TLR4<sup>-/-</sup> mice with 10<sup>7</sup> bone marrow cells from TLR4-sufficient mice that were capable (TLR4<sup>+/+</sup>) or incapable (TLR4<sup>+/+</sup> RAG2<sup>-/-</sup>) of reconstituting TLR4 in the T cell compartment. Eight weeks later, these mice received our standard costimulation blockade and skin transplantation protocol with or without a co-injection of LPS. If LPS were acting exclusively through TLR4 on T cells, then mice reconstituted with RAG2<sup>-/-</sup> bone marrow should exhibit prolonged allograft survival. Conversely, if LPS were acting on non-lymphoid cells, then mice reconstituted with RAG2<sup>-/-</sup> bone marrow should exhibit shortened allograft survival similar to mice reconstituted with wildtype bone marrow.

In support of our earlier observation (**Fig 12**), the prolonged allograft survival induced by costimulation blockade (MST=63 days, n=3) was shortened by LPS (MST=10 days, n=5; **Table V**) in TLR4<sup>+/+</sup> mice. Additionally, irradiated TLR4<sup>-/-</sup> mice that received no bone marrow exhibited prolonged skin allograft survival after treatment with costimulation blockade (MST= 56 days, n=3) or costimulation blockade and LPS (MST>120 days, n=4; **Table V**). TLR4<sup>-/-</sup> mice reconstituted with wildtype TLR4<sup>+/+</sup> (MST=96 days, n=2) or TLR4<sup>+/+</sup> RAG2<sup>-/-</sup> bone marrow (MST=98 days, n=3) exhibited prolonged skin allograft survival following treatment with costimulation blockade (**Table V**). Interestingly, LPS was able to shorten skin allograft survival when TLR4<sup>-/-</sup> mice were reconstituted with wildtype TLR4<sup>+/+</sup> (MST=10 days, n=3) or TLR4<sup>+/+</sup> RAG2<sup>-/-</sup>

(MST=10 days, n=4) bone marrow (**Table V**). This indicates that the expression of TLR4 in the non-lymphoid APC compartment is sufficient for LPS to shorten skin allograft survival in costimulation blockade-treated mice.

**TABLE V: TLR4 IS NOT REQUIRED ON HOST T CELLS**

Recipient Strain	Bone Marrow Donor	LPS	Graft Survival (days)	MST (days)
<i>TLR4</i> <sup>+/+</sup>	<i>None</i>	-	59, 61, 63, 63, 74, 84, >119	63
<i>TLR4</i> <sup>+/+</sup>	<i>None</i>	+	10, 10, 10, 10, 12	10
<i>TLR4</i> <sup>-/-</sup>	<i>None</i>	-	10, 56, 103	56
<i>TLR4</i> <sup>-/-</sup>	<i>None</i>	+	95, 108, >132, >132	>120
<i>TLR4</i> <sup>-/-</sup>	<i>TLR4</i> <sup>+/+</sup>	-	70, 123	96
<i>TLR4</i> <sup>-/-</sup>	<i>TLR4</i> <sup>+/+</sup>	+	10, 10, 17	10
<i>TLR4</i> <sup>-/-</sup>	<i>TLR4</i> <sup>+/+</sup> <i>RAG2</i> <sup>-/-</sup>	-	63, 98, 117	98
<i>TLR4</i> <sup>-/-</sup>	<i>TLR4</i> <sup>+/+</sup> <i>RAG2</i> <sup>-/-</sup>	+	10, 10, 10, 17	10

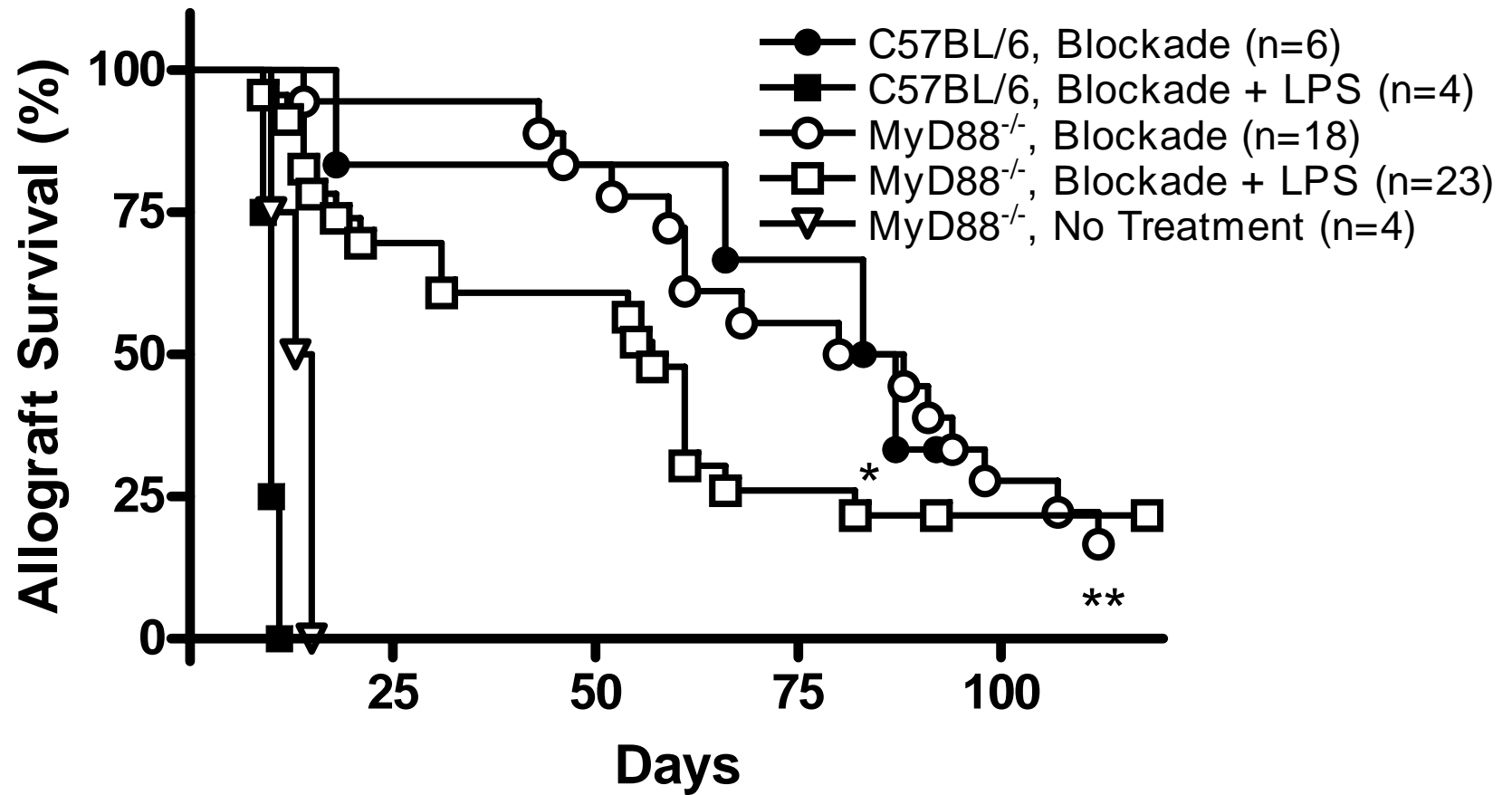
**Table V Legend:** C57BL/10ScSn (*TLR4*<sup>+/+</sup>) and C57BL/10ScN (*TLR4*<sup>-/-</sup>) mice were irradiated with 350 cGy and given 1 x 10<sup>7</sup> bone marrow cells from the indicated donor strain. Eight weeks later, mice were treated with our standard costimulation blockade protocol, with or without injection of LPS on the day of DST administration. All groups received BALB/c skin allografts on day 0. Skin allograft survival times are indicated for each individual mouse.

#### 4. *LPS shortens skin allograft survival by activating the host MyD88 pathway*

The finding that LPS shortens skin allograft survival by activating host TLR4 next led us to determine which components of the TLR4 signaling pathway were required. TLR4 has signaling pathways that act both dependently and independently of the adaptor molecule MyD88 (148). We therefore sought to determine whether the MyD88 pathway was required for LPS to shorten skin allograft survival. To test this, C57BL/6 and B6.MyD88<sup>-/-</sup> mice were treated with costimulation blockade in the presence or absence of a co-injection with LPS.

C57BL/6 and B6.MyD88<sup>-/-</sup> mice treated with costimulation blockade alone exhibited prolonged allograft survival with MSTs of 85 days (n=6) and 84 days (n=18) respectively (**Fig 14**). As expected, treatment with LPS shortened skin allograft survival in C57BL/6 mice (MST=9.5 days, n=4, p<0.01 vs. DST and anti-CD154 treated C57BL/6; **Fig 14**). In contrast, LPS failed to significantly impair allograft survival in B6.MyD88<sup>-/-</sup> mice treated with costimulation blockade (MST=57 days, n=23, p= N.S. vs. DST and anti-CD154 treated B6.MyD88<sup>-/-</sup>; **Fig 14**). Untreated B6.MyD88<sup>-/-</sup> mice rapidly rejected their skin allografts (MST=14 days, n=4; **Fig 14**). These data demonstrate that LPS requires the MyD88-dependent pathway to shorten skin allograft survival prolonged by costimulation blockade.

**FIGURE 14: LPS SHORTENS ALLOGRAFT SURVIVAL IN A MYD88-DEPENDENT MANNER**



**Figure 14 Legend:** C57BL/6 or B6.MyD88<sup>-/-</sup> mice were given BALB/c DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of LPS on day -7 relative to skin grafting on day 0. All groups received BALB/c skin allografts on day 0. \*Skin allograft survival in the C57BL/6 group treated with costimulation blockade was significantly prolonged vs. comparably treated mice co-injected with LPS (p<0.01). \*\*Survival between the B6.MyD88<sup>-/-</sup> group treated with costimulation blockade and costimulation blockade plus LPS was not significantly different (p=N.S.).

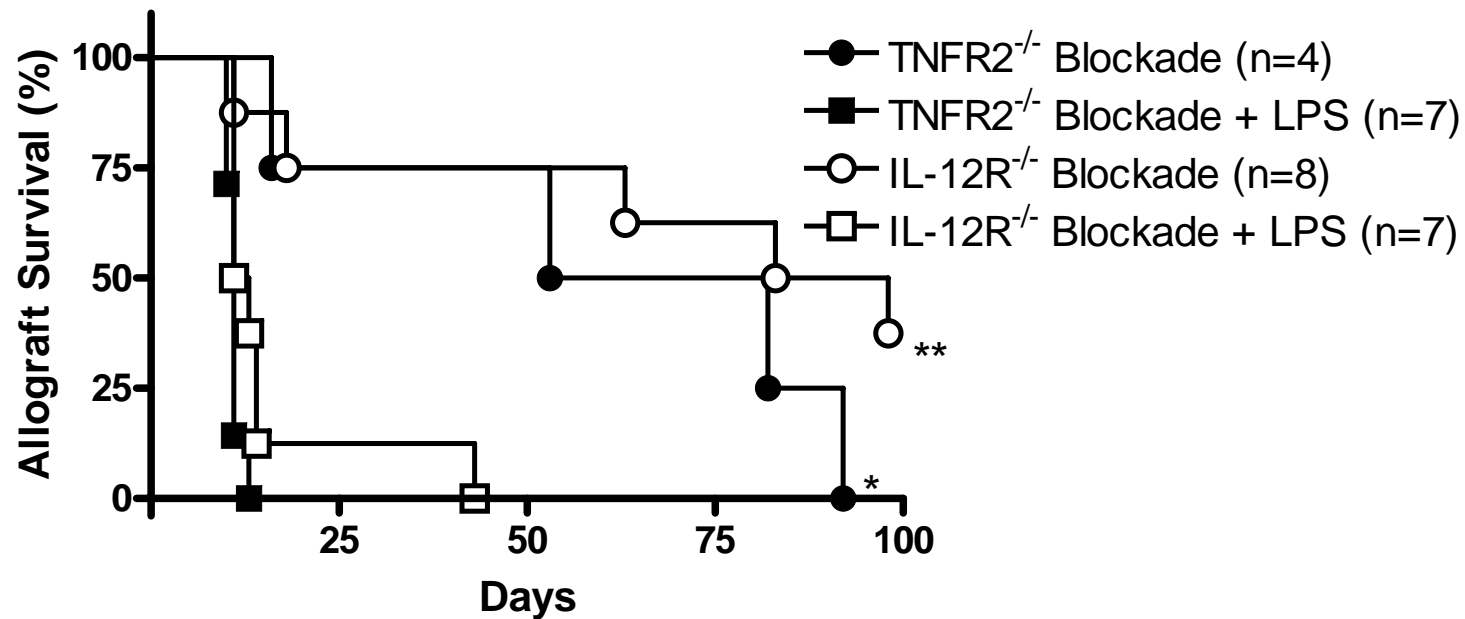
### 5. *IL-12R and TNFR2 are not required for LPS to shorten allograft survival*

The MyD88-dependent pathway is critical for TLR-mediated production of certain pro-inflammatory cytokines such as IL-12 and TNF $\alpha$ . The critical role of MyD88 in shortening allograft survival, and the essential role of CD8<sup>+</sup> T cells in the rejection process, suggests that LPS might provide a survival signal to CD8<sup>+</sup> T cells by inducing IL-12 or TNF $\alpha$  production. CD8<sup>+</sup> T cells express IL-12R (53) and TNFR2 (55,56), and their cognate cytokines can provide “signal 3” to CD8<sup>+</sup> T cells. Signal 3 enhances CD8<sup>+</sup> T cell activation, survival and the generation of effector function (53,55,56,149,150). To address the role of these cytokine receptors in shortening allograft survival, B6.IL-12R<sup>-/-</sup> and B6.TNFR2<sup>-/-</sup> mice were treated with costimulation blockade in the presence or absence of LPS co-administration.

Treatment of B6.TNFR2<sup>-/-</sup> mice with costimulation blockade led to prolonged skin allograft survival (MST=68 days, n=4), which was shortened by LPS treatment (MST=11 days, p<0.005; **Fig 15**). We observed a similar result in a preliminary experiment using TNFR1-deficient mice. Allograft survival induced by costimulation blockade in B6.TNFR1<sup>-/-</sup> (MST=103 days, n=3) was shortened by LPS (MST=10 days, n=2). Similarly, treatment of B6.IL-12R<sup>-/-</sup> mice with costimulation blockade led to prolonged skin allograft survival (MST=91 days, n=8) that was also shortened by LPS treatment (MST=12 days, n=4, p<0.005; **Fig 15**).

These data suggest that neither signaling through TNFR2 nor IL-12R is required for LPS to shorten allograft survival in costimulation blockade-treated mice.

**FIGURE 15: TNFR2 AND IL-12R ARE NOT REQUIRED FOR LPS TO SHORTEN ALLOGRAFT SURVIVAL**



**Figure 15 Legend:** B6.TNFR2<sup>-/-</sup> and B6.IL-12R<sup>-/-</sup> mice were given BALB/c DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of LPS on day -7 relative to skin grafting on day 0. All groups received BALB/c skin allografts on day 0. \* Skin allograft survival in costimulation blockade-treated B6.TNFR2<sup>-/-</sup> mice was significantly prolonged (MST=68 days, n=4) as compared to allograft survival in mice co-injected with LPS (MST=11 days, p<0.005). \*\* Skin allograft survival in costimulation blockade-treated B6.IL-12R<sup>-/-</sup> mice significantly prolonged (MST=91 days, n=8) as compared to allograft survival in mice co-injected with LPS (MST=12 days, n=4, p<0.005).

## 6. LPS does not impair deletion by upregulating CD80/86 on the DST

Our lab has previously shown that costimulation blockade impairs the upregulation of the key costimulatory molecule CD86 on donor APCs in DST (60). Immunologists have proposed that the failure of donor APCs to upregulate key costimulatory molecules is a primary mechanism for the deletion of alloreactive CD8<sup>+</sup> T cells. Therefore, we wanted to determine whether LPS required the upregulation of the key costimulatory molecules CD80 and CD86 in order to prevent CD8<sup>+</sup> T cell deletion. We have shown that LPS does not act directly on donor cells to prevent the deletion of transgenic alloreactive CD8<sup>+</sup> T cells that recognize alloantigen directly (**Table IV**); however, it remains possible that host-produced cytokines activate donor APCs by an indirect mechanism. To test this, we treated KB5 synchimeric mice with C57BL/6 or B6.CD80/86<sup>-/-</sup> DST and anti-CD154 mAb with or without a co-injection of LPS.

Reaffirming our previous findings, C57BL/6 DST and anti-CD154 mAb treatment lead to robust deletion of the transgenic alloreactive CD8<sup>+</sup> T cell population (84.5 ± 10.8%, n=4) in the peripheral blood 6 days after treatment (**Table VI**). This deletion was significantly impaired by co-injection with LPS (-36.2 ± 12.9%, n=4, p<0.00001; **Table VI**). Interestingly, the deletion induced by CD80/86<sup>-/-</sup> DST and anti-CD154 mAb (86.0 ± 9.63%, n=4) was similarly impaired by LPS (-15.6 ± 34.6%, n=4, p<0.01 vs. CD80/86<sup>-/-</sup> DST and anti-CD154 group, p=N.S. vs. C57BL/6 DST, anti-CD154 and LPS group; **Table VI**).

These data demonstrate that LPS does not require direct or indirect induction of CD80 and CD86 on the DST in order to impair the deletion of alloreactive CD8<sup>+</sup> T cells that recognize antigen directly.

**TABLE VI: LPS DOES NOT IMPAIR DELETION BY UPREGULATING CD80/86 ON THE DST**

DST Strain	LPS	Chimerism Pre-treatment (%)	Chimerism 6d post-treatment (%)	N	Deletion (%)
<i>C57BL/6</i>	-	2.07 ± 0.57	0.30 ± 0.20	4	84.5
<i>C57BL/6</i>	+	2.19 ± 0.87	2.92 ± 0.92	4	-36.2*
<i>CD80/86<sup>-/-</sup></i>	-	1.67 ± 0.33	0.25 ± 0.19	4	86.0
<i>CD80/86<sup>-/-</sup></i>	+	1.68 ± 0.28	1.92 ± 0.56	4	-15.6**

**Table VI Legend:** KB5 synchimeric mice were treated with C57BL/6 DST and anti-CD154 mAb on day 0 and anti-CD154 mAb on day +3, with or without injection of LPS on day 0 relative to recovery of blood for analysis on day 6. Peripheral blood mononuclear cells were collected, stained and analyzed as described in Table II. \*p<0.00001 vs. C57BL/6 DST and anti-CD154 group. \*\*p<0.01 vs. CD80/86<sup>-/-</sup> DST and anti-CD154 group; p=N.S. vs. C57BL/6 DST, anti-CD154 and LPS group.

## Summary

LPS impairs costimulation blockade-induced allograft survival by activating the host MyD88-dependent TLR4 pathway. However, the shortened allograft survival does not require the MyD88-dependent cytokines IL-12 and TNF $\alpha$ , nor does it require the induction of CD80 and CD86 on the donor DST.

## **CHAPTER VI: LPS AND POLY(I:C) IMPAIR ALLOGRAFT SURVIVAL THROUGH AN IFN- $\alpha/\beta$ -DEPENDENT MECHANISM**

### **Introduction**

We have discovered that LPS shortens allograft survival by activating the host MyD88-dependent TLR4 pathway. This is particularly interesting because other TLR agonists such as poly(I:C), which impair allograft survival, do not signal through the MyD88 pathway and possess TLR-independent signaling mechanisms. In this chapter we explore the important differences and similarities between LPS and poly(I:C) that may have biologically significant implications for potential therapeutic strategies.

Unlike LPS, the dsRNA mimetic poly(I:C) has TLR-dependent (97) and TLR-independent signaling capabilities and signals through several different classes of receptors, including the RNA helicase receptors RIG-I (109) and MDA5 (110), and the protein kinase PKR (108). We therefore determined whether poly(I:C) requires its cognate receptor, TLR3, to shorten allograft survival induced by costimulation blockade.

Poly(I:C) is also known to be a more potent inducer of IFN- $\alpha/\beta$  than LPS. We therefore explored the requirement for IFN- $\alpha/\beta$  in shortening allograft survival, and the biological significance that the difference in IFN- $\alpha/\beta$ -induction might have in a complex biological response.

## Results

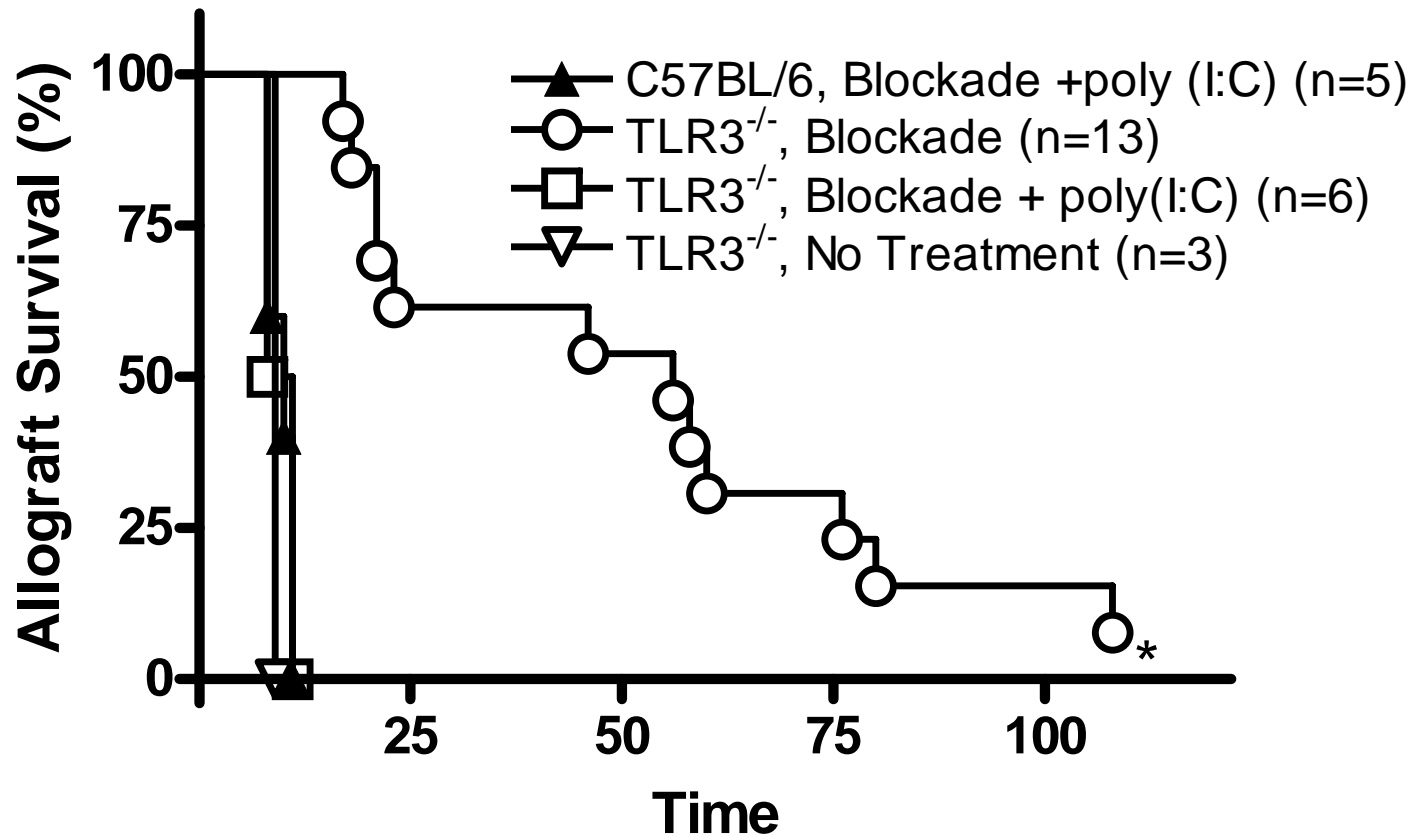
### 1. *Poly(I:C) does not require TLR3 to shorten skin allograft survival*

Poly(I:C) mediates anti-viral activities through several distinct classes of receptors, including the Toll-like family member TLR3 (97), the RNA helicase receptors RIG-I (109) and MDA5 (110), and the serine-threonine kinase PKR (108). Since we have demonstrated that LPS acts in a TLR-dependent manner to shorten allograft survival, we wanted to determine if the same held true for poly(I:C). To address this, C57BL/6 or B6.TLR3<sup>-/-</sup> mice were treated with costimulation blockade with or without the co-injection of 50 µg of poly(I:C) on day -7 relative to skin transplantation on day 0.

B6.TLR3<sup>-/-</sup> mice treated with costimulation blockade exhibited prolonged allograft survival with an MST of 56 days (n=13; **Fig 16**). Confirming our previously published observation (70,77), C57BL/6 mice treated with costimulation blockade and poly(I:C) rapidly rejected their skin allografts (MST=10 days, n=5, p<0.01 vs. DST and anti-CD154 treated C57BL/6; **Fig 4, 16**). Interestingly, poly(I:C) also shortened allograft survival in B6.TLR3<sup>-/-</sup> mice treated with costimulation blockade (MST=10 days, n=6, p<0.0001 vs. DST and anti-CD154 treated B6.TLR3<sup>-/-</sup>; **Fig 16**).

These data document that poly(I:C), unlike LPS, does not require its cognate TLR to shorten skin allograft survival induced by costimulation blockade.

**FIGURE 16: POLY(I:C) DOES NOT REQUIRE TLR3 TO SHORTEN SKIN ALLOGRAFT SURVIVAL**



**Figure 16 Legend:** C57BL/6 or B6.TLR3<sup>-/-</sup> mice were given BALB/c DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of LPS on day -7 relative to skin grafting on day 0. All groups received BALB/c skin allografts on day 0. \*p<0.001 vs. all other groups.

## *2. LPS and poly(I:C) impair islet allograft survival*

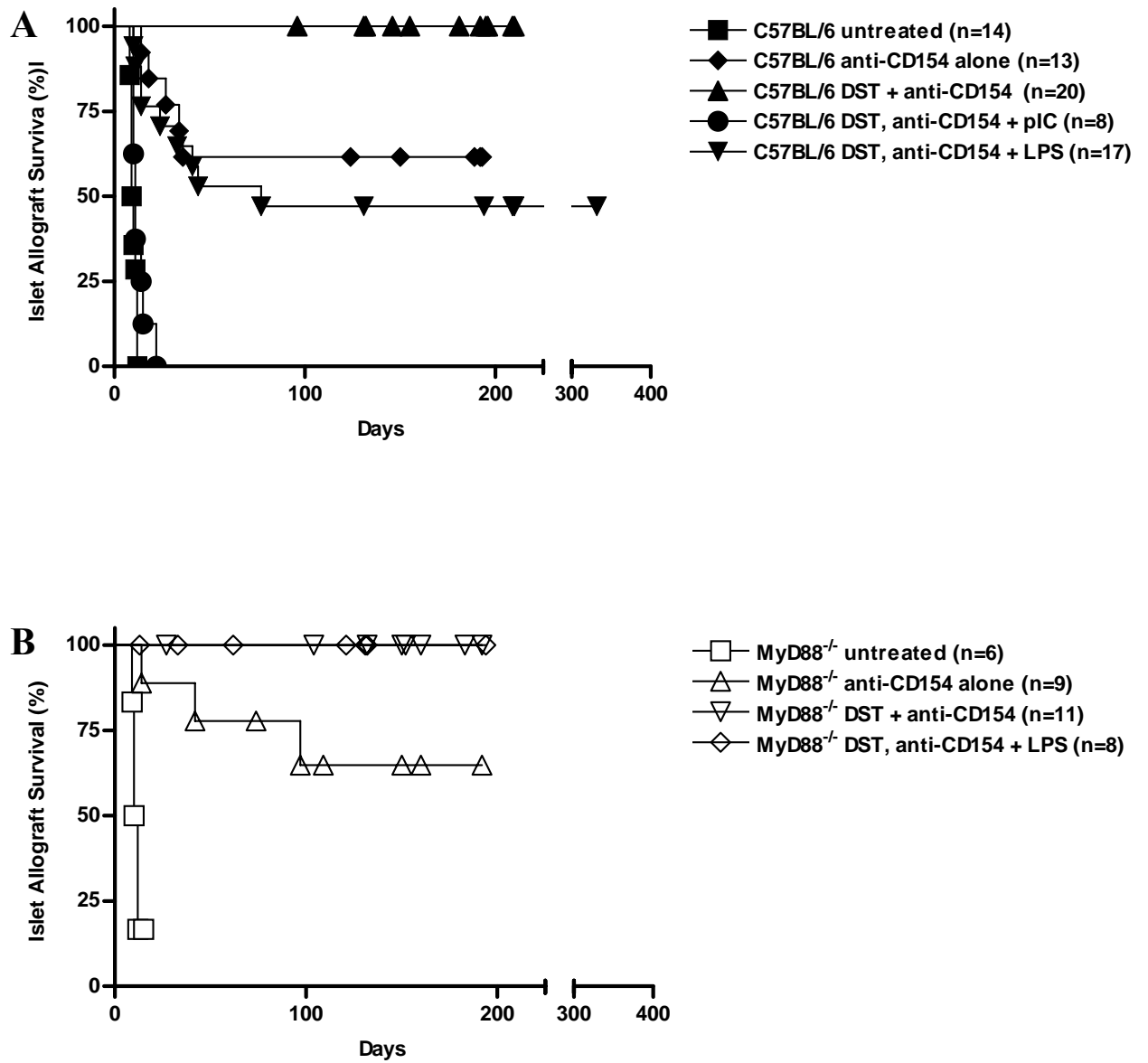
The efficacy of costimulation blockade in prolonging allograft survival is often tissue-dependent. For example, anti-CD154 mAb alone is capable of inducing permanent islet allograft survival in approximately 40% of treated mice (57); however, it fails to prolong the survival of skin allografts in the absence of DST (65). Based on these observations, we next determined whether LPS and poly(I:C) were capable of shortening islet allograft survival in C57BL/6 mice. Chemically diabetic C57BL/6 mice were randomized into five groups. Three groups received the costimulation blockade treatment described earlier for skin transplantation, one of which was co-injected with 100 µg of LPS on day -7 and another of which was co-injected with 50 µg of poly(I:C) on day -7. A fourth group received only the four injections of anti-CD154 mAb, and a final group received no preconditioning at all. All five groups were transplanted with BALB/c islets on day 0.

BALB/c islet allografts survived in all C57BL/6 mice treated with costimulation blockade until the experiment was terminated at 180 days (n=20; **Fig 17A**). The prolonged islet allograft survival induced by DST and anti-CD154 mAb was completely eliminated in C57BL/6 mice by poly(I:C) treatment (MST=11 days, n=8,  $p<0.0001$  vs. DST and anti-CD154 treated C57BL/6) but, surprisingly, it was only partially shortened by LPS treatment (MST=77 days, n=17,  $p<0.001$  vs. DST and anti-CD154 treated C57BL/6,  $p<0.0001$  vs. DST, anti-CD154 and poly(I:C) treated C57BL/6; **Fig 17A**). These data suggest that 50 µg of poly(I:C) renders mice completely refractory to costimulation blockade-induced islet allograft survival, whereas 100 µg of LPS renders mice only partially resistant.

Since we observed that MyD88 was required for LPS to abrogate skin allograft survival, we examined whether the same was true for islet allografts. As expected, islet allografts survived in all B6.MyD88<sup>-/-</sup> mice treated with costimulation blockade until the experiment was terminated at 180 days (n=11; **Fig 17B**). Islet allograft survival was not shortened by LPS in B6.MyD88<sup>-/-</sup> mice (MST>180 days, n=8, p=N.S. vs. DST and anti-CD154 mAb treated B6.MyD88<sup>-/-</sup>, p<0.05 vs. DST, anti-CD154 and LPS treated C57BL/6; **Fig 17B**). This observation indicates that LPS requires host MyD88 to shorten islet allograft survival in mice treated with costimulation blockade, in support of our findings in the skin allograft model.

Untreated B6.MyD88<sup>-/-</sup> mice rapidly rejected islet allografts (MST=11 days, n=6; **Fig 17B**) with kinetics similar to those of C57BL/6 mice (MST=9.5 days, n=14, p=N.S.; **Fig 17A**). In addition, B6.MyD88<sup>-/-</sup> mice treated with anti-CD154 mAb alone (MST>180 days, n=9; **Fig 17B**) exhibited islet allograft survival that was similar to that observed in C57BL/6 mice treated with anti-CD154 mAb alone (MST>180 days, n=13, p=N.S.; **Fig 17A**). These data suggest that MyD88 is not required for islet allograft rejection.

**FIGURE 17: LPS AND POLY(I:C) IMPAIR ISLET ALLOGRAFT SURVIVAL**



**Figure 17 Legend:** Chemically diabetic C57BL/6 (A) or B6.MyD88<sup>-/-</sup> (B) mice were untreated, injected with anti-CD154 mAb on days -7, -4, 0, and +4, or injected with DST and anti-CD154 mAb on the same schedule with or without injection of poly(I:C) or LPS on day -7 relative to islet transplantation on day 0. All groups received BALB/c islet allografts on day 0. (A) Islet allograft survival in C57BL/6 mice treated with DST and anti-CD154 mAb plus LPS was significantly shorter than that achieved in mice treated with DST and anti-CD154 mAb ( $p < 0.001$ ) and significantly longer than in mice treated with DST and anti-CD154 mAb plus poly(I:C) ( $p < 0.0001$ ). (B) Islet allograft survival in B6.MyD88<sup>-/-</sup> mice treated with DST and anti-CD154 mAb was not significantly different from that achieved in B6.MyD88<sup>-/-</sup> mice treated with DST and anti-CD154 mAb plus LPS ( $p = \text{N.S.}$ ).

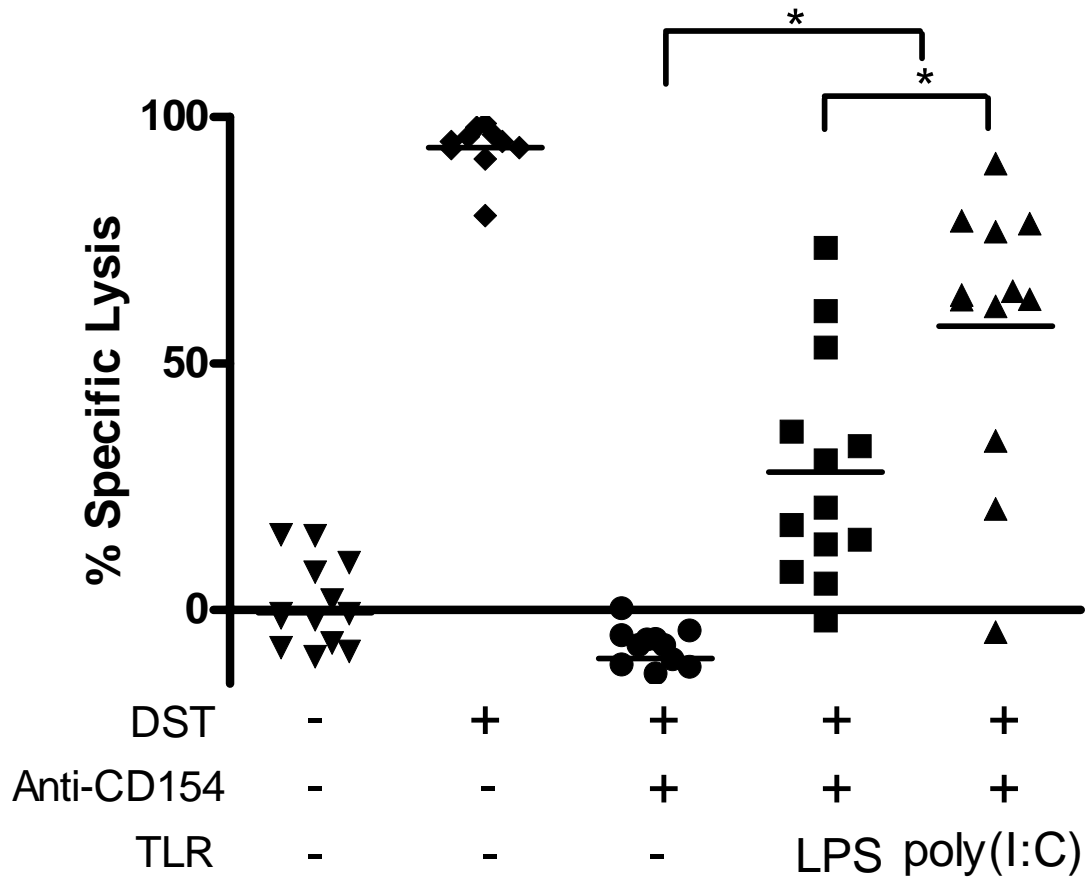
### 3. LPS and poly(I:C) prime an allo-specific CTL response

The observation that that LPS has a more modest effect on islet allograft survival than poly(I:C) led us to determine whether these agonists differ in their ability to prime alloreactive CD8<sup>+</sup> T cells in the presence of costimulation blockade. Primed mice are resistant to tolerance induction by costimulation blockade (76,151); therefore it is possible that both agonists prevent the deletion of alloreactive CD8<sup>+</sup> T cells, but differ in their ability to prime effector function. To address this, C57BL/6 mice were treated with our standard costimulation blockade protocol; however, instead of receiving a skin or islet allograft on day 0, mice were analyzed for the presence of primed alloreactive CD8<sup>+</sup> T cells, as determined by their ability to rapidly kill allogeneic target cells using an *in vivo* cytotoxicity assay (137,139). This assay detects cytotoxic activity of NK cells and effector/memory alloreactive CD8<sup>+</sup> T cells.

To detect alloreactive CD8<sup>+</sup> T cell cytotoxicity in the absence of NK cell-mediated killing, mice treated with costimulation blockade with or without co-administration of TLR agonists 6 days earlier were depleted of NK-cells using a depleting anti-NK1.1 mAb. The NK-depleted mice were injected 24 h later with an equal number of allogeneic and syngeneic splenocytes that were labeled with different concentrations of CFSE, which allowed us to differentiate the two populations based on fluorescence intensity (137). Spleens were harvested 4 hours later and analyzed by flow cytometry for the two CFSE-positive populations. The relative depletion of the allogeneic population was used to calculate the level of *in vivo* cytotoxic activity as an indicator of CD8<sup>+</sup> effector T cell activity. Specific lysis was calculated as described in *Materials & Methods*.

We observed that animals treated with DST and anti-CD154 mAb exhibited low cytotoxic activity ( $-9.87 \pm 7.71\%$ ,  $n=13$ ), while animals treated with DST alone exhibited near-complete killing of the allogeneic cell population ( $93.8 \pm 5.29\%$ ,  $n=10$ ; **Fig 18**). Surprisingly, animals treated with costimulation blockade and 100  $\mu\text{g}$  of LPS exhibited only a marginal level of cytotoxic activity to the allogeneic targets ( $28.0 \pm 22.8\%$ ,  $n=12$ ; **Fig 18**). In contrast, animals treated with costimulation blockade and 50  $\mu\text{g}$  of poly(I:C), exhibited a two-fold higher level of cytotoxic activity to allogeneic targets ( $57.6 \pm 27.5\%$ ,  $n=12$ ; **Fig 18**) as compared to animals treated with costimulation blockade and LPS ( $p<0.01$ ). These data suggest that poly(I:C) is more effective at priming an allo-specific CTL response in mice treated with costimulation blockade than is LPS at the doses of the agonists we administered.

**FIGURE 18: LPS AND POLY(I:C) PRIME AN ALLO-SPECIFIC CTL RESPONSE**



**Figure 18 Legend:** C57BL/6 mice were untreated, injected with DST on day -7, injected with DST on day -7 and anti-CD154 mAb on days -7, -4, 0, and +4, or injected with DST and anti-CD154 mAb on the same schedule with or without injection of poly(I:C) or LPS on day -7 relative to performing the *in vivo* cytotoxicity assay on day 0. Data are presented as the percent specific lysis, which was calculated as described in *Materials & Methods*. Each symbol represents an individual animal from one of five independent experiments. \*p<0.01.

#### 4. *Poly(I:C) induces more IFN- $\alpha/\beta$ than LPS in a TLR3-independent manner*

The difference in *in vivo* cytotoxic activity detected in mice treated with costimulation blockade and LPS and mice treated with costimulation blockade and poly(I:C) led us to explore the differential effects of these two agonists. It is possible that these two agonists differ in their ability to induce the production of IFN- $\alpha/\beta$ , a potent immunomodulator of CD8<sup>+</sup> T cells that has been shown to mature APCs and provide a third signal for T cell activation (54). Poly(I:C) stimulates IFN- $\alpha/\beta$  production through several receptors, including TLR3 (97) and RIG-I (109). In contrast, LPS acts exclusively through TLR4. We therefore hypothesized that poly(I:C) and LPS may result in differential priming of alloreactive CD8<sup>+</sup> T cells as a result of differences in their ability to induce the production of IFN- $\alpha/\beta$ .

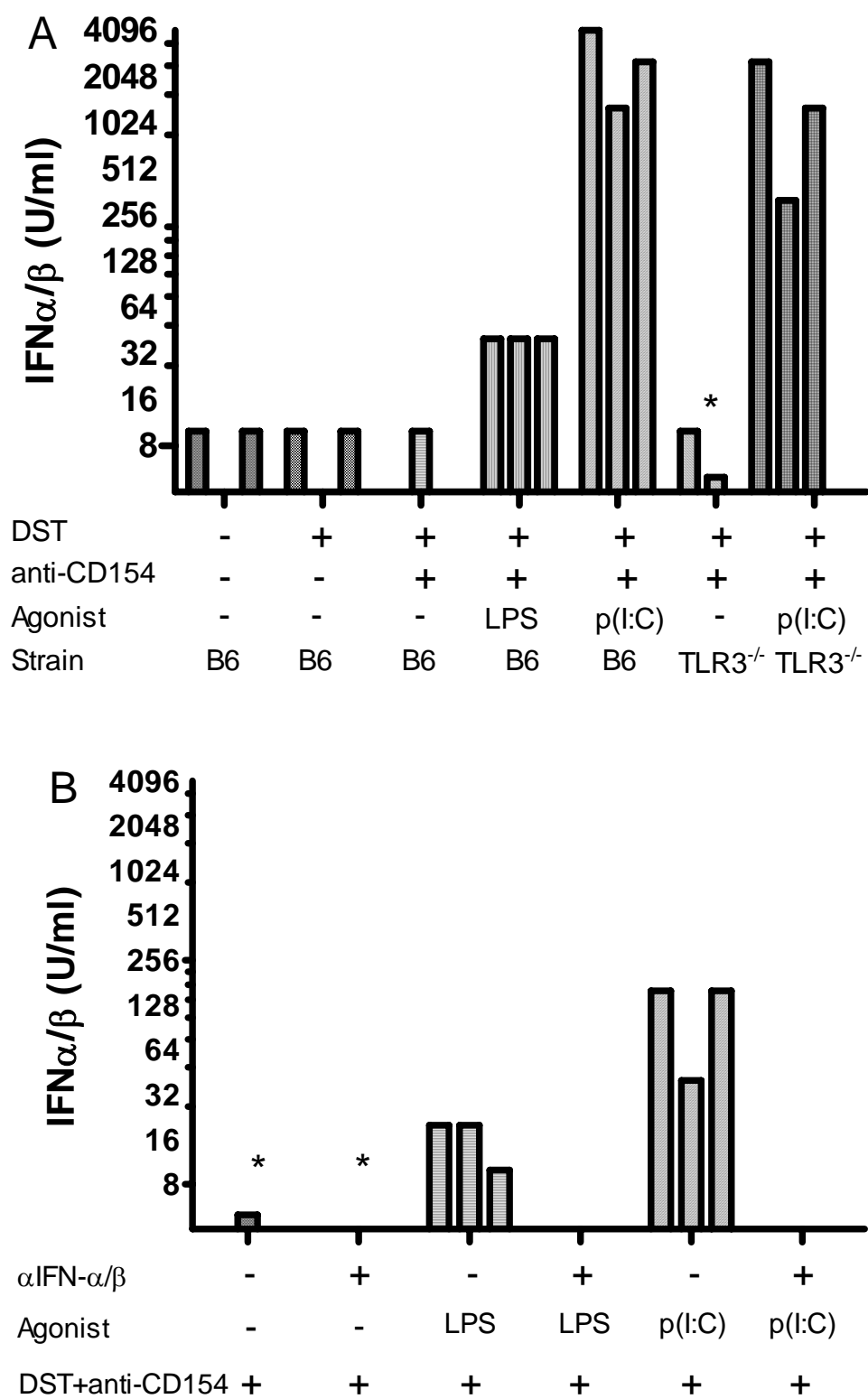
To quantify IFN- $\alpha/\beta$  levels, we performed a standard IFN- $\alpha/\beta$  bioassay (135,136). Sera were obtained from mice 16 hours after treatment with costimulation blockade and 100  $\mu$ g of LPS or 50  $\mu$ g of poly(I:C). Sera were serially diluted and added to mouse L-929 cell cultures overnight. Cells were then infected with VSV and observed for cytopathic effects (CPE) 48 hours later. IFN- $\alpha/\beta$  titers were defined as the reciprocal of the dilution that provided 50% protection against CPE from VSV infection.

We detected minimal IFN- $\alpha/\beta$  ( $\leq 10$  U/ml) in untreated mice, mice treated with DST and mice treated with DST and anti-CD154 mAb (**Fig 19A**). In contrast, we detected high levels of IFN- $\alpha/\beta$  in mice treated with DST, anti-CD154 mAb and poly(I:C) (Range 1280-5120 U/ml, n=3; **Fig 19A**). In support of our hypothesis, LPS induced a much lower level of IFN- $\alpha/\beta$  in animals treated with DST and anti-CD154 mAb (40 U/ml, n=3; **Fig 19A**).

Surprisingly, poly(I:C) induced high levels of IFN- $\alpha/\beta$  in B6.TLR3<sup>-/-</sup> mice treated with DST, anti-CD154 mAb and poly(I:C) (Range 320-2560 U/ml, n=3; **Fig 19A**). This is consistent with our earlier observation that poly(I:C) shortens skin allograft survival in TLR3-deficient mice treated with costimulation blockade.

To confirm that the inhibitory activity of the sera was due to the presence of IFN- $\alpha/\beta$ , we collected sera from a second cohort of C57BL/6 mice treated with costimulation blockade and LPS or poly(I:C). Prior to serial dilution, one of two replicates was blocked with a combination of one kilounit (KU) of neutralizing anti-mouse IFN- $\alpha$  and one KU of neutralizing anti-mouse IFN- $\beta$ . Similar to our previous experiment, we observed a higher level of IFN- $\alpha/\beta$  in sera obtained from mice treated with costimulation blockade and poly(I:C) (Range 40-160 U/mL, n=3; **Fig 19B**) than in the sera of mice treated with costimulation blockade and LPS (Range 10-20 U/mL, n=3; **Fig 19B**). The inhibitory activity was completely abrogated in all samples treated with neutralizing anti-IFN $\alpha$  and anti-IFN- $\beta$  antibodies (**Fig 19B**), indicating that the inhibitory activity is the specific result of IFN- $\alpha/\beta$ .

FIGURE 19: POLY(I:C) MORE POTENTLY INDUCES IFN- $\alpha/\beta$  THAN LPS

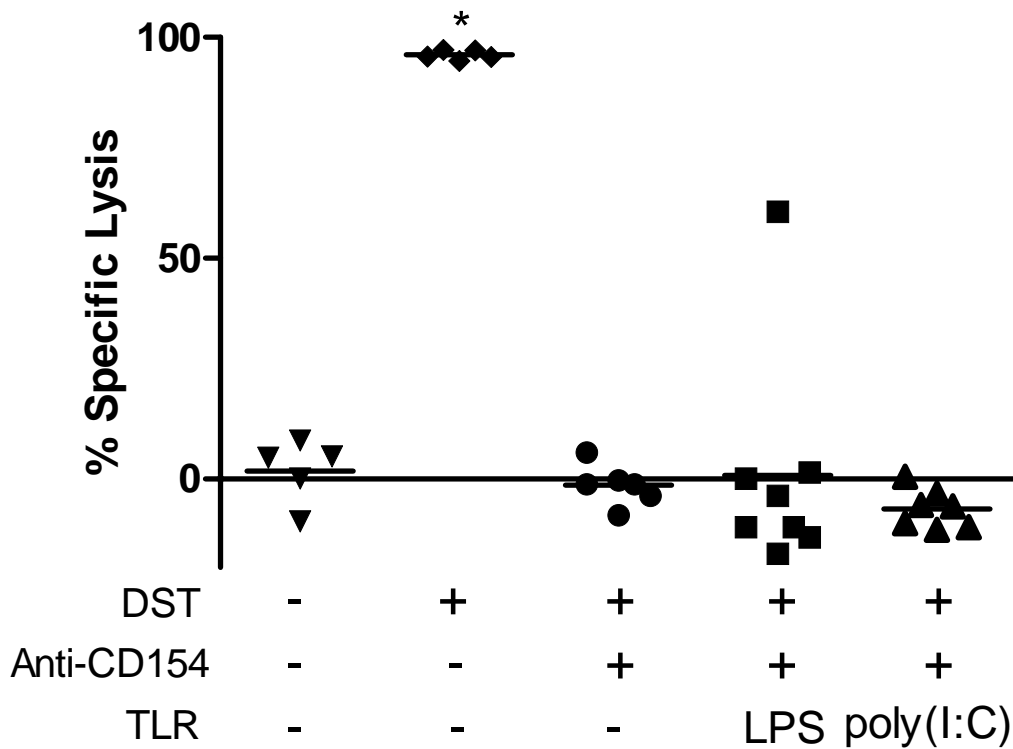


**Figure 19 Legend:** (A) Sera were collected from C57BL/6 or B6.TLR3<sup>-/-</sup> mice 16 hours after receiving the indicated treatment. Sera were serially diluted across a 96-well plate and incubated with L-929 cells for 24 hours prior to VSV infection. IFN- $\alpha/\beta$  titers were calculated as the reciprocal of the dilution that protected L-929 cells from CPE caused by VSV infection. The limit of detectability was 5 U/mL. (B) Sera were collected from a second cohort of C57BL/6 mice 16 hours after treatment with DST, anti-CD154 mAb and the indicated agonist. Sera were serially diluted across a 96-well plate, in the presence or absence of one KU of neutralizing anti-mouse IFN- $\alpha$  and one KU of neutralizing anti-mouse IFN- $\beta$  as indicated. IFN- $\alpha/\beta$  titers were calculated as indicated in panel A. \*n=2, all others n=3. Each bar represents an individual serum sample obtained from one mouse.

### 5. *IFN- $\alpha/\beta$ is required for LPS and poly(I:C) to prime an allo-specific CTL response*

We next wanted to link the difference in the amount of IFN- $\alpha/\beta$  produced by LPS and poly(I:C) to the differential priming of an allo-specific CTL response. To address this, we repeated the *in vivo* cytotoxicity assay in B6.IFN-RI<sup>-/-</sup> mice, which lack the type I IFN receptor (IFN-RI) responsible for IFN- $\alpha/\beta$  signaling. Similar to C57BL/6 mice, animals treated with costimulation blockade demonstrated no killing of the allogeneic cell population ( $-1.38 \pm 4.65\%$ , n=6), while animals treated with DST alone exhibited near-maximal killing ( $96.0 \pm 1.05\%$ , n=5,  $p < 0.0001$  vs. all other groups; **Fig 20**). Strikingly, B6.IFN-IR<sup>-/-</sup> mice treated with costimulation blockade and LPS ( $0.85 \pm 25.0\%$ , n=6) or poly(I:C) ( $-6.71 \pm 4.40\%$ , n=6; **Fig 20**) demonstrated no evidence of allogeneic killing (p=N.S. vs. DST and anti-CD154 treated group). These data demonstrate that IFN- $\alpha/\beta$  is essential for the allo-specific CTL activity observed in mice treated with costimulation blockade and LPS or poly(I:C).

**FIGURE 20: IFN- $\alpha/\beta$  IS ESSENTIAL FOR LPS AND POLY(I:C) TO PRIME AN ALLO-SPECIFIC CTL RESPONSE**



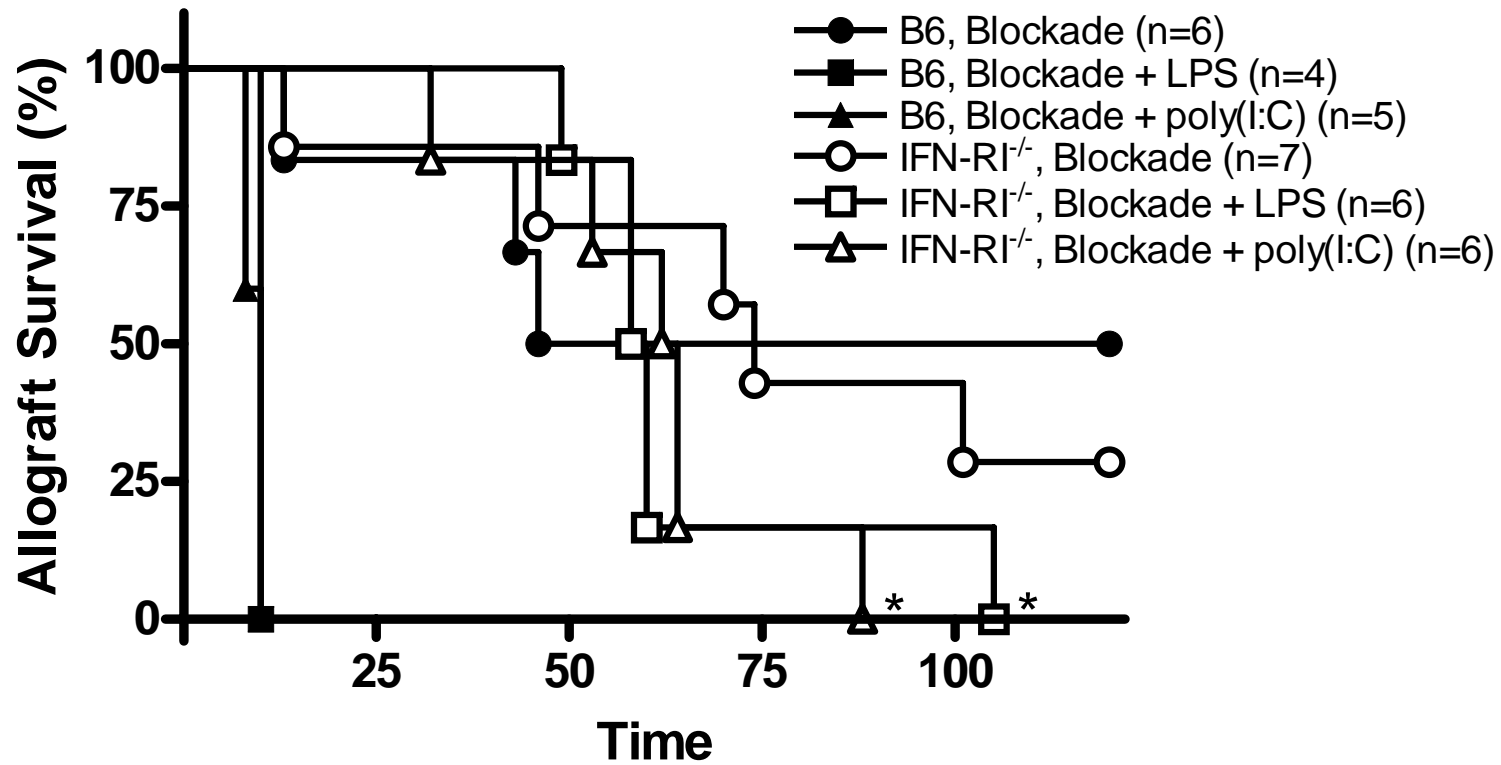
**Figure 20 Legend:** B6.IFN-RI<sup>-/-</sup> mice were given the indicated treatment on day-7 relative to performing the *in vivo* cytotoxicity assay on day 0. Data are presented as the percent specific-lysis, which was calculated as described in *Materials & Methods*. Each symbol represents an individual determination (n=5 to 8). \* DST treated group was significantly different from all others (p<0.0001).

## 6. *IFN- $\alpha/\beta$ is required for LPS and poly(I:C) to shorten skin allograft survival*

The observation that the allo-specific CTL activity generated by costimulation blockade and LPS or poly(I:C) treatment requires IFN- $\alpha/\beta$  led us to determine whether skin allograft rejection was also dependent on IFN- $\alpha/\beta$ . C57BL/6 or B6.IFN-RI<sup>-/-</sup> mice were treated with our standard costimulation blockade protocol and co-injected with 50  $\mu$ g of poly(I:C) or 100  $\mu$ g of LPS at the time of DST administration.

C57BL/6 and B6.IFN-RI<sup>-/-</sup> mice treated with costimulation blockade exhibited prolonged allograft survival with MSTs of >85 days (n=6) and 72 days (n=7; **Fig 21**), respectively. In agreement with our earlier observations (**Fig 4**), C57BL/6 mice treated with costimulation blockade and LPS (MST=10 days, n=4) or poly(I:C) (MST=10 days, n=5; **Fig 21**) rapidly rejected their skin allografts. In contrast, neither LPS (MST=59 days, n=6) nor poly(I:C) (MST=63 days, n=6; **Fig 21**) significantly shortened skin allograft survival in B6.IFN-RI<sup>-/-</sup> mice (p=N.S. vs. DST and anti-CD154 treated B6.IFN-RI<sup>-/-</sup>). These data demonstrate that LPS and poly(I:C) shorten skin allograft survival in mice treated with costimulation blockade by inducing the production of IFN- $\alpha/\beta$ .

**FIGURE 21: IFN- $\alpha/\beta$  IS ESSENTIAL FOR LPS AND POLY(I:C) TO SHORTEN ALLOGRAFT SURVIVAL**



**Figure 21 Legend:** C57BL/6 or B6. $\text{IFN-RI}^{-/-}$  mice were given BALB/c DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of LPS or poly(I:C) on day -7 relative to skin grafting on day 0. All groups received BALB/c skin allografts on day 0. \*  $p=\text{N.S.}$  compared to costimulation blockade-treated  $\text{IFN-RI}^{-/-}$  mice.

### *7. IFN- $\beta$ impairs alloreactive CD8<sup>+</sup> T cell deletion and shortens skin allograft survival*

We have established that IFN- $\alpha/\beta$  is essential for LPS and poly(I:C) to shorten skin allograft survival induced by costimulation blockade. We next hypothesized that IFN- $\alpha/\beta$  was sufficient on its own to prevent the deletion of alloreactive CD8<sup>+</sup> T cells and shorten skin allograft survival in mice treated with costimulation blockade. To test this, we treated KB5 synchimeric mice with our standard costimulation blockade protocol, with or without a co-injection of  $2 \times 10^4$  or  $5 \times 10^4$  U of IFN- $\beta$ . Blood was collected from mice before treatment and one day prior to transplantation with a C57BL/6 skin allograft on day 0, and analyzed for levels of the transgenic alloreactive CD8<sup>+</sup> T cells.

As expected, animals treated with costimulation blockade showed a dramatic reduction ( $95.6 \pm 1.53\%$ , n=4) in the number of transgenic alloreactive CD8<sup>+</sup> T cells in the blood 6 days after treatment (**Table VII**). In support of our earlier data, LPS impaired this deletion, leading only to a  $19.7 \pm 31.0\%$  (n=4) decrease ( $p < 0.01$  vs. costimulation blockade-treated group; **Table VII**). Interestingly, the injection of  $2 \times 10^4$  U of IFN- $\beta$  impaired deletion in costimulation blockade-treated mice, resulting in a  $63.1 \pm 15.3\%$  reduction (n=4,  $p < 0.01$  vs. costimulation blockade-treated group and  $p = \text{N.S.}$  vs. costimulation blockade and LPS treated group; **Table VII**).

In a second experiment, a higher dose of IFN- $\beta$  more dramatically impaired deletion. Mice injected with  $5 \times 10^4$  U of IFN- $\beta$  showed only a  $23.5 \pm 30.4\%$  (n=4; **Table VII**) reduction in alloreactive CD8<sup>+</sup> T cell number. This was significantly lower than the reduction observed in mice treated with costimulation blockade alone ( $92.0 \pm 1.83\%$ ,  $p < 0.01$ ; n=4; **Table VII**) and was similar to the reduction observed in mice

treated costimulation blockade and LPS ( $4.34 \pm 22.2\%$ ,  $p=\text{N.S.}$ ; **Table VII**). These data demonstrate that type I IFN alone can impair the deletion of alloreactive CD8<sup>+</sup> T cells in mice treated with costimulation blockade.

Strikingly, the prolonged skin allograft survival induced by costimulation blockade (MST=30 days,  $n=4$ ) was shortened by the injection of  $2 \times 10^4$  U of IFN- $\beta$  (MST=12 days,  $n=4$ ,  $p<0.01$  *vs.* costimulation blockade-treated group; **Table VII**). This is similar to the allograft survival observed in mice treated with costimulation blockade and LPS (MST=10 days,  $n=3$ ,  $p=0.01$  *vs.* costimulation blockade-treated group,  $p=\text{N.S.}$  *vs.* costimulation blockade and IFN- $\beta$  treated group; **Table VII**). Together, these data demonstrate that IFN- $\beta$  can impair the deletion of alloreactive CD8<sup>+</sup> T cells and shorten allograft survival induced by costimulation blockade.

**TABLE VII: IFN- $\beta$  IMPAIRS DELETION AND SHORTENS ALLOGRAFT SURVIVAL**

Exp	Group	DST	$\alpha$ CD154	Treatment	N	Chimerism Pre-treatment (%)	Chimerism 6d post-treatment (%)	Deletion (%)	Graft Survival (days)	MST (days)
A	1	Yes	Yes	None	4	$8.97 \pm 1.24$	$0.40 \pm 0.14$	$95.6 \pm 1.53^*$	22, 22, 38, 43 <sup>‡</sup>	30 <sup>‡</sup>
	2	Yes	Yes	LPS	3	$8.21 \pm 2.63$	$6.22 \pm 2.35$	$19.7 \pm 31.0^{**}$	10, 10, 13, 13	12
	3	Yes	Yes	$2 \times 10^4$ U IFN- $\beta$	4	$8.58 \pm 1.50$	$3.31 \pm 1.73$	$63.1 \pm 15.3$	10, 10, 12	10
B	4	Yes	Yes	None	4	$7.19 \pm 1.35$	$0.58 \pm 0.19$	$92.0 \pm 1.83^\dagger$	ND	ND
	5	Yes	Yes	LPS	4	$8.28 \pm 0.91$	$7.78 \pm 1.21$	$4.34 \pm 22.2^{\dagger\dagger}$	ND	ND
	6	Yes	Yes	$5 \times 10^4$ U IFN- $\beta$	4	$7.97 \pm 0.52$	$6.02 \pm 2.24$	$23.5 \pm 30.4$	ND	ND

**Table VII Legend:** KB5 synchimeric mice were treated with DST on day -7; anti-CD154 mAb on days -7, -4, 0, and +4; with or without injection of IFN- $\beta$  or LPS on day -7 relative to skin grafting on day 0. All mice were bled for analysis of CD8<sup>+</sup>DES<sup>+</sup> cells on day -1 relative to transplantation with C57BL/6 skin allografts on day 0. Percentage of transgenic alloreactive CD8<sup>+</sup> T cells in the blood was determined as described in Table II. Allograft survival times and MSTs are indicated in days. ANOVA analysis showed an overall significant difference among chimerism levels,  $p < 0.00001$ . \* $p < 0.01$  vs. groups 2 and 3. \*\* $p = \text{N.S.}$  vs. group 3.  $^\dagger p < 0.01$  vs. group 5 and  $p < 0.05$  vs. group 6.  $^{\dagger\dagger} p = \text{N.S.}$  vs. group 3.  $^\ddagger p \leq 0.01$  vs. groups 2 and 3. ND= Not Done.

## Summary

In contrast to the findings in our skin allograft system, the effect of LPS on islet allograft survival in mice treated with costimulation blockade is intermediate. Consistent with our results in the skin graft model system, the effect of poly(I:C) is dramatic. The difference in effects of LPS and poly(I:C) appear to result from their differential ability to prime an adequate allo-specific CTL response in mice treated with costimulation blockade. Strikingly, the two agonists differ greatly in their ability to induce IFN- $\alpha/\beta$ , which is both necessary and sufficient to shorten skin allograft survival in mice treated with costimulation blockade.

## **CHAPTER VII: MCMV REQUIRES THE ADAPTOR MOLECULE MYD88 TO SHORTEN SKIN ALLOGRAFT SURVIVAL**

### **Introduction**

We have shown that the induction of IFN- $\alpha/\beta$  at the time of costimulation blockade treatment shortens skin allograft survival in mice treated with costimulation blockade. We have also demonstrated that the induction of IFN- $\alpha/\beta$  may be TLR-dependent or –independent. In this chapter, we determine the extent to which LCMV and murine cytomegalovirus (MCMV) depend on TLRs to shorten allograft survival.

The immune response to the dsDNA herpesvirus MCMV has been shown to rely heavily upon the activation of the MyD88/TLR9 pathway (152,153). However, MCMV has been reported to mediate other actions through the TLR2 (104,154) and TLR3/TRIF pathways (100). We therefore tested the ability of MCMV to shorten allograft survival in mice deficient in TLR2 and the TLR-adaptor molecule MyD88.

## Results

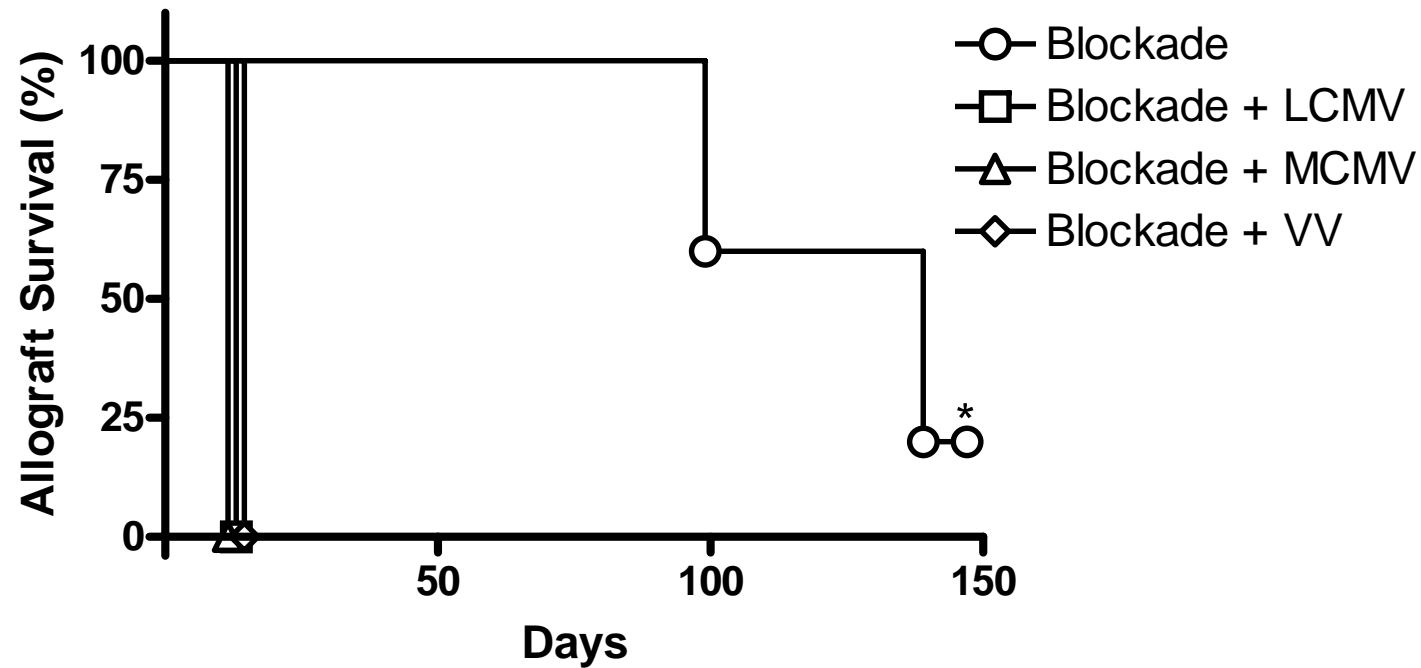
### 1. *Viruses shorten allograft survival and impair alloreactive CD8<sup>+</sup> T cell deletion*

Having shown that agonists to TLR2, TLR3, TLR4, and TLR9 impair the efficacy of our costimulation blockade protocol, we next tested whether a diverse repertoire of viruses shorten allograft survival and impair alloreactive CD8<sup>+</sup> T cell deletion. In order to test this, we treated KB5 synchimeric mice with our standard costimulation blockade protocol and randomized the mice into four groups. One group received no additional treatment, while the three remaining groups were infected i.p. on day – 7 with LCMV, MCMV, or vaccinia virus (VV).

As expected, KB5 synchimeric mice treated with costimulation blockade exhibited robust skin allograft survival (MST=139 days, n=5; **Fig 22**). In contrast, mice treated with costimulation blockade and infected with LCMV (MST = 13 days, n=4), MCMV (MST = 13 days, n=5), or VV (MST = 13 days, n=5) acutely rejected their skin allografts (p<0.01 vs. uninfected mice).

To determine whether the acute rejection observed in the virus-infected mice correlated with an impaired deletion of alloreactive CD8<sup>+</sup> T cells, we quantified the percentage of transgenic KB5 alloreactive CD8<sup>+</sup> T cells in peripheral blood in the four groups. KB5 synchimeric mice treated with costimulation blockade exhibited a marked depletion of alloreactive CD8<sup>+</sup> T cells, from  $5.26 \pm 0.84\%$  prior to treatment to  $0.83 \pm 0.32\%$  6 days after treatment (**Table VIII**). This deletion was substantially impaired by all viruses tested (p<0.0001 vs. costimulation blockade alone; **Table VIII**).

**FIGURE 22: VIRUS INFECTION SHORTENS SKIN ALLOGRAFT SURVIVAL**



**Figure 22 Legend:** KB5 synchimeric mice were given C57BL/6 DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of the indicated virus on day -7 relative to skin grafting on day 0 as described in *Materials and Methods*. All groups received C57BL/6 skin allografts on day 0. \* $p < 0.01$  vs. all other groups.

**TABLE VIII: VIRUS INFECTION IMPAIRS ALLOREACTIVE CD8<sup>+</sup> T CELL DELETION**

<b>Virus</b>	<b>Chimerism Pre-treatment (%)</b>	<b>Chimerism 6d post-treatment (%)</b>	<b>N</b>	<b>Deletion (%)</b>
<i>None</i>	5.26 ± 0.84	0.93 ± 0.30	10	82.2 ± 5.14*
<i>LCMV</i>	5.14 ± 1.06	2.47 ± 0.58	10	50.4 ± 14.0
<i>MCMV</i>	4.94 ± 1.42	3.39 ± 0.99	10	27.5 ± 20.8
<i>VV</i>	5.35 ± 1.24	4.46 ± 0.59	10	13.4 ± 15.4

**Table VIII Legend:** KB5 synchimeric mice were treated with C57BL/6 DST and anti-CD154 mAb on day 0 and anti-CD154 mAb on day +3, with or without injection of the indicated virus on day 0 relative to recovery of blood for analysis on day 6. Peripheral blood mononuclear cells were collected and the percentage of DES<sup>+</sup>CD8<sup>+</sup> transgenic T cells was determined as described in Table II. ANOVA showed an overall significant difference of  $p < 0.000001$ . \*  $p < 0.0001$  vs. all other groups.

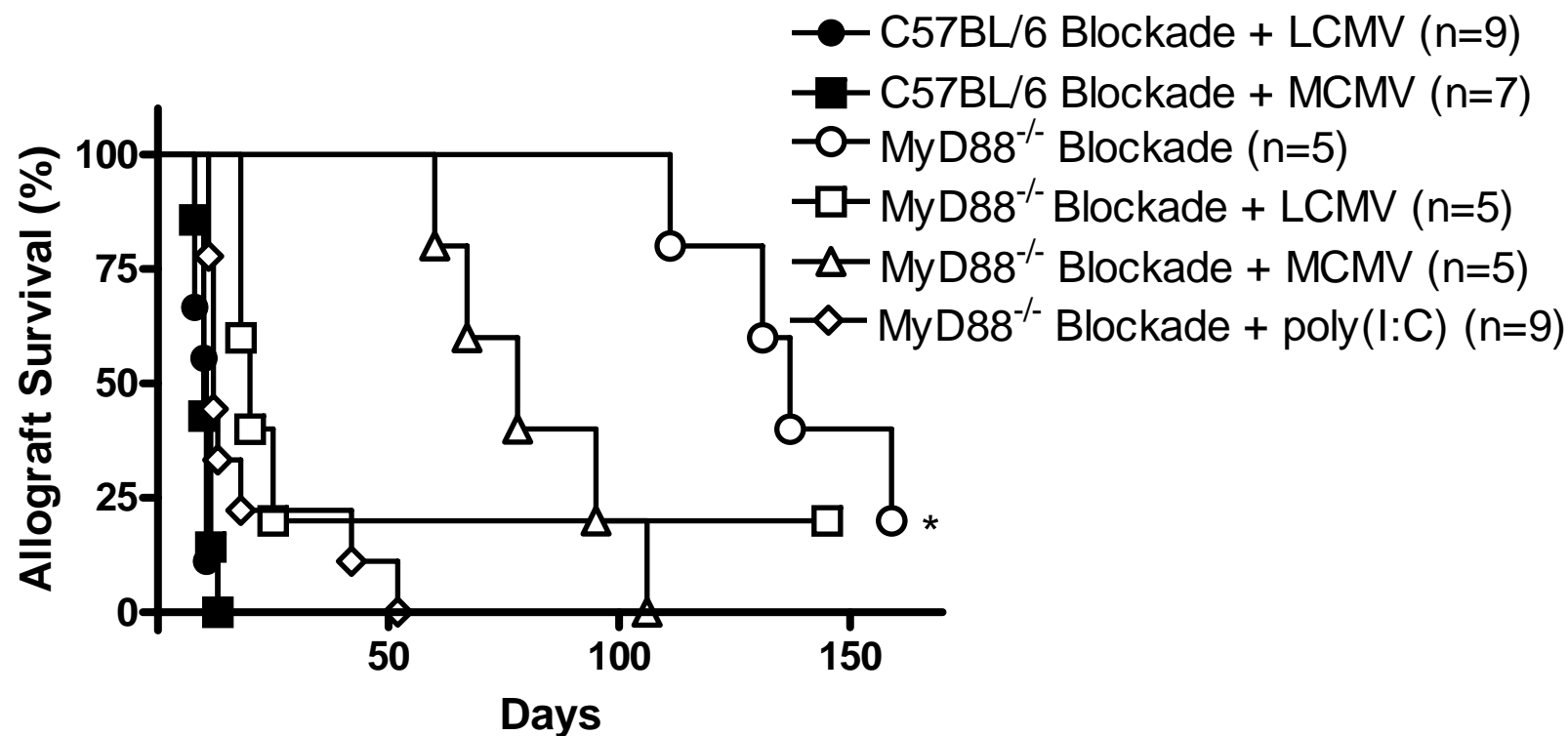
## 2. *Viruses shorten allograft survival through a MyD88-dependent mechanism*

We hypothesized that viruses shorten skin allograft survival in part by activating TLRs. As a generic test of the importance of TLRs, we employed mice deficient in the common TLR adaptor molecule MyD88. Three groups of B6.MyD88<sup>-/-</sup> mice were treated with our standard costimulation blockade protocol and transplanted with skin allografts. One group was not treated further. A second group was infected with MCMV on the day of DST. A third group was infected with LCMV on the day of DST.

Confirming our earlier observation, costimulation blockade treatment prolonged skin allograft survival in B6.MyD88<sup>-/-</sup> mice (MST=137 days, n=5; **Fig 23**). This survival was moderately shortened in mice infected with LCMV (MST=20 days, n=5, p=N.S. vs. costimulation blockade-treated MyD88<sup>-/-</sup>) or MCMV (MST=78 days, n=5, p<0.02 vs. costimulation blockade-treated MyD88<sup>-/-</sup>; **Fig 23**). In contrast, allograft survival was dramatically shortened by injection with poly(I:C) (MST=12 days, n=9, p<0.001 vs. costimulation blockade-treated MyD88<sup>-/-</sup>; **Fig 23**).

Strikingly, allograft survival was significantly longer in LCMV- and MCMV-infected B6.MyD88<sup>-/-</sup> mice compared to comparably treated C57BL/6 control mice. C57BL/6 mice rejected their skin allografts more rapidly than their B6.MyD88<sup>-/-</sup> counterparts when infected with either LCMV (MST=11 days, n=9, p<0.001 vs. LCMV-infected costimulation blockade-treated MyD88<sup>-/-</sup>) or MCMV (MST=10 days, n=7, p=0.001 vs. MCMV-infected costimulation blockade-treated MyD88<sup>-/-</sup>; **Fig 23**). These data suggest that MyD88, and consequently TLRs, may play an important role in allograft survival shortened by viruses.

**FIGURE 23: VIRUSES SHORTEN ALLOGRAFT SURVIVAL IN A MYD88 DEPENDENT MANNER**



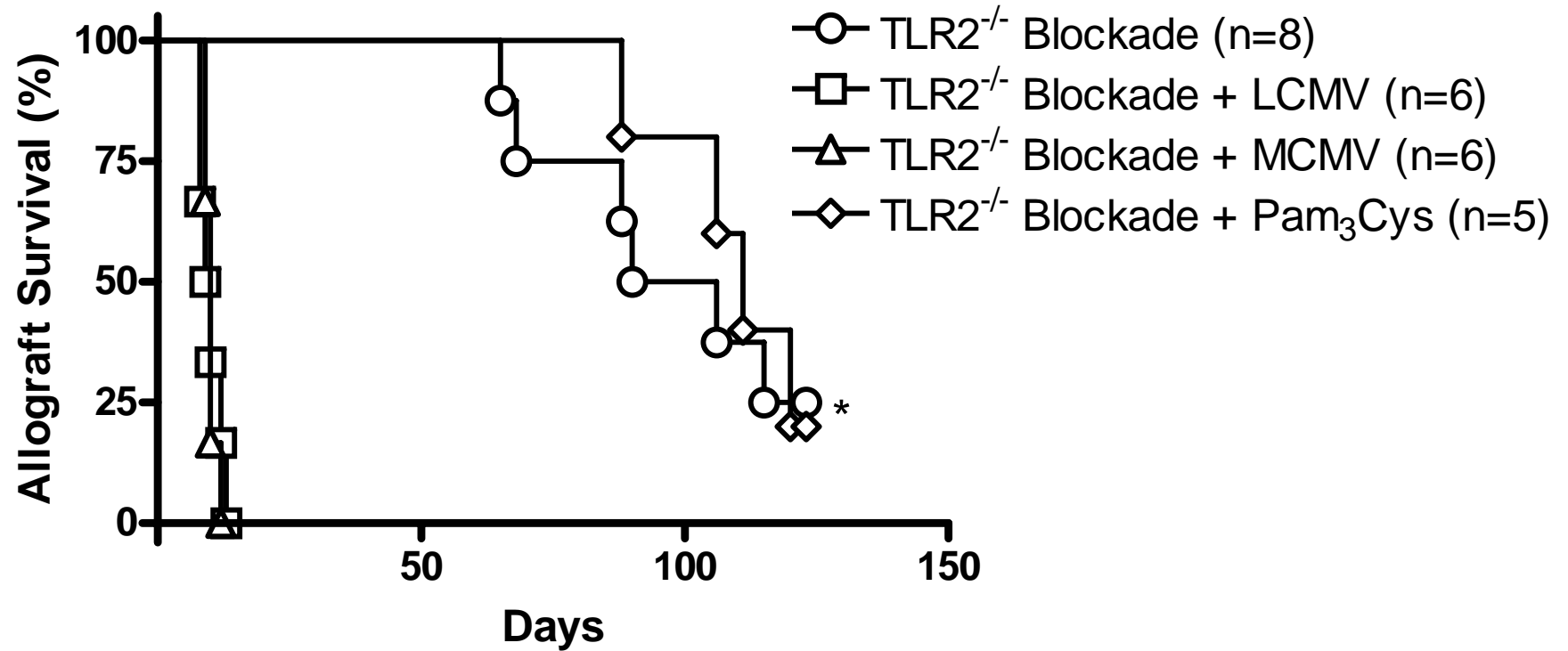
**Figure 23 Legend:** C57BL/6 or B6.MyD88<sup>-/-</sup> mice were given BALB/c DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of poly(I:C) or the indicated virus on day -7 relative to skin grafting on day 0. All groups received BALB/c skin allografts on day 0. \*p<0.02 compared to costimulation blockade and MCMV treated group; p=N.S. compared to costimulation blockade and LCMV treated group.

### *3. MCMV does not shorten skin allograft survival by activating TLR2*

LCMV shortened skin allograft survival in MyD88-deficient mice, whereas the effect of MCMV was intermediate. These data suggest that MCMV shortens allograft survival in part by activating a TLR that signals through the MyD88-dependent pathway. TLR2 is a candidate TLR that recruits the adaptor molecule MyD88, and is known to recognize MCMV (104,154). In order to test whether MCMV shortens allograft survival by activating TLR2, three groups of B6.TLR2<sup>-/-</sup> mice were treated with our standard costimulation blockade protocol and transplanted with BALB/c skin allografts. One group was not treated further. A second group was infected with MCMV on the day of DST. A third group was infected with LCMV on the day of DST.

Similar to wildtype C57BL/6 mice (**Fig 4**), B6.TLR2<sup>-/-</sup> mice treated with costimulation blockade exhibited prolonged allograft survival (MST=98 days, n=9; **Fig 24**). Skin allograft survival was shortened by infection with LCMV (MST=10 days, n=6, p<0.0001 vs. costimulation blockade-treated B6.TLR2<sup>-/-</sup>) or MCMV (MST=10 days, n=6, p=0.0001 vs. costimulation blockade-treated B6.TLR2<sup>-/-</sup>), but not the purified TLR2 agonist Pam<sub>3</sub>Cys (MST=111 days, n=5, p=N.S. vs. costimulation blockade-treated B6.TLR2<sup>-/-</sup>; **Fig 24**). These data document that MCMV shortens skin allograft survival through a TLR2-independent, MyD88-dependent mechanism.

**FIGURE 24: MCMV AND LCMV SHORTEN ALLOGRAFT SURVIVAL IN TLR2-DEFICIENT MICE**



**Figure 24 Legend:** B6.TLR2<sup>-/-</sup> mice were given BALB/c DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of the indicated virus or Pam<sub>3</sub>Cys on day -7 relative to skin grafting on day 0. All groups received BALB/c skin allografts on day 0. \* p<0.0001 compared to costimulation blockade and LCMV or MCMV treated groups; p=N.S. compared to costimulation blockade and Pam<sub>3</sub>Cys treated group.

#### *4. MCMV and LCMV do not require IL-18 or IL-1R to shorten allograft survival*

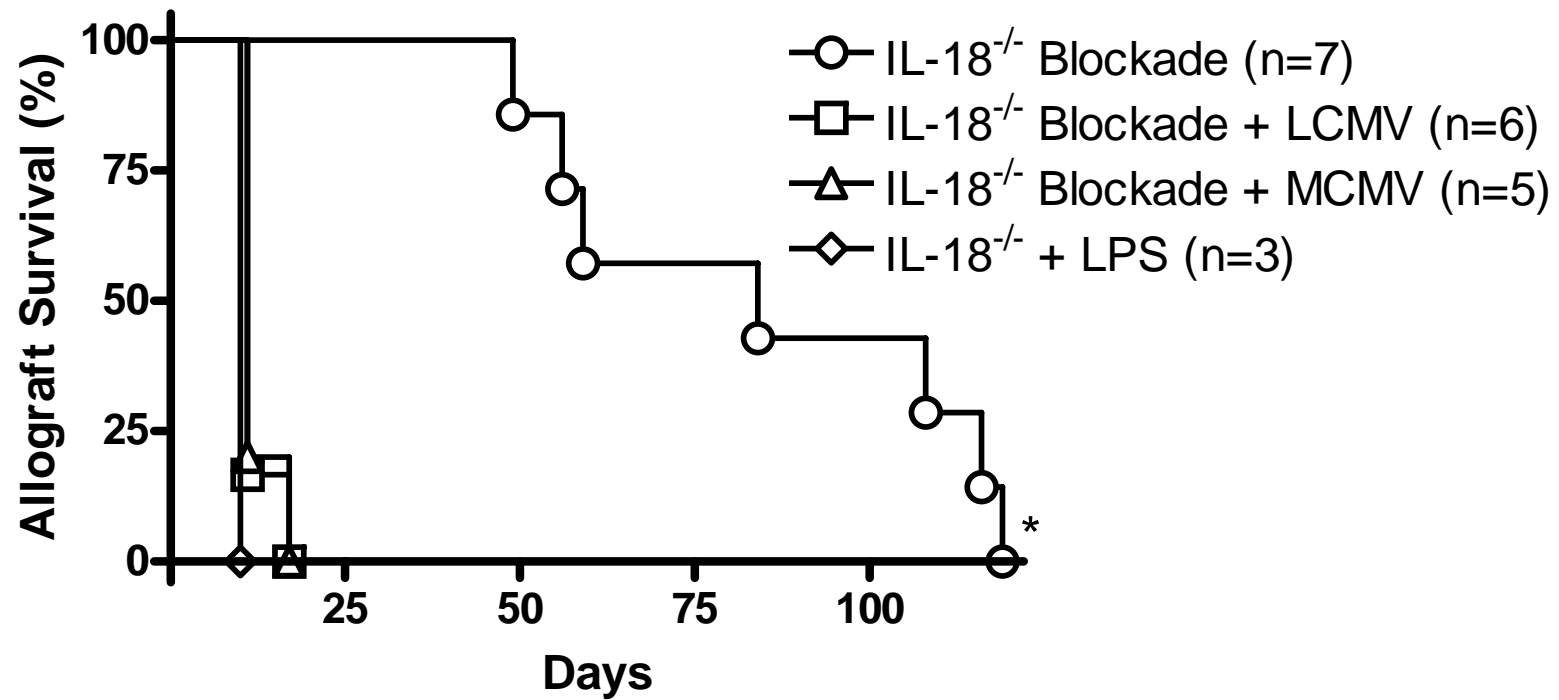
We have interpreted the finding that infection with LCMV and MCMV shortens allograft survival to a lesser degree in MyD88<sup>-/-</sup> mice than wildtype mice to indicate that these viruses shorten allograft survival by activating TLRs. However, IL-1R and IL-18R also signal through the adaptor molecule MyD88. To rule out the possibility that LCMV and MCMV shorten allograft survival by inducing IL-1 or IL-18, IL-1R<sup>-/-</sup> and IL-18<sup>-/-</sup> mice were each divided into three groups that were treated with our standard costimulation blockade protocol and transplanted with BALB/c skin allografts. One group was not treated further. A second group was infected with MCMV on the day of DST. A third group was infected with LCMV on the day of DST.

Costimulation blockade prolonged skin allograft survival in IL-18<sup>-/-</sup> (MST=84 days, n=4; **Fig 25**) and IL-1R<sup>-/-</sup> mice (MST=38 days, n=3; **Fig 26**). In contrast, costimulation blockade treatment failed to prolong allograft survival in IL18<sup>-/-</sup> mice that were injected with LPS (MST=10 days, n=3, p<0.01 vs. costimulation blockade-treated B6.IL-18<sup>-/-</sup>), LCMV (MST=11 days, n=6, p<0.0005 vs. costimulation blockade-treated B6.IL-18<sup>-/-</sup>) or MCMV (MST=11 days, n=5, p<0.0005 vs. costimulation blockade-treated B6.IL-1R<sup>-/-</sup>; **Fig 25**). Costimulation blockade treatment also failed to prolong skin allograft survival in IL-1R<sup>-/-</sup> mice infected with LCMV (MST=10 days, n=6, p<0.01 vs. costimulation blockade-treated B6.IL-1R<sup>-/-</sup>) or MCMV (MST=20 days, n=6, p<0.05 vs. costimulation blockade-treated B6.IL-1R<sup>-/-</sup>; **Fig 26**). It should be noted, however, that skin allograft survival in mice infected with MCMV appears to be more dependent on IL-1R compared to mice infected with LCMV (p<0.05). Nevertheless, skin allograft

survival is significantly shorter in MCMV-infected B6.IL-R<sup>-/-</sup> mice than B6.MyD88<sup>-/-</sup> mice (p<0.01, **Fig 23**).

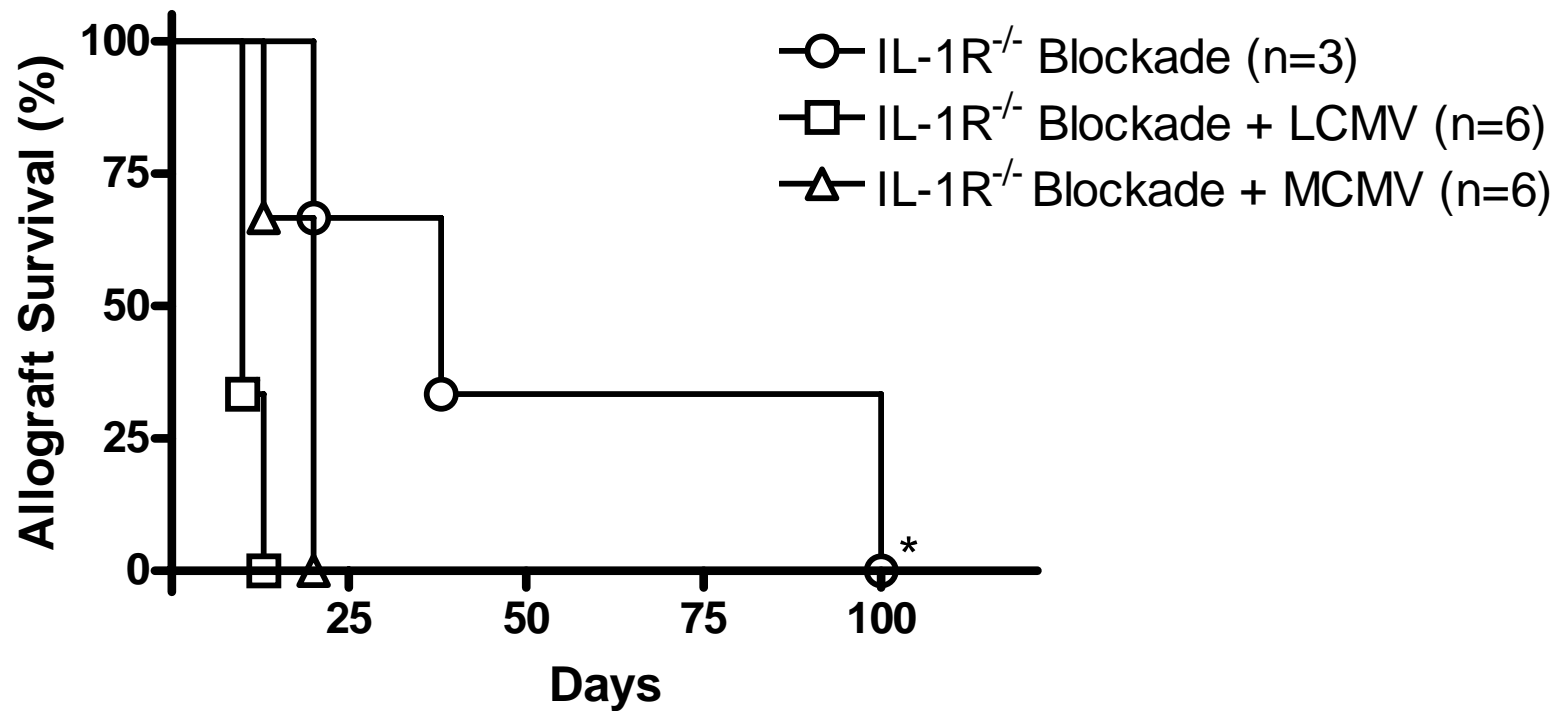
Taken together, these data suggest that LCMV and MCMV shorten allograft survival through a mechanism that is reliant on MyD88, but independent of IL-1R and IL-18R. However, we have not performed this experiment in double-knockout mice and cannot exclude the possibility that IL-1R and IL-18R have redundant functions in this system.

**FIGURE 25: LCMV AND MCMV SHORTEN ALLOGRAFT SURVIVAL IN IL-18<sup>-/-</sup> MICE**



**Figure 25 Legend:** B6.IL-18<sup>-/-</sup> mice were given BALB/c DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of the indicated virus or LPS on day -7 relative to skin grafting on day 0. All groups received BALB/c skin allografts. \*p<0.01 compared to all other groups.

**FIGURE 26: LCMV AND MCMV SHORTEN ALLOGRAFT SURVIVAL IN IL-1R<sup>-/-</sup> MICE**



**Figure 26 Legend:** B6.IL-1R<sup>-/-</sup> mice were given BALB/c DST and anti-CD154 mAb according to our standard costimulation blockade protocol with or without injection of the indicated virus on day -7 relative to skin grafting on day 0. All groups received BALB/c skin allografts on day 0. \*p<0.05 compared to all other groups.

## Summary

We demonstrate that a variety of RNA and DNA viruses impair the deletion of alloreactive CD8<sup>+</sup> T cells and shorten skin allograft survival induced by costimulation blockade. Interestingly, both LCMV and MCMV shorten allograft survival through a mechanism that is partially dependent on MyD88 and largely independent of IL-1R and IL-18R signaling. Together, these data suggest that TLRs may play a critical role in the shortened skin allograft survival observed in mice infected with virus at the time of costimulation blockade.

## CHAPTER VIII: DISCUSSION

### Activation of TLRs Prevents Transplantation Tolerance Induction

Costimulation blockade protocols are effective in animal models, but it will be important for their implementation in the clinic to determine how environmental perturbants impair their efficacy. Our group has previously shown that LCMV-infection at the time of initial treatment with DST and anti-CD154 mAb shortens skin allograft survival (72); however, the mechanism underlying this observation is not known. We hypothesize that LCMV activates innate immunity through TLRs, a hypothesis consistent with the fact that mice deficient in the TLR-associated adapter molecule MyD88 do not mount a robust anti-LCMV T cell response and fail to clear the virus (155). TLR activation has many of the same consequences for APCs as CD40 activation, including upregulation of the costimulatory molecules CD80 and CD86 and the production of pro-inflammatory cytokines (156). Therefore, the activation of TLRs may mature APCs and complete alloreactive T cell activation in the presence of a CD40-CD154 blockade.

We provide proof of principle for our hypothesis by demonstrating that agonists of TLR2 (Pam<sub>3</sub>Cys), TLR3 (Poly(I:C)), TLR4 (LPS), and TLR9 (CpG DNA) shorten allograft survival (**Fig 4, 5**) and impair alloreactive CD8<sup>+</sup> T cell deletion (**Table II**) in mice treated with costimulation blockade. This outcome is identical to the one observed in mice infected with LCMV, MCMV or VV (**Fig 22, Table VIII**). The observation that each of these TLR agonists leads to the same outcome is interesting, but not surprising. Despite the diversity of signaling pathways employed by TLRs (**Fig 3**), they frequently have shared biological outcomes (96). For example, the induction of pro-inflammatory

cytokines is at least partially dependent on the activation of I $\kappa$ B; however, the upstream signaling leading to I $\kappa$ B activation differs among several of the TLRs. TLR2 activates I $\kappa$ B through the MyD88 pathway; TLR3 activates I $\kappa$ B through the MyD88-independent TRIF pathway; and TLR4 activates I $\kappa$ B through both the MyD88 and TRIF pathways (96).

We first addressed whether TLR agonists are impairing the deletion of alloreactive CD8<sup>+</sup> T cells by preventing their apoptosis during the initial stages of activation, or by enhancing the expansion of a subset of cells that survives. The TLR4 agonist LPS does not increase the capacity of antigen to stimulate alloreactive CD8<sup>+</sup> T cells. Alloreactive CD8<sup>+</sup> T cells are activated (**Fig 10**) and proliferating (**Fig 11**) in mice treated with DST and anti-CD154 mAb in the presence or absence of LPS. In fact, the co-administration of LPS decreases the overall percentage of activated (CD44<sup>hi</sup>) and proliferating (Ki-67<sup>+</sup>) alloreactive CD8<sup>+</sup> T cells at 72 h in mice treated with costimulation blockade. This result may be the product of increased competition for antigen in mice treated with costimulation blockade and LPS, which possess an approximately 6-fold higher number of alloreactive CD8<sup>+</sup> T cells 72 h after treatment compared to mice treated with costimulation blockade alone (**Table III**).

We initially speculated that the reduced frequency of activated, proliferating cells in mice treated with costimulation blockade and LPS is associated with protection of these cells from apoptosis. It has been reported that activation and proliferation are molecularly linked to apoptosis (157-159). Interestingly, the increased frequency of proliferating cells in mice treated with costimulation blockade is associated with an increase in apoptosis as determined by annexin V staining (**Fig 8, 9**). Additionally,

alloreactive CD8<sup>+</sup> T cells from mice treated with LPS alone had a naïve CD44<sup>lo</sup> Ki-67<sup>-</sup> phenotype similar to that observed in untreated mice, confirming that activation requires antigen (DST) and that LPS does not “non-specifically” activate T cells.

It is notable that we did not observe a statistical difference in annexin V staining within the non-transgenic CD8<sup>+</sup> T cell population between untreated mice and mice treated with costimulation blockade (**Fig 9**). We would expect 0.1-10% of the non-transgenic cells to be alloreactive (19-21), and that these cells undergo apoptosis after treatment with costimulation blockade. Anecdotally, we did observe a higher number of individual CD8<sup>+</sup> T cells staining highly for annexin V in mice treated with costimulation blockade than in untreated control mice (**Fig 8A**). It is therefore likely that non-transgenic alloreactive CD8<sup>+</sup> T cells are undergoing apoptosis, but that our assay is not sensitive enough to detect such a small population as being statistically relevant. The background level of annexin V staining in the non-transgenic population, approximately 5-7% in untreated animals, may conceal a population that falls at the lower end of our estimate for non-transgenic alloreactive CD8<sup>+</sup> T cells.

The fact that costimulation blockade does not prevent the initial activation of alloreactive CD8<sup>+</sup> T cells suggests that LPS is inducing an essential factor that determines whether the antigen-activated CD8<sup>+</sup> T cells will survive or undergo cell death, and perhaps determines the outcome of allograft survival or rejection. In support of this, we demonstrate that LPS decreases the frequency of apoptotic alloreactive CD8<sup>+</sup> T cells in the spleen at 72 h after treatment with costimulation blockade (**Fig 8, 9**). There was no significant difference in apoptosis within the non-transgenic CD8<sup>+</sup> T cell population among the treatment groups, indicating that the apoptosis induced by costimulation

blockade is an antigen-specific effect. The decrease in apoptotic cell number also correlates with a decrease in active caspase-3 expression, indicating that LPS protects cells from costimulation blockade-induced apoptosis.

The changes in the absolute number of alloreactive CD8<sup>+</sup> T cells during the 96 h period following treatment with costimulation blockade (**Table III**) support a mechanism whereby LPS prevents apoptosis and promotes the antigen-specific expansion of alloreactive CD8<sup>+</sup> T cells. Specifically, LPS prevents the early antigen-dependent loss of these cells 24 h after treatment with costimulation blockade, before cells have started to proliferate. LPS also leads to the antigen-specific expansion of these cells at 72 h after treatment. Together, these data indicate that LPS prevents the early apoptosis and promotes the later expansion of alloreactive CD8<sup>+</sup> T cells.

The small decrease in alloreactive CD8<sup>+</sup> T cell number in LPS-treated mice, regardless of costimulation blockade treatment, is probably due to IFN- $\alpha/\beta$  production. It has been previously established that IFN- $\alpha/\beta$  leads to the attrition of CD8<sup>+</sup> T cells after stimulation with poly(I:C) (114). The attrition that we observed is smaller in size and scope than what has been observed with poly(I:C) (114), which is consistent with our observation that LPS induces significantly less IFN- $\alpha/\beta$  than does poly(I:C) (**Fig 19**).

The allo-specific CD8<sup>+</sup> T cells that are protected from apoptosis are functionally active as evidenced by their ability to rapidly produce IFN- $\gamma$  upon *in vitro* allogeneic stimulation (**Fig 7**). This observation suggests that CD8<sup>+</sup> T cells play a critical role in the rejection of allografts. In support of this hypothesis, we demonstrate that mice deficient in CD8<sup>+</sup> T cells reject skin allografts with similar kinetics when treated with costimulation blockade in the presence or absence of LPS co-injection (**Fig 6**).

The requirement for CD8<sup>+</sup> T cells is somewhat surprising because untreated CD8-deficient mice rapidly reject allografts (160). The fact that CD4<sup>+</sup> T cells are not sufficient to reject a skin allograft after treatment with costimulation blockade and LPS may have several explanations. Firstly, the activation of CD4<sup>+</sup> and CD8<sup>+</sup> T cells is augmented by distinct subsets of pro-inflammatory cytokines (161). It is therefore possible that the cytokine milieu generated by our chosen dose of LPS is sufficient for the preservation of alloreactive CD8<sup>+</sup> T cells, but insufficient for the preservation of alloreactive CD4<sup>+</sup> T cells. We hope to test this hypothesis in our D10.G4.1 transgenic model, which possesses a CD4<sup>+</sup> T cell population that expresses a clonotypic TCR with an allo-specificity for I-A<sup>b</sup> (162).

Secondly, it may be possible that a CD8<sup>+</sup> T cell population is necessary to initiate the allo-immune response induced by TLR agonists. It was recently reported that CD8<sup>+</sup> T cells rapidly produce the pro-inflammatory cytokine TNF $\alpha$  upon stimulation (163). The rapid production of TNF $\alpha$  may play an important role in shaping the immune response and determining cellular differentiation programs. This raises the possibility that LPS shortens allograft survival by promoting the survival of CD8<sup>+</sup> T cells, which rapidly produce pro-inflammatory cytokines that are necessary to generate a pro-rejection T<sub>H1</sub> response as opposed to a tolerogenic T<sub>H2</sub> response.

One potential caveat to our findings in CD8 $\alpha$ -deficient mice is that dendritic cells in these mice do not express CD8 $\alpha$ . However, it is unlikely that the lack of dendritic cells expressing CD8 $\alpha$  accounts for this observation. CD8 $\alpha$ -like dendritic cells can be isolated from CD8 $\alpha$ -deficient mice using alternative surface markers (164), indicating that CD8 $\alpha$  expression is dispensable for their function. These data demonstrate that LPS

shortens skin allograft survival induced by costimulation blockade by preventing the apoptosis of alloreactive CD8<sup>+</sup> T cells.

Similar to TLR agonists, a diverse repertoire of viruses impairs skin allograft survival (**Fig 22**) and alloreactive CD8<sup>+</sup> T cell deletion (**Table VIII**). The viruses include the arenavirus LCMV, the herpesvirus MCMV, and the poxvirus VV. While rapid skin allograft rejection was mediated by each of the viruses, our results with LPS and poly(I:C) suggest that the effects of TLR agonists on islet allograft survival (**Fig 17**) is more variable and may warrant further study in the virus system.

### **TLR-dependent and TLR-independent Mechanisms**

We document that LPS acts exclusively through its cognate receptor, TLR4, to shorten graft survival following costimulation blockade treatment. Interestingly, TLR4 expression is essential on host cells (**Fig 12**), but is dispensable on the DST and graft for both transgenic alloreactive CD8<sup>+</sup> T cell deletion (**Table IV**) and accelerated skin allograft rejection (**Fig 13**). This is striking because transgenic KB5 CD8<sup>+</sup> T cells recognize native antigen by direct presentation (128). These data demonstrate that LPS does not prevent deletion or shorten allograft survival by directly maturing donor H2<sup>b</sup>-expressing APCs.

There are at least two possible mechanisms to explain the observation that LPS requires TLR4 on host but not donor cells. LPS may be providing an activation signal directly to CD8<sup>+</sup> T cells or, alternatively, it may induce host cytokines that lead to complete T cell activation by directly or indirectly maturing donor APCs. The possibility that LPS acts directly on host T cells is unlikely to be the primary mechanism despite the

fact that TLRs are expressed on a subset of T cells (144-147), including regulatory T cells (146). This possibility seems unlikely because a recent report demonstrated that TLR4 activation does not impair regulatory T cell function in tolerated allografts (165). To determine if TLR4 expression on APCs was sufficient for LPS to shorten allograft survival, we reconstituted TLR4<sup>-/-</sup> mice with TLR4<sup>+/+</sup> RAG2<sup>-/-</sup> bone marrow. TLR4<sup>-/-</sup> mice reconstituted with TLR4<sup>+/+</sup> RAG2<sup>-/-</sup> bone marrow, which circulate TLR4<sup>-/-</sup> T cells and a combination of TLR4<sup>-/-</sup> and TLR4<sup>+/+</sup> APCs, exhibited short allograft survival when treated with costimulation blockade and LPS, similar to TLR4<sup>+/+</sup> mice, whereas TLR4<sup>-/-</sup> mice exhibited prolonged allograft survival (**Table V**). These data indicate that TLR4 expression is sufficient on non-lymphoid APCs, and is dispensable on host T cells.

The second possibility, that LPS is inducing host cytokine production, is supported by the fact that LPS requires the MyD88-dependent pathway (**Fig 14**), which is dispensable for the upregulation of costimulatory molecules, but not for the secretion of key cytokines (156). These cytokines may provide a costimulatory “signal 3” directly to CD8<sup>+</sup> T cells, or mature donor APCs to express costimulatory molecules that will facilitate direct antigen presentation. Our data demonstrate that LPS prevents the deletion of alloreactive CD8<sup>+</sup> T cells independent of CD80/CD86 expression on donor DST (**Table VI**), suggesting that it is not through the induction of these costimulatory molecules; however, we have not yet explored the role of alternative costimulatory molecules such as inducible costimulator (ICOS) (166).

In contrast to LPS, which acts exclusively through TLR4, dsRNA is known to activate a wide range of PRRs. These receptors include the TLR-family member TLR3 (97), the serine-threonine kinase PKR (108), and the RNA-helicase receptors RIG-I (109)

and MDA5 (110). While TLR3 has been proposed as an important component in the biological response to poly(I:C) in some cell types (167), we show that it is not required for the *in vivo* induction of IFN- $\alpha/\beta$  (**Fig 19**) or the short skin allograft survival observed in mice treated with costimulation blockade and poly (I:C) (**Fig 16**). This is not entirely surprising because the activation of this diverse repertoire of receptors leads to many of the same biological outcomes. For example, the activation of TLR3 and RIG-I both lead to the activation of NF- $\kappa$ B and IRF3, which lead to the expression of IL-12 and IFN- $\alpha/\beta$ , respectively (111) (109). Furthermore, mice deficient in a single dsRNA receptor still produce IFN- $\alpha$  and IL-12 in response to poly(I:C) stimulation, albeit at reduced levels (168). Therefore, it is possible that the redundancy among the dsRNA-recognition pathways accounts for the observation that, unlike LPS, poly(I:C) acts in a TLR-independent manner.

### **Involvement of the IFN- $\alpha/\beta$ Pathway**

The observation that the effect of LPS on islet allograft survival is intermediate whereas the effect of poly(I:C) is dramatic (**Fig 17**), led us to explore the mechanism underlying the differential effects of these two agonists. The results of additional experiments demonstrate that poly(I:C) possesses a greater ability to prime an allo-specific CTL response than does LPS (**Fig 18**). This observation is particularly surprising because both agonists are extremely effective in preventing alloreactive CD8<sup>+</sup> T cell deletion (**Table II**). This suggests that the outcome of skin allograft survival hinges on the impaired deletion of these cells, while islet allograft survival hinges on the effective priming of these cells. This hypothesis is supported by the fact that almost half

of islet allografts survive in mice treated with anti-CD154 mAb monotherapy (57). Since anti-CD154 mAb monotherapy fails to delete alloreactive CD8<sup>+</sup> T cells (61), these data suggest that islet allografts, but not skin allografts (65), can survive in the presence of alloreactive CD8<sup>+</sup> T cells.

In addition to the ability of poly(I:C) to more potently prime allo-specific CTL activity than LPS, its superior ability to induce IFN- $\alpha/\beta$  (**Fig 19**) suggests that IFN- $\alpha/\beta$  is a critical factor in determining allograft survival. IFN- $\alpha/\beta$  has diverse roles in shaping an immune response depending on the timing of its production and its level of expression. For example, IFN- $\alpha/\beta$  simultaneously matures APCs (94), inhibits T cell proliferation (169), leads to the attrition of naïve and antigen-experienced CD8<sup>+</sup> T cells (114,170), and provides a costimulatory “signal 3” to naïve CD8<sup>+</sup> T cells (54).

The attrition induced by IFN- $\alpha/\beta$  in both naïve and antigen-experienced CD8<sup>+</sup> T cells is antigen-independent (169). This stands in contrast to proliferation, which is selectively inhibited by IFN- $\alpha/\beta$  in non-specific CD8<sup>+</sup> T cells through a STAT1-dependent mechanism (115). This is consistent with our observation that injection of LPS, which induces low levels of IFN- $\alpha/\beta$ , leads to a small and early decrease in the number of naïve alloreactive CD8<sup>+</sup> T cells, followed by antigen-specific expansion (**Table III**). It is likely that the small amount of IFN- $\alpha/\beta$  induced by LPS compared to poly(I:C) explains why we observe a small attrition of CD8<sup>+</sup> T cells following LPS injection (54) when others have observed a more dramatic attrition after poly(I:C) injection (114,169).

It is quite striking that IFN-RI<sup>-/-</sup> mice treated with costimulation blockade fail to generate allo-specific CTL activity following the co-administration of LPS or poly(I:C)

(**Fig 20**). Moreover, these mice do not exhibit the shortened allograft survival observed in wildtype mice treated with costimulation blockade and LPS or poly(I:C) (**Fig 21**). These observations are particularly surprising because only low levels of IFN- $\alpha/\beta$  are detected in the serum of mice injected with LPS.

The critical role that we have identified for IFN- $\alpha/\beta$  is interesting because some TLR agonists that shorten allograft survival, such as the TLR2 agonist Pam<sub>3</sub>Cys, do not stimulate IFN- $\alpha/\beta$  production. However, it is possible that Pam<sub>3</sub>Cys overcomes this deficit by being more effective at suppressing regulatory T cell activity than other TLR agonists. This possibility is supported by a recent report which demonstrated that the TLR2 agonist Pam<sub>3</sub>Cys, but not the TLR4 agonist LPS or the TLR9 agonist CpG DNA, directly suppresses regulatory T cell activity (171). In combination with the possibility that MyD88-dependent cytokines also impair CD8<sup>+</sup> T cell deletion, this might explain how TLR2 activation can shorten skin allograft survival in an IFN- $\alpha/\beta$ -independent manner.

It is important to note that significant cross-talk exists between the IFN-RI, which recognizes IFN- $\alpha/\beta$ , and IFN-RII, which recognizes the type II IFN, IFN- $\gamma$ . IFN-RII signaling is impaired in the absence of IFN-RI (112,126,172), raising a potential caveat to the results we have obtained in IFN-RI<sup>-/-</sup> mice. However, our results are not likely to be caused exclusively by a deficiency in IFN- $\gamma$  signaling because IFN- $\gamma$  is essential to achieve prolonged allograft survival using our costimulation blockade protocol (130). A substantial defect in IFN- $\gamma$  signaling would lead to poor allograft survival, which we did not observe in IFN-RI<sup>-/-</sup> mice.

Importantly, we demonstrate that the injection of IFN- $\beta$  alone can impair alloreactive CD8<sup>+</sup> T cell deletion and shorten skin allograft survival induced by costimulation blockade (**Table VII**). It is striking that the impaired deletion observed when mice are treated with a higher dose of IFN- $\beta$  is almost as dramatic as that observed when mice are treated with LPS or poly(I:C) (**Table II, VII**).

IFN- $\alpha/\beta$  has emerged as a linchpin underlying the shortened allograft survival induced by LPS and poly(I:C). However, it is flanked by two equally important players, the population that is producing IFN- $\alpha/\beta$  and the population that is responding to IFN- $\alpha/\beta$ . Our data do not directly address the identity of these populations, but do provide some insights on which populations may be responsible.

The plasmacytoid DC (pDC) subset, or IFN producing cell (IPC), secretes a large quantity of IFN- $\alpha/\beta$  upon activation (173); however, it can be excluded in the response to LPS and poly(I:C) on the basis that these cells do not express TLR3 or TLR4 (144,173,174) and do not utilize the RIG-I pathway for viral dsRNA recognition (135,175). Therefore, we speculate that a conventional DC (cDC) population is producing IFN- $\alpha/\beta$  in response to these agonists, based on the knowledge that these cells produce IFN- $\alpha/\beta$  in response to dsRNA (175) and TLR4 activation (176) in humans. This may differ for other TLR agonists such as CpG DNA. CpG DNA activates TLR9, which is primarily expressed on pDCs (144,173).

We further document that a host population is responding to IFN- $\alpha/\beta$ . This is demonstrated by the fact that neither LPS nor poly(I:C) shorten allograft survival in IFN-IR<sup>-/-</sup> mice treated with costimulation blockade. It is possible that IFN- $\alpha/\beta$  is providing a signal directly to CD8<sup>+</sup> T cells (54), which we are currently investigating experimentally

by adoptively transferring wild-type CD8<sup>+</sup> T cells into IFN-RI<sup>-/-</sup> mice. However, it is also possible that IFN- $\alpha/\beta$  is acting on host DCs as the initial player in a larger cascade of events (94).

### **Involvement of the MyD88-dependent Pathway**

MyD88 is essential for a female mouse to reject a male skin graft (177), suggesting an important role for MyD88 in the rejection of minor-mismatched allografts. Similarly, we demonstrate that MyD88 is required for LPS to shorten the survival of fully allogeneic skin or islet grafts following costimulation blockade treatment. However, MyD88 is not required for the rejection of fully allogeneic skin or islet grafts in untreated mice. In light of the important role for IFN- $\alpha/\beta$  in impairing the efficacy of costimulation blockade, it is interesting that LPS also requires the MyD88-dependent pathway to shorten skin and islet allograft survival. IFN- $\alpha/\beta$  production in response to LPS is mostly, if not completely, MyD88-independent (94,178,179), suggesting that a second mechanism exists.

We experimentally ruled out an exclusive role for the MyD88-dependent cytokines TNF $\alpha$  and IL-12 (**Fig 15**); however, we have not addressed the possibility that one cytokine can compensate for the lack of the other. IL-12 and TNF $\alpha$  have overlapping functions, including the abilities to activate NF- $\kappa$ B (124,180,181) and provide a costimulatory signal directly to CD8<sup>+</sup> T cells (53,55,56). It is also possible that cytokines such as IL-6 (182) and IL-12 (183) may synergize with MyD88-independent cytokines such as IFN- $\alpha/\beta$  to shorten allograft survival.

IL-6 is an attractive candidate that we have not yet tested. IL-6 is capable of suppressing regulatory T cell generation (184) and function (185). It is therefore possible that the effect of LPS on skin allograft survival requires the impaired generation, recruitment or function of regulatory T cells that are induced by costimulation blockade treatment (130,186,187) (142). Our lab has previously demonstrated that a CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cell population is essential for the induction of tolerance to skin allografts, but not for the deletion of alloreactive CD8<sup>+</sup> T cells, which is mediated by a CD4<sup>+</sup> CD25<sup>-</sup> population (142). It is therefore possible that TLR agonists shorten allograft survival using a two-pronged assault, simultaneously preventing effector T cell deletion and suppressing regulatory T cell generation, recruitment or function. Notably, MyD88 plays a key role in suppressing regulatory T cell function. This was demonstrated in a recent manuscript showing that these cells have increased activity in MyD88-deficient mice (188). A more thorough assessment of the potential role for regulatory T cells and IL-6 in our system is discussed in the following section.

Interestingly, we demonstrate that the common TLR adaptor molecule MyD88 is partially responsible for allograft rejection that is mediated by LCMV and MCMV in costimulation blockade-treated mice (**Fig 23**). These data suggest that MCMV impairs skin allograft survival through a TLR-dependent mechanism. We rule out an exclusive role for TLR2 (**Fig 24**); however, TLR9 has emerged as another, more likely candidate (152,153) that we have not yet tested.

One potential caveat to our observations in B6.MyD88<sup>-/-</sup> mice is the involvement of MyD88 in cytokine receptor signaling pathways, including IL-1R (124,189) and IL-

18R (124). However, we exclude this possibility by demonstrating that viruses shorten skin allograft survival in both IL-1R (**Fig 26**) and IL-18 (**Fig 25**) deficient mice.

### **TLR-mediated Suppression of Regulatory T Cells**

It was recently reported that the TLR agonists Pam<sub>3</sub>Cys and CpG DNA precluded the presence of regulatory T cells in cardiac allografts after treatment with anti-CD154 mAb (190). Since anti-CD154 mAb treatment alone fails to delete alloreactive CD8<sup>+</sup> T cells, this finding suggests that TLR agonists can impair allograft survival by impairing the generation or recruitment of regulatory T cells, thereby allowing the activation and effector function of alloreactive CD8<sup>+</sup> T cells. Although our data do not directly address the role of regulatory T cells, the critical importance of this population in maintaining allograft survival in costimulation blockade-treated mice (142) makes it an important point of discussion.

While TLR activation exerts a considerable effect on regulatory T cells at the time of tolerance induction (190), this effect is not as dramatic at later time-points. Poly(I:C) treatment one day after transplantation fails to shorten allograft survival in costimulation blockade-treated mice (72) and LPS fails to shorten the survival of healed-in allografts in anti-CD154 mAb-treated mice (165). Together, these data suggest that TLR activation impairs allograft survival by preventing the induction or recruitment of regulatory T cells at the time of tolerance induction, rather than suppressing their function in intact allografts at later time points. If TLR agonists shortened allograft survival by suppressing the function of regulatory T cells, which are required to maintain skin

allograft survival (142), then we would have expected TLR agonists to shorten allograft survival regardless of the time that they were injected.

We speculate that TLR-induced cytokines prevent the induction of regulatory T cells. This idea is supported by a recent report which showed that the pro-inflammatory cytokine IL-6 impairs the generation of regulatory T cells that are induced by transforming growth factor (TGF)- $\beta$  (184). TLR activation induces IL-6 through at least two distinct mechanisms. Firstly, IL-6 production is induced by the MyD88-dependent pathway. This supports our hypothesis that LPS impairs regulatory T cells in a MyD88-dependent fashion (185). Secondly, IFN- $\alpha/\beta$  augments IL-6 production in a MyD88-independent manner (191). This suggests that TLR agonists such as poly(I:C), which do not activate the MyD88 pathway, can augment IL-6 production and subsequently impair regulatory T cell generation through their potent induction of IFN- $\alpha/\beta$ .

Our finding that CD8<sup>+</sup> T cells are necessary for LPS to shorten allograft survival is tempered by our knowledge of the important role that regulatory T cells play in maintaining allograft tolerance. Therefore, we hypothesize that TLR activation shortens allograft survival through a bimodal mechanism that simultaneously prevents effector cell deletion and impairs regulatory cell generation.

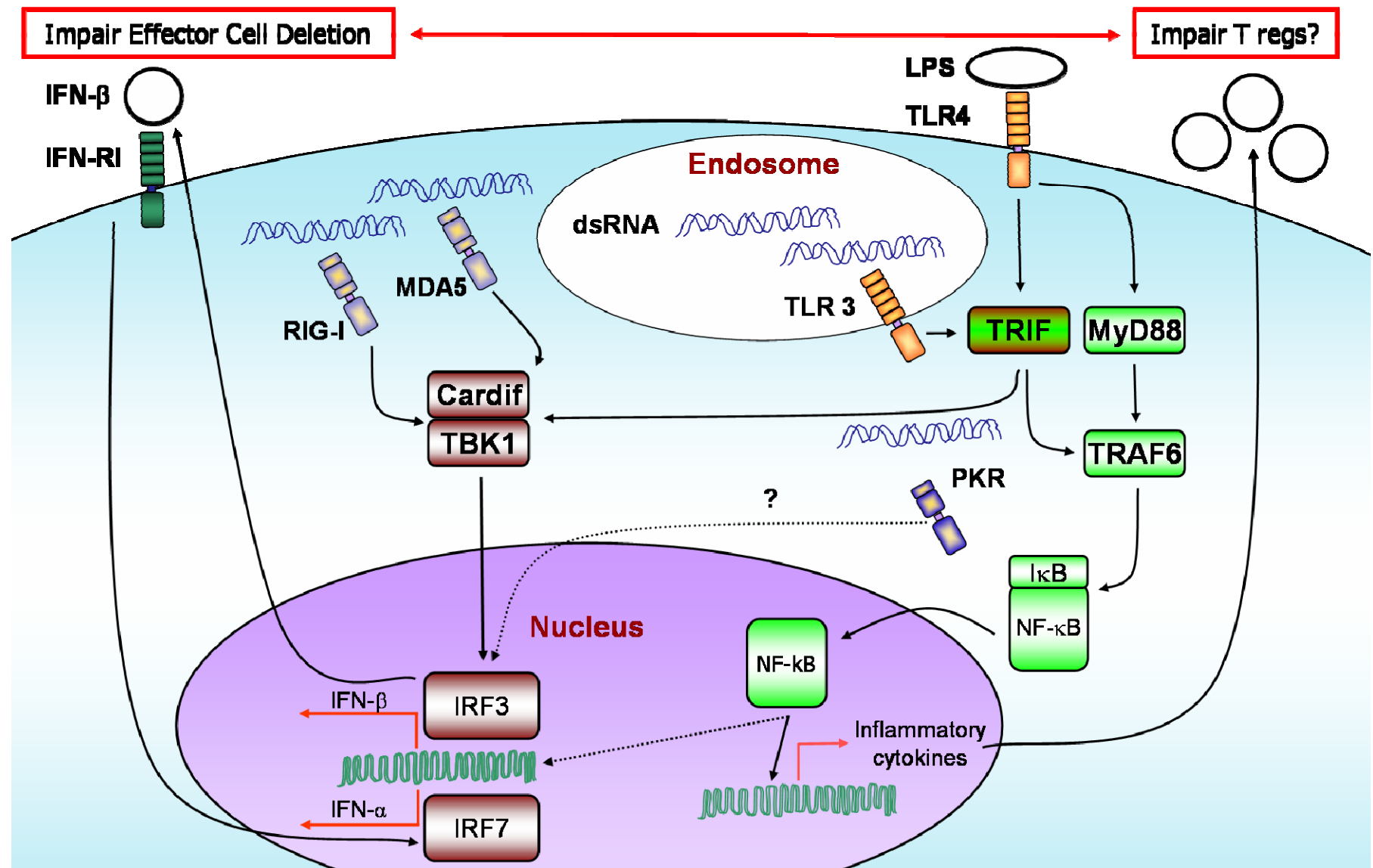
### **Overall Model and Therapeutic Targets**

The ultimate goal of our research is to develop a costimulation blockade protocol that is effective in a “real-world” clinic that is not free of pathogens. We therefore turn our discussion to potential targets for therapeutic intervention that might create a more effective costimulation blockade without compromising its safety. It would be

impractical to target simultaneously dozens of distinct receptors or cell types that respond to diverse microbial motifs, but identifying the common molecular mediators that these receptors signal through might provide viable targets.

On the molecular level, our experimental results suggest that transplantation tolerance is circumvented by signaling through two different pathways. Firstly, it requires the secretion of IFN- $\alpha/\beta$ , demonstrated by our data showing that LPS fails to prime an allo-specific CTL response or shorten skin allograft survival in IFN-RI<sup>-/-</sup> mice treated with costimulation blockade. Secondly, the TLR4 agonist LPS requires the activation of the MyD88-dependent pathway, evidenced by our data showing that LPS fails to shorten skin or islet allograft survival in MyD88<sup>-/-</sup> mice. We present a comprehensive model for how these two pathways might work in concert to counteract the beneficial effects of transplantation tolerance induction in **Fig 27**. Each of these pathways provides unique opportunities and limitations for therapeutic intervention in restoring allograft survival shortened by the activation of the innate immune system by PRRs.

**FIGURE 27: MODEL OF PRR-MEDIATED ABROGATION OF TRANSPLANTATION TOLERANCE**



**Figure 27 Legend:** An overall model for how PRRs abrogate transplantation tolerance induced by costimulation blockade is presented. (1) Components of the IFN- $\alpha/\beta$  pathway are shaded in red. The induction of IFN- $\alpha/\beta$  is initiated by the TLR family member TLR3, the serine-threonine kinase PKR, and the RNA helicases MDA5 and RIG-I. TLR3 activates the Cardif/TBK1 complex in a TRIF-dependent manner, whereas RIG-I and MDA5 activate the same complex in a TRIF-independent manner. TBK1 then activates the transcription factor IRF3, which leads to the transcription of IFN- $\beta$ . IFN- $\beta$  is secreted and signals through the IFN-RI to a) prevent alloreactive CD8<sup>+</sup> T cell deletion induced by costimulation blockade, and b) activate the transcription of IFN- $\alpha$  via a positive feedback loop. (2) Components of the MyD88-dependent pathway are shaded in green. The MyD88-dependent pathway is activated upon TLR4 engagement, and is required for the TLR4 agonist LPS to shorten allograft survival induced by costimulation blockade. The mechanism by which the MyD88-dependent pathway shortens allograft survival remains unresolved; however, we propose that the induction of pro-inflammatory cytokines by the transcription factor NF- $\kappa$ B may impair regulatory T cell function and alloreactive CD8<sup>+</sup> T cell deletion.

The IFN- $\alpha/\beta$  pathway is the most attractive target for therapeutic intervention because it is required for both LPS (TLR-dependent) and poly(I:C) (TLR-independent) to shorten allograft survival. However, targeting the cytokines that make up the IFN- $\alpha/\beta$  family directly is complicated by the fact that there are numerous members, including 13 forms of IFN- $\alpha$  and a single form of IFN- $\beta$ . The identification of the particular subtypes of IFN- $\alpha/\beta$  induced by TLRs is further complicated by the positive IFN- $\alpha/\beta$  feedback loop, where one subtype of IFN- $\alpha/\beta$  can induce the expression of other subtypes (112). Neutralizing host IFN- $\alpha/\beta$  may also increase host morbidity and mortality by impairing the innate antiviral response. However, these impediments would be avoided by targeting signaling molecules upstream of IFN- $\alpha/\beta$  induction.

IFN- $\alpha/\beta$  is induced through the activation of numerous signaling pathways that converge on IRF3 and IRF7, which initiate the transcription of IFN- $\alpha/\beta$  (94) (113). TLR3 and TLR4 both recruit the TRIF complex, which in-turn recruits the Cardif and TBK1 complex to activate the transcription factor IRF3. This stands in contrast to RIG-I and MDA5, which recruit Cardif and TBK1 in a TRIF-independent manner. Despite this difference in TRIF-dependence, both pathways activate IRF3 in a Cardif and TBK1-dependent manner, leading primarily to the transcription of IFN- $\beta$ . The initial burst of IFN- $\beta$  interacts with the IFN-RI on the cell surface, providing a positive feedback loop that activates IRF-7, which in-turn initiates the efficient transcription of IFN- $\alpha$  (94,95).

The critical role for IRF3 and IRF7, as well as the upstream signaling molecules TBK1 and Cardif, in the induction of IFN- $\alpha/\beta$  makes them particularly attractive targets for therapeutic intervention. Resveratrol (3,4',5-trihydroxy-*trans*-stilbene), a phytoalexin found in grapes, has been shown to have strong anti-inflammatory properties (192) linked

to an inhibition of NF- $\kappa$ B activation (193,194). More recently, the molecular targets of resveratrol have been identified as the IFN- $\alpha/\beta$ -inducing TBK1 and TRIF signaling complexes (195). Resveratrol may not be potent enough to abrogate IFN- $\alpha/\beta$  signaling in its natural form; however, a modified derivative or similar inhibitor may be capable of therapeutically blocking the negative influence of IFN- $\alpha/\beta$  on allograft survival. While a resveratrol derivative might be therapeutically effective, a drug that targets IRF7 would be more attractive because a subset of TLRs, including TLR9, activate IRF7 in a TBK1- and TRIF-independent manner (94,196).

The MyD88-dependent pathway is another potential target for intervention. It is essential for the secretion of pro-inflammatory cytokines, including IL-1, IL-6, IL-12 and TNF $\alpha$ , in response to TLR signaling. However, our inability to identify which, if any, of these particular cytokines is responsible for shortened allograft survival limits them as the preferred and accessible extracellular targets. Targeting MyD88 may be challenging, but the polyphenol (-)-epigallocatechin-3-gallate (EGCG), found in green tea, has been shown to impair the pro-inflammatory effects of LPS (197). The inhibition occurs by impairing both the MyD88- and TRIF-dependent pathways (198). Therefore, EGCG, a derivative of EGCG or a small molecule inhibitor of MyD88 may be viable approaches to hampering TLR signaling and prolonging allograft survival in the presence of an active infection.

Naturally, the goal of prolonging allograft survival cannot be achieved in the clinic at the expense of patient safety. Disrupting the normal function of IFN- $\alpha/\beta$  or MyD88 could have devastating immunologic consequences to patients. The molecular obstacles that reduce the efficacy of our costimulation blockade treatment are also

responsible for ensuring patient survival during infection. However, transiently blocking specific immunologic functions may be preferable to a life-long regimen of global immunosuppression. Previous studies have indicated that poly(I:C) does not shorten allograft survival when administered 8 days after costimulation blockade, which is one day after transplantation (70,77). Therefore, it might be possible to target critical immunologic function for several weeks during the peri-transplant period without significantly affecting patient morbidity and mortality.

### **Alternative Mechanisms**

We have thus far focused exclusively on the mechanisms underlying shortened allograft survival induced by viral infection at the time of costimulation blockade. However, our group has previously demonstrated that skin allograft survival is also impaired when animals are infected with LCMV 6-8 weeks prior to treatment (76), or 1 day after transplantation (72). It appears, based on published and unpublished observations, that the mechanisms by which viruses shorten allograft survival differ based on the timing of infection.

LCMV-immune mice, exposed to virus 6-8 weeks prior to costimulation blockade treatment, reject skin allografts with accelerated kinetics (76). This cannot be explained simply by the activation of the IFN- $\alpha/\beta$  or MyD88-dependent pathways because the virus, and resultant cytokine production, have long-since dissipated by the time that animals are treated with costimulation blockade. Instead, we believe that the shortened allograft survival results from a failure to delete virus-specific memory CD8<sup>+</sup> T cells that cross-react with alloantigens (76). This is supported by the long-standing observation

that a population of virus-specific CD8<sup>+</sup> T cells crossreacts with alloantigens (80-82), a characteristic known as heterologous immunity. It has been hypothesized that these memory CD8<sup>+</sup> T cells resist deletion induced by costimulation blockade and precipitate allograft rejection (76,199).

Heterologous immunity may be a powerful obstruction to achieving transplantation tolerance in the clinic. An individual encounters numerous immunologic challenges during his or her lifetime, each generating immunologic memory and a new opportunity for the generation of cross-reactive T cells. The challenge posed by heterologous immunity is complicated by the fact that the sequence of viral infection can substantially alter the immunodominance and outcome of subsequent heterologous challenges (78,200), thus making it impractical to target specific cross-reactive populations. Inducing transplantation tolerance in clinical patients, who are not immunologically naïve, may be difficult. However, a recent report suggests that the drug 15-deoxyspergualin may improve the ability to induce transplantation tolerance in LCMV-immune mice (199).

Mice infected with LCMV one day after transplantation (8 days after costimulation blockade treatment) also reject skin allografts with accelerated kinetics (72). This observation is particularly surprising in light of our data that alloreactive CD8<sup>+</sup> T cells are deleted within 24 h of costimulation blockade treatment. Therefore, LCMV is not shortening allograft survival by preventing the deletion of alloreactive CD8<sup>+</sup> T cells, which has already occurred by the time that mice are challenged with virus. The mechanisms underlying this observation remain unclear, but it is possible that the

rejection occurs through the activation and expansion of LCMV-specific new thymic emigrants that cross-react with alloantigen, or through a CD8-independent mechanism.

### **Conclusions and Unresolved Questions**

We have demonstrated that activation of host TLRs at the time of costimulation blockade treatment shortens allograft survival by impairing the apoptotic deletion of alloreactive CD8<sup>+</sup> T cells through a mechanism that depends on IFN- $\alpha/\beta$ . We also identify a critical role for MyD88, but not IL-12 or TNF $\alpha$ , in allograft survival shortened by the TLR4 agonist LPS. This research identifies several novel therapeutic targets to improve the efficacy of costimulation blockade in the presence of an ongoing infection. However, in order to select the best candidate targets, it will be necessary to elucidate further the signaling pathways involved. In the future, the role of the IFN- $\alpha/\beta$  transcription factors IRF3 and IRF7 in shortening allograft survival and impairing alloreactive CD8<sup>+</sup> T cell deletion should be investigated. It will also be important to examine closely the role that TLRs and the MyD88-dependent pathway play in suppressing regulatory T cell activity. In addition to the data that we present in this dissertation, these experiments will help to achieve our overall goal of successful allograft transplantation in the absence of chronic immunosuppression.

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