TLR Activation Prevents Hematopoietic Chimerism Induced by Costimulation Blockade: A Dissertation

David M. Miller
University of Massachusetts Medical School

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TLR ACTIVATION PREVENTS HEMATOPOIETIC CHIMERISM INDUCED BY COSTIMULATION BLOCKADE

A Dissertation Presented

By

DAVID M. MILLER

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By
David M. Miller

Approved as to style and content by:

_____
Dr. Raymond Welsh, Chair of Committee

_____
Dr. Dale Greiner, Member of Committee

_____
Dr. Hardy Kornfeld, Member of Committee

_____
Dr. Evelyn Kurt-Jones, Member of Committee

_____
Dr. S. Brian Wilson, Member of Committee

_____
Dr. Aldo Rossini,
Thesis Advisor

_____
Dr. Anthony Carruthers,
Dean of the Graduate School of Biomedical Sciences

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ABSTRACT

Costimulation blockade based on a donor-specific transfusion and anti-CD154 mAb is effective for establishing mixed allogeneic hematopoietic chimerism and inducing transplantation tolerance. Despite its potential, recent evidence suggests that the efficacy of costimulation blockade can be reduced by environmental perturbations such as infection or inflammation that activate toll-like receptors (TLR). TLR agonists prevent costimulation blockade-induced prolongation of solid organ allografts, but their effect on the establishment of hematopoietic chimerism has not been reported.

In this dissertation, we hypothesized that TLR activation during costimulation blockade would prevent the establishment of mixed hematopoietic chimerism and shorten skin allograft survival. To test this hypothesis, costimulation blockade-treated mice were co-injected with TLR2 (Pam_3Cys), TLR3 (poly I:C), or TLR4 (LPS) agonists and transplanted with allogeneic bone marrow and skin grafts. Supporting our hypothesis, we observed that TLR agonists administered at the time of costimulation blockade prevented the establishment of mixed hematopoietic chimerism and shortened skin allograft survival.

To investigate underlying cellular and molecular mechanisms, we first determined that LPS administration during costimulation blockade did not increase production of alloantibodies or activate natural killer cells. Similarly, costimulation blockade-treated mice depleted of $CD4^+$ or $CD8^+$ cells did not become chimeric when co-injected with LPS. In contrast, mice depleted of both $CD4^+$ and $CD8^+$ cell subsets were resistant to the effects of LPS.

We next observed that alloreactive T cells were activated by TLR agonists in mice treated with costimulation blockade, and this activation correlated with LPS-induced maturation of donor and host alloantigen-presenting cells. In contrast, TLR4-deficient mice treated with costimulation blockade and LPS did not upregulate costimulatory molecules on their APCs, and mixed chimerism and permanent skin allograft survival were readily achieved. We further observed that injection of recombinant IFN-β recapitulated the detrimental effects of LPS, and that LPS-injected mice deficient in the type I IFN receptor were partially protected. Importantly, alloantigen-presenting cells did not upregulate costimulatory molecules in response to LPS, and mixed chimerism and permanent skin allograft survival were readily established in type I IFN receptor and MyD88 double deficient mice treated with costimulation blockade. We conclude that the TLR4 agonist LPS prevents the establishment of mixed hematopoietic chimerism and shortens skin allograft survival in mice treated with costimulation blockade by inducing the production of type 1 IFN and MyD88-dependent factors that upregulate costimulatory molecules on APCs, leading to the generation of activated alloreactive T cells.

ABBREVIATIONS

APC	antigen presenting cell
APDC	alloantigen-presenting dendritic cell
BM	bone marrow
BMT	bone marrow transplantation
CARD	caspase recruitment domain
CARDIF	CARD adaptor inducing IFN-β
CB	costimulation blockade
cDC	conventional dendritic cell
cTEC	cortical epithelial cell
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DAI	DNA-dependent activator of IFN-regulatory factors
dsRNA	double stranded RNA
DST	donor-specific transfusion
FoxP3	forkhead box P3
GVHD	graft-versus-host disease
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
IFN	interferon
IFNAR1	interferon-α/β receptor
IKK	IκB kinase
IKK-I	inducible IκB kinase
IL	interleukin
I.P.	intraperitoneally
I.V	intravenous
IPS-1	interferon-β promoter stimulator
IRAK	IL-1 receptor associated kinase
IRF	interferon regulatory factory
ISRE	interferon-stimulated response elements
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
mAb	monoclonal antibody
MAL	MyD88-adaptor-like
MAPK	mitogen-activated protein kinase
MAVS	mitochondrial antiviral signaling protein
MCMV	murine cytomegalovirus
MDA5	melanoma differentiation-associated factor-5
MFI	median fluorescence intensity
MHC	major histocompatibility complex
MST	median survival time
mTEC	medullary epithelial cell
MyD88	myeloid differentiation primary response gene-88
NF-κB	nuclear factor κ B
NLR	NOD-like receptor
NOD	nucleotide binding and oligomerization domain
Pam$_3$Cys	Pam$_3$-Cys-Ser-(Lys)$_4$
PAMP	pathogen-associated molecular pattern
PMBC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PFU	plaque forming units
Poly I:C	polyinosinic: polycytidylic acid
PRR	pattern recognition receptor
PV	Pichinde virus
RIG-I	retinoic acid inducible gene I
RIP	receptor-interacting protein
RLR	RIG-I-like receptor
S.D.	standard deviation
SNP	single nucleotide polymorphism
ssRNA	single-stranded RNA
TANK	TNFR-associated factor family member-associated NF-κB activator
TBK-1	TANK-binding kinase 1
TCR	T cell receptor
TGF	transforming growth factor
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor-associated factor
Treg	regulatory T cell
TRIF	TIR-domain-containing adaptor protein inducing IFN-β
VISA	virus-induced signaling adaptor
VV	Vaccinia virus
VSV	vesicular stomatitis virus

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CHAPTER I: INTRODUCTION

History of Transplantation

Early Pioneers

Remarkably, reports of transplantation surgery date as far back as two millennia ago. In the ancient Sanskrit text, the *Sushruta Samhita*, the Indian surgeon Sushruta described methods for reconstructing noses amputed during battle or as a form of punishment using tissue grafts from the patient's own forehead [1,2]. Additional early accounts of rhinoplasty can be found in the works of the Italian surgeon Gaspare Tagliacozzi. In the late 16th century, he succeeded at replacing the lost nose of a patient using the pedicle flap of the person's own arm [3]. Interestingly, Tagliacozzi is reported to have observed that transplanting tissue from allogeneic donors was not tenable. He eloquently wrote that "the singular character of the individual entirely dissuades us from attempting this work on another person" [3]. Given the inchoate understanding of medicine at the time, one can imagine that his impressive observation was only made after unsuccessful attempts that were no doubt painstaking for Tagliacozzi and punishingly painful for the patient.

Despite these extraordinary early attempts, the field of transplantation did not significantly advance until the 20th century. Advancements in anesthesia and surgical technique, including the work done by Alexis Carrel at the turn of the 20th century in improving vascular anastomosis [4], permitted more rigorous investigation by enabling animal, and some human, experiments to be performed. Interestingly, many of the important discoveries in the first decade of the 20th century were made by tumor biologists, studying the outcome of transplanted tumors. In 1912, George Schone

synthesized many of these thoughtful insights into what are now commonly referred to as the "laws of transplantation" [3]. He stated that: 1) Transplantation of tissue from foreign species (xenogeneic) never succeeds. 2) Transplantation of tissue from unrelated members of the same species (allogeneic) typically fails. 3) Transplantation of autologous tissue typically survives. 4) Recipients of allogeneic grafts tolerate the graft for a brief period, before ultimately rejecting the tissue. 5) Recipients of a second allogeneic graft from the same donor reject the second graft more rapidly than they reject the first. 6) Allograft survival is more likely if the donor and recipient have a close "blood relationship".

Pioneering research in the 1940s and 1950s provided both a genetic, as well as immunological, understanding of Schone's laws of transplantation. Work done at the Jackson Laboratory in Maine by George Snell and colleagues, provided the genetic understanding, while research done in England by Peter Medawar's lab helped elucidate the role of the immune system. The seminal discoveries of both of these groups provided the basis for many of the important breakthroughs that were to follow in the second half of the 20th century.

The Advent of Immunogenetics

Working under the aegis of Clarence Little, George Snell discovered an interval of DNA, or 'genetic locus", that was critically related to the survival of transplanted allografts. Utilizing mice that were genetically identical except for a short, defined locus (a.k.a. congenic mice), Snell discovered that mice rejected tissue from their "genetic cousins" if they differed at a specific locus, which he then called H, for histocompatibility

[3]. Following a year-long stay at the Jackson Laboratory by Peter Gorer, Snell and Gorer realized that the H locus, and the blood group locus that Gorer had discovered earlier - known as "antigen II" [5] - were the same [6,7]. Consequently, this genetic locus was renamed "H-2". The genes contained within the murine H-2 locus have proven to be critical determinants of transplantation success, tumor surveillance, and cell-mediated immunity. This genetic region has also come to be known as the major histocompatibility complex (MHC). The "singular character of the individual" that Tagliacozzi so presciently spoke of is largely a manifestation of the uniqueness of each person's MHC. The relationship between polymorphisms in the MHC and allograft success will be discussed later.

Working independently of the immunogeneticists in Maine, the zoologist Peter Medawar and his colleague Thomas Gibson began their work in transplantation biology following World War II. They were commissioned by the War Wounds Committee of the British Medical Research Council to study skin allograft rejection in the hope of improving techniques of skin grafting for soldiers burned during the war [3]. At the time, attempts to replace damaged skin on burn victims with allografts were invariably unsuccessful.

In 1943, Gibson and Medawar published a report detailing the fate of a skin allograft placed on a 22-year-old female burn victim, which provided insight into the mechanism of allograft rejection. Due to the extensive nature of the patient's thermal burns, she required multiple skin grafts from both her own thigh, as well as the thigh of her brother. Over the several months of treatment, the patient had repeat surgeries, including serial

transplants from her brother. This enabled Gibson and Medawar to examine the tenets of Schone's laws. They observed that: 1) tissue transplanted from the patient's own thigh survived; 2) tissue transplanted from the brother's thigh was initially tolerated, but began to degenerate after two weeks (a process known as first-set rejection); and 3) subsequent skin grafts from her brother displayed accelerated rejection (a process known as second-set rejection). These observations led them to conclude that the rejection of foreign tissue was "brought about by a mechanism of active immunization" [8].

These key insights, as well as others acquired from experiments in animal models [9], demonstrated that allograft rejection was mediated by the immune system. This led to the rigorous investigation into how the immune system recognizes foreign tissues, and how it distinguishes "self-tissues" from "non-self tissues". It also prompted the discovery of chemotherapeutic agents that could thwart the immune system's ability to abrogate allograft survival, and made allograft transplantation a therapeutic reality. The development of modern day immunosuppressive agents and their caveats will be discussed later.

Distinguishing Self from Non-self: The Immune Response To Foreign Tissues

Innate Immunity

The human immune system employs an intricate array of mechanisms to distinguish native structures from foreign ones. Structures from evolutionarily distant species (e.g. microbes) are recognized by members of the innate immune system. Often considered as the first line of defense, the innate immune system consists of epithelial barriers,

circulating proteins and effector cells. Innate immunity has been highly conserved throughout evolution, and elements of it exist in all multicelluar organisms [10]. The effector cells of the innate immune system consist principally of monocytes, macrophages, dendritic cells, natural killer (NK) cells, mast cells and neutrophils. These cells possess specific germ-line encoded pattern recognition receptors (PRR) that can discern distinct molecules that are common to groups of related pathogens. These structures, which are also known as pathogen-associated molecular patterns or PAMPs, are typically microbial products that are required for the survival of the microorganism. Thus, PAMPs cannot be easily altered by the microbe as a way of evading the host's defenses. Specific PRRs recognize distinct PAMPs, and have distinguishing expression patterns and signaling pathways, which will be discussed in detail in a later section.

Adaptive Immunity

Most members of the innate immune system, however, are not effective at recognizing structures from closely related species, or from members of the same species. For this purpose, the immune system has evolved a sophisticated system that is often referred to as adaptive or acquired immunity. The adaptive immune system is comprised of T and B-lymphocytes, which possess antigen-specific cell-surface receptors that are generated via rearrangement of their T cell receptor and immunoglobulin genes, respectively. A large repertoire of peptide-specific receptors is produced by this process, allowing the acquired immune system to respond to a wide range of potential pathogens. Due to the enormous diversity produced by gene rearrangement, T and B cells undergo extensive selection processes to ensure to a great degree that the cells that are found

circulating in the body possess receptors that only react to antigens from non-native sources. One practical consequence of this for transplantation is that a recipient's B and T cells can mount immune responses to the genetic differences found on donor tissues. The antigenic differences between recipients and donors are the result of polymorphic genes that are found throughout the population, and are often referred to as alloantigens. There are three immune reactions generated in response to alloantigens, and these typically lead to the rejection of the transplanted graft. These responses are often categorized as hyperacute, acute, and chronic rejection.

Mechanisms of Allograft Rejection

Hyperacute rejection is mediated by circulating antibodies that bind to antigens on the allografts' endothelium, and it can begin minutes to hours after organ transplantation. These antibodies are believed to arise either from prior exposure of the host to tissues from the same donor, or in response to carbohydrate moieties expressed by colonized bacteria that mimic antigens found on the donor [11]. For example, antigens found on common bacteria of the gut closely resemble those of the A, B, O antigens found on erythrocytes and endothelial cells. If donor tissues are not properly matched to the recipient for these antigens they will quickly be rejected. Transplanted tissues subjected to hyperacute rejection rapidly become necrotic, as antibody-mediated thrombotic occlusions develop in the endothelium of the tissues.

In the absence of immunosuppression, all allografts that do not elicit a hyperacute response will be subjected to acute rejection. Acute rejection typically develops after the first week of transplantation, and is characterized by a T cell response directed against

alloantigens. Due to their highly polymorphic nature, the genes located within the locus of the MHC produce the most important alloantigens for acute rejection [12].

There are two distinct mechanisms by which T cells recognize and respond to alloantigens, a process also known as allorecognition. The first mechanism, also called the direct allorecognition pathway, involves host T cells recognizing donor MHC molecules found on the surface of cells of the graft, such as dendritic cells [13,14]. The frequency of T cells with TCRs specific for foreign MHC molecules is believed to be 100-1000 fold higher than the precursor frequency of T cells specific for any self-MHC-foreign-peptide complex [15]. As a consequence, the direct allopresentation pathway produces a robust immune response, and has traditionally been considered the predominate mechanism in acute rejection [16]. In contrast, donor MHC and other minor antigens can be phagocytosed and processed by recipient APCs, and presented to host T cells. This process, also known as indirect allorecognition, is analogous to the presentation of conventional pathogen-derived protein antigens in which self-MHC-foreign peptide complexes on host APCs engage the TCR of host T cells.

The mechanisms of allorecognition can be contravened by modern immunosuppressive chemotherapies, and thus acute rejection can be avoided in a great number of recipients. However, eventually the majority of allografts fail to survive, as they fall victim to chronic rejection. The mechanisms of chronic rejection have not been clearly elucidated; however, it likely results from fibrosis of the graft's vasculature, secondary to chronic immune stimulation.

Despite the inexorable loss of allografts, the ability to preclude acute rejection has

greatly improved the outcome and quality of life of many patients. There are approximately 28,000 organ transplants per year in the U.S [17]. These are only feasible because of the work done in the fields of tissue matching and immunosuppression, which are the focus of the next section.

Clinical Advancements in Transplantation

Tissue Matching

Snell's discovery of the major histocompatibility complex in mice became clinically relevant to humans with the discovery in the late 1950s of the human counterpart, the human leukocyte antigen (HLA), by Jean Dausset [18]. His report of circulating antibodies in the blood of transfusion patients that were directed against leukocyte antigens provided the groundwork for the development of tools that could identify individual HLA antigens. This knowledge, coupled with the discovery of the ABO blood system a half a century earlier by Karl Landsteiner [3], provided physicians with powerful tools to minimize the differences in the donor and recipient's "blood relationship".

In the decades to follow, it would be learned that the HLA complex on chromosome 6 is comprised of two major clusters of genes that are critically related to the outcome of transplanted tissues. These distinct regions of the complex, named class I and class II, possess genes with a vast number of genetic variants, or alleles, within the human population. For transplantation, the three most important genes are HLA-A, HLA-B, and HLA-DR, as minimizing allelic differences at these loci correlate with better clinical

outcomes. Each person possesses a maternal and a paternal copy of these genes. Given the large number of polymorphisms at each of these genes, most individuals inherit different alleles from their parents. Therefore, attempting to match donor and recipients for these six genetic possibilities has become the standard. Even perfect "6/6" matches, however, do not ensure prolonged allograft survival, as variations at other genes, known as minor histocompatibility antigens, can elicit an immune response. Therefore, even with perfect matches, the utilization of immunosuppressive agents is critical to ensure extended allograft survival.

Immunosuppression

Pharmacotherapy and Radiation

Gibson and Medawar's realization that allograft rejection was mediated by the immune system ignited a community of scientists to investigate methods of prolonging allograft survival by suppressing the recipient's immune system. Early attempts at prolonging the survival of allografts were also performed by Medawar and colleagues. At the time, Medawar knew that tissues of mesenchymal origin were the cells that mediated immunological responses. Having observed that the glucocorticoid cortisone had "profound influence on the activity of all mesenchymal tissues", he hypothesized that treatment with cortisone would prolong skin allograft survival in rabbits [19]. His hypothesis did prove correct, as skin graft survival was prolonged. However, the survival was only extended a few days, and the quest for additional modalities continued.

A significant advance came in the late 1950s and early 1960s with the observation

that allograft survival could be significantly extended if the recipient was treated with total body irradiation (TBI). Surgeons in Boston and Paris reported renal allograft function up to a year in patient's treated with sublethal doses of TBI [20]. In fact, using this modality the Boston group of Merril, Murray and Harrison was able to perform the first successful kidney allograft transplantation [21]. However, these cases did little to incite optimism, as the majority of the patients fell subject to infection, and died within weeks of irradiation [21]. Therefore, the pursuit for chemotherapeutics, which could mimic the cytoablative qualities of TBI without the high degree of morbidity and mortality, continued.

In 1959 and 1960, work done by Schwartz and Dameshek [22,23], Meeker [24] and Calne [25] demonstrated that allograft survival could be prolonged by administering the anti-metabolite 6-mercaptopurine (6-MP). After being taken up by cells, 6-MP is converted to thioinosinic acid, which disrupts the *de novo* synthesis of purine nucleotides. This has a profound effect on proliferating cells, such as those participating in an immune reaction. Unfortunately, other proliferating cells such as those of the intestinal epithelium, hair follicle and bone marrow are also significantly damaged. Therefore, less toxic anti-metabolites including azathioprine and mycophenolate mofetil were developed.

The discovery in 1976 that an extract of the fungi *Trichoderma polysporum* (later re-identified as *Tolypocladium inflatum*) had potent and largely selective effects against T lymphocytes, marked another significant advance in the field of transplantation [21,26]. The compound, named cyclosporine, and its successor tacrolimus, disrupt the antigen activation of T cells by inhibiting the molecule calcineurin. The serine/threonine

phosphatase calcineurin plays a critical role in the T cell signaling cascade by activating the transcription factor NFAT. Without NFAT activation, the downstream targets of T cell signaling, such as interleukin (IL)-2, are not produced. Due to their relative specificity to T cells, cyclosporine and tracrolimus are less toxic than myeloablative agents such as 6-MP and azathioprine. Consequently, both are used routinely for kidney, liver, and heart transplantation. However, despite their efficacy and decreased side-effect profile, adverse effects such as increased incidence of neoplasia, nephrotoxicity, hypertension, tremor, hirsutism, and islet-toxicity are not uncommon [27].

Additional attempts to target T cell signaling as a way of extending allograft survival led to the discovery of inhibitors of the protein kinase, mammalian target of rapamycin (mTOR). Sirolimus (rapamcyin), and the related compound everolimus, prevent antigen activation of T cells by first forming a complex with the immunophilin FKBP-12. The sirolimus/everolims-FKBP-12 complex then binds and inhibits mTOR, a key regulator of cell-cycle progression downstream of the IL-2 receptor [27,28]. By preventing T cell proliferation, these agents effectively reduce the host's immune response against the transplanted organ. Unfortunately, adverse effects such as hypercholesterolemia, hypertriglyceridemia, anemia, leukopenia and lymphoid malignancy have been reported [27].

Immunosuppressive Antibodies

Antibodies directed against lymphocyte-specific antigens can also be used as an adjunct to the aforementioned agents. In the 1960s and 1970s, the polyclonal preparation

antilymphocyte globulin (ALG) was used clinically with varying degrees of success. ALG is a mixture of antibodies directed against a variety of lymphocyte cell-surface proteins. The heterologous nature of ALG, coupled with variations in batch-to-batch potency, prompted the development of monoclonal antibodies directed against specific T cell antigens [21]. Antibodies specific for CD3 (OKT3) [29], CD52 (Alemtuzumab) [30], CD11a (Efalizumab) [31], CD25 (Daclizumab and Basiliximab) [32,33] have proven efficacious at suppressing the immune response to alloantigens, and have demonstrated the ability to extend the lifespan of various allografts. Scientists have also utilized monoclonal antibodies to target costimulatory molecules involved in T cell activation in the hope of inducing a state transplantation tolerance, which will be discussed in more detail later.

Immunosuppressive Regimens

The ever-increasing immunosuppressive armament has provided the transplant caregiver with a multitude of potential drug combinations. However, there are three main uses of immunosuppressants. The first is to establish initial acceptance of the allograft and to prevent acute rejection. This is often accomplished with an initial heavy dose of immunosuppression [34]. During the initial induction, a biological agent such as daclizumab, basiliximab, or anti-thymocyte globulin (ATG) may be used. This is then followed typically by a three-drug cocktail that includes a calcineurin inhibitor, such as tacrolimus or cyclosporine, an anti-metabolite such as mycophenolate mofetil, and the glucocorticoid prednisone. The second use of immunosuppressants is maintenance. During the maintenance phase, the level of immunosuppression is reduced to preserve

host defenses, but maintained at a level to prevent rejection. The dosage of prednisone is frequently tapered during this phase. The final use of immunosuppressants is to reverse a rejection episode. High-dose corticosteroids or lymphocyte-depleting antibodies can be employed to quell an acute rejection episode.

Transplantation Tolerance

Although prolonged graft survival can be achieved with modern immunosuppressive regimens, it is clear that all immunosuppressive drugs, even the newer generations of immunosuppressive regimens, are toxic [35,36]. Immunosuppressive drugs are also known to increase the risk of infection and neoplasia [37,38], and their associated side-effects can lead to patient non-compliance [39]. Since most patients eventually reject transplanted allografts either acutely or through a process of chronic rejection [40-42], these deleterious side-effects make organ transplantation a therapy in which the risk/benefit ratio must be carefully weighed.

To overcome issues associated with chronic immunosuppression, investigators have focused on approaches that lead to the induction of tolerance to transplanted organ allografts [43]. Operationally, transplantation tolerance is defined as the survival of a donor allogeneic graft in the absence of immunosuppression. Most transplantation tolerance induction protocols take advantage of information resulting from studies on the natural mechanisms by which the immune system prevents self-reactivity and autoimmune disease. Two major forms of natural tolerance have been identified: central tolerance and peripheral tolerance.

Central Transplantation Tolerance

In 1953, Billingham, Brent and Medawar demonstrated for the first time that the establishment of allogeneic hematopoietic chimerism leads to the induction of central tolerance and permits permanent acceptance of skin allografts [44]. Inspired by the work done in freemartin cattle by Owen in 1945 [45], and the clonal selection theory subsequently proposed by Burnet [46], Medawar demonstrated in mice that the transfer of allogeneic hematopoietic cells *in utero* could induce tolerance to skin transplanted from the original donor later in life [44].

Medawar's demonstration of acquired neonatal tolerance inspired Main and Prehn two years later to recapitulate that phenomenon in adult mice. Their strategy entailed ablating the recipient's immune system with lethal irradiation, reconstituting it with allogeneic bone marrow, followed by transplantation with donor-strain-matched skin allografts [47]. This protocol successfully induced tolerance to skin allografts, conclusively linking the establishment of hematopoietic chimerism with subsequent allograft survival.

Mechanisms of Central Transplantation Tolerance

The mechanisms by which hematopoietic chimerism induces a state of central tolerance are believed to mirror those the body normally uses to prevent autoreactivity and establish a state of self-tolerance. The wide array of pathogen-specific T cells the human body possesses is ultimately the result of random gene rearrangements of the T cell receptor (TCR). The aleatory nature of TCR rearrangement, however, presents a

potential problem, as a number of developing T cells could express receptors that not only react to pathogenic antigens presented on self-MHC, but also self-proteins. The hazard of autoimmunity is mitigated by a selection process in the thymus that ensures the survival of only those T cells that recognize self-MHC (a process known as positive selection) that present foreign peptides (negative selection).

During positive selection, developing T cells that have migrated from the bone marrow interact with cortical thymic epithelial cells (cTECs). MHC class I and class II-expressing cTECs present self-peptides that bind to the TCR of developing T cells (thymocytes), imparting crucial survival signals [48]. TCR gene rearrangement produces a repertoire of immature T cells with a wide range of specificities. Therefore, only a small proportion of the developing T cells possess TCRs that recognize self-peptide/MHC complexes [49]. Cells that survive positive selection display a vast heterogeneity in their specificities. Some express TCRs with low-affinity for self-peptide/MHC, while many have receptors with a high-affinity for self-peptide MHC complexes, and are thus possibly autoreactive. Potentially autoreactive cells are eliminated by a process carried out in the medulla of the thymus, designated negative selection.

The principal mediators of negative selection are bone marrow-derived APCs and resident medullary thymic epithelial cells (mTECs). These cells present maturing thymocytes with an assortment of self antigens. Bone marrow-derived APCs sample and present antigens from the environment, while mTECs have the ability to promiscuously express otherwise tissue-specific antigens [50,51]. Following positive selection,

developing thymocytes migrate to the medulla of the thymus and engage these self-antigen-presenting cells. Those thymocytes with high-affinity TCRs receive a death signal, rapidly undergo cell death, and are effectively purged from the T cell repertoire [52-55].

The mechanisms that govern the clonal deletion of self-reactive T lymphocytes also mediate the elimination of lymphocytes with TCR specificities to alloantigens in hematopoietic chimeras (**Figure 1**). After transplantation into conditioned (immunosuppressed) recipients, allogeneic hematopoietic stem cells (HSCs) home to the bone marrow. Following engraftment, donor HSCs differentiate into a population of antigen-presenting cells (both dendritic cells and macrophages), which can seed the thymus and participate in thymic selection of T lymphocytes. In the thymus, similar to host derived-APCs, those from the donor are able to mediate negative selection by presenting self-antigens. Consequently, host thymocytes with donor-specific TCRs (which can be as high in frequency as 1 in 10^3-10^4 of all circulating T cells [15]) are induced to die [56].

FIGURE 1: CENTRAL TOLERANCE INDUCED BY MIXED CHIMERISM
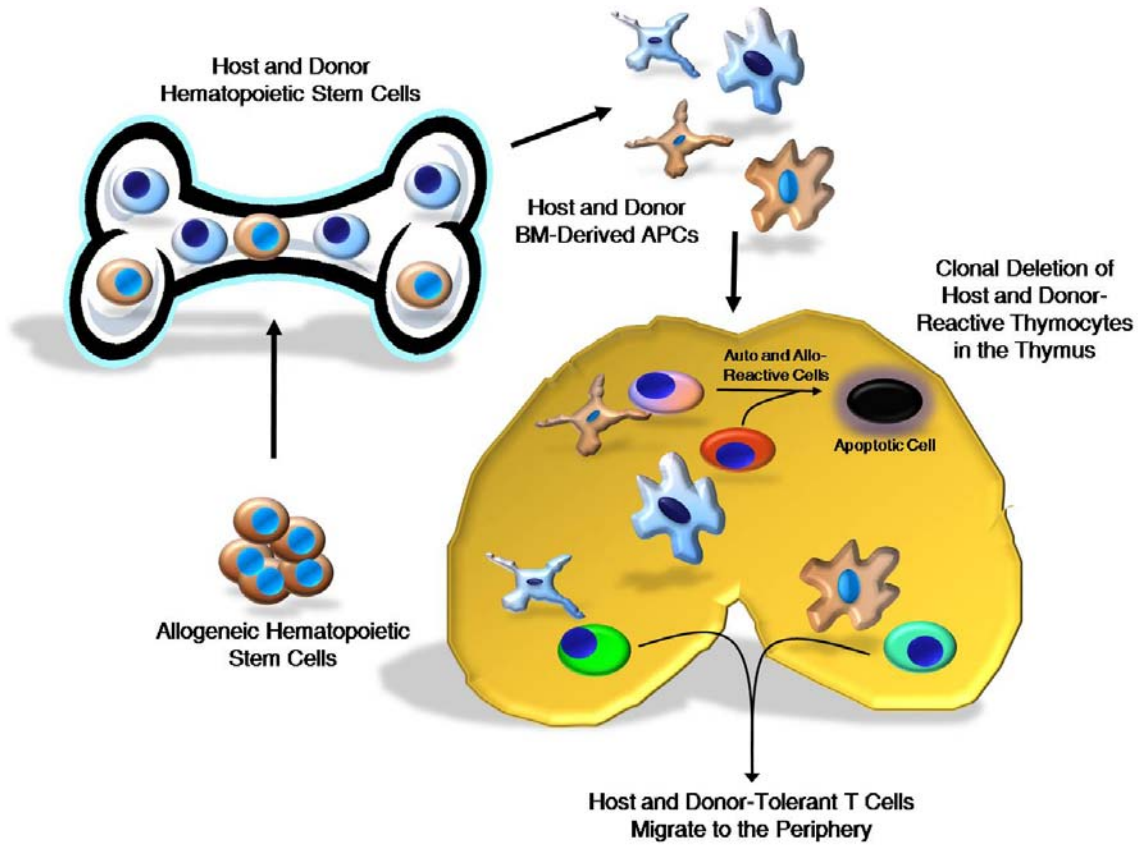
Figure 1. After transplantation, allogeneic hematopoietic stem cells (HSCs) (light brown round cells) home to the bone marrow. Host (light blue round cells) and donor HSCs can differentiate into antigen-presenting cells (stellate cells), which can seed the thymus and participate in thymic selection of T lymphocytes. In the thymus, host bone marrow-derived APCs induce the deletion of donor-derived thymocytes with high-affinity TCRs towards the host (dark red cell). In contrast, donor-derived APCs mediate the negative selection of host thymocytes with donor-specific TCRs. Consequently, mature T cells that emigrate from the thymus and take residence in secondary lymphoid organs in the periphery are tolerant to both host and donor tissues.

Barriers Limiting the Translation of Mixed Chimerism To The Clinic

Despite the robust form of tolerance hematopoietic chimerism induces, stem cell transplantation is not routinely used as an adjunct to solid-organ transplantation. Two primary barriers exist that have slowed its clinical application. The first is the requirement for significant immunosuppression in order for allogeneic stem cells to survive and engraft in the recipient. The second is the development of lethal graft-versus-host disease (GVHD), a condition where lymphocytes in the donor bone marrow incite an immune reaction against the recipient. Therefore, modern conditioning protocols to induce central tolerance have been designed to address the common objectives of 1) establishing hematopoietic chimerism using a relatively benign preconditioning protocol that 2) prevents the development of GVHD.

Hematopoietic Chimerism Induction Protocols

Despite these common objectives, modern conditioning regimens can differ quite significantly in their methodology. In pre-clinical models of hematopoietic chimerism, conditioning regimens span the spectrum from myeloablative protocols, which often entail lethal irradiation and subsequent stem cell rescue, to non-cytoreductive treatments that do not require irradiation (*e.g.* costimulation blockade [57-59]). Myeloablative protocols typically produce a state of full chimerism, in which all of the hematopoietic cells in the recipient are derived from the donor [60]. In contrast, non-cytoreductive regimens produce a state where donor and host hematopoietic cells co-exist [60]. This state is known as mixed chimerism. A third type of conditioning regimen exists between

these two extremes. These protocols are often referred to as non-myeloablative, and are designed to significantly weaken, but not ablate, the recipient's immune system through selective antibody-mediated elimination of specific immune populations (*e.g.* CD4^{+} and CD8^{+} T cells) coupled with targeted irradiation (*e.g.* thymic irradiation) [61]. In clinical trials, successful non-myeloablative approaches have recently been described [62,63]. Stable renal allograft function in recipients for as long as five years after complete withdrawal of immunosuppressive drugs was observed in recipients in which hematopoietic chimerism was established [62,63]. These observations show that in humans, as in rodents, establishment of hematopoietic chimerism is a robust approach for the development of central tolerance and the permanent survival of donor-specific allografts.

Peripheral Transplantation Tolerance

The second major form of tolerance is peripheral tolerance. Different from central tolerance in which hematopoietic chimerism leads to the clonal deletion of antigen-specific cells during development, peripheral tolerance targets pre-existing cells that have already been generated. To induce tolerance in this population, fundamental insights into how naïve antigen-specific T cells become activated have led to protocols designed to prevent this process. Naïve T cell activation is initiated by the interaction of the antigen-specific T cell receptor with a peptide presented by the MHC. This interaction conveys specificity, leading to the activation of only antigen-specific T cells. This signal is often termed "signal 1" **(Figure 2)**. Following TCR-peptide/MHC ligation, a T cell then receives a number of costimulatory signals [64-66]. A key costimulatory signal in this

pathway that permits the activated naïve T cells to become functional effector/memory T cells is provided by CD28-CD80/86 interaction [67], which has often been referred to as "signal 2." In early studies it was shown *in vitro* that T cells that receive signals through their TCR in the absence of engagement of the CD28-CD80/86 costimulation pathway became non-responsive, a state of T cell non-responsiveness often referred to as anergy [43,68]. Following induction of signal 2, cytokines are produced that impart the final signal for T cell activation, and this is termed "signal 3" [65,69,70]. Although these three critical signals are required for the full activation of T cells, additional signals such as those derived from CD40-CD154 interaction can have potent effects on the activation of naïve T cells [71-74] **(Figure 2)**.

Understanding of these mechanisms provided the conceptual basis for the induction of peripheral transplantation tolerance, where the *in vivo* disruption of the costimulatory process – referred to as costimulation blockade – leads to the induction of tolerance in an antigen-specific manner [43].

Costimulation blockade therapies can target several different steps in the process of T cell activation. However, the CD40-CD154 pathway linking signal 1 to signal 2 has been identified to be a critical step in the activation of naïve T cells. Anti-CD154 mAb blocks the interaction between CD154 on the T cell and CD40 on the APC [75,76], and prevents the differentiation of naïve T cells to effector/memory T cells [76,77] **(Figure 2)**.

In peripheral tolerance induction protocols, anti-CD154 monotherapy significantly improves islet [78] and cardiac [79] allograft survival in mice and islet allograft survival

in non-human primates [80-83]. In combination with a donor-specific transfusion (DST), anti-CD154 monoclonal antibody (mAb) induces permanent islet [78] and prolonged skin [84] allograft survival in mice. DST provides selective activation of the alloantigen-specific T cells, and our lab has shown that the subsequent blockade of costimulation by anti-CD154 mAb leads to selective depletion of only the specific alloantigen-reactive CD8$^+$ T cells [85,86]. Another reagent, CTLA4-Ig, binds to the costimulatory molecules CD80/86 on the APC. This blocks its interaction with CD28 on the T cell, preventing signal 2. CTLA4-Ig monotherapy induces the survival of xenogeneic islets [87] and allogeneic cardiac grafts [88]. Not surprisingly, the combination of anti-CD154 mAb and CTLA4-Ig has shown great potential in prolonging skin and cardiac allograft survival in mice [89].

FIGURE 2: COSTIMULATION BLOCKADE
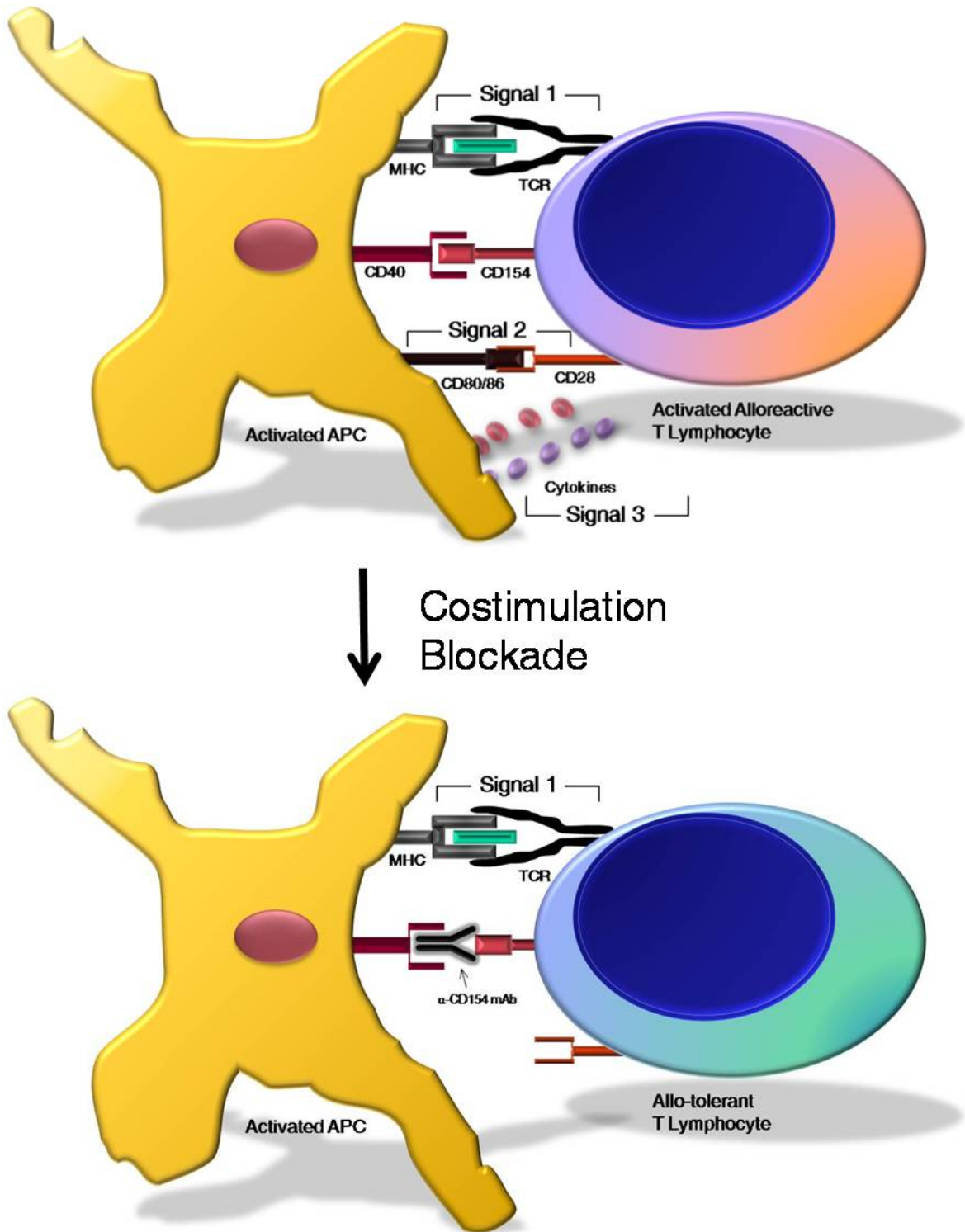
Figure 2. Activation of a T cell involves a series of interactive steps with an APC. The first signal imparts antigen specificity and commences when the TCR engages the antigen/MHC complex presented by the APC and it is commonly referred to as "signal 1." In subsequent steps, the T cell upregulates CD154, which matures the APC by engaging CD40. The next step commonly referred to as "signal 2" involves the upregulation of costimulatory molecules by the mature APC. These activated APCs secrete cytokines, which provide the final activation signals to the T cell; this step is commonly referred to as "signal 3." Protocols based on costimulation blockade can prevent T cell activation by targeting steps in the T cell activation cascade. Anti-CD154 mAb prevents the T cell from licensing the APC by blocking the interaction between CD154 and CD40. This prevents the upregulation of costimulatory molecules and the secretion of stimulatory cytokines, thus depriving the T cell of signals 2 and 3. As a result, of costimulation blockade, the T cell does not develop an activated phenotype, and consequently becomes tolerant to allogeneic antigens.

Costimulation Blockade-Based Strategies for the Induction of Hematopoietic Chimerism

Effective as a peripheral tolerance induction protocol, costimulation blockade protocols based on blockade of the CD40-CD154 pathway have also been used to establish hematopoietic chimerism leading to the generation of central tolerance [57-59]. By establishing multi-lineage hematopoietic chimerism, these non-cytoreductive protocols have proven to promote robust transplantation tolerance to a variety of solid-organ allografts across fully-allogeneic barriers when transplanted several weeks after bone marrow transplantation (BMT) [57,58] or concurrent with BMT [59,90]. Furthermore, because donor-reactivity against the host is dependent on the CD40-CD154 pathway [91], costimulation blockade effectively establishes hematopoietic chimerism in the absence of GVHD [57,58].

Our lab has developed a conditioning regimen for the induction of hematopoietic mixed chimerism that is based on costimulation blockade, and due to its minimal toxicity is amenable to clinical use. By coupling donor specific transfusion with anti-CD154 mAb, Seung *et al.* were able to establish mixed chimerism across fully MHC-disparate barriers by transplanting 50×10^6 bone marrow cells in the absence of radiation [58]. On the first day of tolerance induction (referred to as "day -7"), recipients received 10×10^6 splenocytes from the donor in the form of DST intravenously (i.v), and a 0.5mg dose of anti-CD154 mAb intraperitoneally (i.p). Three days later, another 0.5mg dose of anti-CD154 mAb was given i.p. Four days later (referred to as "day 0"), the recipients received a third 0.5mg dose of anti-CD154 mAb, and were transplanted with donor-strain-matched bone marrow i.v. Four days after transplantation, a final 0.5mg dose of

anti-CD154 mAb was administered. Depending on the genotype of the recipient and the donor, this protocol can produce levels of mixed chimerism that range between 1-10%. Interestingly, permanent tolerance to donor-specific skin allografts transplanted on the same day as the bone marrow, or eight weeks post bone marrow-transplant, can be established in animals that circulate as low as 1% donor-origin peripheral blood mononuclear cells (PBMCs).

Innate Immunity and Transplantation Tolerance

As costimulation blockade protocols move closer to clinical reality, investigations into the interplay between innate and adaptive immunity have intensified. Although alloantigen-specific responses dictated by the adaptive immune system are paramount in transplant rejection, the role of antigen-independent responses by the innate immune system is increasingly being appreciated [92-98]. Transplantation can result in the activation of innate immunity via several mechanisms including tissue damage, ischemia, reperfusion, and through the introduction of pathogens present on colonized organs. Activation of innate immunity leads to the modulation of adaptive immunity, and has been shown to impair transplantation tolerance induction and allograft survival [99-108]. The mechanisms that govern innate immune activation will be the focus of the next section.

Signaling Pathways Involved in Innate Immune Cell Activation

As mentioned earlier, cells of the innate immune, in contrast to B and T lymphocytes, use a limited repertoire of non-rearranged receptors to perform their sentinel functions. These receptors, referred to as pattern recognition receptors (PRRs),

have evolved to recognize conserved pathogen-associated moieties, such as microbial cell-wall components, hypomethylated DNA, and double-stranded RNA (dsRNA) [109]. The three best-characterized PRRs include Toll-like receptors (TLRs), retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs).

TLRs

Members of the TLR family all share a structural homology to the Drosophila receptor Toll, which in 1985, was found to govern control over body pattern formation [110,111]. The toll receptor was demonstrated to control the function of a morphogen protein known as dorsal [112]. Dorsal, a member of the NF-κB family, was found to be a critical determinant in the dorsoventral axis formation in drosophila [112]. In 1996, Lemaitre and colleagues discovered that the toll receptor, when engaged by its putative ligand spatzle, controlled a signaling cascade that regulated the antifungal molecule drosomycin in adult fruit flies [113]. They showed that flies deficient in Toll showed marked susceptibility to disseminated *Aspergillus fumigates* infection, a relatively weak pathogen in insects, highlighting the importance of the toll pathway in fungal immunity. Interestingly, wild-type and Toll mutants demonstrated similar survival to infection with the gram-negative bacterium Escherichia coli, indicating that other pathways than Toll regulated the expression of the Drosophila's antibacterial peptides such as Diptericin [113]. A year later, Ruslan Medzhitov and Charles Janeway discovered the human homologue of Toll [114]. In the decade to follow, 12 additional mammalian TLRs would be discovered [115].

TLRs are found on a vast number of cells, including those of the innate and adaptive immune system. Expression has also been described on non-immune cells, such as those of the endothelium and organ parenchyma. Expression patterns of TLRs, however, vary from tissue to tissue. For example TLR7 and TLR9 - which recognize ssRNA [116,117] and unmethylated CpG-DNA [118], respectively - are thought to be largely restricted to hematopoietic cells such as B cells and conventional (cDC) and plasmacytoid (pDC) dendritic cells [119,120]. In contrast, TLR3, which recognizes dsRNA [121], has a broader distribution, and can also be found on non-hematopoietic cells such as astrocytes, epithelial cells of the cervix, airway, uterus, vagina, intestine, and cornea [119,120,122,123]. In addition, TLRs also differ in their subcellular localization. Certain TLRs, such as TLR1, 2, 4, 5, 6, 10, and 11 are mainly found on the extracellular surface of cells, while TLRs 3, 7, 8, and 9 are restricted to compartments within the cell such as endosomes [124].

Structurally, TLRs are type I integral transmembrane receptors that have a leucine-rich region (LRR) located in their ectodomain, and a Toll/Interleukin (IL)-1 receptor (TIR) domain found on their cytosolic face [115,123]. The LRR domain contains a region of 19-25 tandem leucine-rich repeats, and provides the receptor with its ligand specificity [123]. TLRs with similar primary amino acid sequences have been categorized into five subfamilies: TLR2, TLR3, TL4, TLR5, and TLR9 subfamilies [124]. Members of each subfamily recognize structurally related patterns. For example, those in the TLR9 family - TLR7, 8, and 9 - all recognize distinct nucleic acid moieties, while those in the TLR2 family - TLR1, 2, 6, and 10 - recognize microbial lipids [123]. Interestingly, TLR4 has a

broad range of specificities that include bacterial lipopolysaccharide [125,126], viral glycoproteins [127], as well as host-derived molecules such as heat shock proteins (HSPs) [128], fibronectin [129], and hyaluronic acid [130]. The ligand specificities for the TLRs are summarized in Table 1.

TABLE 1: OVERVIEW OF PRRS: LIGANDS, ADAPTORS, CELL LOCALIZATION, AND ROLE IN TRANSPLANTATION

PRR	Adaptor	Stimulatory Motif	Motif Origin	Tissue Distribution	Subcellular Localization	Role In Transplantation
TLR1/2	MyD88	Triacyl Lipopeptides [131] Zymosan [132]	Bacteria Fungi	B cells, Tregs, Teffs, NK, Monos, pDCs	Cell Surface	1) Activation by Pam$_3$Cys abrogates tolerance induction to skin [133] and cardiac [108] allorafts
TLR2/6	MyD88	Diacyl lipopeptides [134] Zymosan [135]	Mycoplasma Fungi	B cells, Tregs, Teffs, NK, Monos, pDCs	Cell Surface	ND
TLR3	TRIF	dsRNA [121]	Viruses	B cells, NK, various epithelial cells	Endosome	1) Activation by pI:C abrogates tolerance induction to skin allografts [133]
TLR4	MyD88 TRIF	LPS [126] Taxol [136] RSV Fusion Protein [127] HSPs [128,137] Fibronecitn [129] Hyalorinic Acid [130]	Gram (-) Bacteria Plants Virus Host Host Host	B cells, Monos, Tregs	Cell Surface	1) Activation by LPS abrogates tolerance induction to skin allografts [133] 2) Hyporesponsive SNPs are associated with improved survival of lung [138,139] and renal [140-142] allografts
TLR5	MyD88	Flagellin [143]	Bacteria	Tregs, Teffs, NK, Monos	Cell Surface	ND
TLR7	MyD88	ssRNA [116,117]	Viruses	B cells, Tregs, pDCs	Endosome	ND
TLR8	MyD88	ssRNA [116]	Viruses	B cells, Tregs, Monos	Endosome	ND
TLR9	MyD88	CpG-containing DNA [118]	Bacteria and Viruses	B cells, pDCs	Endosome	1) Activation by CpG-DNA abrogates tolerance induction to skin [133] and cardiac [108] allorafts
TLR10	ND	ND	ND	B cells, pDCs	Cell Surface	ND
TLR11	MyD88	Profilin [144]	Uropathogenic Bacteria [145]	ND	Cell Surface	ND
TLR12	ND	ND	ND	ND	Cell Surface	ND
TLR13	ND	ND	ND	ND	Cell Surface	ND
RIG-I	IPS1	dsRNA [146]	Viruses	Various	Cytoplasm	ND
MDA-5	IPS1	dsRNA [147,148]	Viruses	Various	Cytoplasm	ND
NALP3	ASC CARDINAL	Peptidoglycan [149] Uric acid [150] ATP [151]	Bacteria Host Host	Various	Cytoplasm	ND

Upon recognition of its cognate ligand, TLRs activate specific signaling cascades that lead to a potent immunostimulatory response characterized by rapid induction of proinflammatory cytokines, antimicrobial peptides, and the enhanced expression of adhesion and costimulatory molecules [119]. The coupling of pathogen sensing and gene transcription is mediated by a family of TIR containing-adaptor molecules. TLRs, which function as dimers, undergo a conformational change after binding to their ligand. The intracellular TIR domains of each receptor rearrange, come together, and form a new stage for additional signaling molecules that contain the same TIR motif. Five adaptor molecules that bind to the TIR domain of TLRs have been characterized: myeloid differentiation primary response gene-88 (MyD88), TIR-domain-containing adaptor protein inducing IFN-β (TRIF), MyD88-adaptor-like (MAL), TRIF-related adaptor molecule (TRAM), and sterile-α-and armadillo-motif-containing protein (SARM) [152].

MyD88 was the first of the five to be described [153], and it was shown to be indispensable for cytokine induction to a variety of PAMPs [154,155]. It is now known that MyD88 is crucial for signaling of all TLRs with the exception of TLR3, which relies on TRIF [123,124] (**Figure 3**). As a result, TLR signaling pathways are typically referred to as the MyD88-dependent pathway or the MyD88-independent pathway (also called the TRIF pathway). MAL functions as a bridging adaptor for TLR2 and TLR4, aiding in the recruitment of MyD88 to these receptors [152]. TRAM is also thought to be important in TLR4 signaling, as TRAM is required for LPS-mediated expression of cytokines downstream of the MyD88-independent pathway [156]. SARM, in contrast, is believed to be a negative regulator of TLR signaling, largely by inhibiting the function of TRIF

[152].

Both TLR signaling pathways have a common target, the transcription factor Nuclear Factor (NF)-κB. Following recruitment to the TLR, MyD88 recruits the kinases IL-1 receptor-associated kinase (IRAK)-4 and IRAK-1, which subsequently activate tumor necrosis factor receptor-associated factor (TRAF)-6. TRAF-6 stimulates the inhibitor of kappa B kinase (IκK) complex to phosphorylate and inactivate the inhibitor of kappa B (I_kB). After phosphorylation, IkB releases NF-κB and permits its translocation into the nucleus, where it initiates the transcription of numerous inflammatory cytokines and chemokines [123]. For NF-κB activation, the TRIF pathway is thought to signal via two separate pathways. Through an N-terminal region consensus motif, TRIF is able to directly recruit TRAF-6 [157], while its C-terminus can recruit the molecule receptor-interacting protein (RIP)1 [158]. Both TRAF-6 and RIP1 are believed to converge at the level of IκK [152].

The MyD88 and TRIF pathways are also able to couple pathogen recognition to the induction of the type I interferons (IFNα/β). Interestingly, not all TLRs that recruit MyD88 to their TIR domain are able to access this pathway. In fact, only the endosomal TLRs 7, 8, and 9 are able to signal through the MyD88→IFNα/β axis [159]. Following recognition of either ssRNA or unmethylated CpG-DNA, a large complex consisting of MyD88, TRAF-3, TRAF-6, IRAK-4, IRAK-1, IκK-α is recruited to the TLR. This then results in the phosphorylation and activation of interferon regulatory factor (IRF)7 [160-165]. Once activated, IRF7 translocates to the nucleus and binds to interferon-stimulated response elements (ISRE) to induce the expression of type I IFN, as well as other IFN-

inducible genes [159,165,166]. Interestingly, TLR7 and TLR9 activation does not lead to type I IFN induction in all cell types. Stimulation of TLR7 and TLR9 in conventional DCs produces only cytokines that are downstream of the NF-κB pathway, while activation of these receptors in plasmacytoid DCs yields both NF-κB-dependent cytokines and IFN-α [122]. TRIF-mediated induction of type I IFN following TLR3 or TLR4 activation, involves recruitment of TRAF-3 to the N-terminal region of TRIF [160,161,167,168]. TRAF-3 then activates TANK-binding kinase (TBK) 1 [160], which phosphorylates IRF3 and IRF7 leading to the induction of type I IFN [157]. Thus, TLRs constitute a complex family of PRRs that can modulate the immune response to a variety of stimuli using a multitude of signaling pathways.

RLRs and NLRs

In addition to TLRs, members of the RLR and NLR families can link pathogenic perturbations to immune modulation, and thus profoundly affect transplantation outcomes. In contrast to TLRs, RLRs are cytosolic receptors that detect nucleic acids upon viral infection, and are expressed ubiquitously by nucleated cells. Cytosolic RLRs, exemplified by the proteins RIG-I and melanoma differentiation-associated factor-5 (MDA5), recognize double stranded RNA (dsRNA) located in the cytosol following replication by a ssRNA virus, or infection with a dsRNA-genome virus, through interaction with their helicase domains [165]. RLRs contain a caspase recruitment domain (CARD) [146] which link detection of viral dsRNA to the transcription of inflammatory cytokines and IFN-α/β by forming homotypic interactions with the CARD-containing molecule interferon-β promoter stimulator (IPS-1, also known as CARD

adaptor inducing IFN-β (CARDIF), mitochondrial antiviral signaling protein (MAVS), and virus-induced signaling adaptor (VISA)) [169-172]. Activation of IPS-1 triggers TRAF-3, and subsequently members of the IκK family, specifically TBK-1 and IκKε (also known as inducible IκB kinase, IκK-i), to phosphorylate and activate IRF3 and/or IRF7 [160,161,173-176]. As described above, activation of IRF3 and IRF7 results in type I IFN production following their translocation to the nucleus and binding to interferon-stimulated response elements [159,165,166]. RLRs can also activate NF-κB-dependent cytokines, likely through a pathway that involves IPS-1 triggering of FAS-associated death domain-containing protein (FADD) to induce caspase-8/-10 [165].

It has recently been recognized that cytoplasmic sensing of DNA can also trigger IFN-α and IFN-β production [177-179]. This pathway is thought to intersect with the RIG-I and MDA5 pathways at the level of TBK-1 and IKK-I [177], and requires IRF3 for IFN-α/β induction [178]. A candidate cytosolic recognition receptor that senses and is activated by DNA has been described [180]. This receptor, known as DNA-dependent activator of IFN-regulatory factors (DAI), was reported to induce type I IFN upon recognition of bacterial, mammalian and viral DNA [180].

Similar to RLRs and DAI, NLRs consist of a large family of cytosolic immune regulatory receptors that couple the recognition of pathogen-associated patterns to a strong inflammatory response. NOD-like receptors belong to a diverse gene family for which the more unifying name nucleotide-binding domain and leucine-rich repeat containing gene family (NLR) has recently been proposed [181]. Similar to TLRs, NLRs possess leucine-rich repeat domains that serve to detect conserved microbial motifs. They

possess a domain that functions in nucleotide binding and self-oligomerization domain, which was given the appellation NOD. Protein-protein interactions are mediated by CARD, pyrin or baculovirus inhibitor repeat domains, contained in the N-terminus of the receptor [182]. There are currently 23 human and 34 murine NLRs that have been described [182].

Three of the best-characterized NLRs include NOD1, NOD2, and NALP3. NOD1 and NOD2 translate the presence of cytosolic bacterial peptidoglycan molecules into activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways. In contrast, NALP3, also known as cryopyrin, links cytosolic recognition of microbial components such as peptidoglycan, as well as the host molecules ATP and uric acid, to the induction of IL-1β and IL-18 via a molecular complex named the inflammasome [150,151,183]. The inflammasome – which consists of the NLRs NALP3, IPAF or NALP1, the adaptor molecules ASC and CARDINAL, and caspase-1 – has also been reported to produce IL-1β in response to both microbial and host DNA [184]. Hence, the innate immune system has evolved a multitude of mechanisms by which danger signals such as cellular stress and microbial invasion can be translated into a variety of stimuli that active innate immunity and modulate the adaptive immune system during transplantation.

FIGURE 3: PATHOGEN RECOGNITION SYSTEMS
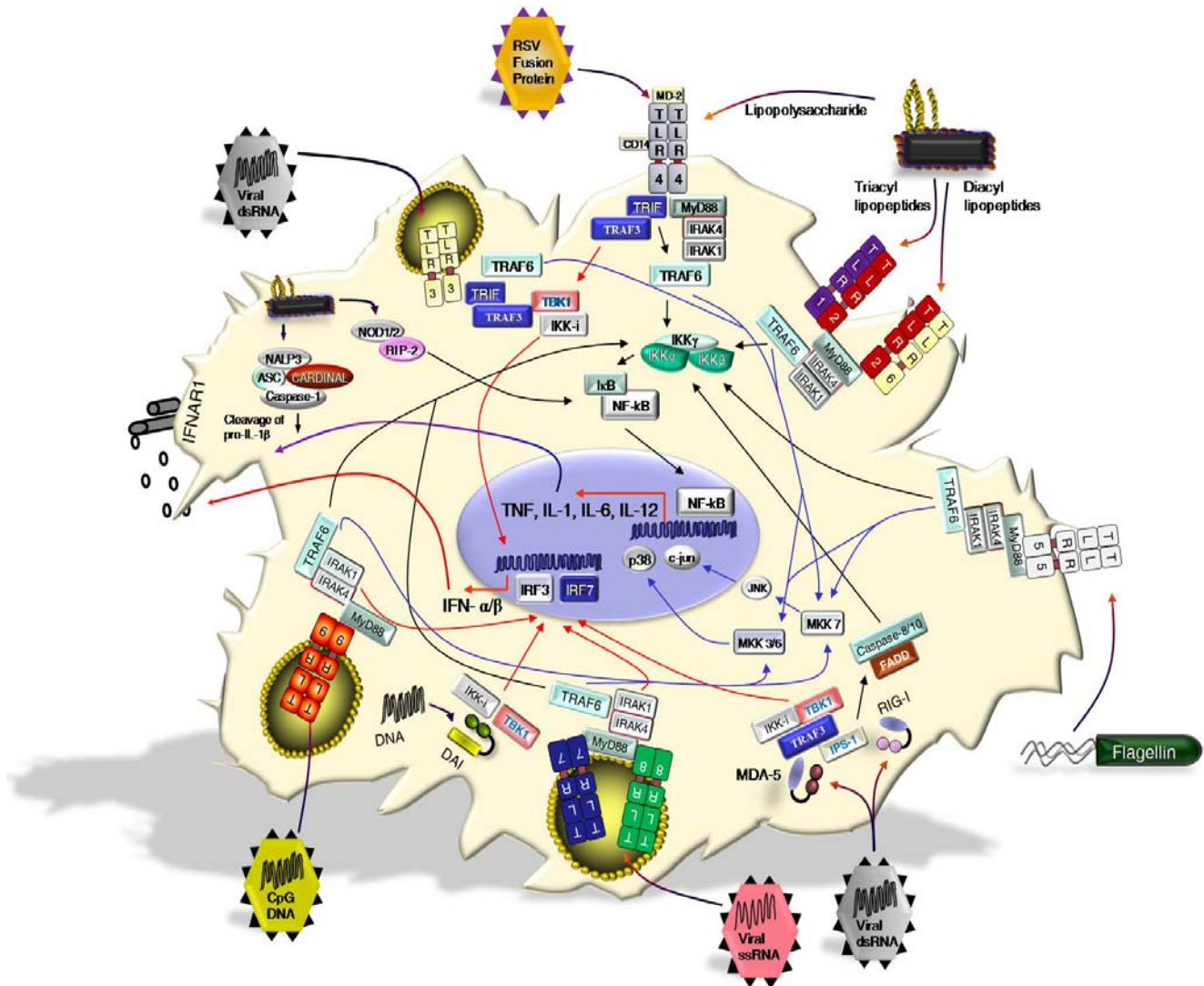
Figure 3. The pathways for select TLRs, RLRs and NLRs are shown. TLRs initiate a vigorous inflammatory response after sensing various PAMPs, such as microbial cell-wall components, hypomethylated DNA, and double-stranded RNA (dsRNA). TLR signaling downstream of the adaptor molecules MyD88 and TRIF results in the transcription of cytokines regulated by NF-κB and the MAP kinase pathway. Select TLRs are also capable of inducing expression of type I IFN. The RNA helicase receptors MDA5 and RIG-I couple the recognition of viral RNA to the induction of pro-inflammatory cytokines and IFNα/β via the adaptor molecule IPS-1. NLRs, such as NOD1 and NOD2 recognize components of the bacterial cell wall, and induce NF-κB activation by triggering the serine/threonine kinase RIP2/RICK. The NLR NALP3 complexes with the adaptor molecules ASC and CARDINAL, and caspase-1 after sensing bacterial peptidoglycan. This complex, known as the inflammasome, catalyzes the processing of pro-IL-1β to the mature form.

PRRs: The Link Between Innate and Adaptive Immunity

In the last several years, the role of PRR signaling in adaptive immunity has been intensively studied. It is evident that activation of these germ-line encoded receptors can have profound effects on the adaptive immune response. Undoubtedly, we have only begun to understand the physiological effects of PRR activation; however, in the next section we will review some of the reported effects of PRR signaling on B cells, regulatory T cells (Treg), and effector T cells (Teff).

Role of PPRs in B-cell Immunity

B cells play a significant role in transplantation by acting as both antigen-presenting cells and as effectors. As described above, B cells contribute to hyperacute rejection of solid organs, and they also pose a significant barrier to the engraftment of allogeneic bone marrow [185]. TLR signaling appears to affect B cell function in a number of ways. First, TLR signals are important in DC maturation and the subsequent activation of helper T cells (described in more detail later), which are crucial to provide stimulatory signals to B cells. Second, TLR signaling on B cells appear to be critical for activation and antibody production. In 2005, Pasare and Medzhitov, showed signaling through TLRs governs several aspects of B cell physiology, including the production of IgM, IgG1, and IgG2 antibodies, and the differentiation into germinal center cells [186]. Interestingly, certain aspects of the B cell phenotype, such as IgE and IgA responses, were shown not to be mediated by TLRs [186].

In addition to regulating differentiation and activation, TLR signaling on B cells may

also dampen immune responses in certain contexts. For example, in 2008, Lampropoulou and colleagues reported that LPS or CpG-DNA-activated B cells could suppress Th1 and Th17 immune responses, and stimulate the recovery of autoimmune experimental encephalomyelitis [187]. Therefore, it is clear that TLR signals can directly affect the phenotype of adaptive immune cells, and thus, the outcome of immune responses.

Role of PRRs in T-cell Immunity

The role of TLRs in T cell function has also received extensive study in recent years. T cells are known to be pivotal players in allograft rejection, and their relationship with antigen presenting cells has been abundantly demonstrated. As described before, TLR activation can affect key steps in T cell activation, namely the upregulation of costimulatory molecules and secretion of stimulatory cytokines by APCs. Signaling downstream of MyD88 also appears to be important in the generation of CD4$^+$ T cell memory responses [188]. In addition, TLRs can profoundly affect the intercourse between effector and regulatory T cells. This has significant ramification for transplantation, as the generation and maintenance of CD4$^+$Foxp3$^+$ Tregs has been shown to be indispensable to the survival of allografts [189-191].

Both Tregs and Teffs have been shown to express various TLRs [192]. Therefore, TLR signaling can affect Treg-Teff interactions by directly activating receptors on either subset. Interestingly, Tregs have been shown to express a more diverse repertoire of TLRs, including TLR1, 2, 4, 5, 6, 7, and 8, while non-Treg cells express only TLR 1, 2, 5, and 6 [192,193]. Consistent with the diverse signaling pathways exhibited by each

TLR, distinct PAMPs appear to induce distinct T cell responses.

For example, Caramalho and colleagues demonstrated that TLR4 activation in conjunction with TCR ligation directly induces murine Treg survival and proliferation *in vitro*, as well as enhances their suppressive function *in vitro* and *in vivo* [192]. Consistent with the idea that TLR activation can produce anti-inflammatory responses as well as pro-inflammatory effects, Crellin *et al.* showed that activation of TLR5 with flagellin enhanced both the expression of the key regulatory transcription factor FOXP3, and the suppressive capacity of human Tregs [193]. Interestingly, Crellin found that TLR4 activation in human Tregs did not reverse their anergic phenotype nor induce proliferation as Caramalho noted in murine Tregs [194]. Consequently, the influence of direct TLR4 stimulation on the Treg phenotype requires further inquiry.

Interestingly, direct TLR signaling on Tregs can also suppress their regulatory abilities and result in enhanced immunity. Building on the observation that the numbers of circulating Tregs are significantly reduced in TLR2 knockout mice [195], Sutmuller and colleagues tested the hypothesis that triggering of TLR2 on Tregs would result in their proliferation [194]. In confirming their hypothesis, they also observed that during TLR2-mediated proliferation, Tregs experienced a moratorium of their suppressive capabilities. *In vivo*, TLR2-mediated cessation of suppression translated into enhanced anti-fungal immunity. Later that same year, Liu and colleagues also reported that TLR2 activation in the absence of APCs induced Tregs to proliferate and temporarily lose their suppressive capacity [196]. In addition, TLR8 activation on Tregs is also capable of reversing their suppressive phenotype. In 2005, Peng *et al.* demonstrated that

pretreatment of Tregs with either ssRNA or guanosine-containing DNA oligonucleotides can abrogate their ability to suppress effector T cells in a TLR8-MyD88-IRAK-dependent fashion [197].

Collectively these studies suggest that regulatory cells have evolved a tailored response to distinct microbes, which allows them to enhance or attenuate immunity given the requirement of the organism. For example, as Sutmuller and colleagues have postulated [194], in the setting of acute infection, direct TLR ligation on Tregs permits the effector arms of the immune system to eradicate the pathogen by reversing suppressive mechanisms. Once the pathogen has been cleared, the lack of microbial components allows Tregs to regain their suppressive phenotype and provide a balance between immunity and tolerance.

Increasing evidence suggests that regulatory mechanisms can also be affected by indirect pathways that do not require Tregs to directly sense PAMPs. In 2003, Pasare and Medzhitov demonstrated that microbial induction of the TLR pathway on DCs can enable effector T cells to overcome regulatory cell-mediated suppression [198]. The authors showed that LPS or CpG-DNA–activated DCs produced soluble mediators that act synergistically to render effector CD4$^+$ T cells refractory to Treg-mediated regulation, permitting activation of antigen-specific T cells in the presence of Tregs. IL-6 was identified as one of the critical cytokines; however, it alone was not capable of blocking suppression of effector CD4$^+$ T cells *in vitro,* suggesting that additional cytokines, such as IL-1 or IL-12, are also required. A year later, Yang *et al.* reported that persistent TLR signals were required for the reversal of Treg-mediated tolerance of CD8$^+$ T cells *in vivo*

[199]. Administration of LPS or CpG-DNA was required for Treg-tolerized CD8$^+$ T cells to proliferate in the presence of cognate antigen. Direct CpG-DNA activation of TLR9 on CD4$^+$ effector cells was also shown to be sufficient to allow their escape from regulatory T cell suppression [200]. Together, these studies suggest that a variety of mechanisms exist by which TLR activation can affect the adaptive immune response and potentially alter transplantation outcomes.

Evidence of TLR-Mediated Modulation of Transplantation

Animal Models

Mounting evidence suggests that transplantation outcomes can be greatly affected by environmental perturbations that activate various PRRs such as tissue damage, ischemia and microbial infection. In 2003, Goldstein and colleagues used a minor antigen mismatch model to investigate the role of TLR2, TLR4 and the adaptor molecule MyD88 in allograft survival [201]. They reported that rejection of male skin grafts by female recipients was dependent on MyD88, as skin grafts from MyD88-deficient male donors survived indefinitely when transplanted on MyD88-deficient recipients. The authors also demonstrated the MyD88 was critical for optimal DC maturation and homing, and for CD8$^+$ T cell priming. Interestingly, both host and donor MyD88 proved important, as graft rejection occurred if either the donor or the host expressed functional MyD88 protein. The role of TLR2 and TLR4 proved less important, as rejection of skin allografts occurred in the absence of TLR2 and TLR4 signaling. A year later, the same group showed that despite the role of MyD88 in DC maturation, T cell priming, and T$_H$1

immunity, fully MHC-mismatched skin and cardiac allografts were rejected with normal kinetics in the absence of MyD88 [202]. These data reveal several important insights. First, in the setting of minor-antigen mismatches, factors downstream of MyD88 signaling are critical. Those factors can be contributed, however, by either host or donor, and seem to regulate DC function. Second, during strong immune responses, such as those seen in fully mismatched allograft rejection, MyD88-independent processes appear to be sufficient for alloimmunity.

To address the role of MyD88-independent pathways in allograft rejection, McKay and colleagues examined the role of TRIF and MyD88 in major histocompatibility and minor antigen mismatch skin graft rejection [203]. Their work showed that the simultaneous absence of MyD88 and TRIF signaling on the donor (H-2^b) resulted in modest but significantly prolonged skin graft survival when transplanted onto wild-type BALB/c (H-2^d) mice. Absence of either signaling pathway by itself, however, did not prolong skin graft survival. This suggests that the MyD88 and TRIF pathways work synergistically to promote rejection of major antigen mismatched allografts. Furthermore, like Goldstein *et al.* [201], McKay attributed the prolonged skin graft survival in this model to the role these synergistic pathways play in the migration of donor cells to draining lymph nodes. Like cells in Goldstein's MyD88$^{-/-}$ animals, donor cells in mice deficient in both MyD88 and TRIF exhibited impaired ability to traffic to draining nodes. Collectively, the data from the above experiments suggest that during allograft rejection, TLR-mediated maturation of DCs plays a critical role in alloreactive T cell priming and, thus, the survival of the graft.

Given the role that TLR signals have in DC function and T cell priming, Thornley and colleagues from our lab hypothesized that direct TLR activation by microbial components would abrogate tolerance induction mediated by costimulation blockade. In 2006, they reported that the activation of either TLR2, TLR3, TLR4 or TLR9 could prevent fully mismatched skin allograft survival established with donor-specific transfusion and anti-CD154 mAb treatment [133]. DST and anti-CD154 mAb conditioning has been shown to induce the deletion of alloreactive CD8$^+$ T cells rapidly after tolerance induction, a process critical for graft acceptance [86]. Activation of TLRs on the day of tolerance induction was shown to rescue alloreactive CD8$^+$ T cells from costimulation blockade-mediated apoptosis [133]. This mechanism proved essential, as removal of CD8$^+$ T cells permitted the survival of allografts in the presence of TLR agonists.

A year later, they also showed that rejection of skin grafts in the same model by LPS was dependent on TLR4 and MyD88, while polyI:C mediated-rejection was not dependent on TLR3 [204]. In addition, a critical role for type I interferon in TLR-mediated abrogation of transplantation tolerance was demonstrated. Mice deficient in the IFN-α/β receptor exhibited prolonged skin allograft survival following DST and anti-CD154 mAb treatment, even in the presence of LPS or poly I:C. Interestingly, similar to LPS and poly I:C, administration of recombinant IFN-β at the time of tolerance induction protected alloreactive T cells from costimulation blockade-induced cell death. These data indicate that production of type I IFN following TLR activation can contribute to the abrogation of transplantation tolerance by preventing the deletion of T cells specific for

the graft. It is also tempting to speculate that in the case of polyI:C, type I IFN induction via redundant mechanisms of nucleic acid sensing, such as the cytosolic helicase MDA-5, are sufficient to prevent peripheral tolerance.

Additional evidence that TLR activation can compromise tolerance induction strategies was provided by Chen and colleagues. In 2006, they reported that treatment with the TLR2 agonist Pam_3Cys or the TLR9 agonist CpG-DNA on the day of transplantation could abrogate anti-CD154 mAb-induced tolerance to fully mismatched cardiac allografts [108]. Interestingly, the effects of TLR activation in this model were not subscribed to alloantibody production or $CD8^+$ T cells, but to $CD4^+$ T cells. In fact, activation of TLR9 at the time of transplant was associated with a decrease in the intra-graft accumulation of regulatory T cells. Whether or not TLR activation prevented the generation of regulatory T cells or affected their migration was not clearly elucidated; although, CpG-DNA was shown to prevent the anti-CD154 mAb-mediated upregulation of the putative Treg-homing ligands CCL17 and CCL22.

In a separate set of studies that year, Walker *et al.* also demonstrated the importance of TLR signaling on peripheral tolerance induction. Using a fully-mismatched skin transplant model where modest allograft survival is established with anti-CD154 mAb and CTLA-4 Ig treatment, the authors demonstrated that MyD88 played a pivotal role [205]. They showed that in the absence of MyD88 signaling on both donor and host cells, costimulation blockade treatment could yield long-term skin allograft survival. The synergistic effect between costimulation blockade and the absence of MyD88 was attributed to a decrease in IL-6 production by DCs, and an increase in susceptibility of

effector T cells to the suppression of regulatory cells. Taken together, these studies show that TLR activation by PAMPs can affect peripheral tolerance induction by several mechanisms, which include: 1) modulating APC function, 2) preventing clonal deletion of donor-specific T cells, and 3) disrupting immune regulation.

Clinical Studies

The role of TLRs in human transplant outcomes has also begun to receive more attention. Polymorphisms in TLR4 have been reported to affect the development of graft-versus-host-disease (GVHD) [206,207], as well as lung [138,139] and renal [140-142] allograft survival. The two most common polymorphisms that have been studied are the Asp299Gly and the Thr399Ile (TRL4 299/399) mutations. The resulting amino acid changes in the extracellular domain of the receptor produce a hyporesponsiveness to LPS, as evidenced by a blunted response to inhaled endotoxin [208].

Endotoxin liberated from the gut during conditioning for BMT has been shown to be an important factor in precipitating GVHD [209]. Consequently, investigators have hypothesized that hyporesponsiveness mutations in either the donor or recipient would correlate with decreased incidence of GVHD. Two reports, however, have produced contrary and inconclusive results. In 2001, Lorenz and colleagues showed that TLR4 mutations correlated with a decrease in risk for GVHD, although the results did not reach statistical significance [206]. In contrast, Elmaagacli *et al.* in a more recent paper claimed that the Thr399Ile mutation was associated with an increase in risk [207]. Therefore, the role of TLR4 signaling in the pathogenesis of GVHD has not been conclusively

established.

On the contrary, TLR4 signaling has been more definitively linked to outcomes in lung and renal allograft transplantation. In a series of studies by Palmer and colleagues, it was demonstrated that both Asp299Gly and Thr399Ile single nucleotide polymorphisms (SNP) in the recipient correlated with a decrease in incidence of acute rejection and the development of obliterative bronchiolitis, a major cause of morbidity in lung transplant patients [138,139]. In support of the hypothesis that induction of pulmonary innate immunity by LPS contributes to allograft pathology, Palmer and colleagues have recently reported that an increase-in-function polymorphism in the promoter of CD14 correlated with a poorer outcome [210]. Increased production of CD14 – a LPS binding protein that is indispensable for TLR4 signaling – in patients with the 159TT polymorphism was associated with higher rates of bronchiolitis obliterans syndrome, a putative manifestation of chronic rejection [211], increased T_H1 immunity, and decreased graft survival. Together, these three reports indicate that allelic variation in pathways that govern pulmonary innate immunity can profoundly affect the development of both acute and chronic rejection in recipients of lung transplants.

Pursuant of a similar hypothesis, Ducloux *et al.* investigated the role of TLR4 polymorphisms in renal transplant patients. Concordant with the lung transplant studies, they found that renal transplant recipients with TLR4 299/399 polymorphisms benefitted from a lower risk of post-transplant atherosclerotic events and acute rejection; however, they suffered from a higher incidence of opportunistic infection [140]. Interestingly, Palmer and colleagues found that improved outcome was imparted only when the

hyporesponsive SNP was found on the donor [141]. No association between acute allograft rejection and recipient polymorphism was demonstrated. In support of a protective role of TLR4 Asp299Gly ((299)G) in recipients, Fekete *et al.* reported a year later that the (299)G SNP was more frequent in renal transplant recipients with long-term acceptance [142]. Furthermore, they documented that SNPs in the putative endogenous TLR4 ligand HSP70s also correlated with renal allograft outcome. The HSPA1B (1267)AA genotype was more common in renal transplant recipients with good renal graft function over 15 years compared to recipients that needed to be consecutively transplanted due to graft failure. Taken together, the above clinical and experimental studies suggest that by modulating both the innate and adaptive immune response, TLRs play an important role in allograft transplantation.

Viral Infection: A Potent Barrier to Transplantation Tolerance

The majority of the aforementioned studies have taken a reductionist approach to investigate how individual microbial components or specific molecules influence transplantation outcomes. However, actual infection stimulates multiple pattern recognition systems such as TLRs, RLRs and NLRs simultaneously. Therefore, it is no surprise that direct infection by virus has been shown to have deleterious effects on the induction of transplantation tolerance. For example, infections with lymphocytic choriomeningitis virus (LCMV) before [104], at the time of [101,106,212], or shortly after [107] costimulation blockade all impair allograft survival. Interestingly, this effect appeared to be virus-specific, as infection with vaccinia virus (VV) and murine cytomegalovirus (MCMV) the day after transplantation did not engender allograft

rejection [107].

Barriers to the induction of hematopoietic chimerism and establishment of central tolerance in the setting of viral infection have also been reported. Anti-CD154 mAb, CTLA4-Ig and busulfan treatment fails to induce bone marrow chimerism and tolerance to skin allografts in the setting of multiple viral infections [103]. Moreover, using a non-myeloablative protocol where anti-CD154 mAb treatment was coupled with sublethal irradiation, Forman *et al.* observed that infection with LCMV on the day of bone marrow transplantation resulted not only in allograft rejection, but also proved lethal to the recipient [105]. Interestingly, conditioned recipients that were infected and given syngeneic bone marrow grafts did not die. Recipients of allogeneic bone marrow died by a type I interferon-dependent mechanism, as mice deficient in the type I interferon receptor survived. The recent deaths of a cluster of human transplant recipients of LCMV-infected organs makes this finding particularly relevant to the safety and efficacy of tolerance induction protocols based on costimulation blockade [213].

TLR activation may abrogate the establishment of hematopoietic mixed chimerism in recipients treated with costimulation blockade

Despite the numerous clinical and experimental studies demonstrating an important role for toll-like receptors in solid-organ transplantation, very little is known about their function in establishing hematopoietic mixed chimerism. Understanding how TLRs affect hematopoietic chimerism induction is critical, however, as it is one of the most promising strategies to induce the acceptance of transplanted allografts in the absence of chronic immunosuppression. Conditioning regimens based on costimulation blockade are

effective at establishing mixed chimerism and inducing transplantation tolerance. However, activation of TLRs has been shown to shorten the survival of solid-organ grafts in recipients treated with costimulation blockade [133,204]. Therefore, we initially asked if perturbations that activate TLRs would also reduce the efficacy of costimulation blockade to establish mixed chimerism. In this dissertation, we show convincing data that TLR activation during tolerance induction prevents the establishment of hematopoietic mixed chimerism and the induction of transplantation tolerance to skin allografts in mice treated with costimulation blockade. In support of this hypothesis, we present data that TLR4 agonists prevent the establishment of mixed chimerism by inducing the production of type 1 IFN and MyD88-dependent factors that upregulate costimulatory molecules on APCs, leading to the generation of activated alloreactive T cells.

CHAPTER II: MATERIALS AND METHODS

Animals

BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice were obtained from Charles River Laboratories (Wilmington, MA), or The Jackson Laboratory (Bar Harbor, ME). C57BL/10ScSnJ ($H\text{-}2^b$, abbreviated as C57BL/10), C57BL/10ScNJ-$Tlr4^{lps\text{-}del}$ ($H\text{-}2^b$, abbreviated as C57BL/10.TLR4$^{-/-}$), and C57BL/6.129S2-$Cd8a^{tm1Mak/J}$ ($H\text{-}2^b$, abbreviated as C57BL/6.CD8$\alpha^{-/-}$) mice were obtained from The Jackson Laboratory and bred at the University of Massachusetts Medical School. C57BL/6.IRF3$^{-/-}$ mice were the gift of Dr. Evelyn Kurt-Jones (University of Massachusetts Medical School). C57BL/6.MyD88$^{-/-}$ (N6, abbreviated as MyD88$^{-/-}$) [214] and C57BL/6.129S2-$Ifnar1^{tm1At}$ (N12, abbreviated as IFNAR1$^{-/-}$) [215] were the gift of Dr. Egil Lien (University of Massachusetts Medical School), who originally obtained the MyD88$^{-/-}$ mice from Dr. Douglas Golenbock (University of Massachusetts Medical School) and the IFNAR1$^{-/-}$ mice from Dr. Jonathan Sprent (Scripps Research Institute, La Jolla, CA). C57BL/6.MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were produced by crossing IFNAR1$^{-/-}$ and MyD88$^{-/-}$ mice, and then performing an F1 intercross. Confirmation of the targeted gene deletions were confirmed by PCR (see below). All animals were certified to be free of murine hepatitis virus, minute virus of mice, mouse adenovirus, Sendai virus, pneumonia virus of mice, ectromelia, LDH elevating virus, polyoma, mouse poliovirus, Reo-3 virus, LCMV, *Mycoplasma pulmonis*, and *Encephalitozoon cuniculi*. All mice were housed in microisolator cages, given *ad libitum* access to autoclaved food, and maintained in accordance with the guidelines of

Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) assays for the presence of targeted mutations in the MyD88 and IFNAR1 genes were performed on cells obtained from the ear. DNA was prepared by incubating cells in 21 µl of buffer containing 50mM Tris-HCl, 10mM EDTA, 2mM NaCl, 1% sodium dodecyl sulfate, and 1mg/ml proteinase K for 20 min at 55oC. Samples were then vortexed and incubated for another 20 min at 55oC. 780µl of water was then added to the samples, which were then boiled for 5 min. One microliter of this sample was amplified for 35 cycles in 50ul of buffer containing 2mM MgCl2, 10mM Tris, 50 mM KCl, 2.5mM dNTPs, 1.5 U Taq polymerase (Boehringer Mannheim, Mannheim, Germany), 1.5 U anti-Taq antibody (Clontech, Palo Alto, CA) and 0.2µM primers.

To detect the MyD88 gene mutation, two separate PCR reactions, using a combination of three primers, were performed. One reaction contained a primer sequence specific for the targeted MyD88 gene (5'- AGA CAG GCT GAG TGC AAA CTT GTG CTG-3') and a primer directed to a sequence downstream of the targeted gene (5'-AGC CTC TAC ACC CTT CTC TTC TCC ACA-3'). This reaction produces a 1000 base pair (bp) PCR product when the wild-type allele is present. The second reaction contained the primer for the neomycin resistance gene contained within the targeting construct (5'-ATC GCC TTC TAT CGC CTT CTT GAC GAG-3') and the primer for the sequence

downstream of the target gene (see above). This reaction produces a 1000 bp PCR produce only when the mutated allele is present. The denaturation, annealing, and extension temperatures were 95°C, 65°C, and 72°C, respectively. The products from each reaction were run separately on a 1% agarose gel, and the genotype was assessed from the presence or absence of the mutated gene PCR product (i.e. the absence of a product from the first reaction, coupled with the presence of a product from the second reaction is indicative of a mouse homozygous for the gene mutation).

To detect the IFNAR1 gene mutation, one reaction using three primer sequences was performed. A forward primer specific for the IFNAR1 gene (5'-AAG ATG TGC TGT TCC CTT CCT CTG CTC TGA-3') and a reverse primer for the IFNAR1 gene (5'-ATT ATT AAA AGA AAA GAC GAG GCG AAG TGG-3') was used in combination with a primer for the neo resistance gene (5'-ATC GAC AAG ACC GGC TTC CAT CCG A-3'). DNA from wild-type mice amplified a PCR product that was 151bp, while DNA from IFNAR1$^{-/-}$ mice amplified a PCR product that was 826bp in length. The denaturation, annealing, and extension temperatures were 95°C, 60°C, and 72°C, respectively.

Antibodies

Purified hamster anti-mouse CD154 mAb (clone MR1) was produced at The National Cell Culture Center (Minneapolis, MN) [216]. FITC-conjugated anti-H-2K^d (clone SF1-1.1) anti-mouse H-2K^b-PE (clone AF6-88.5), anti-mouse CD8α-PerCP (clone 53-6.7), anti-mouse CD8α-Pacific Blue (clone 53-6.7), anti-mouse CD8β-PE (clone H35-

17.2), anti-mouse CD4-PerCP (clone RM4-5), anti-mouse CD4-PE (clone RM4-4), anti-mouse CD3-biotin (clone 145-2C11), anti-mouse CD44-APC (clone IM7), anti-mouse IFN-γ-APC (clone XMG1.2), anti-mouse IFN-γ-PE (clone XMG1.2), anti-mouse CD11a-FITC (clone 2D7), anti-mouse CD11a-PE-Cy7 (clone 2D7), anti-mouse CD49b-PE (clone DX5), allophycocyanin-conjugated Steptavidin, purified anti-Ly49D (clone 4E5), purified anti-mouse CD16/32 (clone 2.4G2) and corresponding isotype control antibodies were purchased from BD PharMingen (San Diego, CA).

Flow Cytometry

Single-cells suspensions were prepared from heparinized whole blood (acquired from the retro-orbital venous plexus) and spleens and washed two times with $Ca^{2+}Mg^{2+}$-free Dulbecco's phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) containing 1.0% fetal clone serum (HyClone, Logan, Utah) and 0.1% sodium azide (Sigma-Aldrich, St. Louis, MO). Cells were then incubated for 10 min with purified αCD16/32 ($100ng/1x10^6$ cells) at 4^oC before incubation for 20 min with fluorescent antibodies. Antibodies were used at a concentration of $0.2ug/1x10^6$ cells unless specifically noted elsewhere. Samples were then treated with FACS Lysing Solution (BD Biosciences Immunocytometry Systems, San Jose, CA), and then washed two times with PBS before fixation in PBS containing 1% paraformaldehyde (Poly-Sciences, Warrington, PA). Labeled cells were analyzed with a FACSCalibur or LSRII instrument (BD Biosciences). For *ex vivo* cell analysis, at least 100,000 events were collected, unless specified elsewhere. FlowJo software (Tree Star, Ashland, OR) was used to analyze flow data after

acquisition.

Donor-Specific Transfusion and Antibody Treatment

Donor-specific transfusion (DST) was prepared as previously described [84]. Briefly, spleens were harvested from euthanized donors and single-cell suspensions were prepared by mechanical disruption. Cells were washed, diluted in PBS and counted with either a hemocytometer or a coulter counter (Beckman Coulter, Inc, Fullerton, CA). Cell viability was assessed using trypan blue exclusion, and viability was >90%. Recipient mice aged 5 to 10 weeks of age were treated with a DST consisting of 10×10^{6} BALB/c spleen cells injected intravenously. DST was given on day −7, and 0.5 mg of anti-CD154 mAb was injected intraperitoneally on days −7, −4, 0, and +4 relative to bone marrow transplantation on day 0. Anti-CD4 mAb (clone GK1.5, 0.5mg) or anti-CD8α (clone 2.43, 0.5mg) was injected intraperitoneally on day −10, −9, and −8 relative to transplantation on day 0. We confirmed >90% $CD4^{+}$ and $CD8^{+}$ T-cell depletion by flow cytometry using antibodies against a noncompeting CD4 (clone RM4–4) epitope and the CD8β chain. Anti-NK1.1 antibody (clone PK136, 25μg) was injected i.p. on day −10 relative to transplantation. We confirmed >90% NK cell depletion by flow cytometry using antibodies against CD49b and Ly-49D.

Bone marrow transplantation

Single cell suspensions of bone marrow were prepared from femurs and tibias, filtered through sterile 70-μm nylon mesh (Becton Dickinson, Franklin Lakes, NJ), washed in PBS and counted with either a hemocytometer or a coulter counter. Cell

viability was assessed using trypan blue exclusion, and viability was >90%. Recipient mice received a single intravenous injection of 50×10^6 bone marrow cells in a volume of 0.5 ml D-PBS, as described previously [58].

Skin transplantation

Full-thickness skin grafts 1-2 cm in diameter were obtained from the trunks of donor mice and transplanted onto the dorsal flanks of recipients as described [58,84]. Briefly, skin was removed from donors euthanized via cervical dislocation and pneumothorax, and the subcutaneous layer was scraped with a scalpel to remove muscle and adipose tissue. Skin was kept on sterile filter paper moistened with RPMI (Invitrogen) at room temperature until transplanted. The dorsal flank of the anesthetized recipient was shaved and washed with 70% ethanol. The graft bed was prepared by removing the epidermis and dermis down to the fascia of the muscle using scissors. A 1-2 cm^2 skin graft was then placed on the graft bed and trimmed to fit the prepared area. Grafts were covered with Vaseline-impregnated gauze and secured with plastic bandage. One week following transplantation, bandages were removed and graft survival was evaluated three times a week by visual and tactile inspection. Graft rejection was defined as the first day on which the entire epidermal layer of the graft was necrotic.

Determination of hematopoietic chimerism

Heparinized whole blood samples were obtained from the retro-orbital venous plexus from anesthetized mice for determination of chimerism. The percentage of donor and host cells expressing MHC class I in chimeric mice was determined by dual-labeling with

antibodies to H-2K^b and H-2K^d as described previously [58]. We defined recipients as chimeric if the percentage of donor-origin peripheral blood mononuclear cells (PBMCs) was >0.10% at 8 weeks.

IFN-α/β Bioassay

IFN-α/β was measured using a standard virus-inhibition bioassay [217]. Briefly, whole blood was isolated from mice 8 and 24 hours after the indicated treatment, and was then centrifuged to obtain serum. The serum was then diluted two-fold across a 96-well plate. Wells were seeded with $3x10^4$ mouse L-929 cells (NCTC clone 929; ATCC, Manassass, VA) and incubated overnight. The following day, each well was infected with $2x10^5$ PFUs of vesicular stomatitis virus (VSV) strain Indiana and incubated for 2 days. The cytopathic effects were evaluated using microscopy, and the IFN-α/β titer was determined as the reciprocal of the dilution that provided 50% protection from cytopathic effects [217].

Preparation and injection of TLR agonists and Recombinant IFN-β

TLR agonists were prepared as previously described [133]. Briefly, LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich) was purified as previously described [218], except that phenol-PBS phase separation was conducted at 2000xg for 30 min to accommodate larger volumes. Repurified (pLPS) was suspended in PBS, and we assumed a 10% loss during purification [218]. pLPS was stored at 4°C until used. Polyinosinic:polycytidylic acid (poly I:C; Sigma-Aldrich) was dissolved in PBS at a concentration of 1 mg/ml. The stock was filtered through a 0.45-μm sterile nylon mesh

(BD Biosciences), and was stored at −20°C until used. Pam$_3$-Cys-Ser-(Lys)$_4$ (Pam$_3$Cys) (EMC Microcollections) was reconstituted in PBS and was stored at -20°C until needed. TLR agonists were injected into the peritoneum (i.p.) of mice at the indicated ligand dose in a volume of 0.5 ml of PBS. Doses were determined to be active in previous publications [133,204]. Recombinant mouse IFN-β was obtained from PBL Interferon Source (Piscataway, NJ) and injected i.p. at the indicted dose on the day of DST and the first injection of anti-CD154 mAb.

Alloantibody Assay

The generation of donor-specific antibodies was determined by flow cytometry. Dilutions (undiluted, 1/10, 1/100) of mouse serum were incubated with BALB/c thymocytes for 20 minutes at 4°C. Cells were washed in PBS and incubated with FITC-conjugated polyclonal anti-mouse immunoglobulin (BD Pharmingen) for 20 minutes. The median fluorescence intensity (MFI) of the samples was determined by flow cytometry.

Intracellular IFN-γ Assay

IFN-γ production was assayed in spleen cells and circulating leukocytes as described previously [133]. Briefly, red blood cells from heparinized whole blood or single-cell spleen suspensions were lysed using 0.84% ammonium chloride. Cells were then incubated for 5 h in Golgiplug (BD Pharmingen) with 10U/ml rIL-2 (R&D Systems) and 1μg purified anti-CD28 mAb (BD Pharmingen) at 37°C in the presence of single-cell suspensions of irradiated, LPS-treated syngeneic (C57BL/6, *H-2^b*) or allogeneic (BALB/c, *H-2^d*) splenocytes (1x10^6 cells per stimulation). Samples were stained with

anti-H-2K^b-PE, anti-CD8α-Pacific Blue, anti-CD4-PerCp, and anti-CD11a-FITC, followed by fixation with BD Cytofix/Cytoperm and staining with anti-IFN-γ-APC.

In vivo Tracking of Donor Specific Transfusion

BALB/c spleens were harvested and prepared into single-cell suspension by mechanical disruption, washed two times in PBS and counted. Splenocytes were incubated at 37°C in 5μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Eugene, OR). After 15 min, the cells were washed three times in PBS and counted. The cells were then suspended in PBS at a concentration of $2x10^7$ cells/ml 0.5 ml was injected into the tail veins of recipients. Sixteen hours later, recipients were euthanized and the splenocytes were recovered. Single-cell suspensions were prepared by mechanical disruption, and the cells washed with PBS two times at room temperature. Samples were stained with LIVE/DEAD blue (Molecular Probes) for 20 min to visualize and exclude dead cells. Cells were then washed with PBS, containing 1.0% fetal clone serum, 0.1% sodium azide, before incubating with αCD16/CD32 and fluorescent antibodies as described above. A LSR II was used to analyze at least $1x10^6$ events.

Statistical Methods

Statistical analyses were made using Graphpad Prizm Software (Graphpad Software, Version 4.0, San Diego, CA). Comparisons of three or more means was performed via one-way analyses of variance (ANOVA), followed by Bonferroni's adjusted unpaired t-tests. Comparisons of two means used unpaired t-tests without assuming equal variance. Skin 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 were considered statistically significant.

CHAPTER III: TLR AGONISTS ACTIVATE ALLOREACTIVE T CELLS AND PREVENT THE ESTABLISHMENT OF HEMATOPOIETIC CHIMERISM AND SHORTEN ALLOGENEIC SKIN GRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE

Introduction:

Mixed hematopoietic chimerism has the potential to be used in lieu of immune-suppression for the induction of long-term survival of solid organ allografts. The current non-myeloablative conditioning regimens that are available for the establishment of mixed chimerism require significant depression of the host's immune system, which can put the recipient at risk of infection and neoplasia. Costimulation blockade-based regimens are an attractive alternative as they are minimally toxic. However, perturbations that activate Toll-like receptors (TLRs) have been shown to reduce the efficacy of costimulation blockade-based regimens in inducing tolerance to solid-organs. Therefore, environmental stressors such as infection and tissue ischemia that activate TLRs could reduce the clinical applicability of costimulation blockade-based protocols. However, the effects of TLR activation on the establishment of mixed chimerism using costimulation blockade-based protocols is not known.

We hypothesized that signaling through TLRs at the time of tolerance induction would prevent the establishment of mixed chimerism. We confirmed this hypothesis by treating animals with various TLR agonists at the start of costimulation blockade conditioning. Animals treated with agonists of TLR2, 3, and 4 at the start of the

costimulation blockade protocol failed to become chimeric and exhibited shortened skin allograft survival. Upon confirmation of this hypothesis, we investigated the cellular mechanisms governing this effect in the hope of establishing a conditioning regimen that is both safe and effective in a clinical setting.

Results:

1. TLR agonists administered at the time of DST and anti-CD154 mAb prevent the establishment of mixed hematopoietic chimerism and shorten skin allograft survival

We first tested the hypothesis that TLR agonists given during tolerance induction with donor-specific transfusion (DST) and anti-CD154 mAb would prevent the establishment of hematopoietic mixed chimerism. To test this, we treated recipient mice with our standard costimulation blockade-based conditioning regimen, with or without the addition of a TLR agonist on the first day of treatment (**Figure 4**). Our tolerance induction protocol consists of a donor specific transfusion of splenocytes on day −7, and four injections of anti-CD154 mAb on days −7, −4, 0, and +4 relative to injection with 50×10^6 donor-matched bone marrow cells and skin graft on day 0. We used two donor-recipient strain combinations: C57BL/6 recipients transplanted with BALB/c DST, bone marrow and skin, and the reverse, BALB/c recipients transplanted with C57BL/6 DST, bone marrow and skin. We observed that the majority of C57BL/6 mice (29/45) treated with BALB/c DST, anti-CD154 mAb and transplanted with BALB/c bone marrow and skin established low levels of stable allogeneic hematopoietic chimerism (1.59 ± 1.81% of the circulating peripheral blood mononuclear cells (PBMCs) detected at 8 weeks post bone marrow transplant were of donor origin; **Figure 5,** group 1). These mice also exhibited prolonged skin graft survival (MST = 144 days). However, subanalysis of the groups revealed that the mice that developed hematopoietic chimerism displayed permanent skin graft survival (MST > 260 days), while those that failed to develop mixed

chimerism exhibited shorter survival (MST = 88 days; p<0.001 vs. treated mice that developed chimerism; **Figure 5**).

Similarly, BALB/c mice treated with our standard conditioning regimen and transplanted with C57BL/6 bone marrow and skin established low levels of chimerism (4.3 ± 1.14% of the PBMCs detected at 8 weeks post BM transplant were of donor origin; **Figure 5,** group 5) and exhibited prolonged skin allograft survival (MST > 49 days; **Figure 5,** group 5). We also observed that chimeric mice developed B cells, T cells, and mononuclear phagocytes of donor origin in the blood, spleen, and bone marrow, indicating multi-lineage cell engraftment (**Table 2**).

In contrast, mice treated with a TLR agonist on the day of tolerance induction (day -7), universally failed to develop chimerism and exhibited shortened skin allograft survival. We observed that costimulation blockade-conditioned C57BL/6 mice transplanted with BALB/c bone marrow, skin, and treated with the TLR4 agonist LPS failed to become chimeric (0/23; p<0.001 vs. group 1), and rapidly rejected BALB/c skin grafts (MST = 11 days; p<0.001 vs. group 1; **Figure 5,** group 2). Demonstrating that this effect was not strain-specific, we observed that BALB/c recipients given LPS, C57BL/6 DST, anti-CD154 mAb and transplanted with C57BL/6 bone marrow and skin also did not become chimeric (0/6, p<0.01 vs. group 5) and displayed short skin allograft survival (MST = 14 days; p<0.001 vs. group 5; **Figure 5**, group 6).

We observed a similar phenotype with recipient mice conditioned with costimulation blockade in the presence of the TLR3 agonist poly I:C, or the TLR1/2 agonist Pam$_3$Cys.

None of the C57BL/6 (0/23, p<0.001 vs. group 1) or BALB/c (0/3, p<0.01 vs. group 5) recipients treated with our standard conditioning regimen and poly I:C on day -7 became chimeric when transplanted with allogeneic bone marrow (Figure 5). In addition, polyI:C-treated recipients exhibited short survival of donor-specific skin allografts (MST = 9 days for C57BL/6 recipients; p<0.001 vs. group 1; MST = 14 days for BALB/c recipients; p<0.001 vs. group 5). Similarly, C57BL/6 mice conditioned with costimulation blockade and given Pam$_3$Cys failed to become chimeric (0/3; p<0.05 vs. group 1), and skin graft survival was short (MST = 10 days; p<0.001 vs. group 1; **Figure 5,** group 4).

Importantly, transplantation of skin allografts was not required for TLR agonists to prevent bone marrow engraftment, as C57BL/6 mice conditioned with DST, anti-CD154 mAb and given BALB/c bone marrow (and no skin graft) became chimeric at 8 weeks (6/7), while those given DST, anti-CD154 mAb, LPS, and BALB/c bone marrow did not (0/7; p<0.01). These data indicate that TLR agonists prevent induction of mixed hematopoietic chimerism and shorten allograft survival in mice treated with DST and anti-CD154 mAb.

FIGURE 4: TRANSPLANTATION PROTOCOL
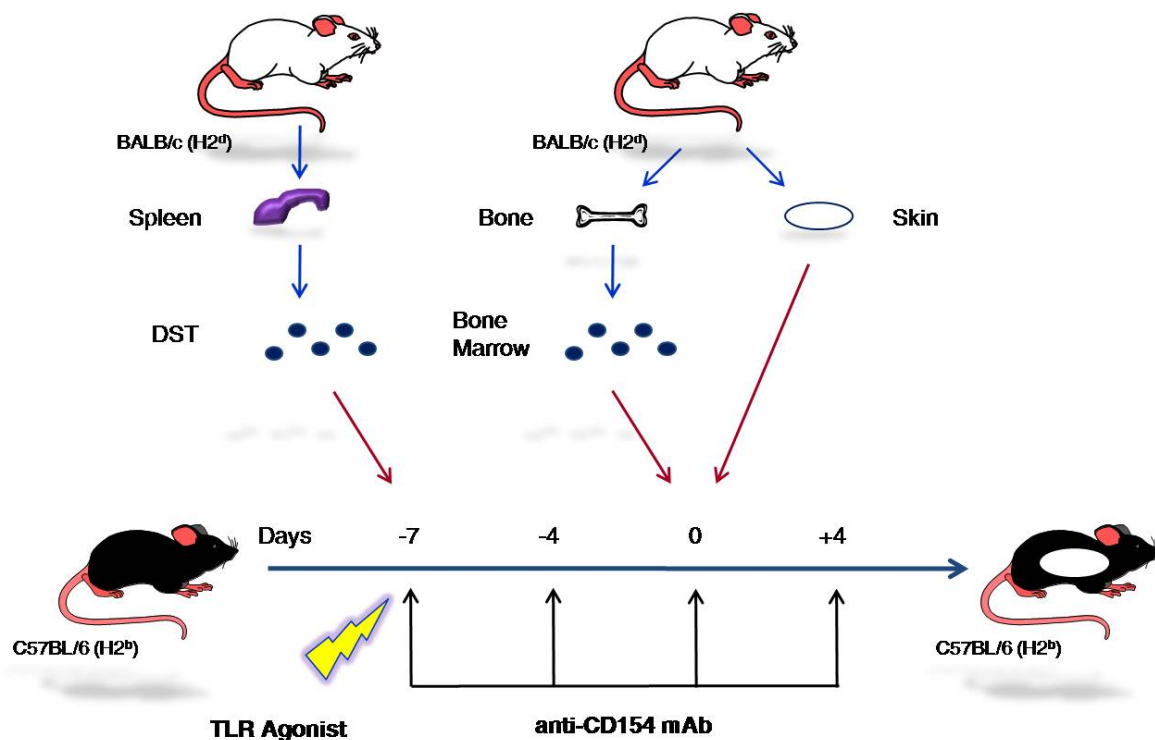
Figure 4. Recipient mice are treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 5×10^7 fully MHC-disparate allogeneic bone marrow cells and skin grafts. This protocol permits the establishment of mixed hematopoietic chimerism and induces permanent skin allograft survival in the majority of recipients. To investigate the effects of environmental perturbants, mice treated with the same conditioning protocol were also treated with an injection of 100ug LPS, 50ug poly I:C, or 100ug of Pam$_3$Cys on day -7, the day of injection of DST and anti-CD154 mAb.

FIGURE 5: TLR AGONISTS PREVENT ALLOGENEIC BONE MARROW ENGRAFTMENT AND SHORTEN ALLOGENEIC SKIN GRAFT SURVIVAL

A

Group	Recipient	Donor	TLR Agonist	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	C57BL/6	BALB/c	None	29/45 (64%)	1.59 ± 1.81	144	88	>260
2	C57BL/6	BALB/c	LPS	0/23 (0%)*	<0.10	11	11	N/A
3	C57BL/6	BALB/c	poly I:C	0/20 (0%)*	<0.10	9	9	N/A
4	C57BL/6	BALB/c	Pam3Cys	0/3 (0%)**	<0.10	10	10	N/A
5	BALB/c	C57BL/6	None	4/4 (100%)	4.3 ± 1.14	>49	N/A	>49
6	BALB/c	C57BL/6	LPS	0/6 (0%)#	<0.10	14	14	N/A
7	BALB/c	C57BL/6	poly I:C	0/3 (0%)#	<0.10	14	14	N/A

B

Donor – BALB/c   Recipient – C57BL/6

C

Donor – BALB/c   Recipient – C57BL/6

D

Donor – C57BL/6   Recipient – BALB/c
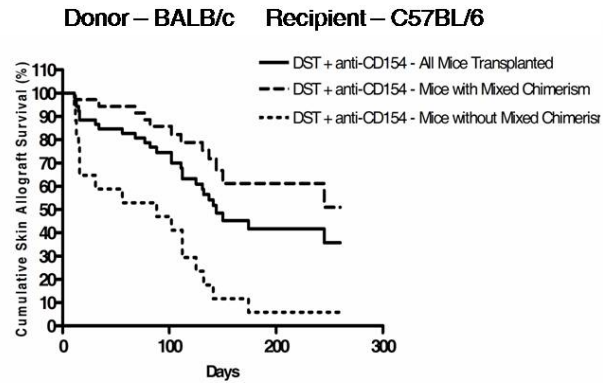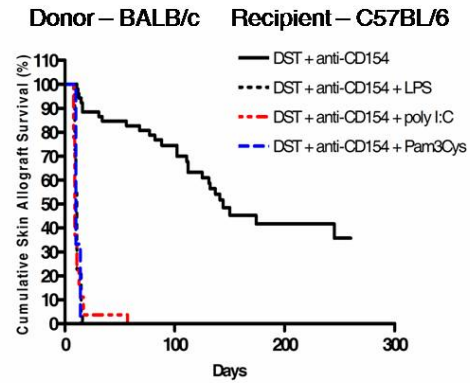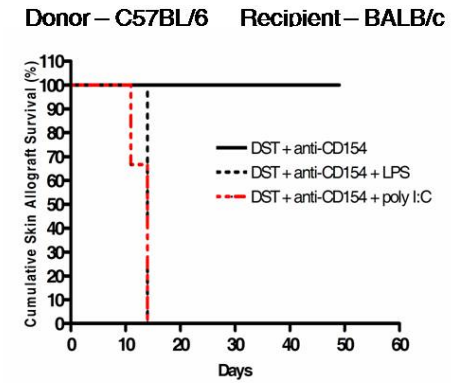
Figure 5. All mice were treated with a DST on day -7 and four injections of 0.5 mg of anti-CD154 mAb on days -7, -4, 0, and $+4$ relative to transplantation on day 0 with 50×10^6 donor-specific bone marrow cells and skin grafts. Mice treated with TLR agonists were given an i.p. injection of 50ug of poly I:C, 100ug LPS, or 100ug of Pam_3Cys on day -7 relative to bone marrow and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection as detected by flow cytometry. % donor-origin PBMCs is the mean $\pm$ S.D. of chimerism levels in chimeric mice. $^*p<0.0001$ vs. group 1; $^{**}p<0.05$ vs. group 1; $^{\#}p<0.01$ vs. group 5 by chi-square analysis. N/A – not applicable. The median survival time (MST) of transplanted skin allografts for each group is displayed in three columns. The column third from the right displays the overall MST of the entire group. The second column from the right is a subset of that group. It shows the MST of skin grafts on treated mice in the group that did not develop mixed chimerism. The column furthest to the right displays the MST of skin grafts on treated mice within the group that developed mixed chimerism. **Panel B:** Kaplan-Meier plots for allogeneic skin graft survival in mice treated with DST and anti-CD154 mAb and transplanted with donor bone marrow and skin (Group 1 from Panel A). The mice have been broken down into three groups to reflect the three columns in Panel A that show skin graft survival data: All of the mice treated (solid line); only treated mice that developed hematopoietic chimerism (dashed line); only treated mice that failed to become chimeric (dotted line). $p<0.001$ for treated mice that became chimeric vs. treated mice that failed to become chimeric. **Panel C-D**. Kaplan-Meier plots for allogeneic skin graft survival

in all mice transplanted with allogeneic bone marrow and skin (i.e. third column from the right in Panel A) is shown. P<0.001 for DST and anti-CD154 mAb vs. all other groups.

TABLE 2: MULTILINEAGE CHIMERISM IS ESTABLISHED IN MIXED CHIMERAS

Tissue	% Chimerism	%CD3+	%CD4+ T cells	%CD8+ T cells	%CD19	%CD11b+ Gr-1+	% CD11b- CD11c+
Blood	5.8 ± 2.6	62.2 ± 2.6	76.05 ± 3.49	20.95 ± 2.9	17.85 ± 7.7	0.067 ± 0.083	1.6 ± 1.8
Spleen	8.14 ± 1.02	56.16 ± 3.5	62.22 ± 1.32	34.45 ± 2.14	31.3 ± 2.6	2.98 ± 0.42	0.89 ± 0.12
Bone Marrow	7.02 ± 1.23	3.64 ± 0.59	0.3725 ± 0.46	0.8675 ± 1.39	23.27 ± 10.67	42.9 ± 8.0	3.2 ± 0.76

Table 2: BALB/c mice were treated with a C57BL/6 DST on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 50×10^6 C57BL/6 bone marrow cells and C57BL/6 skin grafts. At 8 weeks, blood, spleen, and bone marrow were recovered from mice bearing intact skin grafts and analyzed by flow cytometry for percent donor hematopoietic chimerism and the presence of multilineage donor hematopoietic cells. The percentages of $CD3^+$, $CD19^+$, $CD11b^+/Gr-1^+$, $CD11b^-/CD11c^+$ cells represents their proportion of donor ($H-2K^b$) cells that express these phenotypes. The percentages of $CD4^+$ and $CD8^+$ cells represent the proportion of $CD3^+$ cells that express those phenotypes. Data represent mean ± SD of four mice.

2. Alloantibodies are not produced following the administration of LPS during costimulation blockade

Given that B cells express TLRs [124], and anti-alloantibodies are known to induce allograft rejection [219], one mechanism by which TLR activation could prevent mixed chimerism induction is through the stimulation of B cells to produce alloantibodies. To investigate this hypothesis, we looked for anti-donor alloantibodies in the serum of C57BL/6 mice that were: 1) left untreated, 2) primed with BALB/c DST, or 3) given BALB/c DST, anti-CD154 mAb, and grafted with BALB/c bone marrow with or without LPS treatment. As shown in **Figure 6**, completely untreated (naïve) mice had background levels of serum alloantibodies (median fluorescence intensity (MFI) = 4.81 ± 0.87, n=4), whereas mice primed with BALB/c DST developed high levels of alloantibodies (MFI = 45.63 ± 5.6; n=4; $p < 0.001$ vs. untreated; **Figure 6**). In contrast, mice treated with anti-CD154 mAb at the time of DST injection developed only background to very low levels of alloantibodies (MFI = 5.18 ± 0.68; n=6; p=NS vs. untreated, $p < 0.001$ vs. DST primed), even in LPS-treated mice that had rejected BALB/c bone marrow (MFI = 8.59 ± 6.59; n=5; p=NS vs. untreated or DST and anti-CD154 mAb treated, $p < 0.001$ vs. DST primed; **Figure 6**). These data show that: 1) anti-CD154 mAb treatment prevents the generation of donor-specific alloantibodies, and 2) the ability of LPS to prevent the establishment of costimulation blockade-mediated hematopoietic mixed chimerism is not due to the production of alloantibodies.

FIGURE 6: LPS ADMINISTRATION DURING COSTIMULATION BLOCKADE DOES NOT INDUCE ALLOANTIBODY PRODUCTION
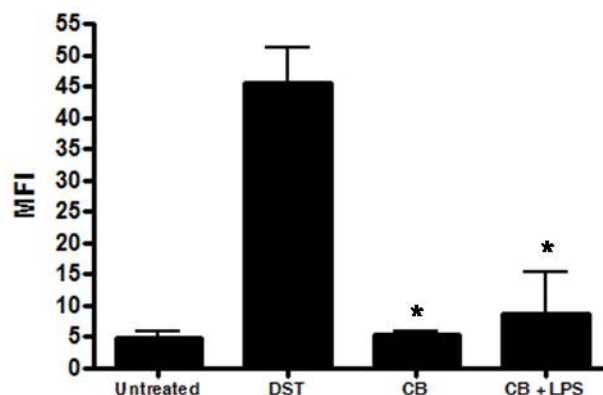
Figure 6. C57BL/6 mice were treated with BALB/c DST and anti-CD154 mAb according to our standard protocol (DST and anti-CD154 mAb without or with co-injection of 100ug LPS on day -7 relative to transplantation of BALB/c bone marrow on day 0. All mice were bled two weeks after transplantation. Serum was analyzed for alloantibody content by flow cytometry. Serum was also taken from untreated mice and mice primed with a single injection of BALB/c splenocytes (DST) to serve as negative and positive controls, respectively for the alloantibody assay. Data are presented as median fluorescence intensity (MFI) plus one standard deviation. Data are pooled from two independent experiment. *$p<0.001$ vs. splenocyte (DST) injected only group; p=NS vs. untreated group; p=NS for DST and anti-CD154 mAb vs. DST, anti-CD154 mAb and LPS.

3. Host NK cells are not required for TLR-dependent prevention of the establishment of mixed chimerism and shortened skin allograft survival in mice treated with costimulation blockade

Based on the absence of alloantibodies in mice treated with DST, anti-CD154 mAb, and LPS, we next investigated the cell populations that are responsible for the TLR-mediated effects. NK cells are known to be a potent barrier to the establishment of hematopoietic chimerism [220-222], and have been shown to express TLRs [223]. Therefore, we postulated that TLR activation at the initiation of costimulation blockade would stimulate host NK cells to become an even more potent barrier to allogeneic bone marrow engraftment. To test this, we depleted C57BL/6 mice of NK cells using anti-NK1.1 mAb, and treated them with our standard costimulation blockade conditioning protocol with or without co-injection of LPS or poly I:C at the time of DST. To confirm depletion of host NK cells, treated mice were bled the day before transplantation, and circulating NK cells were assessed by flow cytometry using antibodies directed against the DX5 and Ly49D receptors. We observed that greater than 90% of host NK cells were depleted following antibody treatment.

In the absence of TLR agonists, the majority of NK-cell depleted mice became chimeric (11/13), circulating low levels of donor-derived leukocytes (2.45 ± 1.16% at 8 weeks post transplant; **Figure 7,** group 4). The mice that became chimeric exhibited permanent skin allograft survival (MST > 221 days; **Figure 7**). The two mice that did not become chimeric exhibited prolonged skin graft survival (MST = 72 days). In contrast,

mice depleted of NK cells and treated with LPS or poly I:C on day -7 failed to become chimeric (0/5 and 0/12, respectively; $p<0.001$ vs. group 4) and both groups exhibited shortened skin allograft survival (MST = 12 days for both groups; p=NS vs. group 2 and 3, respectively; **Figure 7**). These data indicate that host NK cells are not required for LPS or polyI:C to prevent the establishment of mixed hematopoietic chimerism and shorten skin allograft survival in mice treated with costimulation blockade.

FIGURE 7: LPS AND POLY I:C EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE ARE NOT MEDIATED BY NATURAL KILLER CELLS

A

Group	Recipient	Donor	TLR Agonist	NK Depletion	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	C57BL/6	BALB/c	None	No	29/45 (64.0%)	1.59 ± 1.81	144	88	>260
2	C57BL/6	BALB/c	LPS	No	0/23 (0.00%)*	<0.10	11	11	N/A
3	C57BL/6	BALB/c	poly I:C	No	0/20 (0.00%)*	<0.10	9	9	N/A
4	C57BL/6	BALB/c	None	Yes	11/13 (84.6%)	2.45 ± 1.16	>221	72	>221
5	C57BL/6	BALB/c	LPS	Yes	0/5 (0.00%)**	<0.10	12	12	N/A
6	C57BL/6	BALB/c	poly I:C	Yes	0/12 (0.00%)**	<0.10	12	12	N/A

B
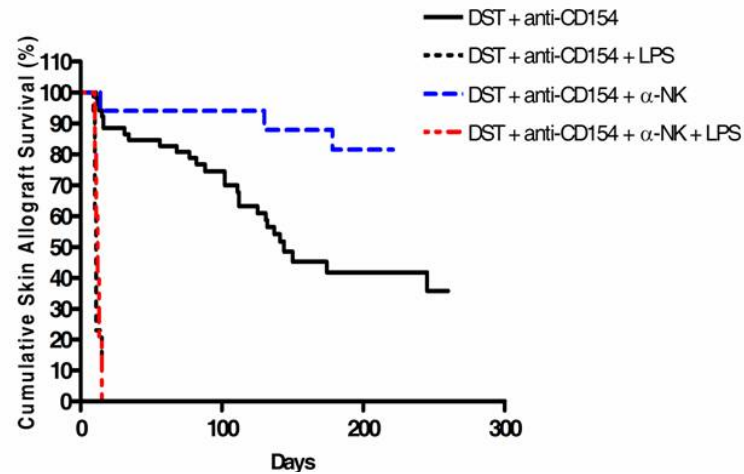
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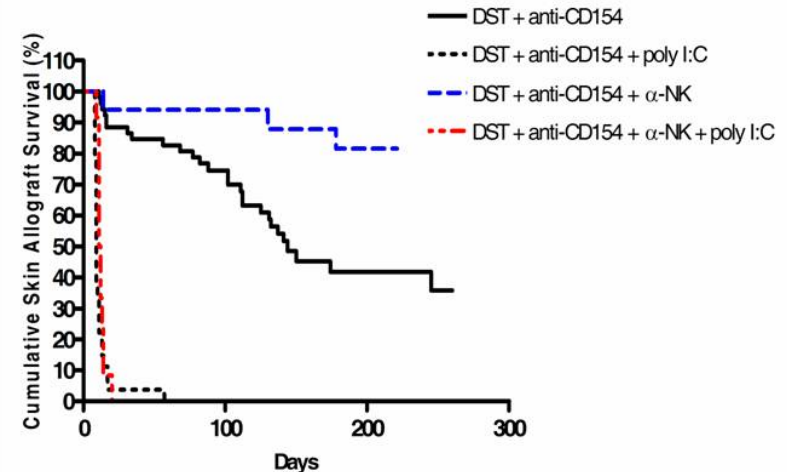
Figure 7. All mice were treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with $50x10^6$ allogeneic bone marrow cells and allogeneic skin grafts. Mice in groups 4-6 were given a 25μg injection of anti-NK1.1 mAb i.p on day −10 relative to bone marrow and skin transplantation. Mice treated with TLR agonist were given either an i.p. injection of 50ug of poly I:C, or 100ug LPS on day -7 relative to bone marrow and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. [*]p<0.0001 vs. group 1; [**]p<0.001 vs. group 4 by chi-square analysis. N/A − not applicable. Skin survival data have been separated into three groups to distinguish chimeric mice from non-chimeric mice. Data from groups 1-3 shown in Figure 5 are reproduced here for ease of comparison with other treatment groups. **Panel B-C**. Kaplan-Meier plots for allogeneic skin graft survival in all mice transplanted with allogeneic bone marrow and skin is shown (third column from the right in Panel A). p<0.01 for DST and anti-CD154 mAb vs. DST, anti-CD154 and anti-NK1.1 mAbs; p=NS for DST, anti-CD154 mAb and LPS vs. DST, anti-CD154, anti-NK1.1 mAbs and LPS; p=NS for DST, anti-CD154 mAb and poly I:C vs. DST, anti-CD154, anti-NK1.1 mAbs and poly I:C.

4. Host CD8$^+$ cells are not required for TLR-dependent prevention of the establishment of mixed chimerism and shortened skin allograft survival in mice treated with costimulation blockade

Previously, our laboratory has shown that TLR-activation can impair the deletion of alloreactive CD8$^+$ T cells in mice treated with DST and anti-CD154 mAb, and that host CD8$^+$ T cells are required for LPS to shorten skin allograft survival in these mice [133]. To determine if CD8$^+$ T cells are also required for the ability of the TLR agonists LPS and poly I:C to prevent the establishment of hematopoietic chimerism, C57BL/6.CD8$\alpha^{-/-}$ mice were treated with our standard conditioning regimen with or without an injection of LPS or poly I:C at the time of DST. As expected, hematopoietic chimerism developed in the majority of C57BL/6.CD8$\alpha^{-/-}$ mice treated with DST and anti-CD154 mAb (12/17; 2.29 ± 1.76% donor PBMCs at 8 weeks post transplant; **Figure 8**, group 1). Skin graft survival was prolonged in all C57BL/6.CD8$\alpha^{-/-}$ mice treated with costimulation blockade (MST = 96 days). However, skin allograft survival was longer in mice that developed mixed chimerism (MST = 136 days) compared to mice that failed to become chimeric (MST = 51 days). Surprisingly, LPS and poly I:C were able to prevent the establishment of chimerism in C57BL/6.CD8$\alpha^{-/-}$ mice treated with DST and anti-CD154 mAb (0/17 and 0/10, respectively; p<0.001 vs. group 1; **Figure 8**, groups 2 and 3). Similarly, skin graft survival was shortened in C57BL/6.CD8$\alpha^{-/-}$ mice treated with LPS or poly I:C (MST = 21 days and 14 days, respectively; p<0.001 vs. group 1).

Given this surprising result, we further tested the role of CD8$^+$ cells using wild-type

C57BL/6 mice depleted of CD8$^+$ cells using an anti-CD8α mAb. C57BL/6 mice were depleted of CD8$^+$ cells and treated with our standard conditioning regimen with or without LPS or poly I:C at the time of DST injection. To confirm depletion of host CD8$^+$ cells, treated mice were bled the day before transplantation, and circulating CD8$^+$ cells were assessed by flow cytometry using an antibody directed against CD8β. We observed that greater than 95% of host CD8$^+$ cells were depleted following antibody treatment. The majority of C57BL/6 mice depleted of CD8$^+$ cells became chimeric (15/17) (0.90 ± 1.13% donor PBMCs at 8 weeks post transplant) and exhibited prolonged skin allograft survival (MST = 144 days**; Figure 8**, group 7). Again, mice depleted of CD8$^+$ cells and treated with LPS or poly I:C on day −7 failed to develop hematopoietic chimerism (0/16 and 0/19, respectively; p<0.001 vs. group 7; **Figure 8**, groups 8 and 9). Skin allograft survival was also shortened in mice treated with LPS and poly I:C and depleted of CD8$^+$ cells compared to CD8-depleted mice not given a TLR agonist (MST = 44 days and 56 days, respectively; p<0.001**; Figure 8,** group 8-9). However, skin graft survival was significantly longer in TLR agonist-treated mice depleted of CD8$^+$ cells compared to mice treated with LPS or poly I:C that had not been depleted of CD8$^+$ cells (p<0.001 for group 8 vs. group 5 and for group 9 vs. group 6). These data indicate that host CD8$^+$ cells are not required for LPS to prevent the establishment of hematopoietic chimerism in mice treated with costimulation blockade. However, CD8$^+$ cells do appear to be important in the TLR-mediated rejection of skin allografts.

FIGURE 8: LPS AND POLY I:C EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE ARE NOT MEDIATED BY CD8^{+} CELLS

A

| Group	Recipient	Donor	TLR Agonist	CD8 Depletion	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	B6.*CD8α*^{-/-}	BALB/c	None	No	12/17 (70.5%)	2.29 ± 1.76	96	51	136
2	B6.*CD8α*^{-/-}	BALB/c	LPS	No	0/17 (0.00%)*	<0.10	21	21	N/A
3	B6.*CD8α*^{-/-}	BALB/c	poly I:C	No	0/10 (0.00%)*	<0.10	14	14	N/A
4	C57BL/6	BALB/c	None	No	29/45 (64.0%)	1.59 ± 1.81	144	88	>260
5	C57BL/6	BALB/c	LPS	No	0/23 (0.00%)**	<0.10	11	11	N/A
6	C57BL/6	BALB/c	poly I:C	No	0/20 (0.00%)**	<0.10	9	9	N/A
7	C57BL/6	BALB/c	None	Yes	15/17 (88.2%)	0.90 ± 1.13	144	72.5	148
8	C57BL/6	BALB/c	LPS	Yes	0/16 (0.00%)#	<0.10	44	44	N/A
9	C57BL/6	BALB/c	poly I:C	Yes	0/19 (0.00%)#	<0.10	56	56	N/A

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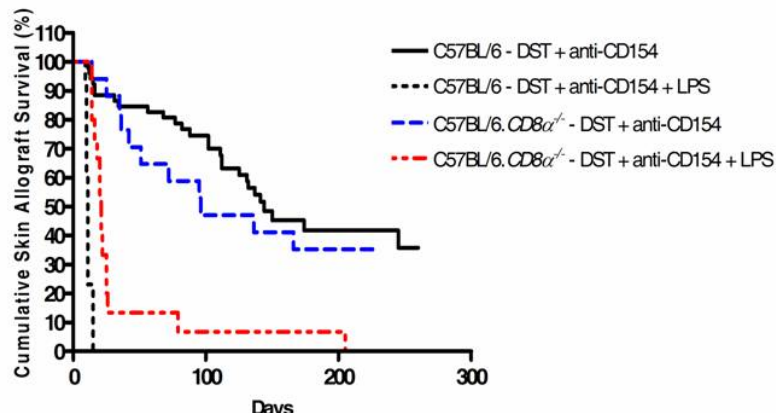
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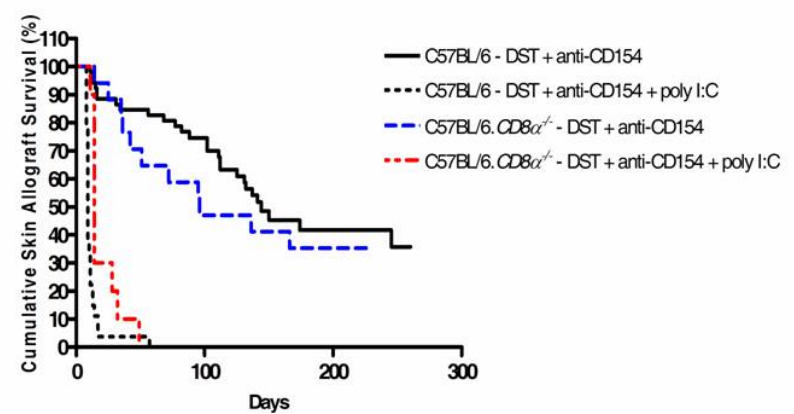
Figure 8. All mice were treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 50×10^6 allogeneic bone marrow cells and allogeneic skin grafts. Mice in groups 7-9 were given three doses of 0.5mg anti-CD8α mAb i.p. on days −10, −9, and −8 relative to bone marrow and skin transplantation. Mice treated with TLR agonist were given an i.p. injection of 50ug of poly I:C, or 100ug LPS on day -7 relative to bone marrow and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin PBMCs 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.001 vs. group 1; **p<0.001 vs. group 4; $^\#$p<0.001 vs. group 7 by chi-square analysis. N/A – not applicable. Skin survival data have been separated into three groups to distinguish chimeric mice from non-chimeric mice in regards to skin MST. Data from groups 1-3 shown in Figure 5 are reproduced here (as groups 4-6) for ease of comparison with other treatment groups. **Panel B-E**. Kaplan-Meier plots for allogeneic skin graft survival for the entire cohort of mice conditioned and transplanted with bone marrow and skin is shown (third column from the right in Panel A). Data for skin graft survival in wild-type mice shown in Figure 5 is shown here for ease of comparison. **Panel B:** p=NS for DST and anti-CD154 mAb in C57BL/6 vs. DST and anti-CD154 mAb in C57BL/6.CD8α$^{-/-}$; p<0.001 for DST, anti-CD154 mAb, and LPS in C57BL/6 vs. DST, anti-CD154 mAb, and LPS in C57BL/6.CD8α$^{-/-}$; **Panel C:** p=NS for DST, anti-CD154 mAb, and poly I:C in C57BL/6 vs. DST, anti-CD154 mAb, and poly I:C in C57BL/6.CD8α$^{-/-}$. **Panel D:** P=NS for DST

and anti-CD154 mAb vs. DST, anti-CD154 and anti-CD8 mAbs; $p<0.001$ for DST, anti-CD154 mAb, and LPS vs. DST, anti-CD154 and anti-CD8 mAbs and LPS; **Panel E:** $p<0.001$ for DST, anti-CD154 mAb and poly I:C vs. DST, anti-CD154 and anti-CD8 mAbs and poly I:C.

5. Host CD8^{+} and NK cells are not required for TLR-dependent prevention of the establishment of mixed chimerism and shortened skin allograft survival in mice treated with costimulation blockade

The fact that mixed chimerism was not established in mice treated with costimulation blockade and TLR agonists in the absence of either NK or CD8^{+} cells does not exclude the possibility that either population by itself could mediate these effects . To test this, mice were depleted of both NK and CD8^{+} cells and treated with DST and anti-CD154 mAb with or without LPS and poly I:C at the time of DST injection. We confirmed depletion of both populations using flow cytometry as described before. In the absence of TLR agonist treatment, most mice developed hematopoietic chimerism (7/8) and exhibited higher levels of circulating donor-origin leukocytes (3.67 ± 1.23% donor PBMCs at 8 weeks post transplant) as compared to mice not depleted of NK and CD8^{+} cells; (1.59 ± 1.81%, p<0.01; **Figure 9**, group 4). These mice also displayed prolonged skin graft survival (MST = 81 days; **Figure 9**, group 4). In contrast, co-injection of LPS at the time of DST prevented the establishment of mixed chimerism (0/9; p<0.001 vs. group 4) and shortened skin allograft survival (MST = 28 days; p<0.001 vs. group 2; p<0.01 vs. group 4; **Figure 9**, group 5). Simultaneous injection of poly I:C at the time of DST similarly prevented mixed chimerism (0/14; p<0.001 vs. group 4) and shortened skin allograft survival (MST = 21 days; p<0.001 vs. group 3 and 4; **Figure 9**, group 6). Again, as seen with depletion of only CD8^{+} cells, skin allograft survival in the presence on TLR agonists was slightly prolonged in mice depleted of both NK and CD8^{+} cells as

compared to mice treated with LPS or poly I:C, but not depleted of NK and CD8$^+$ cells. Taken together, these data indicate that host NK cells and CD8$^+$ cells are not required for either LPS or poly I:C to prevent the establishment of hematopoietic chimerism, but CD8$^+$ cells likely play a role in the rapid rejection of skin allografts in the setting of TLR activation.

FIGURE 9: LPS AND POLY I:C EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE ARE NOT MEDIATED BY CD8^{+} CELLS OR NATURAL KILLER CELLS

A

Group	TLR Agonist	NK Depletion	CD8 Depletion	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	None	No	No	29/45 (64.0%)	1.59 ± 1.81	144	88	>260
2	LPS	No	No	0/23 (0.00%)*	<0.10	11	11	N/A
3	poly I:C	No	No	0/20 (0.00%)*	<0.10	9	9	N/A
4	None	Yes	Yes	7/8 (87.5%)	3.67 ± 1.23[#]	81	23	81
5	LPS	Yes	Yes	0/9 (0.00%)**	<0.10	28	28	N/A
6	poly I:C	Yes	Yes	0/14 (0.00%)**	<0.10	21	21	N/A

B
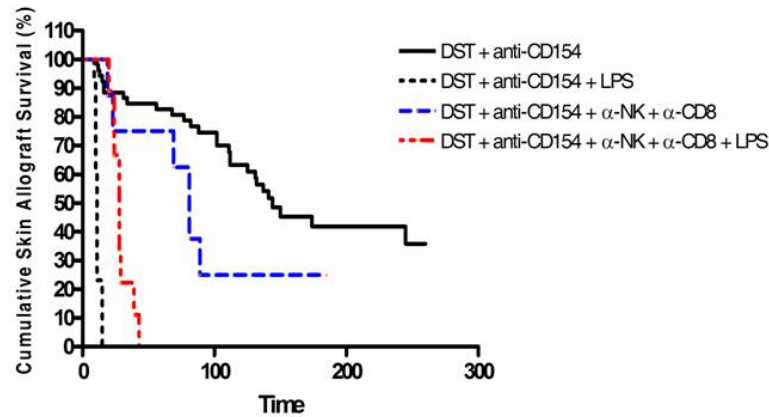
C

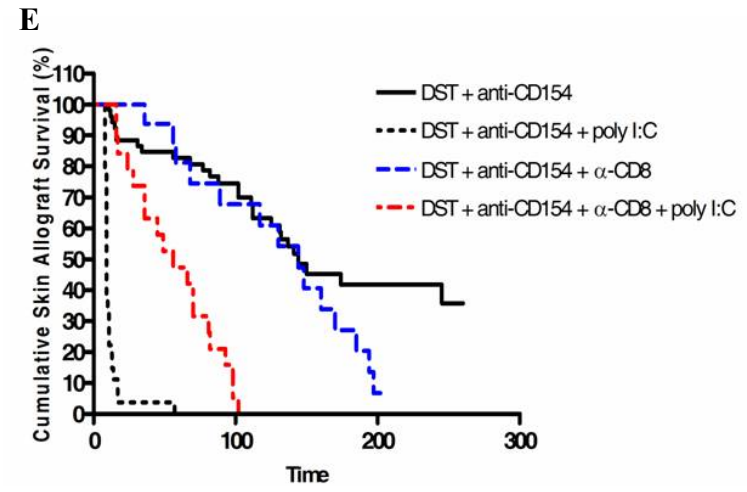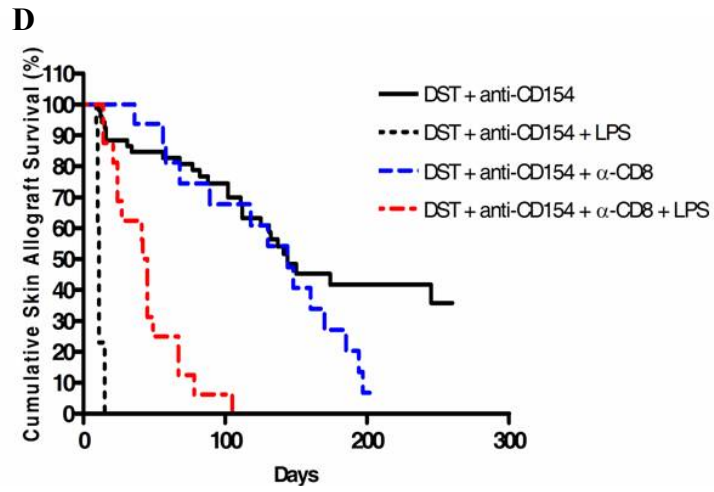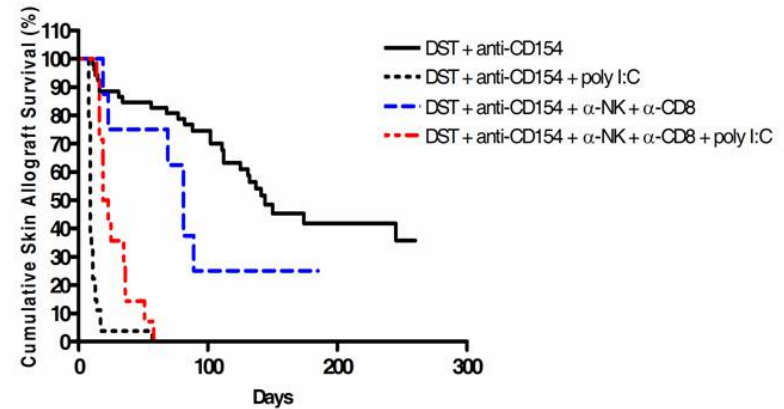**Figure 9.** C57BL/6 mice were treated with a BALB/c donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with $50x10^6$ BALB/c bone marrow cells and BALB/c skin grafts. Mice in groups 4-6 were given an injection of anti-NK1.1 mAb i.p on day -10, and three doses of 0.5mg anti-CD8 mAb i.p. on days -10, -9, and -8 relative to bone marrow and skin transplantation. Mice treated with TLR agonist were given either an i.p. injection of 50ug of poly I:C, or 100ug LPS on day -7 relative to bone marrow and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.0001 vs. group 1; **p<0.001 vs. group 4 by chi-square analysis. #p<0.01 vs. group 1 by unpaired t-test. N/A − not applicable. Skin allograft survival data have been separated into three groups to distinguish chimeric mice from non-chimeric mice. Data from groups 1-3 shown in Figure 5 are reproduced here for ease of comparison with other treatment groups. **Panel B-C.** Kaplan-Meier plots for allogeneic skin graft survival for all mice transplanted with bone marrow and skin is shown (third column from the right in Panel A). Data for skin graft survival in wild-type mice shown in Figure 5 is shown here for ease of comparison. **Panel B**: p=NS for DST and anti-CD154 mAb vs. DST, anti-CD154, anti-NK1.1 and anti-CD8 mAbs; p<0.001 for DST, anti-CD154 mAb, and LPS vs. DST, anti-CD154, anti-NK1.1, anti-CD8 mAbs, and LPS; p<0.001 for DST, anti-CD154 mAb, and LPS vs. DST, anti-CD154, anti-NK1.1, and anti-CD8 mAbs; **Panel C**: p< 0.001 for

DST, anti-CD154 mAb and poly I:C vs. DST, anti-CD154, anti-NK1.1, anti-CD8 mAbs and poly I:C; $p<0.001$ for DST, anti-CD154 mAb, anti-NK1.1, and anti-CD8 mAbs and poly I:C vs. DST, anti-CD154, anti-NK1.1, and anti-CD8 mAbs.

6. TLR4 activation prevents the induction of tolerance in both the CD4$^+$ and CD8$^+$ T cell compartment in mice treated with costimulation blockade

We next wanted to determine if LPS-mediated rejection of BALB/c allografts was dependent on host CD4$^+$ T cells. However, we have previously shown using BALB/c mice as recipients of C57BL/6 bone marrow that CD4$^+$ T cells are required for the establishment of mixed chimerism in mice treated with DST and anti-CD154 mAb, presumably because they are required for tolerizing the alloreactive CD8$^+$ pool [90]. To determine if CD4$^+$ cells were also required for the establishment of mixed chimerism in the reciprocal strain combination (C57BL/6 mice as recipients, and BALB/c mice as donors), mice were depleted of CD4$^+$ cells using the GK1.5 mAb. Consistent with our previously published results, most mice depleted of CD4$^+$ cells did not become chimeric (1/12; p<0.001 vs. group 1) and exhibited short skin graft survival (MST = 46 days; p<0.001 vs. group 1**; Figure 10**, group 3). Not surprisingly, all mice depleted of CD4$^+$ cells and treated with DST, anti-CD154 mAb, and LPS also failed to become chimeric (0/12) and displayed short skin survival (MST = 18 days; p<0.01 vs. group 2; **Figure 10**, group 4). Interestingly, although the skin graft survival exhibited by these mice was short, it was longer than mice treated with DST, anti-CD154 mAb and LPS but not depleted of CD4$^+$ cells (p<0.01 vs. group 3). These data indicate that CD4$^+$ cells are important for both chimerism induction and skin graft survival. It is also likely, given the slight prolongation in skin survival time of mice depleted of CD4$^+$ cells and treated with LPS compared to mice treated with LPS but not depleted of CD4$^+$ cells, that CD4$^+$ cells also

participate in the acute rejection of skin allografts after TLR activation.

Therefore, because mixed chimerism could not be established in recipients treated with TLR agonists in the absence of either CD4$^+$ or CD8$^+$ cells, we hypothesized that TLR activation broke tolerance in both compartments and, consequently, both subsets would have to be depleted in order to establish mixed chimerism in mice treated with costimulation blockade and LPS. To test this hypothesis, we depleted C57BL/6 mice of both CD4$^+$ and CD8$^+$ cells and then treated the mice with our standard costimulation blockade protocol with or without co-injection of LPS. CD4 and CD8-depleted mice developed low levels of hematopoietic chimerism (4/4; 2.42 $\pm$ 0.55% donor-origin PBMCs at 8 weeks post transplant) and exhibited permanent skin graft survival (MST >228 days**; Figure 10**, group 5). In support of our hypothesis, low levels of hematopoietic chimerism (1.14 $\pm$ 0.35% donor-origin PBMCs at 8 weeks post transplant) was observed in all CD4 and CD8-depleted mice given LPS at the time of DST (4/4**; Figure 10**, group 6). Interestingly, the level of chimerism in CD4 and CD8-depleted mice given LPS was significantly lower than CD4 and CD8-depleted mice not given LPS ($p<0.05$). Nevertheless, mice depleted of CD4$^+$ and CD8$^+$ cells and given LPS at the time of DST exhibited prolonged skin allograft survival (MST = 228). These data suggest that TLR4 activation at the time of tolerance induction prevents mixed chimerism by modulating both alloreactive helper and cytotoxic T cells.

Importantly, mice depleted of CD4$^+$ and CD8$^+$ cells but not conditioned with DST and anti-CD154 mAb did not become chimeric (0/4) and displayed short skin graft

survival (MST = 34 days) when transplanted with BALB/c bone marrow and skin (**Figure 10**, group 7). This indicates that costimulation blockade plays an important role in the mixed chimerism induction protocol even when $CD4^+$ and $CD8^+$ T cells are absent.

FIGURE 10: LPS EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE IS MEDIATED BY BOTH CD8$^+$ AND CD4$^+$ CELLS

A

Group	TLR Agonist	CD4 Depletion	CD8 Depletion	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	None	No	No	29/45 (64.0%)	1.59 ± 1.81	144	88	>260
2	LPS	No	No	0/23 (0.00%)*	<0.10	11	11	N/A
3	None	Yes	No	1/12 (8.33%)*	0.58	46	37	189
4	LPS	Yes	No	0/12 (0.00%)	<0.10	18	18	N/A
5	None	Yes	Yes	4/4 (100%)	2.42 ± 0.55	>228	N/A	>228
6	LPS	Yes	Yes	4/4 (100%)	1.14 ± 0.35 ##	228	N/A	228
7	None	Yes	Yes	0/4 (0.00%)**	<0.10	34	34	N/A
8	LPS	Yes	Yes	0/3 (0.00%)#	<0.10	32	32	N/A

B
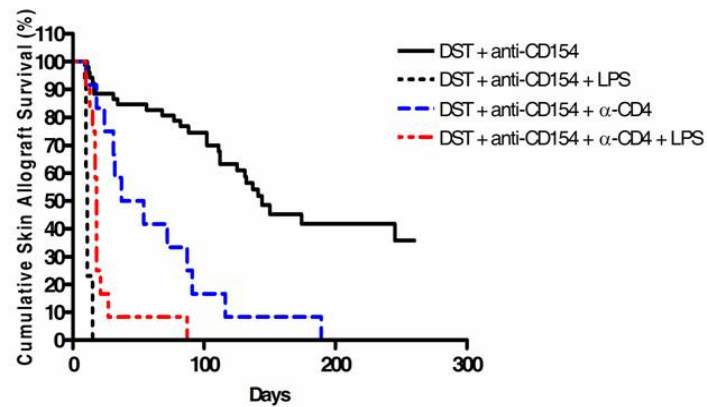
C
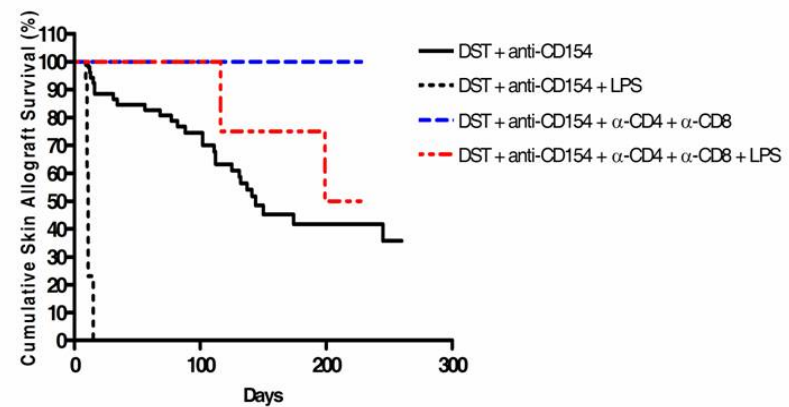
Figure 10. C57BL/6 mice were treated with a BALB/c donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 50×10^6 BALB/c bone marrow cells and BALB/c skin grafts. Mice in groups 7-8 were transplanted with BALB/c bone marrow and skin according to the standard protocol, but were not conditioned with DST and anti-CD154 mAb. Mice in groups 3-8 were given three doses of 0.5mg anti-CD4 mAb i.p. on days −10, −9, and −8 relative to bone marrow and skin transplantation. Mice in groups 5-8 were given three doses of 0.5mg anti-CD8 mAb i.p. on days −10, −9, and −8 relative to bone marrow and skin transplantation. Mice treated with a TLR4 agonist were given an i.p. injection of 100ug LPS on day -7 relative to bone marrow and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. [*]$p<0.001$ vs. group 1; [**]$p<0.01$ vs. group 5; [#]$p<0.01$ vs. group 6; [##]$p<0.05$ vs. group 5 by chi-square analysis. N/A − not applicable. Skin survival data have been separated into three groups to distinguish chimeric mice from non-chimeric mice. Data from groups 1-2 shown in Figure 5 are reproduced here for ease of comparison with other treatment groups. **Panel B-C**. Kaplan-Meier plots for allogeneic skin graft survival is shown (third column from the right in Panel A). **Panel B.** $p<0.001$ for DST, anti-CD154 mAb vs. DST, anti-CD154 and anti-CD4 mAbs; $p<0.001$ for DST, anti-CD154, and LPS vs. DST, anti-CD154, anti-CD4 mAbs and LPS. **Panel C**. p=NS for DST and anti-CD154 mAb vs. DST, anti-

CD154, anti-CD8, and anti-CD4 mAbs; p<0.001 for DST, anti-CD154 mAb and LPS vs.
DST, anti-CD154, anti-CD8, anti-CD4 mAbs and LPS; p=NS for DST, anti-CD154, anti-
CD8 and anti-CD4 mAbs vs. DST, anti-CD154, anti-CD8, anti-CD4 mAbs and LPS.

7. *LPS administration leads to the generation of effector/memory alloreactive CD8⁺ T cells in mice treated with costimulation blockade*

Given that the removal of host CD8$^+$ T cells was required for the establishment of chimerism, we next investigated whether alloreactive effector/memory CD8$^+$ T cells were generated in mice treated with LPS, costimulation blockade, and transplanted with allogeneic bone marrow. To investigate this, we performed *ex vivo* intracellular staining for IFN-γ on splenocytes isolated from C57BL/6 mice treated with our standard conditioning regimen and transplanted with BALB/c bone marrow and skin with or without LPS treatment. In this assay, recovered splenocytes are incubated with either syngeneic or donor-specific stimulator cells, and the production of IFN-γ by CD8$^+$CD11a^{high} T cells is a marker of an effector/memory phenotype [224]. We observed that splenocytes isolated from control mice exhibited essentially background levels of BALB/c-reactive IFN-γ-producing CD8$^+$CD11a^{high} T cells two weeks after bone marrow and skin transplantation. In contrast, mice given a LPS injection at the time of DST developed high levels of IFN-γ-producing CD8$^+$CD11a^{high} T cells following stimulation with BALB/c splenocytes **(Figure 11)**. These data indicate that in contrast to transplanted mice treated with only DST and anti-CD154 mAb, mice given costimulation blockade and LPS generate donor-specific effector/memory CD8$^+$ T cells.

FIGURE 11: LPS TREATMENT INDUCES THE GENERATION OF EFFECTOR/MEMORY ALLOREACTIVE CD8^{+} T CELLS

A

B
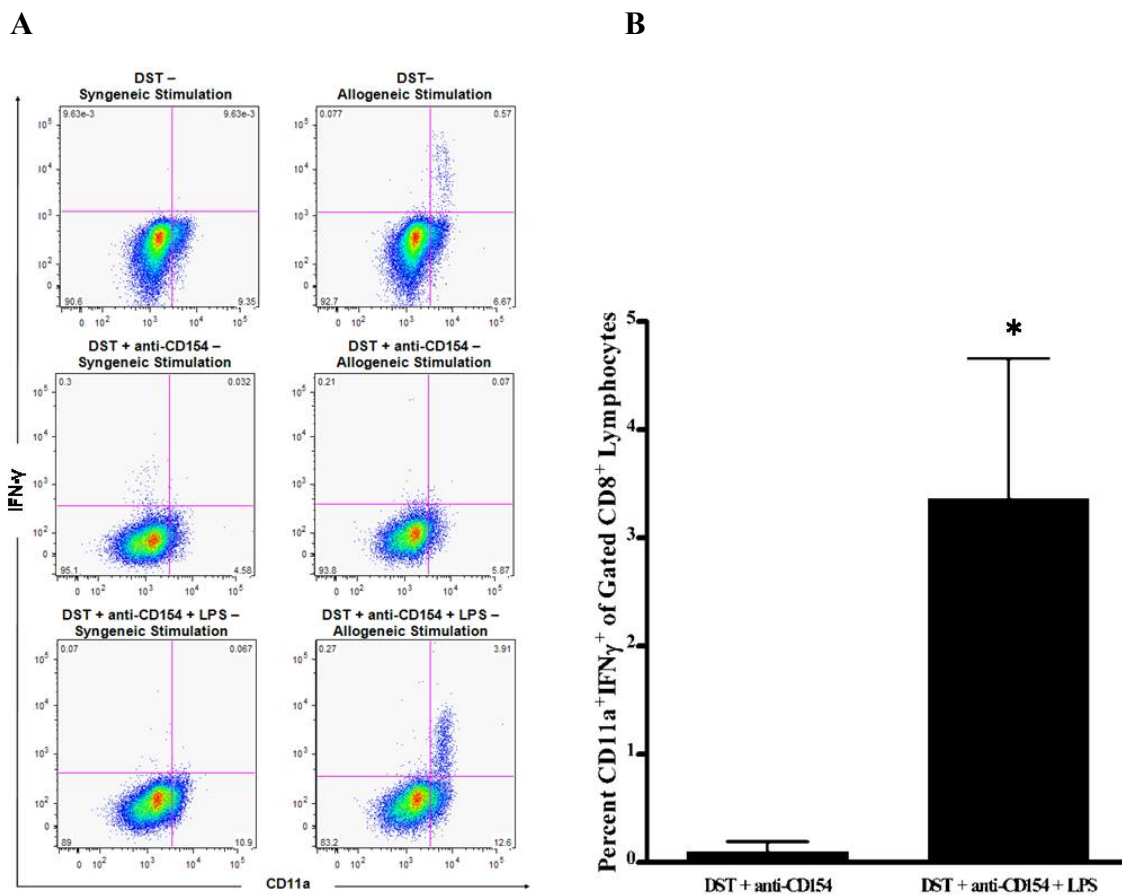
Figure 11. C57BL/6 mice were treated with BALB/c DST and anti-CD154 mAb according to our standard protocol with or without co-injection of 100μg LPS on day −7 relative to transplantation of BALB/c bone marrow and skin on day 0. All mice were bled two weeks after transplantation. **Panels A-B**: Peripheral blood cells were recovered two weeks after transplantation and stimulated *in vitro* for 5 hr with either irradiated syngeneic (H-2^b) or allogeneic BALB/c (H-2^d) splenocytes and their production of IFNγ was quantified by flow cytometry. **Panel A:** Representative flow dot plots showing CD11a and IFNγ expression in gated CD8$^+$ lymphocytes. As a positive control, C57BL/6 mice were primed to H-2^d by injection of BALB/c splenocytes (DST) seven days prior to blood cell recovery. **Panel B:** The mean + one standard deviation of the percentage of CD8$^+$ lymphocytes producing IFNγ is shown. Data are representative of two independent experiments with at least three mice per group. *p=0.012 by unpaired t-test.

8. LPS upregulates costimulatory molecules on cells in the DST in mice treated with anti-CD154 mAb

The observation that LPS promotes the generation of alloreactive CD8$^+$ T cells after treatment with costimulation blockade prompted us to evaluate the potential mechanisms of T cell activation. Given that allogeneic antigens are presented for recognition by host alloreactive T cells via either direct or indirect presentation, we next investigated whether LPS treatment may modulate either of these pathways. We have previously shown that anti-CD154 mAb prevents upregulation of CD80 on APCs in the DST, and this was associated with prolonged skin allograft survival [225]. Therefore, we hypothesized that LPS treatment at the time of DST may act to increase the maturation status of the DST, making it more immunogenic.

To investigate the activation status of cells in the DST following administration of LPS, spleen cells from C57BL/6 mice were analyzed by flow cytometry 15 hr after treatment with BALB/c DST, anti-CD154 mAb, with or without LPS administration at the time of DST (**Figure 12**). Gating on H-2K^{b-}H-2K^{d+} (donor) cells, the DST of mice treated with anti-CD154 mAb and LPS had a modest, but statistically significant increase in the expression of CD80 (MFI=9.6±0.8, n=4) compared to mice treated with anti-CD154 mAb but not LPS (MFI=8.3±0.4; n=4; p=0.025; **Figure 12**). Expression of CD86 was enhanced two-fold on the DST of mice treated with anti-CD154 mAb and LPS (MFI = 36.0±1.1, n=4) compared to mice treated with anti-CD154 mAb but not LPS (MFI = 17.6±3.4; n=4; p < 0.0001). These data suggest that LPS increases the expression of

costimulatory molecules on cells of the DST, even in the presence of anti-CD154 mAb.

FIGURE 12: LPS TREATMENT RESULTS IN THE UPREGULATION OF COSTIMULATORY MOLECULES ON THE DONOR-SPECIFIC TRANSFUSION IN MICE TREATED WITH ANTI-CD154 MAB
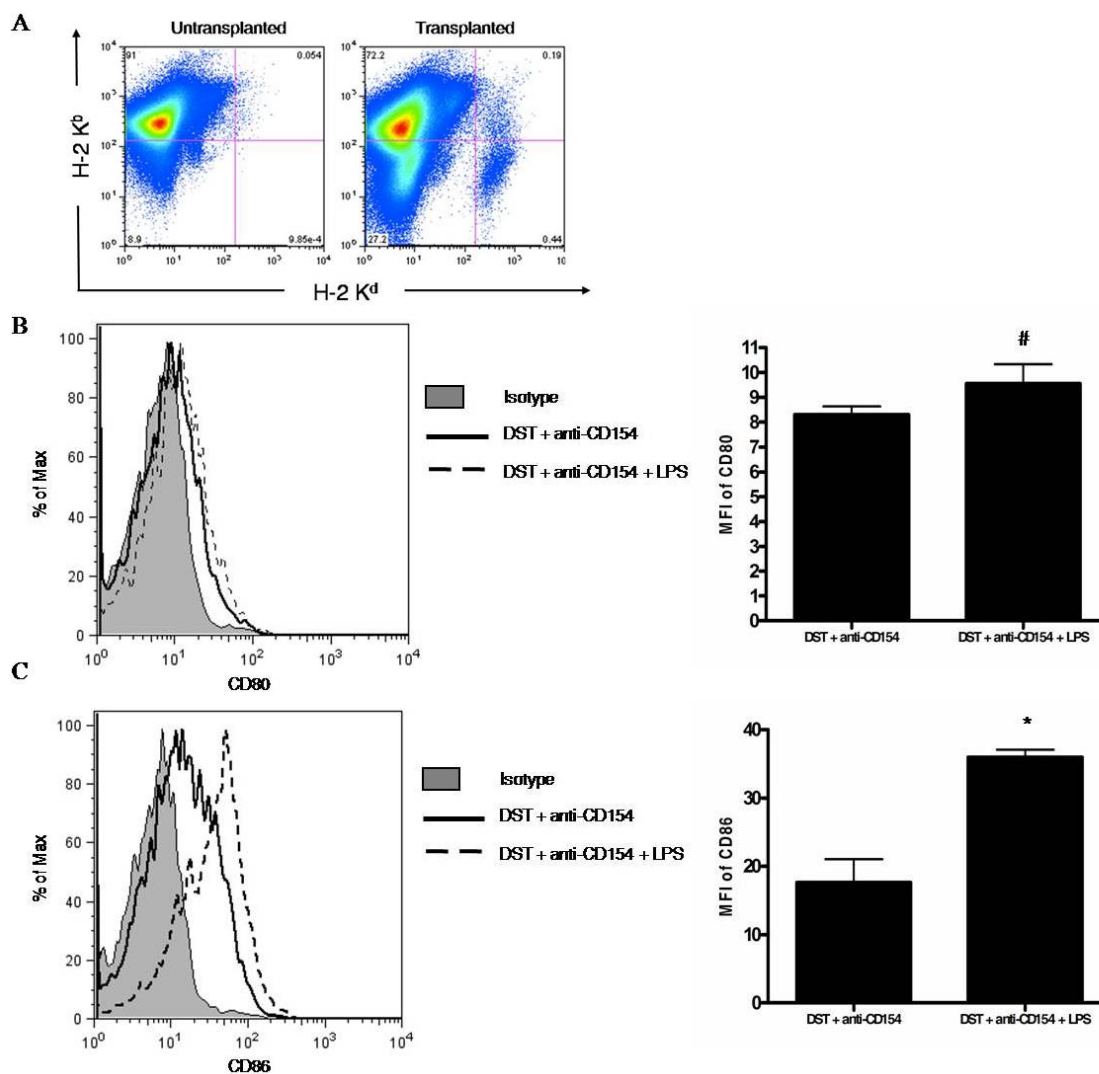
Figure 12. C57BL/6 mice were injected with 10x10^6 BALB/c DST and 0.5mg of anti-CD154 mAb with or without an i.p. injection of LPS. Fifteen hours later, splenocytes were recovered and stained with antibodies to H-2K^b (host), H-2K^d (donor), and CD80 or CD86 and analyzed by flow cytometry. **Panel A:** Representative flow dot plots showing host H-2K^b and donor H-2K^d staining. A group of non-transplanted mice were used as negative controls for H-2K^d staining. **Panels B-C:** Left panels show a representative histogram of the MFI of CD80 (B) and CD86 (C) expression on the H-2K^{b-}H-2K^{d+} (donor) cells that are seen in Figure 5A. These cells represent cells in the BALB/c DST. The right panels are histograms that summarize the MFI plus one standard deviation of CD80 (B) and CD86 (C) expression. Data contains four mice per group. $^#$p=0.025; *p<0.0001.

9. LPS upregulates expression of costimulatory molecules on host alloantigen-presenting dendritic cells

Although LPS treatment appears to increase expression of CD80/86 on the DST, and thus may play a role in abrogating tolerance, Thornley *et al.* have observed that LPS treatment can still prevent the deletion of alloreactive CD8^{+} T cells even when DST genetically deficient in CD80/86 expression is transfused [204]. This suggests that LPS must be mediating other effects that modulate tolerance induction. This led us to hypothesize that LPS administration may be acting to mature host antigen-presenting cells (APCs) that have picked up and processed alloantigen. To investigate this, we adapted a protocol from Iyoda *et al.* [226] to track cells within the donor specific transfusion after it was injected into the host. BALB/c splenocytes were labeled with 5μM of the dye CFSE and injected into C57BL/6 mice that were treated with anti-CD154 mAb with or without co-injection of LPS. Using flow cytometry, this method allows us to detect host DCs that have phagocytosed the CFSE-labeled DST, as these are FITC^{+} cells that are expressing host MHC class I (H-2K^{b+}) and the dendritic cell marker CD11c (the gating scheme used to analyze host alloantigen-presenting DCs can be seen in **Figure 13**). Using the Y-Ae mAb, Iyoda *et al*. determined that nearly all of the CFSE-labeled DCs were indirectly presenting alloantigen [226].

To analyze the maturation status of host alloantigen-presenting DCs following LPS treatment, splenocytes were harvested 16 hours after injection of the CFSE-labeled DST and LPS, and were analyzed by flow cytometry for the expression of CFSE, host class I

(H-2K^b), CD11c, CD8α and CD86. Similar to the findings of Iyoda *et al*., we observed

that essentially all of the alloantigen-containing host DCs were CD8α^+ [226] (**Figure 13**).

FIGURE 13: GATING STRATEGY TO ANALYZE HOST ALLOANTIGEN-PRESENTING DENDRITIC CELLS

A.   Input Cells – CFSE-labeled DST
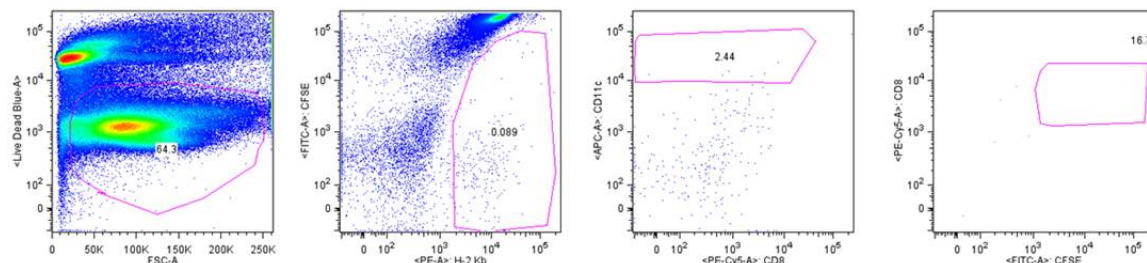
B.   Splenocytes recovered from an untransplanted animal
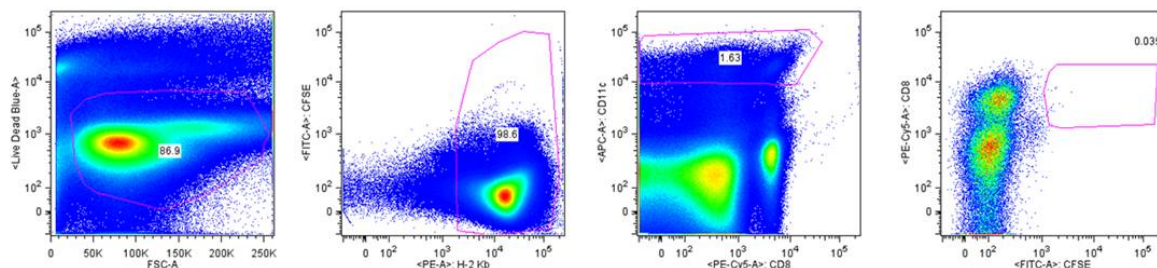
C.   Splenocytes from an animal transplanted with CFSE-labeled DST
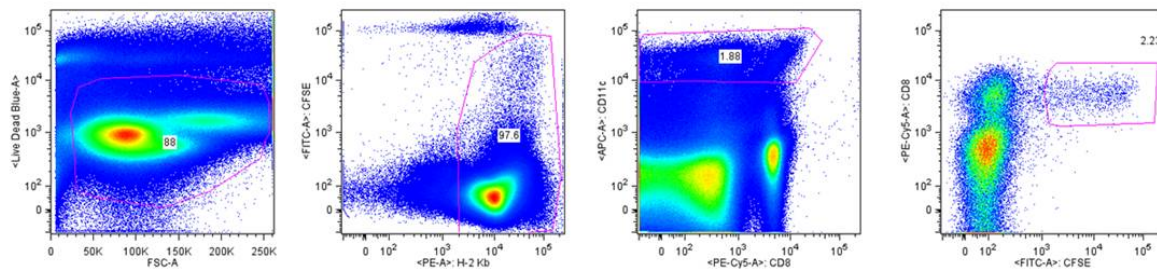
D.   Back gate of CFSE$^+$ DCs from Panel C

Viability Gate   Host Cell Gate   Dendritic Cell Gate   apDC Gate
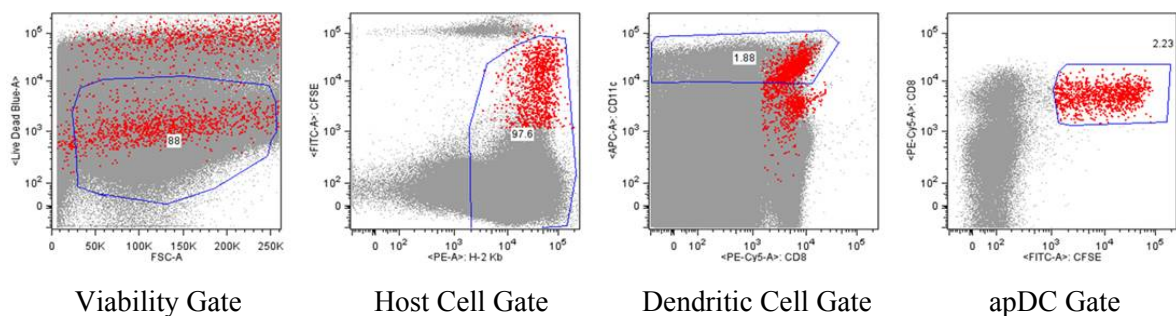
Figure 13. C57BL/6 mice were injected with 10x10^6 CFSE-labeled BALB/c DST and 0.5mg of anti-CD154 mAb with or without an i.p. injection of LPS. Fifteen hours later splenocytes were harvested, stained with Live/Dead blue, antibodies to H-2K^b (host), CD8α, CD11c, and CD86 and analyzed by flow cytometry. For each panel, the dot plot on the left shows the cells that were considered "viable" based on Live/Dead blue staining. The dot plot second from the left shows the cells that were considered of host origin based on staining with H-2K^b. The dot plot second from the right show the cells that were considered dendritic cells based on staining with CD11c. The dot plot on the right shows dendritic cells that have engulfed CFSE$^+$ DST. **Panel A**. Representative flow cytometry dot plots of the input cell, *i.e.* CFSE-labeled BALB/c splenocytes. **Panel B**. Representative flow cytometry dot plots of splenocytes from an uninjected C57BL/6 mouse. These splenocytes do not contain a CFSE$^+$ population. **Panel C**. Representative flow cytometry dot plots of splenocytes from a C57BL/6 mouse injected with CFSE-labeled BALB/c DST. **Panel D**. A "back gate" of the CFSE$^+$CD11c$^+$H-2K^{b+} cells from panel C. This panel indicates that the host alloantigen-presenting DCs are found in the mid-to-upper right of the "Host Cell Gate" (second panel from the left). That population is only present in the spleen of animals transfused with DST.

Gating on host $H-2K^{b+}CD11c^{+}CD8\alpha^{+}CFSE^{+}$ cells, we determined that TLR4 ligation results in a marked upregulation of both class I (MFI = 92,104.0 ± 6419; n=4) and CD86 (MFI = 6487.0 ± 829.5; n=4) compared to non-LPS treated controls (MFI of $H-2K^{b}$ = 54,565.0 ± 8207.0; n=4; p< 0.001; **Figure 14A**; MFI of CD86 = 2398.0 ± 458.3; n=4; p< 0.001; **Figure 14B**).

Given that poly I:C and Pam$_3$Cys also prevent the establishment of transplantation tolerance to allogeneic bone marrow and skin, we hypothesized that treatment with those agents at the time of DST would also result in the maturation of host alloantigen presenting DCs. To investigate this, we performed the above experiment, substituting LPS with either poly I:C or Pam$_3$Cys. We observed that similar to LPS, poly I:C also increased the expression of both host MHC class I $H-2K^{b}$ (MFI = 88,678.0 ± 13,528; n=4; p<0.001 vs. DST and anti-CD154 mAb; **Figure 14A**) and CD86 (MFI = 4,314 ± 754.0; n=4; p<0.01 vs. DST and anti-CD154 mAb; **Figure 14B**) on host alloantigen presenting DCs. Interestingly, although poly I:C and LPS were equivalent in their ability to upregulate class I expression, they differed in the degree to which they induced the expression of CD86. LPS increased the CD86 expression nearly ~1.5x more than poly I:C (p<0.01). Pam$_3$Cys treatment also resulted in an increase in MHC class I expression on alloantigen presenting DCs (MFI = 93,195.0 ± 4,215; n=4; p<0.001 vs. DST and anti-CD154 mAb; **Figure 14A**). CD86 expression was induced with Pam$_3$Cys treatment (MFI = 3,546 ± 440.6; n=4; p<0.05 vs. DST and anti-CD154 mAb; **Figure 14B**); however, like poly I:C, it was markedly lower than that seen by LPS treatment (p<0.001).

FIGURE 14: TLR ACTIVATION ENHANCES HOST APDC MATURATION
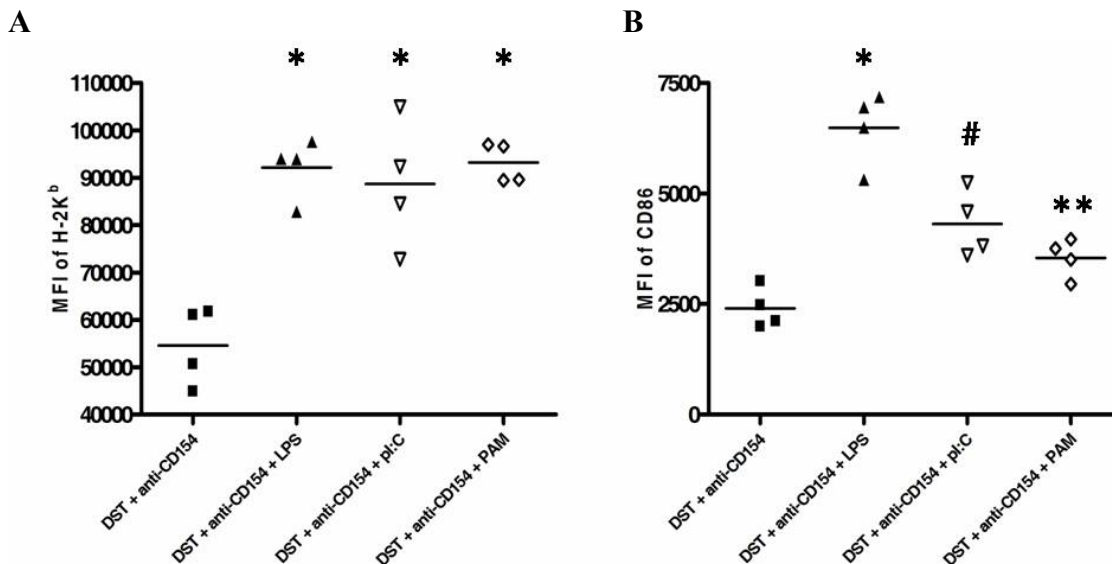
Figure 14. C57BL/6 mice were injected with 10×10^6 CFSE-labeled BALB/c DST and 0.5mg of anti-CD154 mAb with or without an i.p. injection of 100ug LPS, 50ug poly I:C (pI:C), or 100ug of Pam$_3$Cys (PAM). Sixteen hours later splenocytes were harvested, stained with Live/Dead blue, and antibodies to H-2K^b (host), CD8α, CD11c, and CD80 or CD86 and analyzed by flow cytometry. **Panel A**: Histogram of the MFI of the MHC class I molecule H-2K^b. *$p<0.001$ vs. DST and anti-CD154 mAb by Bonferroni's multiple comparison test. **Panel B**: Histogram of the MFI of CD86. *$p<0.001$ vs. DST and anti-CD154 mAb; # $p<0.01$ vs. DST and anti-CD154 mAb; **$p<0.05$ vs. DST and anti-CD154 mAb by Bonferroni's multiple comparison test.

Summary:

We have shown that activation of TLRs at the initiation of tolerance induction by costimulation blockade prevents the establishment of mixed hematopoietic chimerism and significantly shortens skin allograft survival. This effect does not correlate with the production of alloantibodies, nor is it dependent on host NK cells. TLR4 activation, however, did appear to break costimulation blockade-mediated tolerance in both the CD8$^+$ and CD4$^+$ alloreactive T cell compartments, as removal of both populations were required to establish chimerism when LPS was administered at the initiation of tolerance induction. We further document that activated alloreactive CD8$^+$ T cells were generated in mice treated with costimulation blockade plus a TLR agonist. We also observed that following TLR activation, APCs of the DST and the host significantly upregulated costimulatory molecule expression. Therefore, we speculate that alloreactive T cell activation may be the result of TLR-enhancement of both the direct and indirect allorecognition pathways.

CHAPTER IV: LPS PREVENTS THE ESTABLISHMENT OF MIXED

HEMATOPOIETIC CHIMERISM IN MICE TREATED WITH

COSTIMULATION BLOCKADE THROUGH A MECHANISM THAT INVOLVES

HOST TLR4, IFNAR1 AND MYD88

Introduction:

We have shown that activation of the immune system by microbial components can prevent the ability of costimulation blockade-based protocols to induce mixed hematopoietic chimerism. Host $CD4^+$ and $CD8^+$ T cells are required for this effect, and are likely activated by DCs that have been matured by activation through their TLRs. However, the molecular mechanisms that control this process are not known. In this chapter, we address several important questions. 1) Is ligation of host TLR required for the ability TLR agonists to prevent the establishment of mixed hematopoietic chimerism induction in mice treated with costimulation blockade? 2) What TLR signaling cascades are involved in preventing the establishment of mixed chimerism? 3) Does TLR activation at time points other than during the initiation of costimulation blockade also prevent the establishment of mixed chimerism?

LPS administration at the time of tolerance induction matures DCs in the DST as well as DCs in the host. Therefore, it is possible that activation of TLR4 on either cell population is sufficient to prevent establishment of chimerism and to shorten skin allograft survival by providing alloreactive T cells with the necessary costimulatory molecules for activation even in the presence of anti-CD154 mAb. TLR4 signals through

two distinct pathways that can stimulate pro-inflammatory programs in immune cells, the MyD88-dependent and the MyD88-independent pathways. Finally, the effects of TLR signaling are transient, and therefore, activation at various time points during conditioning may result in different outcomes. We addressed these questions by introducing LPS a day before tolerance induction, and one day following transplantation.

Results:

1. TLR4 expression is required on host cells for LPS to prevent the establishment of chimerism in mice treated with costimulation blockade

Our laboratory has shown that LPS-induced rejection of skin allografts in the setting of costimulation blockade was dependent on engaging TLR4 on host cells [133]. To address whether engagement of TLR4 on host cells was also required for LPS to prevent the induction of mixed chimerism, C57BL/10 and C56BL/10.*TLR4*$^{-/-}$ mice were treated with BALB/c DST, anti-CD154 mAb, and given BALB/c bone marrow and skin allografts with or without co-injection of LPS at the time of tolerance induction.

Treatment of wild-type C57BL/10 mice with costimulation blockade led to chimerism (2/6) and prolonged skin graft survival (MST=70 days), which was prevented by administration of LPS (0/8 became chimeric; MST=10; p<0.05 **Figure 15**). In contrast, C57BL/10.*TLR4*$^{-/-}$ mice treated with DST and anti-CD154 mAb in the presence or absence of LPS became chimeric at similar frequencies (6/7 mice treated with LPS became chimeric *vs*. 7/10 mice not treated with LPS; p=NS) and exhibited prolonged skin graft survival (MST>176 for LPS treated mice vs. MST = 129 for control mice; p=NS; **Figure 15**). These data suggest that similar to the results seen by Thornley *et al.* with LPS and skin alone [133], host TLR4 expression is required for LPS treatment to prevent the establishment of mixed hematopoietic chimerism in mice treated with costimulation blockade.

We next hypothesized that in the absence of host TLR4 expression, LPS treatment at

the start of costimulation blockade would not result in the upregulation of costimulatory molecules on host APCs that was observed in wild-type mice (**Figure 12**). To test this hypothesis, C57BL/10 and C57BL/10.TLR4$^{-/-}$ mice (H-2^b) were injected with CFSE-labeled BALB/c splenocytes (H-2^d) and treated with anti-CD154 mAb with or without LPS injection. Sixteen hours after treatment, splenocytes were recovered and analyzed for the expression of host MHC class I (H-2K^b), CD80 and CD86. We observed that in contrast to wild-type mice, mice deficient in TLR4 did not upregulate the expression of H-2K^b, CD80 or CD86 (**Figure 15C, D**, and **E**, respectively). These data document that in the absence of TLR4 signaling, LPS administration at the time of DST does not result in the maturation of host alloantigen-presenting DCs.

FIGURE 15: LPS EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE ARE DEPENDENT ON HOST TLR4

A

Group	Recipient	Donor	TLR Agonist	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	C57BL/10	BALB/c	None	2/6 (33.3%)	1.59 ± 1.81	70	70	93
2	C57BL/10	BALB/c	LPS	0/8 (0.00%)	<0.10	10	10	N/A
3	TLR4$^{-/-}$	BALB/c	None	7/10 (70.0%)	2.98 ± 1.84	129	18	152
4	TLR4$^{-/-}$	BALB/c	LPS	6/7 (85.7%)	2.50 ± 1.00	>176	10	>176

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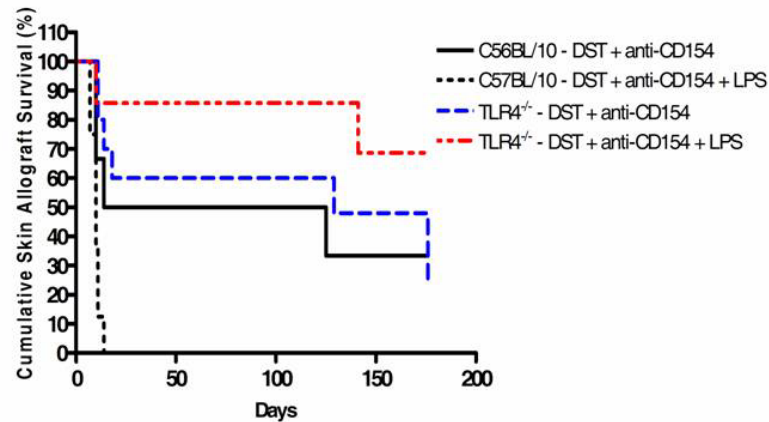
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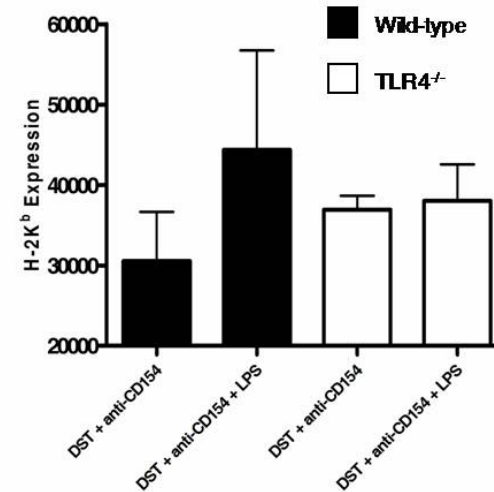
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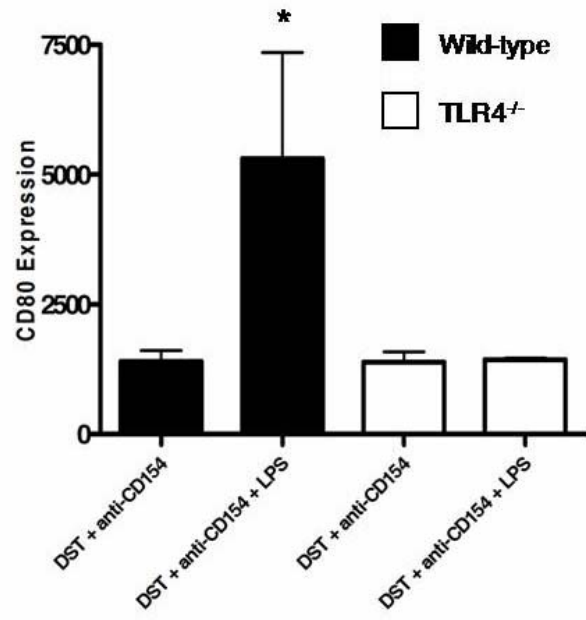
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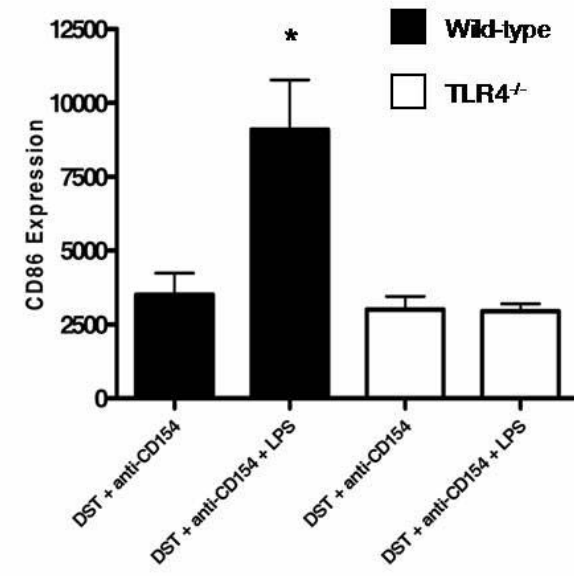
Figure 15. **Panels A-B**. All mice were treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 50×10^6 allogeneic bone marrow cells and allogeneic skin grafts. Mice treated with a TLR agonist were given an i.p. injection of 100ug LPS on day -7 relative to bone marrow cell and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.0001 vs. group 1; **p=NS vs. group 3 by chi-square analysis. N/A − not applicable. Skin graft survival data have been separated into three groups to distinguish chimeric mice from non-chimeric mice in regards to skin MST. Data from groups 1-2 shown in Figure 5 are reproduced here for ease of comparison with other treatment groups. **Panel B**. Kaplan-Meier plots for allogeneic skin graft survival for the entire cohort of mice conditioned and transplanted with bone marrow and skin is shown (third column from the right in Panel A). p=NS for DST and anti-CD154 mAb vs. DST, anti-CD154 mAb and LPS in TLR4$^{-/-}$ mice. **Panels C-E**. Mice were injected with 10×10^6 CFSE-labeled BALB/c DST, 0.5mg of anti-CD154 mAb and an i.p. injection of 100ug LPS. Sixteen hours later splenocytes were harvested, stained with Live/Dead blue, and antibodies to H-2K^b (host), CD8α, CD11c, and CD80 or CD86 and analyzed by flow cytometry. Each group contains four mice. **Panel C:** Histogram of the MFI of the class I molecule H-2K^b. **Panel D**: Histogram of the MFI of

CD80. *p<0.001 vs. all other groups. **Panel E**: Histogram of the MFI of CD86. *p<0.001

vs. all other groups.

2. IL-12p40 expression is not required for LPS to prevent the establishment of chimerism in mice treated with costimulation blockade

The finding that LPS prevents the establishment of mixed chimerism by signaling through TLR4 expressed on host APCs led us next to investigate which cytokines downstream of the TLR4 signaling pathway may be required. LPS is a known inducer of interleukin (IL)-12, a cytokine essential for the optimal differentiation of naïve T cells into IFN-γ-producing T_H-1 cells [227,228]. Furthermore, IL-12 is also thought to be important in the activation of naïve CD8$^+$ T cells, perhaps by acting as "signal 3" in the T cell activation cascade [70] (**Figure 1**). IL-12 is a heterodimeric cytokine comprised of two subunits (p35 (IL-12α) and p40 (IL-12β)) linked by two disulfide bonds [228]. The IL-12p40 subunit is essential for the production of IFN-γ in response to LPS [229,230]. Therefore, to examine the role of IL-12, we treated mice deficient in IL-12p40 with our standard costimulation blockade and transplantation protocol with or without LPS.

Treatment of C57BL/6.IL-12p40$^{-/-}$ mice with costimulation blockade produced low levels of mixed hematopoietic chimerism (2.42 ± 1.03% donor-origin PBMCs at 8 weeks post transplant) in 3 of 5 recipients (**Figure 16**, group 3**)**. These mice also exhibited prolonged skin graft survival (MST = 164 days). In contrast, C57BL/6.IL-12p40$^{-/-}$ mice treated with DST, anti-CD154 mAb and LPS did not develop mixed chimerism (0/5, p<0.05 vs. group 3), and skin survival was short (MST = 11 days, p<0.001 vs. group 3**; Figure 16**). These data suggest that IL-12 signaling is not required for LPS to prevent the establishment of hematopoietic chimerism and shorten skin allograft survival in mice

treated with costimulation blockade.

FIGURE 16: LPS EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE ARE NOT DEPENDENT ON HOST IL-12p40

A

Group	Recipient	Donor	TLR Agonist	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	C57BL/6	BALB/c	None	29/45 (64.0%)	1.59 ± 1.81	144	88	>260
2	C57BL/6	BALB/c	LPS	0/23 (0.00%)*	<0.10	11	11	N/A
3	IL-12p40$^{-/-}$	BALB/c	None	3/5 (60.0%)	2.42 ± 1.03	172	130	>216
4	IL-12p40$^{-/-}$	BALB/c	LPS	0/6 (0.00%)**	<0.10	11	11	N/A

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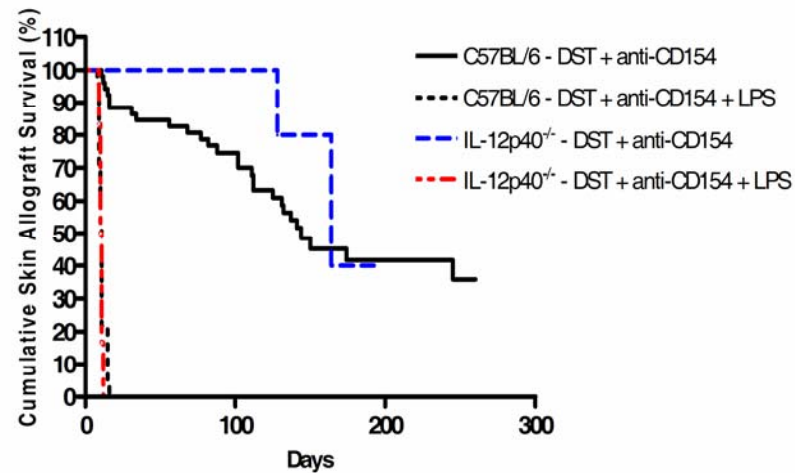
Figure 16. All mice were treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 50×10^6 allogeneic bone marrow cells and allogeneic skin grafts. Mice treated with TLR agonist were given an i.p. injection of 100ug LPS on day -7 relative to bone marrow cell and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.0001 vs. group 1; **p<0.05 vs. group 3 by chi-square analysis. N/A − not applicable. Skin survival data have been separated into three groups to distinguish chimeric mice from non-chimeric mice in regards to skin MST. Data from groups 1-2 shown in Figure 5 are reproduced here for ease of comparison with other treatment groups. **Panel B**. Kaplan-Meier plots for skin allograft survival for the entire cohort of mice treated with costimulation blockade and transplanted with bone marrow and skin is shown (third column from the right in Panel A). p<0.01 for DST and anti-CD154 mAb vs. DST, anti-CD154 mAb and LPS in IL-12p40$^{-/-}$ mice.

3. Host TLR4→Myd88 axis is not required for LPS to prevent the establishment of chimerism in mice treated with costimulation blockade

The finding that host IL-12p40 was not required for LPS to prevent the establishment of chimerism led us to next investigate which signaling molecules in the TLR4 cascade were necessary for the detrimental effects of LPS. LPS can activate innate immunity by signaling through two distinct pathways following ligation of TLR4, the myeloid differentiation factor-88 (MyD88)-dependent pathway, and the Toll/Interleukin-1 receptor domain-containing adaptor protein inducing interferon-β (TRIF) pathway [123]. Therefore, we first determined whether the MyD88 pathway was required for LPS to prevent the establishment of hematopoietic chimerism in mice treated with costimulation blockade. To investigate this hypothesis, we transplanted C57BL/6.MyD88$^{-/-}$ mice with allogeneic bone marrow after treatment with DST and anti-CD154 mAb with or without co-injection of LPS. The majority of C57BL/6.MyD88$^{-/-}$ mice not treated with LPS developed mixed chimerism (4/5; $2.30 \pm 2.47\%$ donor-origin PBMCs at 8 weeks post transplant; Figure 17, group 3). Interestingly, however, mice treated with LPS did not (0/9; $p=0.0015$; Figure 17 group 4). These data suggest that host MyD88 is not required for the ability of LPS to prevent the establishment of mixed chimerism in mice treated with costimulation blockade.

We then asked why the effects of LPS were still evident in C57BL/6.MyD88$^{-/-}$ mice. We reasoned that in the absence of MyD88-dependent cytokines, LPS treatment may still induce the maturation of host alloantigen-presenting DCs. To determine this,

C57BL/6.MyD88$^{-/-}$ mice were injected with CFSE-labeled BALB/c splenocytes and treated with anti-CD154 mAb with or without LPS injection. Sixteen hours after treatment, splenocytes were recovered and analyzed for the expression of host MHC class I (H-2K^b), and CD86. We observed that similar to wild-type mice, but in contrast to TLR4$^{-/-}$ mice, mice deficient in MyD88 did upregulate the expression of H-2K^b and CD86 in response to LPS treatment. LPS-treated C57BL/6.MyD88$^{-/-}$ mice had a greater than two fold increase in the expression of H-2K^b ($p<0.001$; Figure 17B) and CD86 ($p<0.001$; Figure 17C) when compared to non-LPS treated C57BL/6.MyD88$^{-/-}$ mice. We also observed that the expression of CD86 in LPS-treated MyD88$^{-/-}$ mice was slightly decreased compared to wild-type mice treated with LPS ($p<0.05$). These data indicate that LPS can upregulate expresssion of CD86 in the absence of MyD88, although full maturation may require a contribution from MyD88-dependent factors.

FIGURE 17: LPS EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE ARE NOT DEPENDENT ON HOST MYD88

A

| Group	Recipient	Donor	TLR Agonist	Chimerism Frequency	% Donor-origin PBMCs (8 wk)
1	C57BL/6	BALB/c	None	6/9 (66.7%)	2.43 ± 1.37
2	C57BL/6	BALB/c	LPS	0/7 (0.00%)*	<0.10
3	MyD88$^{-/-}$	BALB/c	None	4/5 (80%)	2.30 ± 2.47
4	MyD88$^{-/-}$	BALB/c	LPS	0/9 (00.0%)**	<0.10

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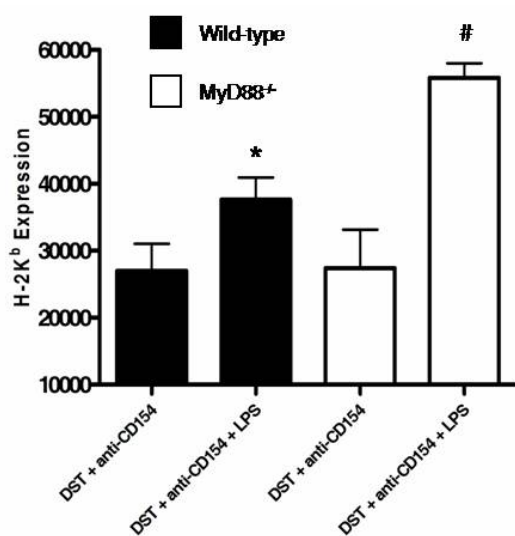
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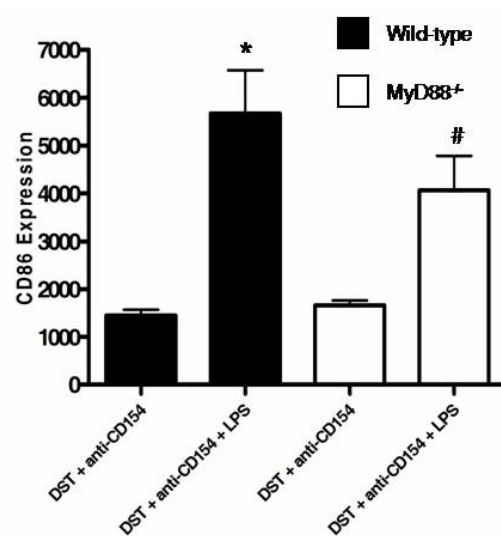
Figure 17. Panel A. All mice were treated with a donor-specific transfusion on day -7 and four injections of 0.5 mg of anti-CD154 mAb on days -7, -4, 0, and +4 relative to transplantation on day 0 with 50×10^6 allogeneic bone marrow cells. Mice treated with TLR agonist were given an i.p. injection of 100ug LPS on day -7 relative to bone marrow cell and skin transplantation. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.01 vs. group 1; **p<0.01 vs. group 3, and p=NS vs. group 2 by chi-square analysis. **Panels B-C**. Mice were injected with 10×10^6 CFSE-labeled BALB/c DST, 0.5mg of anti-CD154 mAb and an i.p. injection of 100ug LPS. Sixteen hours later splenocytes were harvested, stained with Live/Dead blue, and antibodies to H-2K^b (host), CD8α, CD11c, and CD86 and analyzed by flow cytometry. Each group contains four mice. **Panel B:** Histogram of the MFI of the class I molecule H-2K^b. *p<0.05 vs. all other groups; $^\#$p<0.001 vs. all other groups. **Panel C:** Histogram of the MFI of CD86. *p<0.05 vs. MyD88$^{-/-}$ mice treated with DST, anti-CD154 mAb and LPS, p<0.001 vs. both MyD88$^{-/-}$ and wild-type mice treated with DST and anti-CD154 mAb; $^\#$ p<0.05 vs. wild-type mice treated with DST, anti-CD154 mAb and LPS, p<0.001 vs. both MyD88$^{-/-}$ and wild-type mice treated with DST and anti-CD154 mAb.

4. Signaling through the Type 1 IFN receptor is important for LPS and Poly I:C, but not Pam₃Cys, to prevent the establishment of chimerism in mice treated with costimulation blockade

The observation that the adaptor molecule MyD88 was not required for LPS to prevent the establishment of mixed chimerism prompted us to hypothesize that the MyD88-independent (TRIF) pathway may be involved. TLR4 signaling through the TRIF pathway leads to the upregulation of the type I interferon, IFN-β, and it has been hypothesized that TLR4 induction of IFN-β is entirely dependent on the MyD88-independent pathway [159]. Furthermore, our laboratory has also shown that signaling through the type 1 IFN receptor is important for LPS to prevent the induction of tolerance to skin allografts in mice treated with costimulation blockade [204]. Therefore, to test our hypothesis that signaling through the type 1 IFN receptor is also important for LPS to prevent the establishment of bone marrow chimerism, we treated mice deficient in the type 1 IFN receptor (IFNAR1) with DST, anti-CD154 mAb , BALB/c bone marrow and skin with or without co-injection of LPS. The majority of C57BL/6.IFNAR1$^{-/-}$ mice treated without LPS developed mixed chimerism (13/15 developed chimerism; $3.52 \pm 2.14\%$ donor-origin PBMCs at 8 weeks post transplant) and chimeric mice exhibited permanent skin allograft survival (MST >218 days; **Figure 18**, group 4). Interestingly, in contrast to wild-type and MyD88$^{-/-}$ mice, a subset of IFNAR1$^{-/-}$ mice treated with LPS became chimeric (6/17; p <0.01 vs. wild-type animals treated with LPS (group 2)) and chimeric mice exhibited permanent skin allograft survival (MST >218 days; **Figure 18**,

group 5). However, the percentage of IFNAR1^{-/-} mice treated with LPS that became chimeric was significantly lower than that achieved in IFNAR1^{-/-} mice not treated with LPS (p=0.008). These data suggest that although signaling through the type I IFN receptor is important for LPS-mediated rejection, other mediators may be involved.

Because the TLR3 agonist poly I:C is also a known inducer of type I IFN, we next used IFNAR1^{-/-} mice to investigate the hypothesis that signaling through the type 1 IFN receptor was also required for poly I:C to prevent the establishment of mixed chimerism in mice treated with costimulation blockade. Similar to LPS, we observed that a subset of IFNAR1^{-/-} mice treated with poly I:C at the start of the conditioning regimen became chimeric (5/12) and chimeric mice exhibited permanent skin graft survival (MST > 218; **Figure 18**, group 6). The frequency of chimerism was different from IFNAR1^{-/-} mice treated without a TLR agonist (p<0.05), again suggesting the involvement of other mediators.

Since the type I IFN receptor appeared to be playing an important role in the detrimental effects of both LPS and poly I:C, we next tested if signaling through the type 1 IFN receptor was also important in effects induced by Pam₃Cys. TLR2 activation is not believed to induce type I interferons [123], but still shortens skin allograft survival in mice treated with costimulation blockade [204]. Therefore, we hypothesized that Pam₃Cys would still inhibit the establishment of mixed chimerism in IFNAR1^{-/-} mice treated with costimulation blockade. In support of this hypothesis, we observed that mice deficient in signaling through the type I IFN receptor did not become chimeric when

treated with Pam$_3$Cys (0/4; p<0.001 vs. group 5), and skin allograft survival was short (MST = 10 days; p<0.0001 vs. groups 5; **Figure 18**, group 8). These data suggest that signaling through the type 1 IFN receptor has an important role in preventing the establishment of mixed chimerism and shortening skin allograft survival in mice treated with costimulation blockade and given TLR3 and TLR4 agonists, but not a TLR2 agonist.

FIGURE 18: LPS AND POLY I:C BUT NOT PAM$_3$CYS EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE ARE DEPENDENT ON SIGNALING THROUGH THE TYPE 1 IFN RECEPTOR

A

| Group	Recipient	Donor	TLR Agonist	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	C57BL/6	BALB/c	None	29/45 (64.0%)	1.59 ± 1.81	144	88	>260
2	C57BL/6	BALB/c	LPS	0/23 (0.00%)*	<0.10	11	11	N/A
3	C57BL/6	BALB/c	poly I:C	0/20 (0.00%)*	<0.10	9	9	N/A
4	C57BL/6	BALB/c	Pam3Cys	0/3 (0%)**	<0.10	10	10	N/A
5	IFNAR1$^{-/-}$	BALB/c	None	13/15 (87.0%)	3.52 ± 2.14 $^{\#}$	>218	28	>218
6	IFNAR1$^{-/-}$	BALB/c	LPS	6/17 (35.0%)##	2.12 ± 1.11	80	14	>218
7	IFNAR1$^{-/-}$	BALB/c	poly I:C	5/12 (41.7%)$	2.22 ± 0.97	84	16	>218
8	IFNAR1$^{-/-}$	BALB/c	Pam3Cys	0/4 (0.00%)$$	<0.10	10	10	N/A

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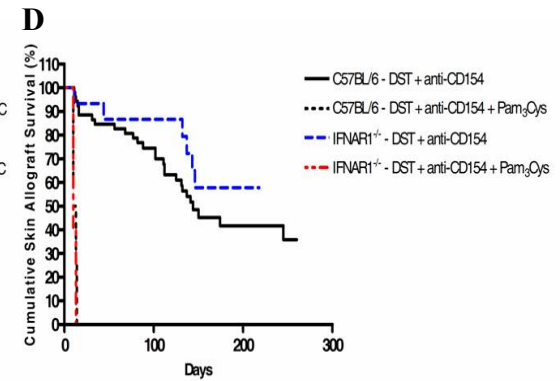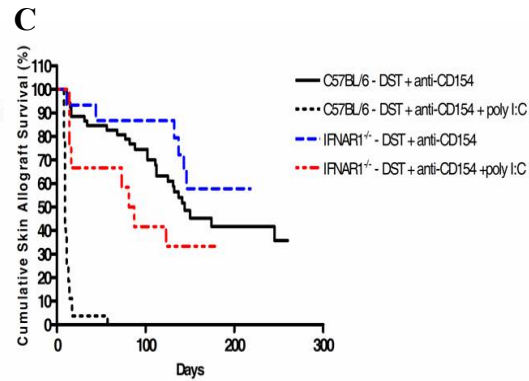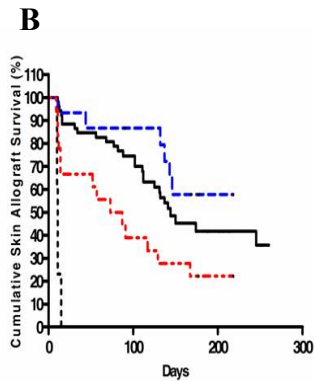
Figure 18. All mice were treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 50×10^6 allogeneic bone marrow cells and allogeneic skin grafts. Mice treated with TLR agonist were given either an i.p. injection of 50ug of poly I:C, 100ug LPS, or 100ug of Pam$_3$Cys on day -7 relative to bone marrow cell and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin PBMCs 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.0001 vs. group 1; **p<0.05 vs. group 1; $^{##}$p<0.01 vs. group 2 and vs. group 5; $^{\$}$p<0.01 vs. group 3 and p<0.05 vs. group 5; $^{\$\$}$p<0.001 vs. group 5 by chi-square analysis. $^{#}$p<0.01 vs. group 1 by unpaired t-test. N/A – not applicable. Skin survival data have been separated into three groups to distinguish chimeric mice from non-chimeric mice in regards to skin graft survival. Data from groups 1-4 shown in Figure 5 are reproduced here for ease of comparison with other treatment groups. **Panel B-D**. Kaplan-Meier plots for allogeneic skin graft survival for the entire cohort of mice conditioned and transplanted with bone marrow and skin is shown (i.e., third column from the right in Panel A). **Panel B**. p<0.0001 for IFNAR1$^{-/-}$ mice treated with DST, anti-CD154 mAb and LPS vs. wild-type (WT) mice treated with DST, anti-CD154 mAb and LPS; p<0.05 for IFNAR1$^{-/-}$ mice treated with DST, anti-CD154 mAb and LPS vs. WT mice treated with DST, anti-CD154 mAb and vs. IFNAR1$^{-/-}$ mice treated with DST and anti-CD154 mAb. **Panel C.** p<0.0001 for IFNAR1$^{-/-}$ mice treated with DST, anti-CD154 mAb and poly I:C vs. WT mice treated with DST, anti-CD154

mAb and poly I:C. **Panel D**. p=NS for IFNAR1$^{-/-}$ mice treated with DST, anti-CD154

mAb and Pam$_3$Cys vs. WT mice treated with DST, anti-CD154 mAb and Pam$_3$Cys.

5. *LPS-induced activation of alloreactive CD8$^+$ T cells is dependent on signaling through the type 1 IFN receptor*

We have shown that LPS administration at the initiation of costimulation blockade results in: 1) an increase in the maturation state of host alloantigen-presenting dendritic cells (**Figure 12**), and 2) the generation of effector/memory alloreactive CD8$^+$ T cells (**Figure 11**). Therefore, the observation that mixed chimerism could develop in LPS-treated mice that were deficient in the type I IFN receptor led us to hypothesize that there would be an attenuation of host DC activation following TLR4 activation in these mice. To address this, wild-type C57BL/6 and C57BL/6.IFNAR1$^{-/-}$ mice were injected with CFSE-labeled BALB/c splenocytes and treated with anti-CD154 mAb with or without LPS injection. Fifteen hours after treatment, splenocytes were recovered and analyzed for the expression of H-2K^b and CD86. We observed that alloantigen-presenting DCs from mice deficient in type I IFN signaling exhibited significantly lower expression of both molecules after injection of LPS as compared to alloantigen-presenting DCs from wild-type mice. (MFI of H-2K^b = 69,793 ± 7,852 *vs.* 92,104 ± 6,419, respectively; p<0.01; **Figure 19A,** Experiment 1) (MFI of CD86 = 4,650 ± 1,066 *vs.* 6,487 ± 829, respectively; p<0.01; **Figure 19B**, Experiment 1). The expression of both molecules, however, was higher in IFNAR1$^{-/-}$ mice treated with LPS when compared to their non-LPS-treated controls (p<0.05). Thus, in the absence of signaling through the type I IFN receptor, LPS treatment can induce alloantigen presenting DC maturation, although the degree of maturation appears to be attenuated.

We next hypothesized that decreased DC activation in the absence of signaling through the type I IFN receptor may prevent the generation of alloreactive CD8$^+$ effector/memory T cells. To test this, we performed intracellular flow cytometry for IFNγ on CD8$^+$ splenocytes isolated from C57BL/6 and C57BL/6.IFNAR1$^{-/-}$ mice one week after treatment with our standard conditioning regimen with or without co-administration of LPS at the initiation of costimulation blockade. Interestingly, we observed that mice deficient in signaling through the type I IFN receptor did not develop effector/memory CD8$^+$ T cells when exposed to LPS, whereas wild-type C57BL/6 mice did (**Figure 19C**). These data suggest that signaling through the type I IFN receptor is required for priming of alloreactive CD8$^+$ effector/memory T cells in mice treated with costimulation blockade.

FIGURE 19: LPS-INDUCED ACTIVATION OF ALLOREACTIVE CD8^{+} T CELLS IS DEPENDENT ON SIGNALING THROUGH THE TYPE 1 IFN RECEPTOR
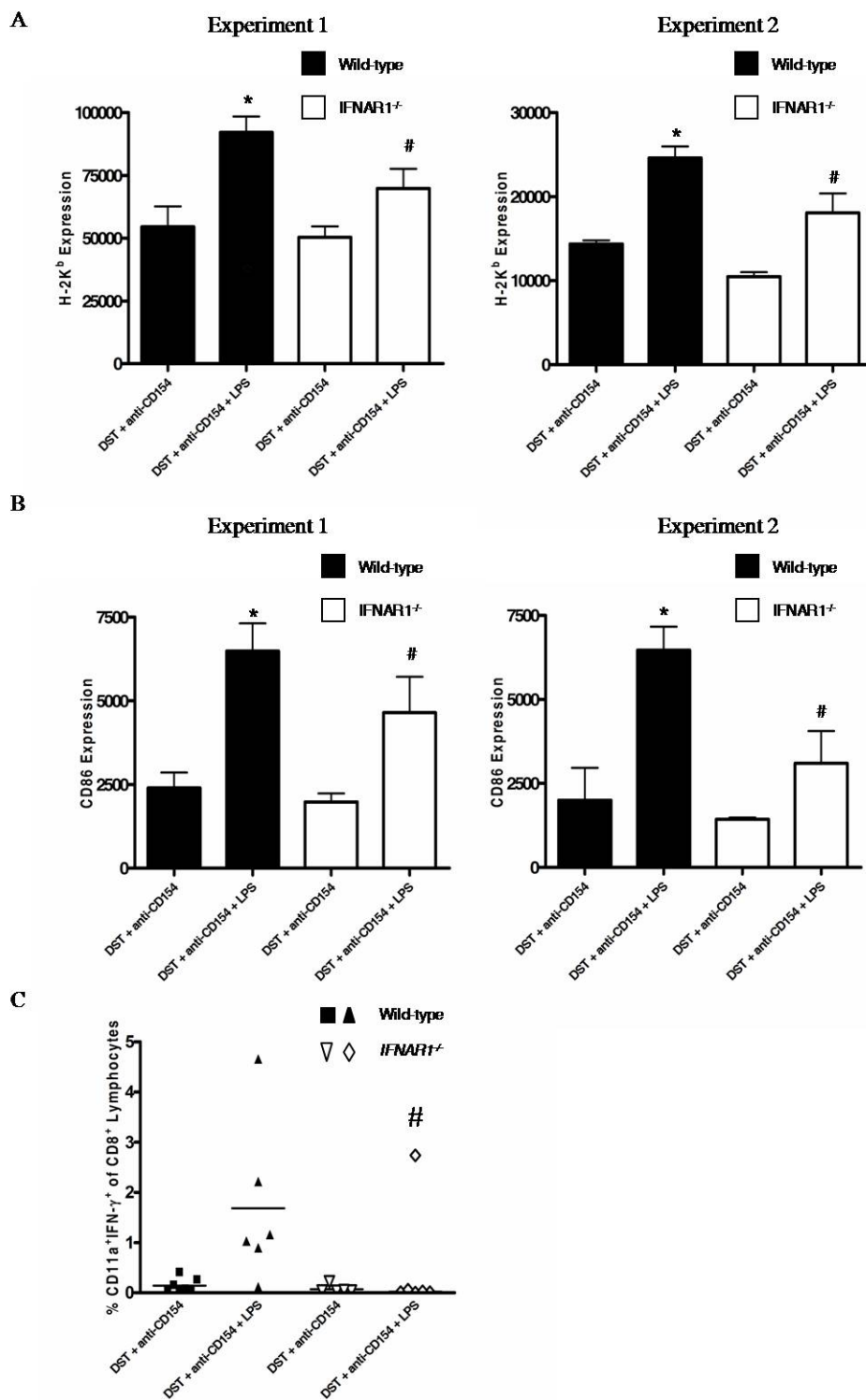
Figure 19. Panels A-B: C57BL/6 and IFNAR1$^{-/-}$ mice were injected with 10x10^6 CFSE-labeled BALB/c DST and 0.5mg of anti-CD154 mAb with or without an i.p. injection of LPS. Fifteen hours later splenocytes were harvested, stained with antibodies to H-2K^b, CD8α, CD11c, and CD86 and analyzed by flow cytometry. Left panel show a representative histogram of the MFI of CD86 expression on the H-2K^{b+}CD11c$^+$CD8α$^+$CFSE$^+$ cells. These cells represent host DCs that have phagocytosed CFSE-labeled DST. **Panel A:** Histogram of the MFI of the MHC class I molecule H-2K^b. *p<0.01 vs. all other groups; $^\#$p=NS vs. wild-type mice treated with DST and anti-CD154 mAb, p<0.01 vs. wild-type mice treated with DST, anti-CD154 mAb and LPS, p<0.05 vs. IFNAR1$^{-/-}$ mice treated with DST and anti-CD154 mAb . **Panel B:** Histogram of the MFI of CD86. *p<0.05 vs. IFNAR1$^{-/-}$ mice treated with DST, anti-CD154 mAb and LPS, p<0.001 vs. both IFNAR1$^{-/-}$ and wild-type mice treated with DST and anti-CD154 mAb. $^\#$p<0.01 vs. both IFNAR1$^{-/-}$ and wild-type mice treated with DST and anti-CD154 mAb. **Panel C:** All mice were treated with a BALB/c DST, bone marrow and anti-CD154 mAb according to our standard protocol with or without an i.p. injection of 100μg LPS. Splenocytes were harvested one week after bone marrow transplantation, stimulated *in vitro* with either irradiated syngeneic (H-2^b) or allogeneic (H-2^d) splenocytes, and analyzed by flow cytometry for intracellular IFNγ production. The percentage of CD8$^+$ lymphocytes producing IFNγ is shown (bar represents the mean). Only wild type mice treated with DST, anti-CD154 mAb and LPS had IFNγ-producing alloreactive CD8$^+$ lymphocytes in the spleen. Mice deficient in type I IFN receptor signaling did not

generate IFNγ-producing alloreactive CD8$^+$ lymphocytes when injected with LPS during costimulation blockade. $^\#$p<0.05 *vs*. wild type mice treated with DST, anti-CD154 mAb and LPS. P=NS *vs*. IFNAR1$^{-/-}$ treated with DST and anti-CD154 mAb.

6. *LPS treatment results in a transient induction of type I IFN*

The observation that signaling through the type I IFN receptor was important for LPS-mediated bone marrow rejection prompted us to investigate the kinetics of IFN-α/β induction by LPS in the presence or absence of costimulation blockade. We first quantified serum levels of IFN-α/β in wild-type C57BL/6 mice following LPS administration using a standard IFN-α/β bioassay [217]. Serum was collected 8 and 24 hours after treatment with PBS, or DST and anti-CD154 mAb with or without co-injection of 100ug of LPS. The serum was then diluted serially in wells containing mouse L-929 cells and incubated overnight. The following day, each well was infected with vesicular stomatitis virus (VSV) and incubated for 2 days. The cytopathic effects were evaluated using microscopy, and the IFN-α/β titer was determined as the reciprocal of the dilution that provided 50% protection from cytopathic effects.

IFN-α/β was not detected in untreated mice (n=3), and only low levels were detected in mice treated with DST and anti-CD154 mAb at 8 hours after treatment (5.0±3.9 U/ml, n=7), and dropped to nearly undetectable levels at 24 hours (0.8±2.1 U/ml; **Figure 20**). In contrast, mice treated with DST, anti-CD154 mAb and LPS had three-fold higher levels of IFNα/β (15.4±6.0 U/ml; n=5; p<0.01; **Figure 20**) 8 hours after treatment compared with mice treated with DST and anti-CD154 mAb. We also observed that levels of IFNα/β were greater at 24 hours in mice treated with DST, anti-CD154 mAb and LPS as compared with untreated controls (6.6±2.5 U/ml; p<0.01; **Figure 20**). However, the levels 24 hours after LPS treatment were not different from those attained 8 hours after

treatment with DST and anti-CD154 mAb.

FIGURE 20: LPS TREATMENT RESULTS IN A TRANSIENT INDUCTION OF TYPE I INTERFERON
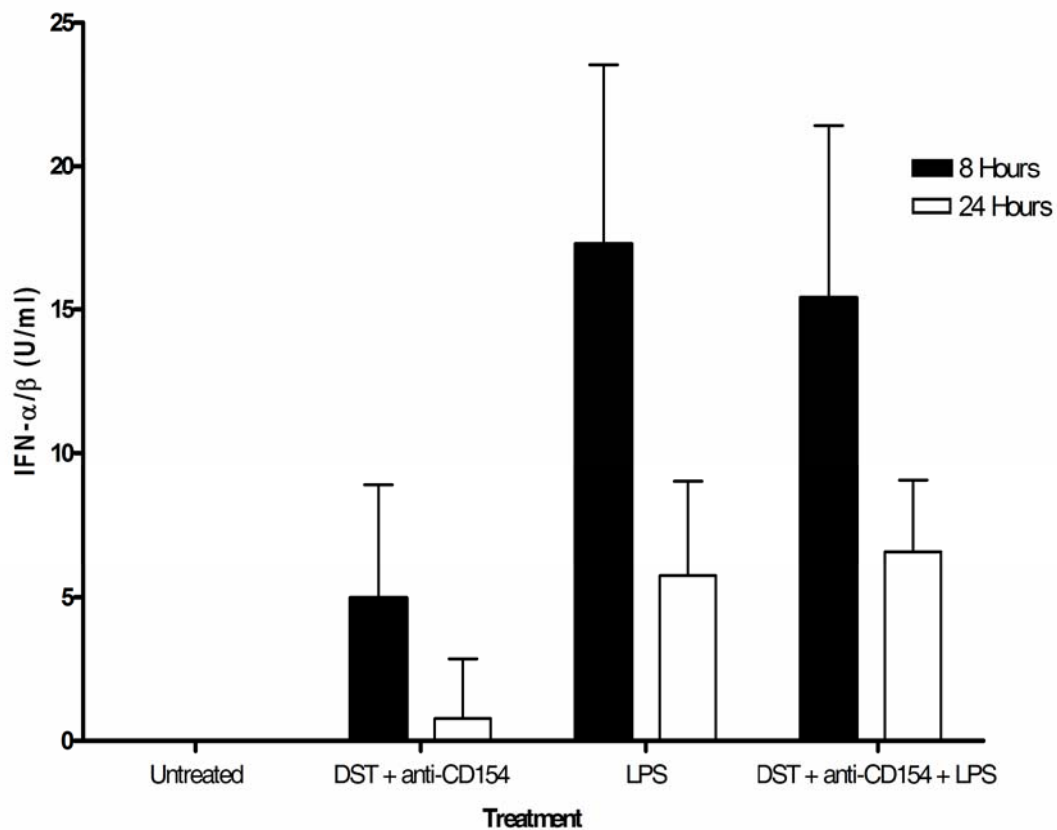
Figure 20: Serum was collected from C57BL/6 mice 8 and 24 hours after indicated treatment. The IFN-α/β titer was determined as the reciprocal of the dilution that protected L-929 cells from cytopathic effect by VSV infection. Sera from untreated mice did not exhibit any protection. Data are presented as mean + S.D.

*7. **LPS-mediated effects on the establishment of mixed chimerism in mice treated with costimulation blockade is dependent on the time of administration and correlates with the transient spike in serum IFN-α/β***

Given the important role of type I IFN, and our observation that LPS treatment appeared to induce a transient spike in IFN-α/β, we hypothesized that LPS administration 24 hours prior to DST and anti-CD154 mAb would not prevent the induction of chimerism. To test this, three cohorts of C57BL/6 mice were treated with DST and anti-CD154 mAb and given BALB/c bone marrow. One cohort was not treated further, one cohort was given LPS on the day of DST (day -7), and one cohort was given LPS on day −8 (24 hours prior to DST and anti-CD154 mAb). Interestingly, we observed that mice treated with LPS on day −8 became chimeric at the same frequency (6/7) as mice not treated with LPS (6/7), while mice given LPS on day −7 did not become chimeric (0/7; p=0.0012; **Figure 21**). These data suggest that the detrimental effects of LPS are transient, and correlate with the production of IFN-α/β in response to TLR4 activation. The data do not exclude, however, the role of other inflammatory mediators that may have a similar expression pattern as type I interferon.

Based on the observation that the effects of LPS were temporally dependent, we next determined whether TLR4 activation at other time points could prevent the establishment of chimerism in mice treated with costimulation blockade. Indeed, we found that mice treated with DST, anti-CD154 mAb and bone marrow did not become chimeric if LPS was given 1 day after bone marrow transplantation (0/7; **Figure 21**, group 4). This

suggests that costimulation blockade-based protocols for chimerism induction are sensitive to TLR-activation during the conditioning regimen, but not before.

FIGURE 21: KINETICS OF LPS ADMINISTRATION

Group	Recipient	Donor	TLR Agonist	Chimerism Frequency	% Donor-origin PBMCs (8 wk)
1	C57BL/6	BALB/c	None	6/7 (85.7%)	3.2 ± 1.84
2	C57BL/6	BALB/c	LPS (Day-8)	6/7 (85.7%)	4.93 ± 2.04
3	C57BL/6	BALB/c	LPS (Day -7)	0/7 (0%)*	<0.10
4	C57BL/6	BALB/c	LPS (Day +1)	0/7 (0%)*	<0.10

Figure 21. All mice were treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 50×10^6 allogeneic bone marrow cells. Mice treated with TLR agonist were given either an i.p. injection of 100ug LPS on day -8 (group 2), day -7 (group 3) or day +1 (group 4) relative to bone marrow cell transplantation. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.01 vs. group 1 and group 2 by chi-square analysis. N/A, not applicable.

8. *IFN-β is sufficient to prevent the establishment of mixed chimerism in mice treated with costimulation blockade*

Following the observation that: 1) signaling through the type I IFN receptor is important for LPS-mediated effects on establishment of bone marrow chimerism, 2) IFN-α/β production correlates with the window for which LPS treatment prevented chimerism induction, and 3) administration of recombinant IFN-β at the time of tolerance induction impairs the deletion of alloreactive T cells [204], we next postulated that administration of recombinant IFN-β would be sufficient to prevent the establishment of mixed chimerism. To determine this, we treated C57BL/6 mice with BALB/c bone marrow and skin grafts, and our standard conditioning regimen with or without 5.0×10^4 or 7.5×10^4 units of IFN-β on day -7. Only one of three mice treated with 5.0×10^4 units of IFN-β became chimeric, however, two of three mice did exhibit prolonged skin graft survival (MST >252; **Figure 22**, group 2). Mice treated with 7.5×10^4 units of IFN-β on day -7 uniformly failed to become chimeric (0/4; $p < 0.05$ vs. group 1), and skin survival was short (MST = 36; **Figure 22**, group 3). These data suggest that IFN-β by itself is a sufficient barrier to the establishment of chimerism and prolongation of skin allograft survival when given at the time of tolerance induction.

FIGURE 22: IFN-β IS SUFFICIENT TO PREVENT THE INDUCTION OF MIXED HEMATOPOIETIC CHIMERISM IN MICE TREATED WITH COSTIMULATION BLOCKADE

A

Group	Recipient	Donor	IFN-β	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	C57BL/6	BALB/c	None	6/10 (60.0%)	1.98 ± 2.34	>252	35	>252
2	C57BL/6	BALB/c	5.0×10^4 Units	1/3 (33.3%)	2.65	>252	181	>252
3	C57BL/6	BALB/c	7.5×10^4 Units	0/4 (0.00%)*	<0.10	36	36	N/A

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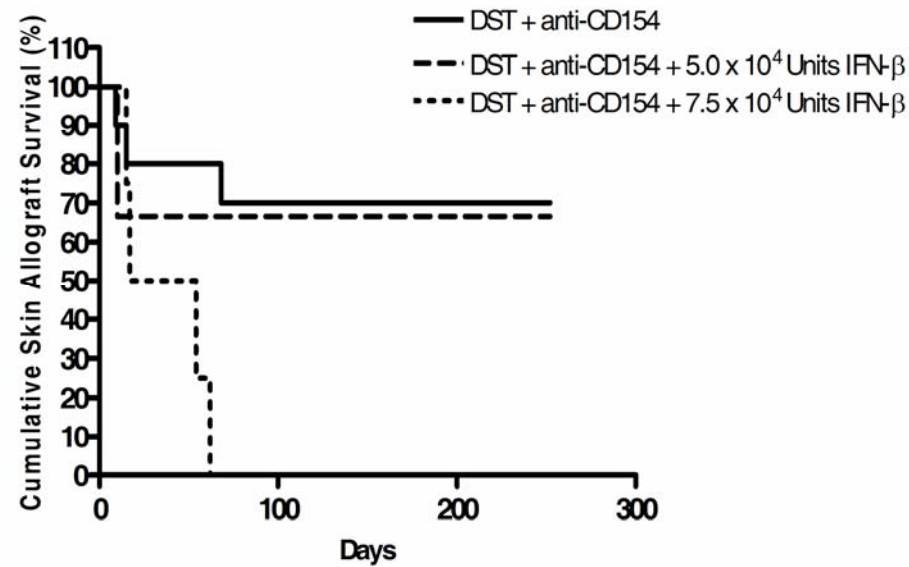
Figure 22. All mice were treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 50×10^6 allogeneic bone marrow cells and allogeneic skin grafts. Mice treated with recombinant IFN-β were given an i.p. injection of the indicated amount on day -7 relative to bone marrow cell and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.05 vs. group 1 by unpaired t-test. N/A − not applicable. Skin survival data have been separated into three groups to distinguish chimeric mice from non-chimeric mice in regards to skin MST. **Panel B**. Kaplan-Meier plots for allogeneic skin graft survival for the entire cohort of mice treated with costimulation blockade and transplanted with bone marrow and skin is shown (i.e., third column from the right in Panel A). p<0.05 for DST and anti-CD154 mAb vs. DST, anti-CD154 mAb and 7.5×10^4 Units IFN-β

9. Host IRF3 is not required for LPS to prevent the establishment of mixed hematopoietic chimerism in mice treated with costimulation blockade

The observation that type I interferon can pose a significant barrier to chimerism induction and allograft survival led us to investigate further the molecular mechanisms involved in LPS-mediated effects. We have previously shown that: 1) host signaling through the type I IFN receptor is important in LPS-mediated rejection, and 2) IFN-β itself can recapitulate the effects of TLR agonists. However, we have not demonstrated that type I IFN induction by LPS is required to prevent mixed chimerism. To investigate this, we examined the role of interferon regulatory factor (IRF)3. IRF3 has been shown to be essential for the induction of IFN-β in DCs following TLR4 activation [159,231]. Therefore, we treated C57BL/6.IRF3$^{-/-}$ mice with our standard conditioning regimen with or without LPS, and transplanted them with BALB/c bone marrow and skin. All of the IRF3$^{-/-}$ mice transplanted in the absence of LPS developed hematopoietic chimerism at levels slightly higher than wild-type mice (5.38 ± 3.45% donor-origin PBMCs at 8 weeks post transplant; $p<0.001$ vs. group 1), and exhibited prolonged skin graft survival (MST = 125 days; **Figure 23,** group 3). In contrast, none of the C57BL/6.IRF3$^{-/-}$ mice treated with LPS became chimeric ($p<0.001$ vs. group 3; **Figure 23**, group 4). Skin graft survival in C57BL/6.IRF3$^{-/-}$ mice treated with LPS was significantly shortened compared to C57BL/6.IRF3$^{-/-}$ mice not treated with LPS (MST = 20 days; $p<0.05$ vs. group 3; **Figure 23**, group 4). However, skin graft survival was prolonged as compared to wild-type mice treated with LPS ($p<0.01$). These data indicate that host IRF3 is not required for LPS to

mediate its effects in mice treated with costimulation blockade but IRF3 does seem to have a modest role in the kinetics of LPS-mediated skin graft rejection.

FIGURE 23: LPS EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE ARE NOT DEPENDENT ON HOST IRF3

A

| Group	Recipient	Donor	TLR Agonist	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	C57BL/6	BALB/c	None	29/45 (64.0%)	1.59 ± 1.81	144	88	>260
2	C57BL/6	BALB/c	LPS	0/23 (0.00%)*	<0.10	11	11	N/A
3	IRF3$^{-/-}$	BALB/c	None	8/8 (100.0%)	5.38 ± 3.45 [#]	125	N/A	125
4	IRF3$^{-/-}$	BALB/c	LPS	0/7 (0.00%)**	<0.10	20	20	N/A

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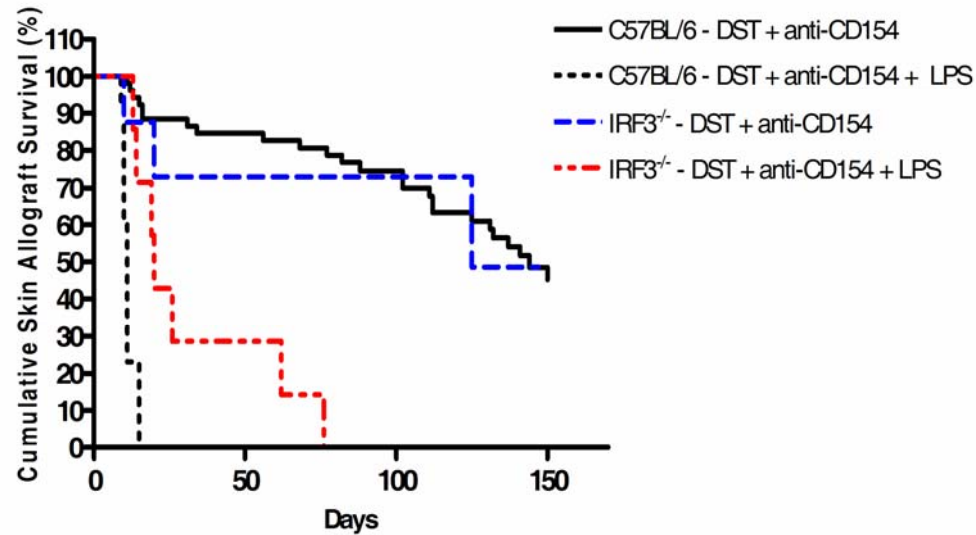
Figure 23. All mice were treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with 50×10^6 allogeneic bone marrow cells and allogeneic skin grafts. Mice treated with TLR agonist were given an i.p. injection of 100ug LPS on day -7 relative to bone marrow cell and skin transplantation. **Panel A**. Hematopoietic chimerism was defined as >0.10% donor-origin peripheral blood mononuclear cells (PBMCs) 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.0001 vs. group 1; **p<0.001 vs. group 3; and p=NS vs. group 3 by chi-square analysis. #p<0.001 vs. group 1 by unpaired t-test. N/A − not applicable. Skin survival data have been separated into three groups to distinguish chimeric mice from non-chimeric mice in regards to skin graft survival. Data from groups 1-2 shown in Figure 5 are reproduced here for ease of comparison with other treatment groups. **Panel B**. Kaplan-Meier plots for allogeneic skin graft survival for the entire cohort of mice conditioned and transplanted with bone marrow and skin is shown (i.e., third column from the right in Panel A). p<0.01 for IRF3$^{-/-}$ mice treated with DST, anti-CD154 mAb and LPS vs. wild-type mice treated with DST, anti-CD154 mAb and LPS; p=0.0158 for IRF3$^{-/-}$ mice treated with DST and anti-CD154 mAb vs. IRF3$^{-/-}$ mice treated with DST, anti-CD154 mAb and LPS.

10. Host MyD88 and IFN-α/β signaling are required for LPS to prevent the establishment of mixed hematopoietic chimerism in mice treated with costimulation blockade

The observation that IFNAR1$^{-/-}$ mice treated with LPS became chimeric at a frequency that was significantly lower than IFNAR1$^{-/-}$ mice not treated with LPS (**Figure 18**), led us to conclude that other molecules in the TLR4 pathway were important. We hypothesized that in the absence of IFN-α/β signaling, downstream mediators of the TLR4→MyD88 axis might be sufficient to prevent the establishment of chimerism with allogeneic bone marrow. However, we have previously shown that in the absence of host MyD88, bone marrow chimerism could not be established when LPS was given at the time of DST, presumably because in those mice the TLR4→IFN-β axis was intact. Therefore, to examine if in the absence of signaling through the type 1 IFN receptor that MyD88-dependent cytokines were important, we created mice that were deficient in both the adaptor molecule MyD88 and the type 1 IFN receptor. C57BL/6.MyD88$^{-/-}$ and C57BL/6.IFNAR1$^{-/-}$ mice were mated, and a F1 intercross was performed to isolate mice deficient in both molecules. MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were identified by PCR of ear DNA as described in the material and methods.

C57BL/6.MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were treated with our standard costimulation blockade protocol with or without co-injection of LPS, and transplanted with BALB/c bone marrow and skin. The majority of the MyD88$^{-/-}$IFNAR1$^{-/-}$ mice transplanted in the absence of LPS developed hematopoietic chimerism at levels slightly higher than wild-

type mice (5/7 developed chimerism; $4.13 \pm 0.98\%$ donor-origin PBMCs at 8 weeks post transplant; $p<0.05$ vs. group 1; **Figure 24A**, group 3), and exhibited prolonged skin graft survival (MST >116 days; **Figure 24A,** group 3). Similar to TLR4$^{-/-}$ mice, a cohort of MyD88$^{-/-}$IFNAR1$^{-/-}$ mice treated with LPS also developed mixed chimerism (5/8; $p<0.001$ vs. group 2, $p =$ NS vs. group 3), and displayed prolonged skin allograft survival (MST >116 days; **Figure 24A,** group 4). Importantly, in the absence of both proteins, mice not conditioned with costimulation blockade did not become chimeric (0/3), and rapidly rejected skin allografts (MST = 11). These data suggest that both signaling through the type I IFN receptor and mediators downstream of MyD88 are important for LPS-mediated effects in mice treated with costimulation blockade.

11. MyD88 and IFN-α/β signaling are required for the maturation of alloantigen-presenting DC following TLR4 ligation

We next hypothesized that in the absence of host MyD88 and signaling through the IFN receptor, LPS activation of TLR4 would not stimulate host alloantigen-presenting DCs to mature. To test this hypothesis, MyD88$^{-/-}$IFNAR1$^{-/-}$ mice were injected with CFSE-labeled BALB/c splenocytes and treated with anti-CD154 mAb with or without LPS injection. Sixteen hours after treatment, splenocytes were recovered and analyzed for the expression of H-2K^b and CD86. We observed that alloantigen-presenting DCs from mice deficient in both MyD88 and type I IFN signaling did not upregulate CD86 after injection of LPS (MFI of CD86 = 3,801 ± 384; n=5; p = NS vs. non-LPS-treated controls; **Figure 24D**). Interestingly, we did observe the LPS treatment did result in a slight increase in MHC class I expression in the absence of MyD88 and IFNAR (MFI of H-2K^b = 80,171 ± 14,105; n=5) compared to non LPS-treated MyD88$^{-/-}$IFNAR1$^{-/-}$ mice (p <0.05; **Figure 24C**). However, this level of expression was not different from tolerant, wild-type mice not treated with LPS. These data suggest that host DCs require both type I IFN signaling as well as the adaptor molecule MyD88 to upregulate costimulatory molecules following phagocytosis of alloantigen in the context of TLR4 activation. However modest increases in MHC class I may occur in the absence of either signaling pathway.

FIGURE 24: LPS EFFECTS ON BONE MARROW CHIMERISM AND SKIN ALLOGRAFT SURVIVAL IN MICE TREATED WITH COSTIMULATION BLOCKADE ARE DEPENDENT ON HOST MYD88 AND TYPE 1 IFN RECEPTOR EXPRESSION

A

Group	Recipient	Donor	TLR Agonist	Chimerism Frequency	% Donor-origin PBMCs (8 wk)	MST of skin grafts in transplanted mice (days)	MST of skin grafts in non-chimeric mice (days)	MST of skin grafts in chimeric mice (days)
1	C57BL/6	BALB/c	None	29/45 (64.0%)	1.59 ± 1.81	144	88	>260
2	C57BL/6	BALB/c	LPS	0/23 (0.00%)*	<0.10	11	11	N/A
3	MyD88$^{-/-}$IFNAR1$^{-/-}$	BALB/c	None	5/7 (71.4%)	4.13 ± 0.98 [#]	>116	50	>116
4	MyD88$^{-/-}$IFNAR1$^{-/-}$	BALB/c	LPS	5/8 (62.5%)**	5.37 ± 1.08 [#]	>116	57	>116

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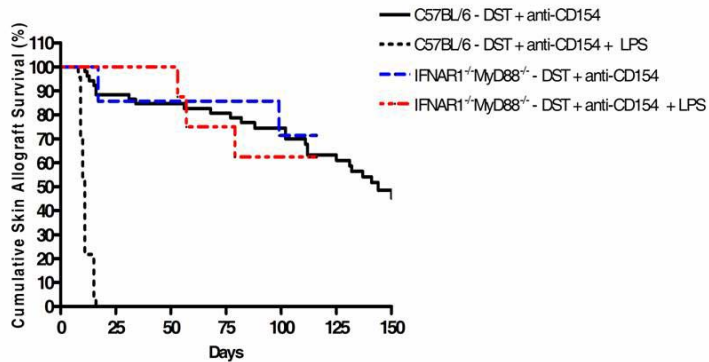
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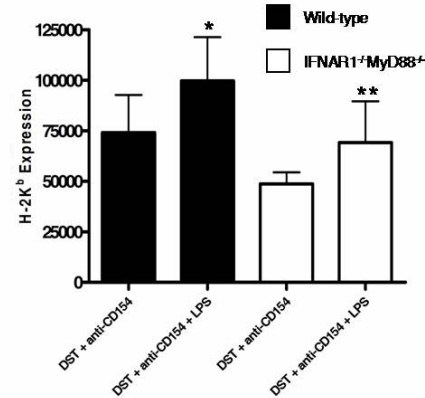
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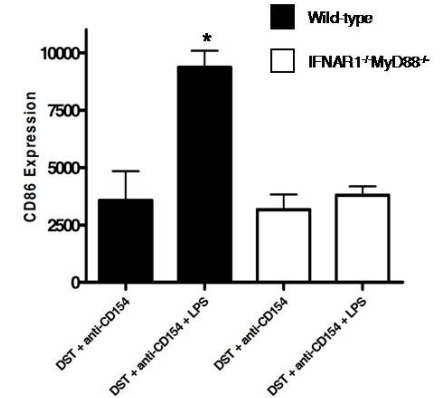
Figure 24. **Panels A-B.** All mice were treated with a donor-specific transfusion on day −7 and four injections of 0.5 mg of anti-CD154 mAb on days −7, −4, 0, and +4 relative to transplantation on day 0 with $50x10^6$ allogeneic bone marrow cells and allogeneic skin grafts. Mice treated with TLR agonist were given an i.p. injection of 100ug LPS on day -7 relative to bone marrow and skin transplantation. **Panel A:** Hematopoietic chimerism was defined as >0.10% donor-origin PBMCs 7-8 weeks after bone marrow injection. % donor-origin PBMCs is the mean ± S.D. chimerism levels in chimeric mice. *p<0.0001 vs. group 1; **p<0.001 vs. group 2 by chi-square analysis. #p<0.05 vs. group 1 by unpaired t-test. N/A – not applicable. Skin graft survival data has been separated into three groups to distinguish chimeric mice from non-chimeric mice. Data from groups 1-2 shown in Figure 5 are reproduced here for ease of comparison with other treatment groups. **Panel B:** Kaplan-Meier plots for allogeneic skin graft survival for the entire cohort of mice conditioned and transplanted with bone marrow and skin is shown (i.e., third column from the right in Panel A). p<0.01 for MyD88$^{-/-}$IFNAR1$^{-/-}$ mice treated with DST, anti-CD154 mAb and LPS vs. wild-type mice treated with DST, anti-CD154 mAb and LPS. **Panels C-D**: Maturation of host DCs in response to LPS treatment is dependent on signaling through the type 1 IFN receptor and host MyD88. Mice were injected with $10x10^6$ CFSE-labeled BALB/c DST, 0.5mg of anti-CD154 mAb and an i.p. injection of 100ug LPS. Sixteen hours later splenocytes were harvested, stained with Live/Dead blue, and antibodies to H-2K^b (host), CD8α, CD11c, and CD86 and analyzed by flow cytometry. Each group contains at least four mice. **Panel C:** Histogram of the MFI of the

class I molecule H-2K^b. *p<0.01 vs. DST and anti-CD154 mAb treated MyD88$^{-/-}$ IFNAR1$^{-/-}$ mice. **p<0.05 vs. DST and anti-CD154 mAb treated MyD88$^{-/-}$IFNAR1$^{-/-}$ mice. **Panel D**: Histogram of the MFI of CD86. *p<0.001 vs. all other groups.

Summary:

We have demonstrated that LPS treatment on the day of tolerance induction prevents the establishment of mixed chimerism and transplantation tolerance by signaling through the host TLR4 receptor. Signaling through the MyD88-independent pathway was sufficient to prevent the establishment of mixed hematopoietic chimerism. Administration of recombinant IFN-β was sufficient to recapitulate the effects of LPS on establishment of bone marrow chimerism and skin allograft survival. We then demonstrated that signaling through the host type I IFN receptor was important for LPS-mediated effects, although additional MyD88-dependent mediators were also involved. We further demonstrated that LPS treatment prior to costimulation blockade induction did not prevent the establishment of mixed chimerism or shorten skin allograft survival, but that administration of LPS the day after bone marrow transplantation did.

CHAPTER V: DISCUSSION

TLR Activation Prevents the Establishment of Mixed Hematopoietic Chimerism in Mice Treated with Costimulation Blockade

Hematopoietic mixed chimerism has proven to induce a robust form of transplantation tolerance. Conditioning regimens based on costimulation blockade to induce mixed chimerism provide a promising strategy while also being minimally toxic. However, costimulation blockade-based regimens to induce tolerance to solid organs have reduced effectiveness in the setting of perturbations that activate the TLR system, such as infection. We originally hypothesized that similar stimuli would reduce the efficacy of costimulation blockade-based protocols to establish mixed chimerism. In support of this hypothesis, we demonstrated that administration of agonists to TLR2 (Pam$_3$Cys), TLR3 (polyI:C), and TLR4 (LPS) on the day of tolerance induction universally prevented the establishment of mixed hematopoietic chimerism (**Figure 5**). As a readout for mixed chimerism-induced transplantation tolerance to solid organs, our protocol includes simultaneous grafting of donor-specific skin allografts. We observed that TLR activation on the first day of the conditioning regimen also shortened skin allograft survival (**Figure 5**).

TLR Activation Preventing the Establishment of Mixed Chimerism is Not Dependent on Production of Alloantibodies or on NK Cells

We first addressed the possibility that activation of TLRs at the start of tolerance induction may prevent bone marrow engraftment by activating B cells to produce alloantibodies. Donor-specific antibodies are a potent barrier to allograft survival

[232,233] and could be generated in response to direct stimulation of TLRs on B cells or via T cell help. Our observation that TLR-mediated rejection was not accompanied by a rise in alloantibodies (**Figure 6**) was somewhat surprising, given that TLR activation has been shown to both promote T-dependent B-cell responses [186] and abrogate costimulation blockade-induced T cell tolerance [108,133]. However, CD154-CD40 interactions have been demonstrated to be essential for T-dependent antibody responses [234-236], such as those generated against alloantigens [232]. Therefore, it is likely that despite receiving a stimulatory signal through TLRs, blocking CD154-CD40 interactions using anti-CD154 mAb precludes the generation of alloantibodies by B cells. This hypothesis is supported by recent work done by Chen and colleagues [108]. In a model where tolerance is induced with anti-CD154 mAb, they found that CpG-induced rejection of cardiac allografts was also independent of alloantibodies. Another possibility is that direct TLR activation of B cells may have resulted in the production of non-specific antibodies by a polyclonal population, and because our assay is specific for antibodies directed against alloantigens, an increase in overall immunoglobulin was not detected. In this population, antibodies with low-affinity to allogeneic antigens may have been present. However, without CD154-CD40 interaction, affinity maturation did not take place, and high-affinity alloantibody was not detected.

We then addressed the role that host natural killer cells had in TLR-mediated effects on establishment of hematopoietic chimerism. NK cells have a complicated function in transplantation. They are known to present a potent barrier to the establishment of hematopoietic chimerism; however, their role in solid organ acceptance appears to be

more complex. Although NK cells are cytotoxic to cells that do not express self MHC molecules [237], they are not sufficient to reject solid organs directly, as evidenced by the survival of allografts in recipients that lack T and B lymphocytes. Nonetheless, NK cells have been shown to promote allograft rejection by inducing inflammation early in the rejection process, which acts to facilitate adaptive immune responses [92,238]. Interestingly, despite their role as sentinels guarding against foreign insults, their function in promoting tolerance to allografts is only now being appreciated [239,240]. For example, NK cells were shown to facilitate tolerance induction by minimizing direct allorecognition through eliminating donor-derived APCs [240]. NK cells express various TLRs, and can be stimulated directly by TLR agonists [223,241], or indirectly via the secretions of cytokines produced by other TLR-stimulated cells such as macrophages [242]. Therefore, we hypothesized that TLR signaling would promote NK activation, and in effect raise the barrier to hematopoietic engraftment. In contrast, however, we observed that NK cells were not required for LPS or poly I:C to prevent the establishment of mixed chimerism (**Figure 7 and 8**). Despite not being required, TLR-activated NK cells could still be sufficient to prevent establishment of mixed chimerism. This possibility, however, was excluded because mixed chimerism could be established following administration of TLR4 agonists when CD4$^+$ and CD8$^+$ cells, but not NK cells were removed (**Figure 10**).

TLR Activation Breaks Tolerance in the CD4$^+$ and CD8$^+$ T Cell Compartments

We began to address the role that T cells have in preventing the establishment of mixed chimerism by examining the CD8$^+$ T cell compartment. Previously, our lab had

demonstrated that costimulation blockade induces the deletion of alloreactive CD8$^+$ T cells prior to transplantation [86]. This tolerance-promoting mechanism is compromised, however, by the administration of TLR agonists [133]. Here, we show that activation of TLR4 during costimulation blockade not only prevents the deletion of alloreactive CD8$^+$ T cells, but also results in the generation of effector CTLs with donor-specificity, as evidenced by *ex vivo* production of IFNγ upon restimulation with alloantigen (**Figure 11**). Although tolerance in the CTL compartment can be broken by TLR activation, we observed that removal of CD8$^+$ cells was not sufficient to establish mixed chimerism in the setting of TLR activation, suggesting that an additional barrier was present (**Figures 8 and 9**). We hypothesized that TLR activation abrogated costimulation blockade-induced tolerance in the CD4$^+$ compartment as well, and this would explain why removing CTLs was not enough to permit bone marrow engraftment. This was supported by the observation that TLR4 triggering did not prevent the establishment of mixed chimerism if CD4$^+$ and CD8$^+$ cells were both depleted (**Figure 10**). The fact that mixed hematopoietic chimerism could only be established in the absence of both T cell subsets indicates that either subset is sufficient to prevent mixed chimerism. This mechanism is interesting because it differs from what is observed with solid organ allografts such as skin [133]. This point will be discussed in more detail in a later section.

It is also important to note that in the absence of DST and antiCD154 mAb treatment, depletion of CD4$^+$ and CD8$^+$ cells was not sufficient to establish mixed chimerism in our system. We speculate that the requirement of costimulation blockade in this setting is due to the NK cell barrier to hematopoietic engraftment. In unpublished

data, we have found that DST and anti-CD154 mAb treatment significantly lowers NK cytotoxicity during the peri-transplant period, perhaps due to transient depletion of CD154^{+} NK cells [74]. However, in addition, costimulation blockade is also important to supplement T cell depletion strategies because it is effective at inhibiting the graft's immune response against the host [58,243]. In the absence of costimulation blockade, mixed allogeneic chimerism can result in graft versus host disease [243-245].

TLR Agonists As Adjuvants

We have shown that TLR-activation can abrogate costimulation blockade-induced tolerance in both CD4^{+} and CD8^{+} T cell pools. Nevertheless, the precise cell population(s) that is targeted by TLR agonists has not been identified. One possibility is that T cells, which do express certain TLRs, could be directly stimulated by TLR engagement. TLR activation could prevent tolerance induction either by directly activating effector cells, or by indirectly by inhibiting regulatory T cell activity. However, these possibilities seem unlikely to explain why T cell tolerance is broken upon LPS treatment for two reasons. First, effector CD4^{+} T cells do not express TLR4 [192], and not surprisingly, LPS treatment does not induce downstream molecules of the TLR4 pathway such as IkB and p38 in effector T cells [246]. Second, specific activation of TLR4 on regulatory T cells is reported to either enhance their suppressive capabilities [192], or have no effect on their phenotype [193,194]. For these reasons, TLR agonists likely activate alloreactive T cells indirectly by modulating other populations. We speculate that APCs are the most likely target of TLR activity.

The prevailing hypothesis for costimulation blockade-induced graft tolerance in our

system is that conditioning with a transfusion of donor cells in the anti-CD154 mAb-suppressed environment results in a preemptive state of hyporesponsiveness in the donor-reactive T cell pool prior to the transplantation of the immunogenic allograft. The state of hyporesponsiveness towards the graft is thought to be largely due to early abortive expansion that results in the deletion of the majority of the alloreactive T cell pool, and renders the remaining cells nonresponsive [85,86,189,247]. This process is thought to be initiated by the presentation of allopeptides via either the direct or indirect allorecognition pathways [77]. One hypothesis postulates that direct recognition of APCs in the DST by host alloreactive T cells leads to hyporesponsiveness because anti-CD154 mAb prevents adequate activation of the DST. Consequently, donor-reactive T cells do not receive the necessary costimulation required for full activation. Work from our lab has supported this hypothesis, as we have shown that down regulation of the costimulatory molecule CD80 on the DST was required for costimulation blockade-induced tolerance to islet allografts [225]. Using a transgenic model, in which B cells of the DST expressed CD80 under the control of the IgM promoter, it was shown that recipients of allogeneic islets could not be tolerized when cells of the DST expressed high levels of CD80. Others have shown a critical requirement for indirect presentation of alloantigen for tolerance induction [77,248]. This hypothesis contends that cells of the DST are quickly rendered apoptotic and phagocytosed by immature host DCs following transfer. Co-administration of anti-CD154 mAb prevents the maturation of the host alloantigen-presenting DCs and, consequently, alloreactive T cells are tolerized by a mechanism that closely mimics those used to maintain peripheral self-tolerance [249]. TLR activation at the time of DST

transfer could be interfering with either, or both, of these mechanisms. We addressed both of these possibilities experimentally, and these will be discussed in the next section.

Role of Direct Alloantigen Presentation

We observed that APCs in the DST upregulated the costimulatory molecules CD80 and CD86 in response to LPS administration (**Figure 12**). Therefore, it is possible that TLR activation increases the immunogenicity of the DST, which leads to alloreactive T cell priming and rescue from costimulation blockade-mediated apoptosis. TLR-mediated maturation of APCs within the DST could result from direct receptor engagement, or through the secretion of cytokines by TLR-stimulated cells of the host. We observed that mixed chimerism and long-term skin graft survival could be established in the presence of costimulation blockade and LPS when only the host was deficient in TLR4 (**Figure 15**). This would suggest that activation of APCs within the DST by direct ligand-receptor interaction is not sufficient to prevent tolerance induction. This does not exclude the possibility that in wild-type animals, the production of cytokines by endotoxin-stimulated host cells could activate APCs in the graft, which may be a sufficient barrier. However, previous work by our lab indicates that maturation of APCs in the DST may not be required for the effects of LPS on naïve allospecific T cells. Thornley *et al.* showed that expression of the costimulatory molecules CD80 and CD86 on the cells of DST was not required for LPS to prevent the deletion of alloreactive CD8$^+$ T cells in mice treated with costimulation blockade [204]. This suggests that: 1) there are either alternative costimulatory molecules expressed by APCs in the DST that prime donor-specific CTLs, or 2) antigen presentation by host APCs matured by direct TLR stimulation is playing an

important role.

TLR Activation Enhances Indirect Presentation

To address the effects of TLR activation on indirect allopresentation, we used a CFSE-labeling system that allowed us to track the DST and examine the phenotype of host DCs that had engulfed the transferred cells. We showed that activation of TLR2, 3, or 4 on the day of tolerance induction led to a rapid and marked upregulation of MHC class I and costimulatory molecules on host DCs that had phagocytosed CFSE-labeled DST (**Figure 14**). Therefore, we speculate that TLR activation on the day of tolerance induction significantly alters the context in which allopeptides are presented to the alloreactive T cell compartment. Instead of mimicking the mechanism of cross-tolerance to self-antigens and inducing transplantation tolerance, TLR-licensed APCs deliver an immunogenic signal to alloreactive T cells that prevents tolerization (**Figure 25**). This is supported by our data with mice deficient in various signaling molecules in the TLR4 pathway. We observed that the effects of LPS were lost in mice deficient in both MyD88 and the type I IFN receptor or TLR4. Chimerism was established in these mice in the presence of LPS, and interestingly, host alloantigen-presenting DCs in these mice did not upregulate costimulatory molecules. In contrast, activation of TLR4 did prevent tolerance induction in mice deficient in only MyD88 or the type I IFN receptor, and host alloantigen presenting DCs were matured to a significant extent. It is important to note that although mice deficient in type I IFN signaling did upregulate MHC class I and CD86 in response to LPS, these mice were partially protected, while the absence of MyD88 provided no protection. Host alloantigen presenting DCs in IFNAR1 knockout

mice may have been less mature, as their expression of the MHC class I molecule was decreased compared to LPS treated wild-type controls, whereas this was not seen with MyD88$^{-/-}$ mice. This could suggest that a critical threshold of costimulation is needed to activate alloreactive T cells, and that this threshold is reached more effectively in the absence of MyD88 than in the absence of signaling through the type 1 IFN receptor. It is also possible that the reason for this difference depends on other mechanisms, independent of allopresentation by host DCs. We will discuss these possibilities in the next section.

FIGURE 25: TLR-LICENSED APCS ACTIVATE ALLOREACTIVE T CELLS
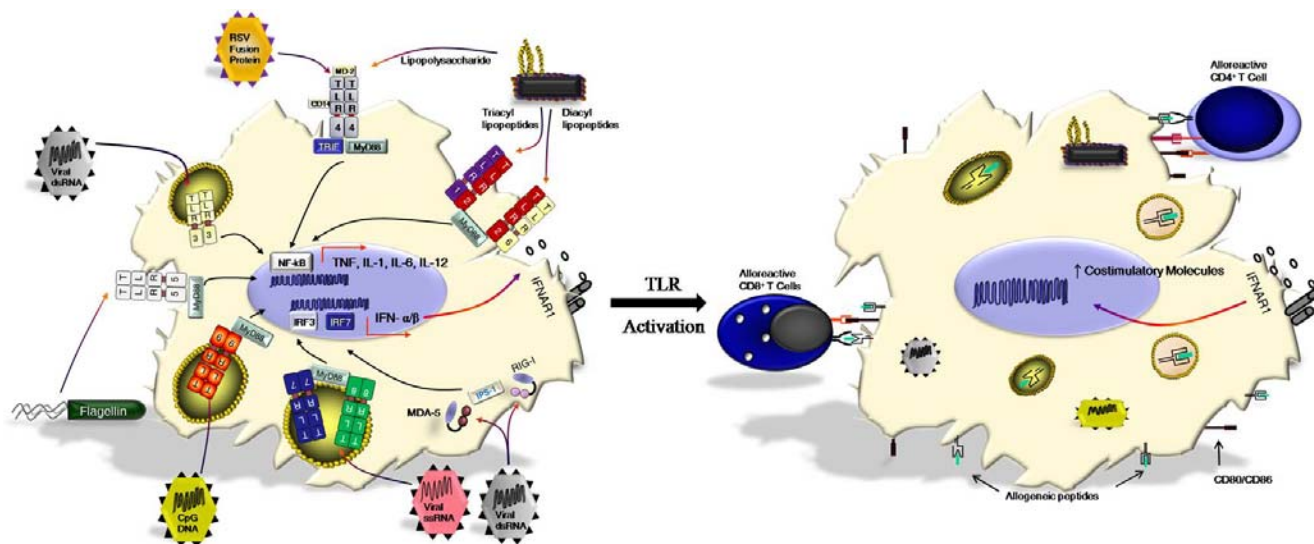
Figure 25: Upon TLR activation, APCs produce inflammatory cytokines such as IFN-α/β, TNF-α and IL-6. These cytokines activate alloantigen-processing APCs in a paracrine or autocrine fashion to upregulate MHC class I and II, as well as costimulatory molecules, such as CD80 and CD86. The heightened expression of costimulatory molecules elicits the proliferation and differentiation of alloreactive T cells, in effect circumventing the effects of costimulation blockade.

Role of Regulatory T Cells

In addition to enhancing the maturation of alloantigen-presenting DCs, TLR activation may prevent tolerance induction by inducing cytokines on accessory cells that feedback onto T cells. In addition to providing stimulatory cytokines to effector T cells (Teff), TLR-induced mediators may modulate Teff – Treg interactions. Although we did not address this experimentally, regulatory T cells are thought to have a vital role in tolerance induction [90,189], and thus we turn our discussion briefly to this possibility. TLR activation could impair regulation via several mechanisms. First, TLR-induced cytokines could act in *trans* to render alloreactive effector T cells refractory to Treg-mediated suppression (**Figure 26**). This idea is supported by work from Pasare *et al*. demonstrating that ligation of TLRs on DCs enabled effector T cells to overcome suppression by $CD4^+CD25^+$ regulatory cells [198]. They reported that secretion of soluble mediators (principally IL-6) by TLR-activated DCs blocked the suppressive effect of Tregs, permitting activation of antigen-specific T cells in the presence of regulatory T cells.

Second, engagement of TLRs could also prevent the differentiation of regulatory T cells through the upregulation of cytokines that affect naïve T cell differentiation. Studies have shown that $CD4^+$ T cells develop a $FoxP3^+$ regulatory T cell phenotype when they are activated in the presence of TGF-β [250]. However, when $CD4^+$ T cells are activated in the presence of TGF-β and IL-6, this regulatory phenotype is suppressed and the cells develop a pro-inflammatory TH17 cell phenotype [251]. Therefore, TLR activation may

precipitate allograft rejection by preventing the generation of Tregs following costimulation blockade and instead favor development of pro-inflammatory effector T cells (**Figure 26**).

Third, TLR signals could impair the recruitment of regulatory T cells to the allograft. This is supported by recent data demonstrating that activation of TLR2 or TLR9 prevented the anti-CD154 mAb-mediated induction of Treg-associated chemokines in cardiac allografts, and decreased accumulation of intra-graft Tregs [108] (**Figure 27A**).

Finally, direct activation of TLR2 on Tregs has been shown to induce expansion of regulatory T cells, while simultaneously inhibiting their suppressive capability [194] (**Figure 26A**). This mechanism may explain why Pam_3Cys, but not LPS or poly I:C, was able to completely prevent tolerance induction in mice deficient in signaling through the type 1 IFN receptor (**Figure 18**). Since LPS and poly I:C cannot directly inhibit Treg function, they may require type I IFN for their adjuvanticity. Pam_3Cys, in contrast, may not require signaling through the type 1 IFN receptor to prevent tolerance, because it can directly inhibit regulatory T cells from suppressing alloreactive effectors.

Hence, TLR activation may trigger allograft rejection by compromising key regulatory mechanisms such as preventing the recruitment of Tregs to the site of the allograft, precluding the generation of regulatory T cells by costimulation blockade, enabling alloreactive T cells to escape Treg-mediated suppression, as well as by directly inhibiting the suppressive capability of regulatory cells. Consequently, we propose that activation of the TLR system by environmental perturbations prevents chimerism induction by breaking T cell tolerance via a multimodal mechanism that involves both the

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licensing of alloantigen-presenting DCs and the impairment of regulatory mechanisms.

FIGURE 26: TLR ACTIVATION ABROGATES REGULATORY MECHANISMS
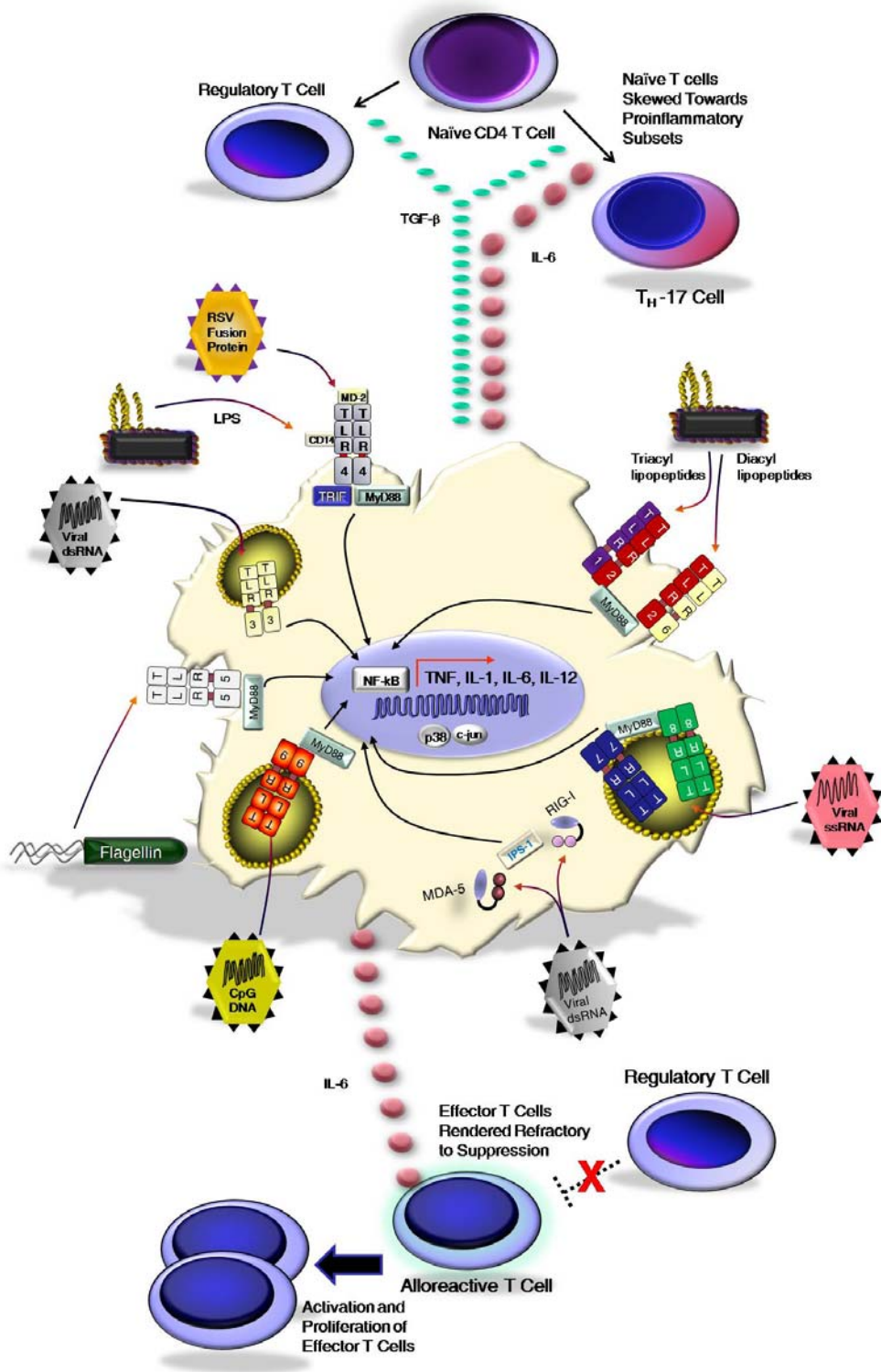
Figure 26: Regulatory T cells play a crucial role in transplantation tolerance to allogeneic organs. Regulatory mechanisms that prevent immune attack on allogeneic tissues may be compromised in the setting of TLR activation by several mechanisms. Release of inflammatory cytokines by TLR-activated cells can prevent the differentiation of uncommitted naïve CD4$^+$ T cells into Tregs. Naïve CD4$^+$ T cells can differentiate into regulatory T cells in the presence of TGF-β. However, in the presence of TGF-β and proinflammatory cytokines such as IL-6, and perhaps IL-21, naïve T cells can be skewed to turn into effector T cells such as the IL-17-producing TH17 cells. In a separate mechanism, release of cytokines such as IL-6 by activated APCs can render alloreactive effector cells refractory to suppression by regulatory T cells.

FIGURE 27: TLR ACTIVATION INHIBITS T$_{REG}$ RECRUITMENT AND T$_{REG}$-MEDIATED SUPPRESSION
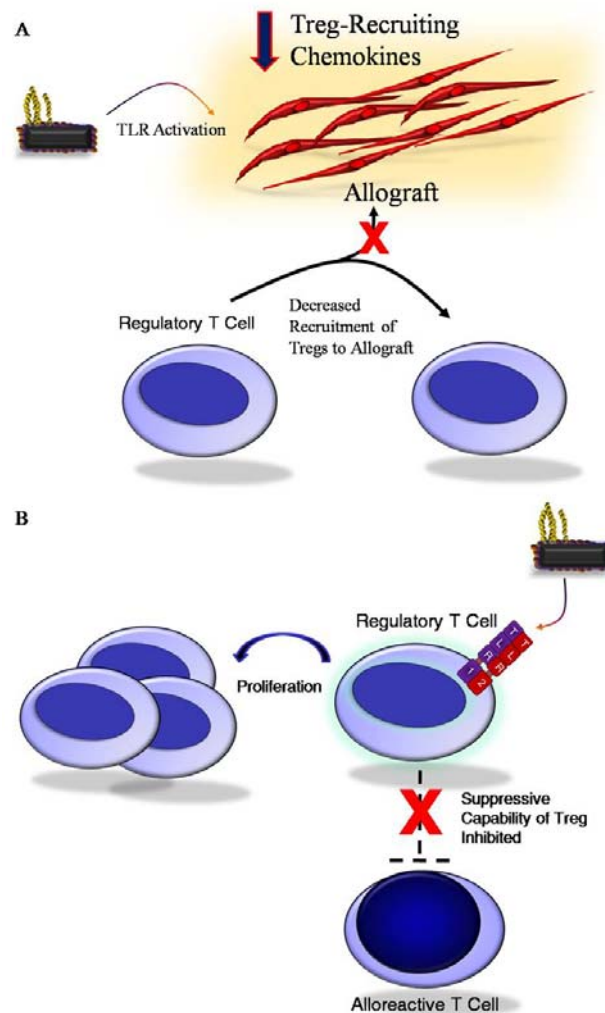
Figure 27: Regulatory T cells may be affected by TLR activation by two additional mechanisms. **Panel A**: TLR activation may decrease the recruitment Tregs to the allograft by decreasing the expression of Treg-recruiting chemokines such as CCL17 and CCL21. **Panel B**: Direct activation of certain TLRs, such as TLR2, can induce proliferation of regulatory T cells, while simultaneously inhibiting their suppressive phenotype.

Role of TLR Signaling Pathways

In order to identify additional therapeutic targets to improve the efficacy of costimulation blockade-based chimerism induction protocols in the setting of TLR perturbation, we examined the molecules involved in the TLR signaling cascade. In doing so, we observed a critical role for both the adaptor molecule MyD88 and for the type I IFN receptor. These observations support a bimodal mechanism whereby ligation of TLRs impairs tolerance induction by both maturating dendritic cells and inhibiting regulatory cells. They also highlight the idea that different mechanisms may underlie costimulation blockade-induced transplantation tolerance to solid organ allografts and those that establish mixed chimerism. We will discuss both concepts in this section.

Role of the MyD88-dependent and MyD88-independent Pathways

The adjuvant effects of LPS are due chiefly to the production of inflammatory cytokines and the maturation of antigen-presenting cells. Cytokine response to TLR4 engagement is almost completely dependent on MyD88, while DC maturation is thought to be principally regulated by the MyD88-independent pathway [252,253]. Similarly, we found that in the absence of host MyD88, LPS treatment increased the expression of the costimulatory molecule CD86 more than 2-fold on host cells that had phagocytosed alloantigens (**Figure 17**). We also documented that recipients deficient in MyD88 could not become chimeric following costimulation blockade conditioning if they were exposed to LPS (**Figure 17**). This would suggest that in the absence of TLR-mediated cytokines, enhanced presentation of allopeptides via the indirect pathway might be sufficient to prevent the establishment of mixed chimerism.

The upregulation of costimulatory molecules following LPS treatment has been shown to be dependent on the adaptor molecule TRIF and the downstream effector type I IFN [253]. Therefore, we hypothesized that the TLR4→TRIF→ IFN-α/β axis would be important for the effect of LPS. In support of this hypothesis, we observed that mice treated with costimulation blockade but lacking the ability to signal through the type I IFN receptor did develop mixed chimerism even in the presence of LPS or polyI:C (**Figure 18**). We also observed a decrease in the number of activated alloreactive CTLs in costimulation blockade and LPS–treated IFNAR1$^{-/-}$ mice compared to wild-type controls, suggesting that type I IFN is important in preventing tolerance in the alloreactive CD8$^+$ T cell compartment (**Figure 19**). These observations correlated with an reduced expression of costimulatory molecules host alloantigen presenting DCs in response to TLR4 agonists (**Figure 18**). Thus, these data are consistent with the idea that mice null for the type 1 IFN receptor are more resistant to the tolerance-abrogating effects of LPS stimulation due to the incomplete maturation of their DCs, which phagocystose and present alloantigen to activate allospecific T cells.

In further support of the role of type I IFNs, we demonstrated that administration of recombinant IFN-β at the start of the conditioning regimen was sufficient to prevent the establishment of mixed chimerism (**Figure 22**). The effect of IFN-β appeared to be dose-dependent, as 5 x 10^4 units/mouse prevented the establishment of chimerism in two thirds of the mice, while 7.5 x 10^4 units/mouse prevented the establishment of mixed chimerism in all of the mice treated (**Figure 22**). Given that type I IFN can directly stimulate APCs to increase expression of costimulatory molecules [253-255], our observations with

recombinant IFN-β are consistent with a mechanism the involves type 1 IFN-dependent upregulation of costimulatory molecules on host APCs. However, it does not exclude the possibility that other mechanisms are involved. Type 1 IFN can affect immune responses in a variety of ways. For example, in addition to directly stimulating upregulation of costimulatory molecules on APCs, type 1 IFN can also enhance cross-presentation of antigen by DCs to CD8$^+$ T cells, which could facilitate priming of alloreactive CTLs [256]. Type I IFN can also directly act on T cells and provide a signal for clonal expansion and proliferation during T cell activation [257]. This role in naïve T cell activation could help explain why mice deficient in type I IFN signaling were partially protected from the effects of LPS, but MyD88$^{-/-}$ mice were not.

Given the important role of type 1 IFN, we were somewhat surprised to find that mice deficient in IRF3 were not protected from the effects of LPS. We had originally hypothesized that because IRF3 is essential for the induction of IFN-β following TLR4 activation with LPS [231], mice lacking IRF3 would be resistant to the effects of LPS, similar to IFNAR1$^{-/-}$ mice. Interestingly, IRF3$^{-/-}$ mice treated with costimulation blockade and LPS uniformly failed to become chimeric (**Figure 23**). Therefore, it appears that type 1 IFN induction by host cells is not required for LPS to prevent the establishment of mixed chimerism. However, the cells of the DST did have functional IRF3; thus, it is possible that type 1 IFN production by donor cells in response to LPS was able to act in *trans* on host cells, which unlike IFNAR1$^{-/-}$ mice, were able to respond to type I IFN.

Interestingly, although we observed that mice deficient in the type 1 IFN receptor became chimeric when exposed to LPS at the start of the conditioning regimen, the

frequency of chimerism was statistically different from IFNAR1$^{-/-}$ mice not given LPS (**Figure 18**). This suggested that an additional mediator was acting synergistically with type 1 IFN to prevent mixed chimerism. We demonstrated, using mice deficient in both MyD88 and IFNAR1, that the additional factor was MyD88 dependent. We observed that MyD88$^{-/-}$IFNAR1$^{-/-}$ mice treated with costimulation blockade and LPS exhibited similar frequencies of mixed chimerism as their non LPS-treated controls (**Figure 24**). We also observed that in the absence of both proteins, host alloantigen-presenting cells did not upregulate the costimulatory molecule CD86 in response to LPS (**Figure 24**). These data indicate that a mediator(s) downstream of MyD88 acts synergistically with type 1 IFN to prevent tolerance induction. Our lab has observed that IL-12 (**Figure 16** and [133]) and TNF-α [133] are not required for the effects of LPS on tolerance induction, making these cytokines unlikely candidates. IL-6, however, remains an attractive possibility that we have not yet examined. As described earlier, IL-6 has been shown to impair the induction of regulatory T cells, decrease their recruitment to the site of the graft, and render effector T cells refractory to the effects of Treg-mediated suppression. An important caveat to our observation in MyD88 and IFNAR1 double knockout mice is that MyD88 is also crucial for the signaling downstream of the IL-1 and IL-18 receptors [214,258]. Therefore, the additional mediator(s) could be extrinsic to the TLR4→MyD88 axis.

Different Mechanisms May Control Tolerance to Hematopoietic and Solid-Organ Allografts

There is mounting evidence that different tissues vary in their susceptibility to tolerance induction by costimulation blockade. For example, survival of cardiac and islet

allografts can be significantly prolonged with anti-CD154 mAb monotherapy, whereas skin allografts cannot [78,79,84]. Data also suggest that the mechanisms that control the abrogation of tolerance induction in the context of TLR activation may differ between tissues as well [108,133]. Our studies with mixed chimerism induction appear to extend this observation. For example, our laboratory documented previously that when transplanted alone, skin allograft survival could be prolonged with costimulation blockade when LPS was co-administered on the first day of the conditioning regimen if the host lacked either MyD88 or IFNAR1 [204]. This indicates that a synergy between the MyD88-dependent and the MyD88-independent pathways are required to prevent tolerance to skin allografts. In contrast, we observed that a synergy was not required, as either pathway could contribute to prevent the establishment of mixed chimerism. This suggests that the mechanisms to induce tolerance to hematopoietic grafts are more sensitive to environmental perturbations than are those required to prolong solid organ grafts such as skin allografts.

Our examination of the cellular mechanisms revealed similar differences between hematopoietic and solid organ grafts. Previously, our laboratory has shown that the abrogation of costimulation blockade-induced tolerance to skin allografts following LPS treatment was solely dependent on host $CD8^+$ cells [133], while another group found that CpG-mediated rejection of cardiac allografts was solely dependent on host $CD4^+$ T cells [108]. Here we show the neither population is the sole mediator of the effects of LPS, as either subset is sufficient to prevent the establishment of mixed chimerism. Given that more immunosuppression is required for the induction of mixed chimerism by

costimulation blockade in the setting of TLR signaling, these data suggest further that the barrier to hematopoietic cell engraftment is higher than the barrier to solid-organ acceptance.

Many explanations have been proposed to account for the differences in tissue susceptibility to allograft rejection. These include differences in the immunogenicity of the tissue, the presence of tissue-specific antigens in the graft, and differences in the susceptibility of various tissues to T cell-mediated rejection [191,259]. Reasons to account for the differences in the susceptibility to rejection of various tissues in the context of TLR activation are not known. One reason that hematopoietic allografts can be acutely rejected in the absence of CTLs following TLR ligation, but skin allografts cannot, could reflect a differential susceptibility to helper T cell effector mechanisms. For example, activated CD4$^+$ T cells may contribute to the acute rejection of hematopoietic grafts by secreting IL-2 to enhance NK cytotoxicity. NK cells are potent killers of allogeneic hematopoietic cells, but are not effectively cytolytic towards solid allografts [92].

Another explanation could be that hematopoietic grafts, unlike solid organ grafts, must not only evade the host's immune system, but also home to a particular niche in the recipient. In order for long-term mixed chimerism to develop, pluripotent hematopoietic stem cells must engraft in specific niches in the bone marrow. Recent work has shown that migration of hematopoietic stem cells is affected by fluctuations in soluble mediators such as CXCL12, which can be modulated by stressors such as LPS [260]. Interestingly, activated macrophages have been shown to produce noradrenaline [261], a substance that

can potentially inhibit hematopoietic stem cell homing to the bone marrow by decreasing CXCL12 expression in bone marrow stromal cells [260]. Therefore, activation of macrophages via direct LPS stimulation or via CD4^{+} T cell licensing may increase the barrier to hematopoietic cell engraftment by precluding donor hematopoietic stem cells from establishing a niche in the recipient's bone marrow. Therefore, although the precise mechanisms that distinguish the host's response to hematopoietic and solid organ allografts in the setting of TLR activation are not known, it does appear that the barrier to mixed chimerism is higher than that of solid-organ allografts.

TLR-Activation After Transplantation

We have until now focused our discussion solely on the mechanisms governing the establishment of mixed chimerism and the effects following administration of TLR agonists at the start of costimulation blockade. However, stimuli that activate the TLR system, such as infection, may occur at any time during the peri-transplant period. It has been shown that skin allograft survival in mice treated with costimulation blockade can be impaired by viral infection the day after transplantation [107]. Similarly, we observed that LPS treatment 1 day after bone marrow transplantation completely prevented the establishment of mixed chimerism (**Figure 21**). Although we did not experimentally address the mechanisms involved, several are possible.

Similar to TLR-activation at the start of costimulation blockade, LPS treatment on day +1 could also affect regulatory mechanisms or disrupt homing of hematopoietic stem cells to the stem cell niche. LPS treatment the day after transplantation may also raise the barrier to hematopoietic cell engraftment by activating host natural killer cells. Long-term

stable chimerism in mixed allogeneic chimeras suggests that donor and host NK cells are mutually tolerant. In fact several studies have shown that NK cell tolerance does develop in mixed allogeneic chimeras [220,262]. Proposed mechanisms include modulation of NK activating and inhibitory receptors, clonal deletion of donor and host-reactive NK cells, and anergy [263]. The kinetics involved in these mechanisms have not been fully elucidated; however, it appears that NK cell tolerance to donor cells may not develop within 24 hours of transplantation. Treatment with LPS the day after bone marrow transfer prevents the establishment of mixed chimerism, perhaps by directly activating NK cells to kill the donor hematopoietic stem cells. TLR activation could also raise the NK cell barrier indirectly by inhibiting Tregs. Recent evidence suggests that $CD4^+CD25^+$ Tregs can modulate NK cell function *in vivo*. Barao *et al.* demonstrated that the adoptive transfer of Tregs could prevent NK cell-mediated rejection of allogeneic bone marrow via a TGF-β-dependent mechanism [264]. It therefore possible, that TLR activation immediately following bone marrow transplantation could prevent engraftment directly by activating NK cells, or indirectly by inhibiting Treg-mediated regulation of both T cells and NK cells.

It is also interesting to note that a discord exists between tolerance induction to allogeneic hematopoietic cells and skin in regards to TLR activation the day after transplantation. Previously, our lab showed that poly I:C treatment one day after skin allograft transplantation does not prevent costimulation blockade-induced allograft survival [107]. Similarly, in ongoing experiments in our laboratory, we have observed that costimulation blockade-conditioned mice simultaneously transplanted with

allogeneic bone marrow and skin that have been treated with LPS on day +1 do not become chimeric, but do not acutely reject their skin grafts. This observation further supports the idea that hematopoietic and solid organ allografts in mice treated with costimulation blockade differ in their susceptibility to TLR-mediated effects.

Targets for Therapeutic Intervention

The ultimate objective of our research is to create a conditioning regimen that is both maximally effective at establishing mixed allogeneic chimerism and transplantation tolerance, and minimally toxic to the recipient. We have shown that our costimulation blockade-based protocol is sensitive to a variety of environmental perturbants that could be encountered in the clinic. Consequently, we turn our discussion towards potential strategies to improve its efficacy.

We have used a reductionist approach to study the effect individual microbial components have on costimulation blockade. However, it is likely that during clinical transplantation, many stimuli will be present and, consequently, multiple pathways will be activated. For example, transplantation of colonized tissues such as skin will introduce multiple pathogen associated molecular patterns, but it could also induce danger associated molecular patterns (DAMPs), such as heat-shock proteins, secondary to tissue injury. Any bacteria present on the allograft will activate multiple receptors, which as we have seen, can reduce the efficacy of costimulation blockade via distinct mechanisms. It is also probable that the timing of the insult, in addition to its nature, will prove critical. As we have proposed, perturbations that occur following transplantation may operate through distinct mechanisms. Given the pleiotropic nature of the challenge, it is likely

that multiple targets will need to be addressed.

One possibility would be to augment the conditioning regimen with depleting antibodies directed against specific immune populations. We have demonstrated that in the absence CD4$^+$ and CD8$^+$ cells, mixed chimerism could be established in the presence of LPS treatment. Therefore, the costimulation blockade protocol could be supplemented with a short course of depleting antibodies directed against these subsets. Although the transient removal of T cells is undoubtedly preferable to a lifetime of chronic immunosuppression, even a temporary loss of T cells may put the patient at risk of opportunistic infection. Therefore, targeting components of the TLR system may be a more favorable approach.

The increased awareness of the role of TLRs in a variety of pathological conditions has generated a deep interest in the development of compounds that contravene the TLR system. By combining compounds that inhibit both cell surface receptors and downstream molecules, it may be possible to buttress the tolerance-promoting effects of costimulation blockade, without significantly increasing the risk to the patient. We will now turn our discussion to a few potential candidates.

Receptor Inhibitors

Given the indispensible role of host TLR4 in the LPS-mediated rejection of allogeneic hematopoietic cells, specific antagonists of the LPS receptor may work as a successful adjunct to costimulation blockade. The synthetic lipid A analog eritoran (E-5564), a potent antagonist of TLR4, has emerged an interesting candidate (**Table 3**). It is

currently being investigated for its potential therapeutic use in gram-negative bacterial sepsis, and has also shown efficacy at mitigating the inflammatory response to cardiac ischemia-reperfusion injury [265-267]. Eritoran infusion into healthy patients was demonstrated to inhibit the TNF-α response to LPS *ex vivo* [268], verifying both its safety and efficacy in humans. Antibodies directed against TLR4 [269] and the TLR4/MD-2 complex [270] have also been demonstrated to be effective at blunting the immune response to LPS.

TLR4 can also be indirectly targeted through administration of the secosteroid hormone vitamin D3, or the NADPH oxidase inhibitor diphenylene iodonium (DPI). Treatment of monocytes with 1α,25-dihydrocholecalciferol (Vitamin D3) was shown to significantly impair LPS-induced TNF-α production and NF-κB translocation [271]. Endotoxin hyporesponsiveness was attributed to a vitamin D receptor-dependent downregulation of TLR4 [271]. In contrast, DPI was shown to induce hyporesponsiveness to LPS by impairing the recruitment of TLR4 to lipid rafts, an early event in the immune cascade [272]. The inhibition of NADPH-oxidase by DPI prevented the generation of reactive oxygen species (ROS), a critical step in the trafficking of TLR4 to membrane rafts [272].

Blockade of the LPS-binding protein CD14 has also shown efficacy at reducing the pathophysiological effects of LPS in animal models [273,274]. Treatment with the anti-CD14 mAb IC14 was shown to be well-tolerated and attenuated the LPS-response in a small subset of patients [275]. However, a recent phase I clinical trial for patients with severe sepsis did not reveal any statistical differences in clinical outcome between

patients treated with IC14 mAb or placebo [276]. Hence, there are multiple strategies under investigation that attenuate TLR4 activation, including competitive inhibitors such as lipid A analogs and monoclonal antibodies, as well as indirect inhibitors that can either prevent TLR4 expression or trafficking to the membrane.

Although we did not directly implicate the role of TLR7 and TLR9 in our model, activation of TLR9 has been shown to prevent costimulation blockade-mediated tolerance to cardiac and skin allografts [108,133]. In all likelihood, agonism at these receptors would also prevent tolerance to allogeneic hematopoietic cells. Immunoregulatory sequences (IRS) with specificity for TLR7 and 9 have been demonstrated to inhibit IFN-α production by plasmacytoid DCs, and to ameliorate symptoms of disease in an animal model of lupus [277,278]. Given the critical role of type I IFN in our system, these oligonucleotide-based inhibitors may also be beneficial adjuncts.

Signaling Inhibitors

Targeting each individual receptor of the TLR system concomitantly as a therapeutic strategy could prove untenable; consequently, utilizing therapies that inhibit molecules common to multiple receptors might be an alternative approach. The indispensable role of MyD88 in the signaling of many receptors makes it an attractive target for inhibition. Several compounds have been shown to effectively inhibit MyD88-dependent signaling (**Table 3**). The synthetic peptide-mimic ST2825 disrupts the homotypic interactions of the TIR domains of MyD88, preventing the adaptor from dimerizing [279]. Consequently, the compound interferes with recruitment of IRAK-1 and IRAK-4 to MyD88, and oral administration of the peptide has been demonstrated to inhibit MyD88-

depenedent production of IL-6 in mice [279]. The TIR family member ST2 has also shown to inhibit TLR4 signaling by sequestering MyD88 and the adaptor Mal [280].

Inhibitors of molecules downstream of MyD88 may also be useful. The polyphenol component of green tea, (-)-epigallocatechin-3-gallate (EGCG), is a well known anti-inflammatory and chemoprotective agent, and has also been shown to attenuate LPS-induced TNF-α production and abrogate endotoxin-mediated lethality in mice [281]. The protective effects were partly due to inhibition of IKK, a key activator of NF-κB downstream of MyD88 [282]. Importantly, EGCG was also shown to inhibit TBK1, a kinase downstream of TRIF and IPS-1 [283]. TBK1 is critical for the activation of IRF3 and IFR7, and the subsequent induction of type 1 IFN. Given the important role for type I IFN in preventing of the establishment of mixed chimerism in mice treated with costimulation blockade, targeting molecules upstream of type 1 IFNs may also prove useful. In addition to EGCG, the polyphenol component of grapes 3,4',5-trihydroxy-*trans*-stilbene (resveratrol), has potent anti-inflammatory properties [284] that have been attributed to its inhibition of TBK1 and RIP1 [283]. Hence, these polyphenolic compounds, or more powerful derivatives of them, may prove useful alone, or in concert with other compounds that can inhibit TLR signaling.

Although the aforementioned agents may be useful at improving the efficacy of protocols based on costimulation blockade, they may also significantly weaken the patient's ability to defend against infection. Therefore, the risk/benefit ratio of their use must be carefully weighed.

TABLE 3: OVERVIEW OF TLR-TARGETED THERAPEUTICS

Compound	Target	Comments
Eritoran	TLR4	Synthetic Lipid A analog [285]
5E3	TLR4/MD-2	mAb directed against TLR4/MD-2 complex [270]
15C1	TLR4	mAb directed against TLR4 [269]
Vitamin D3	TLR2 & 4	Downregulates expression of TLR2 and TLR4 [271]
Diphenylene iodonium	NADPH Oxidase	Impairs ligand-induced recruitment of TLR4 to lipid rafts by inhibiting reactive oxygen species formation [272]
IC14	CD14	mAb directed against CD14 [275]
IRS945	TLR7 & 9	Immunoregulatory sequence antagonizes TLR7 and TLR9 [278]
ST2825	MyD88	Prevents dimerization of TIR domains [279]
A46R & A52R	MyD88	Open-reading frames of vaccinia virus inhibited MyD88-dependent signaling [286]
ST2	MyD88 and Mal	TIR-family member inhibits TLR4 signaling by sequestering MyD88 and Mal [280]
EGCG	TBK1& IKK	Polyphenol component of green tea inhibits TBK1 [283] and IKK [282]
Resveratrol	TBK1 & RIP1	Polyphenol component of grapes inhibits TBK1 and RIP1 [283]

Table 3: A table of selected compounds with known effects on the TLR system that may be useful as adjuncts to costimulation blockade-based regimens for tolerance induction. EGCG, (-)-epigallocatechin-3-gallate. IKK, IκB kinase. MAL, MyD88-adaptor-like. MyD88, myeloid differentiation primary response gene-88. RIP1, receptor-interacting protein 1.TBK-1, TANK-binding kinase 1.

Conclusions and Future Directions

Hematopoietic mixed chimerism can be used to induce a robust form of transplantation tolerance to a variety of allografts. Our laboratory has demonstrated that mixed hematopoietic chimerism can be established using a minimally toxic conditioning regimen based on costimulation blockade. In this dissertation, we have shown that the efficacy of the induction protocol can be significantly affected by perturbations that activate toll-like receptors. It appears that perturbations at the start of the regimen, as well as immediately following transplantation, can abrogate the establishment of hematopoietic chimerism. We have identified a crucial role for host alloreactive CD4^{+} and CD8^{+} cells, the adaptor molecule MyD88 and type I IFNs, but not for IL-12 or IRF3 following activation of TLR4 by LPS. Our research has highlighted several potential cellular and molecular targets to improve the effectiveness of costimulation blockade-based protocols for the induction of mixed chimerism. However, the precise cellular target(s) of TLR agonists have not been identified. Demonstrating a more precise role for host APCs, as well as the function of regulatory T cells following TLR activation in our system will be important. In addition, further research needs to be performed with additional microbial components, as we have evidence that distinct PAMPs operate through distinct mechanisms. Therefore, to extrapolate our results to setting of the clinic, additional studies combining agonists to simulate an actual infection or direct study of microbes or viruses will need to be performed. We hope that these additional insights, paired with those from this dissertation, will help enable costimulation blockade-induced mixed chimerism to become a practical strategy to achieve long-term survival of

allogeneic organs without chronic immunosuppression.

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