Toll-like receptors and their role in transplantation

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1. ABSTRACT

The innate immune system is an ancient, conserved pathogen response system that lays the foundation for self/non-self discrimination. The cells of the innate immune system are responsible for recognizing the highly conserved molecular motifs of microbial pathogens and represent the first line of immunological defense as well as contributing to the activation of the adaptive immune response. Toll-like receptors are a family of 13 germline-encoded receptors on antigen presenting cells, T cells and various non-lymphoid tissues that are critically important for innate immune function and inflammatory responses. Furthermore, numerous clinical and experimental animal studies have demonstrated the importance of Toll-like receptors as well as members of their signaling pathways in the setting of organ transplantation, where endogenous ligands may play a significant role. Toll-like receptor signaling has the capacity to inhibit transplantation tolerance. A complete understanding of the relationship between Toll-like receptor signaling and transplantation tolerance is essential to modifying, reducing or abrogating immune suppression as well as improving patient outcomes.
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2. INTRODUCTION

2.1. Adaptive Immunity

From birth until death, humans and animals are continuously exposed to a variety of microbial pathogens which have the potential to threaten their continued survival. Clearly, the greatest protection from the onslaught of such pathogens is the immune system, which was subdivided into two major categories shortly after the birth of immunology as a field of scientific study (1). The first and albeit more well known category is adaptive immunity, which includes the study of receptor development and diversity, the interactions between the various cells of the immune system and immune memory (1). The complex system of adaptive immune function is exclusively found in jawed, higher vertebrate species (it is absent in lower vertebrates) and centers around the T and B cells, which mediate the immune response through their randomly developed, highly sophisticated and high fidelity antigen receptors that, upon the presentation of cognate antigen by antigen presenting cells (APCs), are able to clonally subdivide and mount a robust effector response to the invading pathogen via cellular response or antibody production (2, 3). However impressive its performance, the adaptive immune system is not the “alpha and omega” of immune function. Indeed, plant and invertebrate species successfully fend off microbial invasion without the assistance of an adaptive immune system. Higher vertebrate species, which possess adaptive immunity, experience potential complications due to the clonal expansion and differentiation required prior to the mounting of a successful immune response (2). This can take four to seven days, which is a significant and potentially lethargic time delay when one considers the rapid replication capacities of many microbial invaders (2). This brings us to the second arm of the immune response, known as innate immunity.

2.2. Innate Immunity

The innate immune system reflects a much more ancient pathogen response system whereby constitutive, conserved molecular motifs of microbial pathogens are recognized by a variety of cells (including APCs) and intracellular sensors (2). These cells and sensors represent the first line of defense in the immune system, detecting both the presence and nature of the microbial pathogen (2, 4). Indeed, it is currently accepted that adaptive immune responses are directly dependent on the antigen presentation and effector class selection functions of APCs (4). However, the innate immune system is not limited to the function of APCs; it also includes the complement system and the self/non-self discrimination conferred by natural killer (NK) cells (4).

The innate immune system was first described over a century ago by the Russian scientist Elie Metchnikoff in his studies of the invertebrate immune response, which revealed the importance of macrophages in cellular responses to disease (2, 5). In fact, Metchnikoff was awarded a Nobel Prize for his pioneering discovery of innate immunity (along with Paul Ehrlich) in 1908. In the last century, discoveries of a variety of dendritic cells (DCs), complement systems and antimicrobial peptides in animals have shed more light on innate immune function (2). Studies of plants and invertebrates have also contributed to the elucidation of innate immunity (2).

Indeed, one of the most interesting discoveries in the realm of innate immunity to date is the existence of Toll-like receptors (TLRs), which are the focus of this review. The discovery of TLRs will first be discussed, followed by a discussion of their pattern-recognition capabilities, structure and general functions. The various TLR signaling pathways will be presented, followed by an analysis of some of the more recent findings on the roles of TLRs in organ transplantation. Specifically, the topics of TLR ligands, ischemia/reperfusion, regulatory T cells, allograft rejection and tolerance induction will be addressed.

3. TOLL-LIKE RECEPTORS: “THE FLIES HAVE IT”

3.1. The Role of Toll in Drosophila

Interestingly, the first finding contributing to the discovery of TLRs was not in the field of immunology. The Toll receptor (note, not Toll-like receptor) was originally identified in Drosophila and functioned as a maternal-effect gene, which assisted in the dorsoventral axis formation in fruit flies (4, 6). Further studies of the Toll receptor determined that it played an essential role in the host defense response to fungal infection by initiating a kinase-mediated protease cascade (analogous to the lectin pathway complement cascade), which results in the production of the antifungal peptide drosomycin (2, 7). In fact, Toll was such an essential element of this pathway that mutant flies without Toll function would rapidly succumb to fungal infection (2). However, Toll mutants demonstrated “wild-type” (normal) resistance to infection by Gram-negative bacteria, establishing a specific and selective response to fungi by the Toll receptor (2). It was later determined that Drosophila had separate signaling cascades which defended the fly against Gram-negative and Gram-positive bacterial infections (2).

3.2. Mammalian Homologues of Toll

Not long after the discovery of Toll-mediated immune functions in Drosophila, mammalian homologues were identified through the pioneering work of Ruslan Medzhitov and Charles Janeway (6). It was Janeway who first hypothesized that the cells of the innate immune system contained germline-encoded receptors (later known as pattern recognition receptors [PRRs]) responsible for the recognition of conserved microbial motifs on invading microbial pathogens, even before the discovery of the mammalian Toll receptor homologues (6). Medzhitov and Janeway discovered these homologues through protein database searches and coined the name “Toll-Like Receptors”. To date, 13 mammalian TLRs have been discovered (3, 8).
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Table 1. Toll-like receptors, ligands, cellular locations and species

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Representative Ligand</th>
<th>Cell Types</th>
<th>Cellular Location</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR1</td>
<td>Triacyl lipopeptides</td>
<td>Macrophages, Many others</td>
<td>Cell surface</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TLR2</td>
<td>Peptidoglycan</td>
<td>Antigen presenting cells, Endothelial Cells</td>
<td>Cell surface</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TLR3</td>
<td>Double-stranded RNA</td>
<td>Dendritic cells, Intestinal epithelium</td>
<td>Intracellular</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TLR4</td>
<td>LPS</td>
<td>Antigen presenting cells</td>
<td>Cell surface</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TLR5</td>
<td>Flagellin</td>
<td>Basolateral intestinal epithelium</td>
<td>Cell surface</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TLR6</td>
<td>Zymosan</td>
<td>Macrophages, Many others</td>
<td>Cell surface</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TLR7</td>
<td>Single-stranded RNA</td>
<td>Antigen presenting cells</td>
<td>Intracellular</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TLR8</td>
<td>Single-stranded RNA</td>
<td>Not determined</td>
<td>Intracellular</td>
<td>Human</td>
</tr>
<tr>
<td>TLR9</td>
<td>CpG</td>
<td>Antigen presenting cells</td>
<td>Intracellular</td>
<td>Human, Mouse</td>
</tr>
<tr>
<td>TLR10</td>
<td>Not Determined</td>
<td>B cells</td>
<td>Not determined</td>
<td>Human</td>
</tr>
<tr>
<td>TLR11</td>
<td>Profilin</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Mouse</td>
</tr>
<tr>
<td>TLR12</td>
<td>Not Determined</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Mouse</td>
</tr>
<tr>
<td>TLR13</td>
<td>Not Determined</td>
<td>Not determined</td>
<td>Not determined</td>
<td>Mouse</td>
</tr>
</tbody>
</table>

4. TOLL-LIKE RECEPTORS IN MAMMALIAN IMMUNITY: THE POWER OF PATTERN RECOGNITION

4.1. Pattern Recognition receptors and pathogen associated molecular patterns

Although PRRs are not unique to Toll-like receptors (i.e. they also include members of the complement pathway and other aspects of innate immunity), they are a critical part of TLR functions (2). As mentioned above, PRRs recognize pathogen associated molecular patterns (PAMPs), which are highly conserved and invariant among microbes of a given class (2). PAMPs as a whole are only produced by microbes and frequently represent a metabolic intermediate, structural component or gene product essential for the particular organism’s survival, which means that organisms of a given class which loose their PAMP (via mutation, etc.) will not survive, greatly augmenting the power of innate recognition via PRRs (2). Of course, there are exceptions to this (such as cytidyl phosphate guanosine oligodeoxynucleotide [CpG] motifs being under-represented in some viral species).

4.2. genetic protection against nucleotide autoimmunity

Although PAMPs allow us to distinguish self from microbial non-self, they include immunostimulatory DNA and RNA motifs that are strikingly similar to our own DNA and RNA. What prevents our bodies from constantly reacting to our own DNA and RNA products? TLRs have an answer for this question too. Although the dual sequestering of DNA and RNA into membrane bound compartments (or degradation upon unexpected release from such compartments) and DNA- and RNA-recognizing TLRs into endosomal compartments may provide some modicum of protection, it is not sufficient to prevent immune activation when excessive de-compartmentalization occurs (i.e. self-DNA and self-RNA release due to cellular damage or viral infection) (9).

CpG motifs are also used to distinguish between microbial and vertebrate DNA (10). CpG motifs are much less frequent in vertebrates as compared to microbes—a condition known as CpG suppression (10). Even when CpG motifs do occur in vertebrates, the base contexts are rarely immunostimulatory and the motifs are usually methylated, which further diminishes any immunogenic effect (10).

In the realm of RNA, poly(A) tails hold the key. Human-derived myeloid dendritic cells are stimulated and matured by bacterial RNA, which does not have poly(A) tails, while vertebrate RNA, which does have poly(A) tails, could not mature them (9). Other post-translational modifications (such as methylation in vertebrate DNA and RNA) may also contribute to the fine-tuning of the TLR-driven innate immune response; the study of the role of such modifications in innate immunity is ongoing (9, 10). It is therefore conceivable that self-DNA and RNA contain other modifications (i.e. secondary structures, chromatin, etc.) that act alone or in concert with those mentioned above to prevent aberrant innate immune activation via TLRs.

4.3. The great diversity of Toll-like receptors and their ligands

As mentioned previously, 13 TLRs have been discovered and a variety of ligands have also been determined (3, 8). The power of TLR pattern recognition can be seen in the diversity of ligands both within and among individual receptors. TLR4, for example, can detect PAMPs from microorganisms as diverse as Gram-negative bacteria, viruses and even Taxol from plants. How are TLRs able to discriminate among all of these different ligands? Some answers have been found in their structure (Table 1).

5. TOLL-LIKE RECEPTORS UP CLOSE: STRUCTURE

Based on their molecular structures, TLRs have been identified as type I integral membrane glycoproteins (3). The extracellular portion of a TLR contains 25 tandem copies of a consensus sequence, leucine-rich repeat domain (LRR) involved in the recognition of PAMP ligands and subsequent signal transduction; they are similar to LRR motifs present on other pattern-recognition proteins (2, 3, 7). The LRRs have a secondary structure composed of a beta strand and an alpha helix connected by a looped segment (3). The LRRs interact to form a horseshoe structure that may function in PAMP recognition (3). However, accessory molecules are likely to be required for TLRs, as mice deficient in CD14 have a significant defect in their ability to recognize LPS through TLR4 (11) (Figure 1).

The cytoplasmic domain of TLRs has considerable sequence homology to cytoplasmic portions of the IL-1 receptor; both are classified together as the Toll/IL-1R (TIR) domain (3). The conserved TIR domain is
about 200 amino acids in length, contains 20-30% sequence conservation among different TIRs and is subdivided into 3 conserved boxes (Box 1, 2 and 3) that vary in size and are critical signaling portions of the molecule, using their side chains for interaction with adapter molecules (3, 12). X-ray diffraction studies of TLRs 1 and 2 have determined that the secondary structure of the TIR domains contain a five-stranded parallel beta sheet surrounded by 5 alpha helices on each side which connect to the beta sheet via loops (12). To date, the structure of the TIR domain in other TLRs has not yet been fully elucidated. Although they have a considerable amount of sequence homology, the large conformational differences seen between TLR1 and TLR2 and the sequence variation seen within the TIR boxes implies that these domains have the potential for significant levels of structural diversity (12). Such structural diversity is likely to contribute to the complex and varied signaling pathways of TLRs, which are described below.

6. TOLL-LIKE RECEPTOR FUNCTION: FINE TUNED IMMUNITY

6.1. Function on the genetic level: microarray studies

As with many other aspects of scientific discovery, the field of TLR immunobiology has become more complex as time goes on. On the genotypic level, microarray experiments were conducted in order to determine which genes are up-regulated or down-regulated due to DC stimulation by a variety of innate immune ligands or their respective pathogens (13). The study found that 166 common genes (primarily composed of immune system and inflammatory genes) are induced as a result of exposure to Escherichia coli, Candida albicans or influenza virus (13). Because these same genes are induced regardless of the pathogen type involved (and therefore the specific TLR stimulated; subsequent experiments implicated at least TLR3 and TLR4) they can be classified as the general response produced by the innate immune system (2, 13). Indeed, additional experiments have determined that this general response can also be induced by stimulation of the IL-1 receptor family which, as mentioned above, also possesses the TIR domain in their cytoplasm (2, 3). Because E. coli and the influenza virus are able to induce an additional set of genes, the implication is that certain microbial species and compounds produce an additional response (along with the general response) tailored to the pathogen in question (13).

Studies conducted with macrophages found that differential induction of cytokine genes is generated based on the specific TLR stimulated (TLR2 or TLR4) (14). Although exhaustive genetic studies have yet to be completed on all cells of the innate immune system, these findings imply that a fine-tuned innate immune response is common.

6.2. Specific cellular locations within and among cells

Looking beyond genetics for more clues, the designation of certain TLRs to specific subsets of cells also gives weight to a fine-tuned response, as only certain cells
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Table 2. Variations Between MyD88-dependent and MyD88-independent Pathways

<table>
<thead>
<tr>
<th>TLRs Involved</th>
<th>MyD88-dependent</th>
<th>MyD88-independent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essential Signaling Molecule Used</td>
<td>MyD88</td>
<td>TRIF</td>
</tr>
<tr>
<td>Activation of NFκB</td>
<td>Standard/“Early Phase”</td>
<td>“Late Phase”</td>
</tr>
<tr>
<td>Substances Produced post-NFκB activation</td>
<td>ALL Inflammatory Cytokines (TNF, IL-6, etc.); Costimulatory molecule upregulation</td>
<td>Interferon (IFN)-alpha and beta; IFN-inducible genes (CXCL10, IRG1, etc.); Costimulatory molecule upregulation</td>
</tr>
</tbody>
</table>

It has been determined that the essential molecule in the MyD88-independent TLR3 and TLR4 pathways is ligand binding and dimerization of TLRs, which allows for the recruitment of downstream signaling molecules (3).

7. TLR SIGNALLING: TWO PATHWAYS, ONE OBJECTIVE

Generally speaking, TLR signaling consists of a myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and a MyD88-independent pathway; an overview of the two pathways is shown in Figure 2. The objective of both pathways is the same: translocation of the transcription factor NF-κB from the cytoplasm to the nucleus, which subsequently leads to the production of inflammatory cytokines, interferons, costimulatory molecules, chemokines and/or other interferon-inducible gene products (3, 6). The speed of NF-κB translocation and subsequent collection of substances produced depends on the pathway taken (MyD88 dependent/independent) (3), shown in (Table 2). Such pathways are believed to generate discrete “signalsomes”, which reflect variations in signal adaptor amalgamations, that occur after TLR stimulation and contribute to the genetic expression induced by TLRs (14).

Note that in addition to NF-κB translocation, the MyD88 independent pathway also leads to the phosphorylation, dimerization and nuclear translocation of interferon regulatory factor (IRF)-3, leading to the induction of type I interferons (IFN alpha and IFN beta) (28).

7.1. MyD88-dependent pathway

MyD88 represents a critical TLR signal adaptor whose TIR region interacts directly with the TIR portion of the TLR after ligand binding (3). Indeed, mice deficient in MyD88 cannot produce inflammatory cytokines in response to TLR2, TLR4, TLR5, TLR7 or TLR9 stimulation, which clearly reflects the importance of MyD88 in this TLR signaling pathway (3, 14). However, further molecular studies with MyD88 knockout mice showed that NF-κB translocation did occur after TLR3 and TLR4 stimulation, but occurred with delayed kinetics, implying that a MyD88-independent signaling pathway existed, although this alternate pathway did not contribute to inflammatory cytokine production (2). The MyD88-dependent pathway is required for the production of inflammatory cytokines and for the upregulation of costimulatory molecules and major histocompatibility complex (MHC) class II in APCs (29, 30).

7.2. MyD88-independent pathway: TRIF

It has been determined that the essential molecule in the MyD88-independent TLR3 and TLR4 pathways is MyD88.
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Figure 2. TLR Signaling Pathways. TLRs signal through 2 major pathway delineations: a MyD88-dependent pathway (TLRs 1, 2, 5, 6, 7 and 9; left side of figure) and a MyD88-independent pathway (TLRs 3 and 4; right side of figure; signal through TRIF). Note that both pathways trigger the same result; translocation of NFκB from the cytoplasm to the nucleus. Additionally, IRF3 induces type 1 interferon production (interferons alpha and beta). Of course, this figure is an oversimplification of the pathways; for more detailed diagrams (including the many intermediates), please see the following sources: (3, 8, 28).

TIR-domain containing adaptor inducing IFN-beta (TRIF), which associates with the TIR domain of TLR4 in a similar fashion to MyD88; TRIF interacts directly with TLR3 (3, 28, 31). The importance of TRIF was confirmed by studies employing in vitro techniques and TRIF knockout mice, which are unable to express IFN-inducible genes upon stimulation by TLR3 or TLR4 ligands (3, 31). Upon stimulation with TLR3 or TLR4 ligands, TRIF knockout mice were defective in their production of IFN-beta and activation of IRF-3 (31). This pathway is required for the upregulation of costimulatory molecules and MHC class II in DCs (29).

7.3. Distinct phases of NF-kappaB translocation
It is believed that the MyD88-dependent pathway is responsible for the “early phase” of NF-kappaB translocation and is required for the production of all proinflammatory cytokines in response to TLR activation, while the MyD88-independent pathway is responsible for the “late phase” of NF-kappaB translocation (as implied by the delayed reaction kinetics described above) which results in both IFN production and the subsequent production of IFN-inducible genes (3, 14).

7.4. Pathway distribution among Toll-like receptors
The MyD88-dependent pathway is used by all TLRs except TLR3, while the MyD88-independent pathway is only used by TLR3 and TLR4 (3, 6, 14). It is important to note that the use of the MyD88-dependent and MyD88-independent pathways by TLR4 is not mutually exclusive; in fact, both pathways must be used in a synergistic fashion for complete gene expression (i.e. production of IFNs and inflammatory cytokines) (3, 6, 14). Indeed, this dual pathway utilization may be the reason why LPS is so immunostimulatory and is such a potent contributor to endotoxic shock (3).

8. THE LINK BETWEEN INNATE AND ADAPTIVE IMMUNITY

One of the main functions of the innate immune system is to activate the adaptive immune system; this is done in a variety of ways. TLRs expressed on B lymphocytes are activated and subsequently result in the triggering of adaptor functions, including the secretion of antibodies (23). B cells express TLR9 and upon stimulation can promote T-dependent antibody responses (30). Furthermore, alloantibodies have been shown to promote allograft rejection (30).

The translocation of NF-kappaB (as a result of TLR stimulation) causes a maturation program in DCs; the cells increase their expression of costimulatory molecules and production of pro-inflammatory cytokines (2, 3, 6, 14).
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As a result, DCs mature and then traffic to the draining lymph nodes, where they have the capacity to activate naïve T cells and elicit an adaptive immune response (3, 6, 14, 32). In addition, DCs produce a variety of cytokines which enable effecter T cells to become refractive to suppression by regulatory T cells (25). Finally, some TLRs which enable effector T cells to become refractive to 14, 32). In addition, DCs produce a variety of cytokines naïve T cells and elicit an adaptive immune response (3, 6, lymph nodes, where they have the capacity to activate as a result, DCs mature and then traffic to the draining lymph nodes, where they have the capacity to activate naïve T cells and elicit an adaptive immune response (3, 6, 14, 32). In addition, DCs produce a variety of cytokines which enable effecter T cells to become refractive to suppression by regulatory T cells (25). Finally, some TLRs which enable effector T cells to become refractive to 14, 32). In addition, DCs produce a variety of cytokines naïve T cells and elicit an adaptive immune response (3, 6, lymph nodes, where they have the capacity to activate

As will be demonstrated below, TLR-induced innate and adaptive immune responses play diverse and important roles in transplantation.

9. TOLL-LIKE RECEPTORS AND ALLOGRAFT RESPONSES: OF MICE AND MEN

9.1. Introduction

Allograft rejection can be placed in two distinct classifications: acute and chronic. Acute rejection is a major detractor of long-term graft survival and mainly occurs within six months of transplantation (33). Episodes of acute rejection can contribute to the occurrence of chronic rejection, which causes gradually progressive graft dysfunction and organ failure (33). Incidents of acute and chronic rejection can occur despite the use of immunosuppressive regimens.

Multiple studies using murine models and human clinical studies have shown a role for TLRs in alloimmunity, which ultimately contributes to graft rejection. It is postulated that the primary role that TLRs play in alloimmunity is the switching on of the adaptive immune system through T cell maturation and activation via antigen presentation by mature, peptide/antigen-loaded DCs. It is currently unclear whether DC maturation in the setting of organ transplantation is due to endogenous or exogenous ligands, or possibly a combination of the two.

In the current section of this review we will describe the upstream innate immune activators in the setting of organ transplantation. We will then describe the role of TLRs in the two main host injury responses to organ allografts: antigen independent, which is largely due to ischemia-reperfusion and alloantigen dependent injury. Next we will elucidate the importance of TLRs and transplantation tolerance. Finally, we will mention what is currently known about TLRs and xenograft responses.

9.2. Endogenous ligands

Although the established function of TLRs is the detection of PAMPs, research has shown that some TLRs identify and mount an immune response to a variety of endogenous ligands in the setting of cellular injury and/or transplantation; this process is known as sterile inflammation (19, 34). A variety of endogenous ligands have been suggested, including heat shock protein (HSP) 60, HSP 70, heparan sulfate, hyaluronan, fibronectin, uric acid, products of proteolytic cascades and intracellular components of ruptured cells (16, 19, 34, 35). The vast majority of these ligands are believed to activate TLR4, while some, such as the HSPs, may activate both TLR2 and TLR4 (16, 35). Such endogenous ligands are commonly referred to as “danger signals”, informing the body of a non-infectious threat; indeed, this could be why TLRs are present on non-immune cells such as endothelial and epithelial cells. However, it is important to note that some of these findings may be complicated by the presence of contaminating LPS (or other TLR ligands); it is important for any study of endogenous TLR ligands to ensure that contamination by interfering ligands is excluded (36).

One of our studies has found a role for hyaluronic fragments (fHA) as innate alloimmune agonists in a murine model and these effects were not confounded by the presence of LPS (34). We found that fHA has the capacity to cause the maturation of DCs (via TLR2- and TLR4-dependent up-regulation of CD40 and CD86 and the TLR4-dependent production of TNF-alpha) (34). Furthermore, fHA-matured DCs are able to induce a significantly more profound T cell response in vitro (via mixed lymphocyte reaction) as compared to rest/immature DCs (34). This response is largely TLR4-dependent and reliant on signaling via Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), a signal adaptor downstream of TLR2 and TLR4. This response is independent of the MyD88 and TRIF adapters (34). Looking at the role of HA in vivo, we found HA accumulates in murine skin grafts 2 weeks post-transplantation (as compared to harvested, non-transplanted donor skin) (34).

Bronchiolitis obliterans syndrome (BOS) is a clinically documented chronic rejection phenomenon in lung transplant recipients and is the major cause of late death after lung transplantation (37). The primary risk factor for the development of BOS is acute allograft rejection (37). Human lung transplant recipients who demonstrate evidence of BOS have higher lung HA levels as compared to BOS-free lung transplant recipients (34, 37). Thus, it is plausible that the immunostimulatory activity of fHA identified in our murine study is also clinically relevant in these patients.

Although several reports have indicated a role for HSP70 in allograft rejection (38-40), our murine studies have found no role in DC maturation in vitro (34). Furthermore, in vivo skin transplant studies using mice with targeted deletions of the inducible form of HSP70 demonstrated no significant role for HSP70 in either graft rejection or Th1 alloimmunity (34, 41). Our use of a murine skin transplant model (as opposed to the use of human (39) or rat (40) models, which predominantly use vascularized systems) may contribute to the differences between these studies, as not all findings are true for all species. There also seems to be some disagreement even within murine models, as both stimulatory and protective roles for HSP70 have been demonstrated previously (38). Furthermore, there may be a highly complicated balance between the cytoprotective and immunostimulatory roles of HSP70 during transplantation. Indeed, HSP70 normally plays a protective, chaperone role during normal cellular function,
and self-HSP reactivity by T cells is tightly controlled (38). The presence of other endogenous TLR ligands could also modulate the immune response to HSP70 (34). Finally, the change in location of HSP70 expression during and after transplantation may contribute to the role it plays in allograft rejection. It has been demonstrated that tumor and viral cells which express surface HSP70 are more prone to immune response (38). Clearly, further studies are warranted to elucidate the complete role of HSP70, in addition to other putative substances, as endogenous ligands for TLRs.

9.3. Exogenous ligands

Although solid organ transplantation is carried out in a clean environment, it is still debatable whether solid organ transplantation is truly a sterile event (42). There is mounting evidence that complete sterility in solid organ transplantation is not achieved due to the presence of exogenous ligands in the donor and recipient. Furthermore, LPS is present in the aerosol of many domestic and work environments (including hospitals); this provides an almost unavoidable possibility that TLR signaling occurs during solid organ transplantation (43).

Cardiac surgery has been found to increase the levels of circulating endotoxin; such ligand exposure is believed to occur in virtually all heart transplant recipients (37, 44). Furthermore, concomitant pulmonary infection at time of transplantation provides exposure to microbial ligands (37). Indeed, TLR4 initiates the innate immune response to Chlamydia pneumoniae and Helicobacter pylori, the two main pathogens implicated in human atherogenesis; such atherogenesis has been found to occur in approximately 3% of the renal transplant population and can contribute to the development of chronic rejection in heart transplants (45, 46). Furthermore, the presence of viruses can modify acute rejection in both experimental and clinical human transplantation models (46). However, there is no direct mechanistic evidence that microbial infections are a primary inhibitor of acute allograft rejection. In support of this a study performed over 30 years ago in germ-free mice demonstrated that such mice can reject skin transplants (47).

9.4. ischemia/reperfusion injury

9.4.1. Introduction

An unavoidable byproduct of transplantation, ischemia/reperfusion injury (IRI) is a common cause of organ dysfunction and can be caused by low flow states, diverse surgical procedures or during organ procurement (16). IRI can cause early organ failure, acute rejection and chronic rejection, making it a significant detriment to transplant survival (16). The duration of donor organ cold ischemia has been found to be a contributor to IRI as cellular and tissue damage is progressively increased during prolonged hypothermic organ preservation (33). Presumably such situations are further exacerbated when the organ is harvested from a cadaveric donor.

Indeed, there is a general consensus on the primary role of innate immunity in IRI, although its role has not yet been fully elucidated (16). Zhai and colleagues believe that the ATP depletion and release of reactive oxygen species (ROS; generated in large amounts by polymorphonuclear cells) may cause an initial wave of inflammatory-independent cell death whose release of cellular contents may subsequently provide endogenous ligands for TLR stimulation (16, 48). Furthermore, the receptor-plasma membrane environment may be physically altered by ROS; causing activation of TLR4 in the absence of any ligand (17). Exogenous ligands could also play an initial or secondary role in IRI-mediated innate immune activation as increased LPS levels have been documented in multiple studies of human liver transplant recipients; gut-derived LPS is believed to play a secondary role in TLR4 activation in the liver, as Zhai and colleagues concluded that gut-derived LPS does not initiate IRI in their clinical model (16).

A working model of TLR involvement in the transplant process was developed by Obhrai and Goldstein in 2006 (42). According to this model, innate immune ligands (both endogenous and exogenous) are released via acute ischemia-reperfusion injury. This results in a primarily antigen-independent acute injury model that has been found to rely on TLR4 (but not TLR2) signaling in liver transplantation and both TLR2 and TLR4 in cardiac models (16, 42, 48). In the liver, the TLR4 activation is believed to occur through an IFN-3-dependent, MyD88-independent pathway, as MyD88 knockout mice suffered significant IRI in an in vivo mouse model, while IFN-3 knockout mice did not (16, 42). This finding is supported by the fact that MyD88 mediates TLR2 and TLR4 signaling while IFN-3 selectively mediates TLR4 signaling; reflecting the pattern of TLR dependence seen in the liver (16). Presented below are some pertinent IRI studies in several organ systems that demonstrate important roles for TLR2 and TLR4 signaling in IRI.

9.4.2 Ischemia/reperfusion injury in the heart

Stimulation of TLR4 (or other TLRs) results in the signaling cascade discussed previously, leading to transcriptionally regulated production of a variety of substances, including cytokines, chemokines, adhesion molecules and other pro-inflammatory mediators along with apoptotic or anti-apoptotic pathway activation (17, 49). One organ demonstrating an impressive example of this is the heart, where injured (including IRI) murine myocardium both have intense TLR4 expression, particularly found in cardiomyocytes (rather than cells of the immune system) (17, 48). When compared to wild type mice, TLR4 knockout mice have consequently demonstrated a decrease in myocardial infarction size associated with a decrease in mitogen-activated protein kinase (MAPK) activation, a decrease in translocation of NF-kappaB and decrease in mRNA expression of IL-1 beta, IL-6 and monocyte chemotactic protein (MCP)-1; all of which demonstrates a novel role for TLR4 in myocardial IRI (17, 48, 49). Oyama and colleagues found that neutrophil accumulation and complement deposition were also TLR4-dependent contributors to the IRI inflammatory response (48). Chong and colleagues believe that the larger infarct size seen in wild type mice may be due to the inflammatory response triggered by IRI, which damages...
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the potentially viable tissue around the initial infarct, thereby increasing its size (17).

As an extension of the work by Chong and colleagues, the same authors investigated the theory that the TLR4 antagonist Eritoran (a clinically safe structural analog of the lipid A portion of LPS that blocks translocation of NF-kappaB) could be used to reduce IRI injury in the heart (49). Indeed, the authors found that Eritoran significantly decreased infarct size by 32% as compared to controls. Eritoran pretreatment also significantly decreased activation of NF-kappaB and subsequent inflammatory cytokine response by IL-1 beta, IL-6 and MCP-1. Interestingly, the authors ensured their mouse model was free from LPS contamination, which indicates that the TLR4 activation by IRI occurs in the absence of LPS. However, it is important to note that LPS can also induce TLR4-dependent left ventricular dysfunction and expression of TNF alpha and IL-1 beta, along with several adhesion molecules (48). Although they did not find evidence of endotoxin contamination in their mouse model, Oyama and colleagues did suggest that part of the IRI inflammatory response could be due to post-operative bacterial infections which can occur during the reperfusion period; such issues may be more relevant in a clinical setting rather than an experimental murine model; obviously opportunistic infections and cytomegalovirus are common problems in hospital settings (48).

It is important to note that Eritoran is not the only TLR4 blocking molecule; Li and colleagues previously reported that glucan phosphate also decreases IRI, albeit in a slightly different fashion (activating the serine/threonine protein kinase Akt and inhibiting apoptosis in addition to NF kappaB activation) (49). These findings are significant as current clinical anti-inflammatory strategies have not yet been able to reduce ischemic injury induced by IRI; transient TLR-based anti-inflammatory strategies may have the potential to improve patient outcomes (48).

9.4.3. Ischemia/Reperfusion Injury in the Liver

In addition to the clinical studies mentioned above, TLR4 plays a role in hepatic IRI in murine and rat models (18, 50-52). The primary cellular mediator of IRI in the liver is the Kupffer cell (a type of macrophage present in hepatic sinusoids which make up 80% to 90% of the fixed macrophage population in the entire body) (52). Kupffer cells initiate a 2-phase model of hepatic injury: activated Kupffer cells release ROS (whose implications were discussed above) (18, 50). The second stage of injury involves the Kupffer- and hepatocyte-mediated production of a variety of pro-inflammatory cytokines (TNF-alpha being the most significant) contributing various positive and negative effects, including hepatic neutrophil recruitment and accumulation, causing hepatocellular damage via destruction of hepatic microcirculation due to blockade of capillary perfusion and protease release (18, 50, 51, 53). Kupffer cells are activated by a large amount of endotoxin released through portal circulation after IR; this causes maturation of the Kupffer cells and a significant increase in TNF-alpha and TLR4 mRNA production (52). Anti-TLR4 antibody significantly decreases TNF-alpha production in IR rats as compared to IR rats that did not receive the antibody (52).

Murine models of partial (ex. incomplete blockade of portal vein inflow) IRI in the liver found significantly decreased levels of TNF-alpha production, plasma alanine aminotransferase (pALT; a marker of hepatic injury) levels and plasma aspartate aminotransferase (pAST) levels in TLR4 knockout mice (as compared to wild type) (18, 50). Wang and colleagues found very small amounts of endotoxin in the portal vein that did not increase as compared to sham controls, which contributed to their conclusion that activation of TLR4 is not due to the increase of circulating endotoxin; Wu and colleagues also found little LPS in peripheral blood post-procedurally (18, 50). Although this finding differs from that of Peng and colleagues, the difference could be due to the use of mice (vs. rats) and a partial (vs. complete) IRI model. Wu and colleagues suggest that while total IRI is preferable for the study of liver damage, portal vein inflow blockade can result in the leakage of bacteria (or their products) into the circulation, leading to high mortality rates and/or complications in clarifying the effects of the IRI independent of the presence of endotoxin due to the surgical procedure (50). It is important to note that in clinical studies, LPS levels are increased in both donors and recipients; Tsoulfas and colleagues found circulating endotoxin levels (20-100pg/ml) in their murine liver transplant model which they state are comparable to reports from clinical liver transplantation (51). Donor LPS levels are theorized to be caused by the multiple injuries and/or cerebrovascular accidents suffered by most donors, which can cause endotoxin translocation from the gut (51). During the anhepatic (intermediate stage between the removal of the recipient's original organ and the implantation of the new, donor organ) stage of transplantation, the recipients are unable to tolerate the released endotoxin due to the lack of Kupffer cells during this time period (51). Therefore, while the partial IRI model may be better for discretely gleaning the TLR4-mediated effects of IRI on the liver, the full IRI model may be more clinically relevant since both full IRI and clinical liver transplantation result in some LPS/endotoxin contamination of the donor and/or recipient.

Although it is unknown whether TLR2 expression in the liver is modulated by Kupffer cells, ionic gadolinium (GdCl3) has been demonstrated to be a potent inhibitor of Kupffer cell activation via depletion of lipid peroxidation, notably without the induction of hepatotoxicity (53). Systemic injection of GdCl3 has been found to significantly down-regulate TLR2 expression, portal vein ALT levels and serum TNF-alpha levels in a mouse model of partial IRI, although the authors are unsure whether inactivation of the Kupffer cells themselves, or simply the cytokines produced by them mediates these effects (53). An important caveat is that the authors did not evaluate TLR4 expression, which has also been shown to contribute to ALT and TNF-alpha levels, as mentioned previously. Furthermore, it would be important to evaluate the effects of GdCl3 in a full IRI model/presence of LPS, as LPS contributes to some of the readouts Zhang and colleagues tested as well. Indeed it has been demonstrated
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that over-expression of TLR2 is capable of conferring LPS-induced NF-kappaB activation in previously unresponsive cells (51). Even so, many agents other than endotoxin can lead to the activation of NF-kappaB, making this a highly complex issue (51).

9.4.4. Ischemia/reperfusion injury in the kidney

Both TLR2 and TLR4 have been shown to play a role in murine and rat models of kidney IRI, which is characterized by tubular necrosis/apoptosis, extracellular matrix degradation and infiltration of monocytes or macrophages into the interstitium (19, 20). Ischemia has been shown to be a major cause of both acute and end stage renal failure, along with causing the induction of acute renal transplant rejection and delayed allograft function (19).

TLR2 has been demonstrated to be up-regulated and/or activated by IRI in multiple models, sometimes in the absence of concordant TLR4 upregulation (51, 53). In mice, TLR2 is constitutively expressed on renal tubular epithelial cells and Bowman capsule epithelium, the expression of which is enhanced as a result of IRI (19). Rat kidneys which had undergone IRI demonstrated enhanced levels of TLR2 and TLR4 mRNA transcript and protein levels, predominantly expressed in the proximal tubules and thick ascending limbs; once again both animal species demonstrated that TLRs are mainly expressed on organ tissues rather than immune cells (20). It is believed that the activation of TLR2 and TLR4 on these epithelial cells leads to the secretion of chemokines which cause phagocyte influx and immune activation, linking innate immunity and toxic tubular cell injury (20). The authors speculate that the activation of innate immunity may either contribute to the activation of adaptive immunity (hinted at by the finding that IRI induced the maturation of dendritic cells in their model) and/or the induction of a more general inflammatory response similar to models of IRI in other organs (20).

IRI damage in the kidney is mediated by renal tubular epithelial cells (TECs) and involves a complex interaction of renal hemodynamics with subsequent tubular injury and resultant inflammatory responses (19). TLR2 is expressed on renal parenchyma and TECs (where it is constitutively expressed in mice), contributing to the inflammatory response to IRI where TLR2 has been found to be up-regulated in vivo (19). TLR2 knockout mice subjected to a kidney model of IRI demonstrate reduced cytokine and chemokine levels 24 hours after injury (19). Notably, the chemokines keratinocyte chemoattractant (KC) and monocyte chemoattractant protein-1 (MCP-1) are found at lower levels in TLR2 knockout mice as compared to wild type mice; these lower chemokine levels contribute to a drop in granulocyte and macrophage infiltration in the TLR2 knockout kidneys, although mutant vs. wild type levels become similar by day 10 post IRI (19). IL-1 beta and IL-6 levels are significantly reduced in TLR2 knockout mice as opposed to wild type mice which have undergone IRI (19). Overall, TLR2 knockout mice demonstrated less severe renal damage and dysfunction as compared to wild type mice (measured via plasma urea and creatinine levels). The primary reasons for these effects are the lowered cytokine and chemokine production as well as the reduced need for cellular infiltrates to phagocytose and remove the less common necrotic and apoptotic cells (19). The reduced numbers of apoptotic TECs and the subsequent finding of less TEC proliferation in TLR2 knockout mice implies both pro-apoptotic and tissue repair functions for TLR2 on renal cells (19). Indeed, TLR2 stimulation by bacterial lipoproteins has been found to induce apoptosis in monocytes (19). It is important to note that the reduced TEC proliferation could simply be a reflection of the reduced tubular damage in TLR knockout mice for the reasons previously mentioned (19). Taking as a whole, Leemans and colleagues state that kidney IRI seems to be mediated by TLR2-expressing renal cells, suggesting that a TLR2 blockade via specific signaling antagonists could have clinical relevance in the treatment and prevention of kidney IRI; this is especially important as no effective treatment is currently available (19).

9.5. The Effect of Toll-like receptor signaling on transplant survival

9.5.1. Introduction

Several publications have demonstrated a role for TLRs in modulation of alloimmunity and transplantation tolerance, often with diverse mechanisms. Note, although they have demonstrated significant roles in allotransplantation, TLR signaling is not the only important immune factor that contributes to transplant rejection. For example, although neither are mutually exclusive to TLR function, it has been demonstrated that polymorphisms in cytokine genes such as transforming growth factor beta (TGF-beta) and IL-10 have been linked to both acute and chronic rejection in renal transplantation (43). Given that other components of innate immunity may play a role in acute allograft rejection, TLRs still have a very important role to play.

9.5.2. Toll-like receptors, regulatory T cells and T cell memory

Regulatory T cells (Tregs) play important roles in immune homeostasis and the development and maintenance of peripheral tolerance, regulating immune responses to a variety of infectious agents (25). Tregs have specific, special homing receptors and are very sensitive to inflammatory cytokines, allowing them to effectively migrate to areas of inflammation (23, 25). The most studied subpopulation of Tregs is the CD4+CD25+FoxP3+; these cells have been shown to be important inducing and maintaining immune tolerance in both experimental models of autoimmunity, alloimmunity and tumor immunity (25). Indeed, depletion of Tregs leads to organ-specific autoimmunity (29). Furthermore, co-transfers of Tregs (along with T effectors) protect from disease via the inhibition of cytoprotective and inflammatory responses (23). These findings suggest that Tregs are important for controlling inflammation in the setting of immunity (23).

Multiple studies have demonstrated a relationship between TLR activation and Treg function (23, 24, 29, 54, 55). TLR activation can either directly or indirectly affect Treg function, as diagrammed in (Figure 3). The indirect method occurs through the activation of DCs, subsequently
Figure 3. Indirect and Direct Methods of TLR-mediated Modulation of Regulatory T cell (Treg) Function. TLR function can either directly or indirectly affect Treg function. The indirect method (left) occurs through the activation of DCs, subsequently leading to a decrease in Treg suppression of effector T cells, who have become refractory to the suppressive effects of Tregs. The direct method (right) involves TLR activation on the Tregs themselves, contributing to an increase in Treg activation and proliferation, followed by various modifications of effector T cell function.

Indirect Method of Treg Modulation

- Stimulation of TLR on APC
- APC stimulation of effector T cell (costimulatory molecule independent; cytokines such as IL-6)
- Effector T cell proliferation increases
- Treg activity decreases

Direct Method of Treg Modulation

- Stimulation of TLR on CD4+CD25+ Treg (via TLR4/5/7/8)
- Treg increases in activity and proliferation followed by variable functions (increase or decrease in suppressor function; may control effector T cell functions early or late)
- Effector T cell activity modified via Treg (ex. IL-2 production decreased)

leading to a decrease in the effectiveness of Tregs to control effector T cell function (29). The direct method concerns TLR activation on Tregs themselves, contributing to an increase in their proliferation and a temporary decrease in function (54, 55). This decrease in function allows the effector T cells to battle the infectious onslaught until the issue is resolved; the regulatory T cells then regain their suppressive functions (possibly through the suppressive activities of Tregs processing self antigen) in order to diminish the residual inflammatory responses and help avoid generalized inflammatory-based immunopathologies (23, 56). It is important to note that other studies have theorized a role for Tregs in suppressing immune responses early on (23).

The indirect method of Treg modulation via TLRs was well studied by Pasare and Medzhitov (29). The authors found that DC stimulation with TLR4 and TLR9 ligands reversed Treg-mediated suppression and allowed normal proliferation of effector T cells. Interestingly, this suppression reversal occurred independently of costimulatory molecule expression by the DCs. Instead, a MyD88-dependent signaling pathway produced secreted factors responsible for the block in suppression. Instead of acting on the Tregs themselves, these secreted factors acted on effector T cells, making them refractory to Treg suppression. Exhaustive studies determined that the secreted factor responsible was IL-6 in combination with another TLR-induced cytokine, possibly IL-12.

Looking at the direct pathway, multiple recent publications have demonstrated an intriguing role for TLRs in effecting the function of regulatory (and non-regulatory) CD4+ T cells (23-25). Work by Caramalho and colleagues in a murine model demonstrates mRNA expression of TLRs 4, 5, 7 and 8 on Treg-containing CD4 T cell subsets (while TLRs 1, 2 and 6 were present in all CD4+ populations analyzed) (23). Furthermore, the authors found that LPS stimulation of CD4+CD25+ Tregs results in activation, proliferation and survival of Tregs in an APC-independent method which is synergistic with TCR stimulation (CD25 is a marker of the IL-2 receptor; CD4+CD25+ cells are a subset of T cells credited with Treg function; this population can also contain activated T effector cells) (25). Furthermore, the dose of LPS required was 10μg/ml, which is much higher than the ng/ml required to activate DCs and other innate immune cells (in our hands, 0.5ng/ml LPS is sufficient to activate bone marrow-derived DCs in vitro). Finally, they found that LPS directly increases Treg activity both in vitro and in vivo. It is interesting to note that LPS does not enhance the survival of T effectors (CD4+ CD44lo) (24). TLR4 expression is restricted to CD4+ T cell subsets with known regulatory function; as such, the authors postulate a possible role for TLR4 as a Treg differentiation marker (23). The restriction of TLR4 expression is substantiated by Gelman and colleagues, who found that murine, activated CD4+ T cells (non-regulatory) did not express TLR4 mRNA (24). However, the authors did note TLR4 mRNA expression in naive CD4+ cells. This discrepancy could be due to the flow sorting techniques used; Caramalho’s group used naive cells based on CD4+ CD45RB sorting (CD45RBlo) while Gelman’s group used naive cells based on CD4+ CD44 sorting (CD44hi) (23, 24).
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TLR5 mRNA expression on human CD4+CD25+ Tregs was noted by Crellin and colleagues and influences the phenotype and function of T effectors (CD4+CD25−) and Tregs (CD4+CD25+) (25). Interestingly, while Treg cells expressed higher amounts of TLR5 mRNA (as compared to effector cells), no difference was noted in TLR5 protein expression, underscoring the importance of looking at both RNA transcript and protein levels (25). The authors theorize that the high levels of TLR5 mRNA in Tregs may function to provide quicker TLR5 protein upregulation upon initial stimulation; this is substantiated by the finding that TLR5 stimulation of Tregs results in a drop in mRNA transcript levels which is correlated with an increase (and then rapid decline) of TLR5 protein expression which may contribute to a negative-feedback mechanism (25). The suppressive effects of TLR5 stimulated Tregs were variable; TLR stimulation enhanced suppression in the absence of APCs, although the presence of APCs caused the opposite effect (25). Notably, unlike LPS (TLR4) stimulation in the Caramalho study, LPS (TLR4) stimulation did not result in Treg proliferation; in fact, it is a better stimulator for the effector cell population; these discrepancies may be due to the use of human T cells (vs. murine T cells) or differing sources of LPS (25). However, Crellin and colleagues noted that other investigators have failed to find a proliferative role for LPS in murine Tregs. Even so, flagellin (TLR5) stimulation and TLR4 ligation do increase the suppressive activity of CD4+CD25+ Tregs, while TLR8 ligation decreases suppressive activity, demonstrating differential effects of TLR ligands on Tregs (25). Future studies are warranted to understand the in vivo implications of these findings.

Although the mechanism of Treg mediated effector T cell suppression is not yet fully elucidated, studies have demonstrated that it is cell-contact dependent and one of the primary roles is to inhibit the production of IL-2 in activated T cells, which normally functions as a survival signal for other T cells and reverses suppressive effects of Tregs (24, 25, 29). However, Pasare and Medzhitov found that IL-2 was not important for TLR-mediated block of suppression by Tregs in their DC-driven in vitro model (29). Regardless of the cytokine(s) involved, a delicate balance must be struck here: inhibition of effector T cell function can be beneficial, especially at the resolution of an immunological onslaught; too much repression or repression at the wrong time could have detrimental effects on immunological responses (29).

TLR agonists have variable effects on IL-2 production and Treg suppression of effector T cells. Crellin and colleagues found that while the addition of flagellin to in vitro T cell cultures does not promote IL-2 production of CD4+CD25+ Tregs, it does enhance IL-2 production by CD4+CD25− effector T cells in a dose-dependent fashion (25). LPS alone does not enhance IL-2 production in CD4+CD25+ Tregs (23). However, LPS in combination with T cell receptor (TCR) triggering (via anti-CD3) induces a low level of IL-2 synthesis; the presence of IL-2 and LPS also maximizes the suppressive effect of these Tregs in vitro (23). Caramalho and colleagues state that this minimal IL-2 production is most likely a protective mechanism, as Tregs demonstrate poor survival in the absence of IL-2. Clearly, this issue is a complex one and future studies should be dedicated to further elucidating these findings and extending them to other TLR agonists.

All this intriguing data begs an important question: if TLRs are already expressed on innate immune cells, why have adaptive immune cells express them? There are many plausible answers to this question. Caramalho and colleagues mention that the expression of TLRs on regulatory T cells may be a clue to the evolutionary steps which developed the adaptive immune system (23). Because endogenous ligands signal through TLR4, Treg control of immune responses may be advantageous when dealing with “self” antigens. Treg TCR repertoires seem to be biased towards self-antigen (23). Indeed, when looking at TLR4 stimulation, the direct method of TLR stimulation (Treg stimulation by TLR signaling) results in an enhanced function of Tregs, while the indirect method (APC stimulation by TLR signaling) results in decreased Treg function (23). Increased Treg function may help suppress activation of naïve, autoreactive or alloreactive T cells by self-peptides inadvertently presented in an inflammatory or transplant context (23). TLR3- and TLR9-activated (APC independent) CD4+ cells (non-Treg) demonstrate enhanced survival (24); enhancement of Treg function may assist in modulating the non-Treg response.

Although the chances of an individual naïve T cell reacting to any given pathogen are about 1:200,000, the probability that the same T cell will recognize foreign MHC is much higher: about 1:10 to 1:100 (57). Thus, it is easy to see how cross-reactive memory T cells can occur: a naïve T cell exposed to a given pathogen eventually forms memory T cells which have the capacity to be quickly activated against a repeat infection and may inadvertently cross-react to alloantigen (57). Furthermore, memory T cells (CD4+ CD45RB+) have been found to express TLR4 (23).

9.5.3. The role of Toll-like receptor 4

Given that various models of T cell deficiency result in graft tolerance, it would seem that the activation of innate immune cells alone is not sufficient for transplant rejection; adaptive immunity is required (44). However, various studies indicate a role for innate immune TLR signaling in allograft rejection. Indeed, it has been shown that monocytes (rather than T cells) are the primary infiltrating cell type in grafts during acute rejection, demonstrating a substantial contribution of innate immune cell types to allograft rejection (44). As mentioned previously, both endogenous and exogenous TLR ligands have the capacity to signal through TLR4. The significance of TLR4 signaling in organ transplantation has been demonstrated in multiple clinical and experimental studies.

The role of two TLR4 polymorphisms (Asp299Gly and/or Thr399Ile; found in the extracellular domain) were examined in human lung transplantation (37). These polymorphisms result in a reduced level of circulating pro-inflammatory cytokines, acute phase reactants and soluble adhesion molecules (37, 46). In
addition, they have been found in approximately 11% of the population studied and contribute to a blunted TLR4 response as the immune response to LPS was decreased in these individuals (37, 45, 46). Furthermore, subjects with the Asp299Gly allele have been found to have an increased risk of infection (including opportunistic infection) in multiple clinical studies (45, 46). Although the presence of one or both of the polymorphisms in the donor organ does not modify the rate of acute allograft rejection (as compared to recipients with neither mutation), there is a significant reduction in acute allograft rejection at six months post-transplant in recipients who possess one or both of the polymorphisms (as compared to recipients with neither mutation) (37). Heterozygosity for either polymorphism was the only factor analyzed which was significantly predictive of an absence of acute rejection 6 months post-transplant (37). This study by Palmer and colleagues was followed up with another by the same authors where they analyzed a larger group of transplant recipients over a longer time and confirmed that heterozygous lung transplant recipients have significantly lower rates of acute rejection sustained over a follow-up of more than 4 years post-transplant (note, no patients had both polymorphisms) (43). Interestingly, despite the presence of TLR4 polymorphisms, the rate of post-operative infectious complications was high but equivalent between the wild type and polymorphic patients (43). The authors pointed to several potential explanations for this finding; prior colonization with infectious agents, post-transplant immunosuppression and redundancy of innate immune signaling may have contributed to the prevalence of post-operative infections (43).

The Asp299Gly and Thr399Ile polymorphisms have been noted to have similar effects in kidney transplantation (46). Clinical studies were conducted on renal transplant recipients with single or multiple TLR4 polymorphisms; the presence of one or both polymorphisms significantly reduces the risk of acute renal rejection (46).

Interestingly, in our murine, minor mismatch (same strain; male donor, female recipient) skin transplant model, the lack of TLR2 signaling contributed only a modest effect on transplant survival, while the lack of TLR4 signaling seemed to have no significant effect (32). While this seems contrary to the findings of the clinical studies mentioned above, it is possible that the chronic immunosuppression used in clinical organ transplantation, differences between human and murine immune responses and the organ-specific responses to TLR signaling may play a role in the variability noted (43). Indeed, it is well established that lung, intestine and skin transplants (which presumably come in more contact with innate immune ligands and pathogens) are more immunogenic than the kidney, heart and pancreas even in the presence of costimulatory molecule inhibition (30).

Work by Samstein and colleagues, who also used a minor- and major-mismatch (tail) skin graft model, corroborated our finding concerning TLR4 signaling in murine skin allograft rejection (35). Instead of pointing to the role of other TLRs in the immune response to allotransplantation, they suggest a role for IL-1 or IL-1 receptors. Although this conclusion is consistent with our own work as we have found that Caspase-1 (ICE) knockout mice (which lack IL-1/IL-18 signaling) demonstrate a modest but significant delay in minor mismatch skin allograft rejection (32), these recipients manifest a significantly more rapid tempo of rejection as compared to MyD88-/- recipients. These results indicate that the phenotype noted in the MyD88-/- recipients must be due to TLR signaling and cannot be solely due to activation via IL-1 and IL-18 (58). Due to the likelihood that there is functional “cross-talk” between and among TLRs (45), it is possible that multiple TLRs contribute to this rejection phenomenon without having to rely on a single receptor. This would be beneficial on the body surface as it presumably comes into contact with multiple, simultaneous microorganisms and ligands, necessitating a complex array of signaling. Disease processes in humans can result from an adverse collection of a variety of genes that cause a collective predisposition to a given condition (43). The complexity of transplant rejection vs. acceptance would imply that a variety of innate and adaptive genetic factors in the donor and recipient contribute to which way the balance tips (43).

The above studies indicate that TLRs are involved in acute allograft rejection. Note, it is likely that there are other innate factors that are TLR independent that may be involved in the host response to organ transplantation. Indeed, several TLR-independent inflammatory mediators (such as CCL19 and CCL21) have been demonstrated to influence donor DC maturation and migration in vivo; thus it is possible that non-TLR ligands influence DC maturation as well (59). Furthermore, different organs may activate different innate immune pathways during acute allograft rejection. Clearly, future studies are warranted to investigate these issues.

9.5.4. The role of MyD88

Our work in a murine skin transplant model has demonstrated an important role for MyD88 in the maturation and migration of DCs, alloimmune priming of CD8+ T cells and subsequent Th1-dependent alloimmune responses in a minor mismatch (same strain; male donor, female recipient) model (58). Our study also indicates that adoptive transfer of activated, wild-type spleen cells restores rejection in the MyD88-/- transplant recipients (58). In our model, an absence of MyD88 in donor or recipient alone led to graft rejection; abrogated graft rejection only occurred if MyD88 was absent from both the donor and recipient (58). We conducted similar studies in a major-mismatch murine skin and heart transplant models and found that, while MyD88 played a significant role in DC function and Th1 immune responses, graft rejection occurred in a MyD88-independent fashion (60). Importantly, in an experimental transplant tolerance model, we found that MyD88 signaling impaired the induction of transplantation tolerance (61).

Work by McKay and colleagues both confirmed our findings and demonstrated a synergistic role for TRIF
and MyD88 adaptors. While inhibition of signaling through MyD88 or TRIF in the C57Bl/6 donor skin alone does not modulate fully mismatched allograft rejection onto BALB/c mice, double knockout (MyD88 and Trif knockout) donor skin survives significantly longer on BALB/c recipients as compared to wild-type donor skin (59). When a less stringent, minor mismatch model is used (multiple minor mismatch), donor skin deficiency in either MyD88 or Trif is sufficient to prolong graft survival (as compared to wild-type donor skin; double knockout donor skin synergistically extended transplant survival (as compared to wild-type donor skin) (59). The increased graft survival was attributed to a decrease in the ability of double knockout donor cells to migrate to the draining lymph nodes of the recipient, as this emigration appears to be the key element in host-anti-donor immune responses (59). In agreement with our work, injection of wild-type donor spleen cells upon transplantation of MyD88/TRIF double knockout skin abrogated the previously noted transplant survival (59). However, in the study by McKay and colleagues, the recipient remained wild-type and thus the recipient DCs were fully sufficient for MyD88/TRIF signaling. Thus, the full impact of TLR signaling on acute allograft rejection was not addressed in this study.

9.5.5. TLR signaling and tolerance induction

Currently, clinical transplantation medical regimens require the chronic use of a plethora of immunosuppressive drugs; such long term immunosuppression has a variety of negative consequences, such as heightened risk of infection, organ toxicity and neoplasia (62). Furthermore, environmental perturbation (such as viral or bacterial infection at the time of costimulatory blockade and/or transplant) can abrogate the effects of the immunosuppressive regimen and cause a rapid allograft rejection (62). As a result, efforts are being made in a variety of research models to provide donor-specific tolerance and allograft survival with a decreased or abrogated need for long-term immunosuppression; TLRs are the focus of some of this work as TLR-mediated signals have been shown to allow effector T cells to overcome steady-state, Treg-mediated suppression in vivo and prevent the therapeutic induction of regulation (30). Although research to elucidate the full mechanisms of the effect of TLRs on regulation is ongoing, recent studies suggest that TLR engagement can alter tolerance through two distinct methods: clonal deletion and Treg suppression (30).

Our work has demonstrated that MyD88 deficiency acts synergistically with costimulatory receptor blockade (anti-CD154 and CTLA4-Ig; inhibit signal 2 from APCs to T cells) in a fully allogeneic and highly immunogenic murine skin transplant model (61). An absence of MyD88 signaling alters the host environment (via modulation of cytokine levels and other inflammatory mediators) and provides an environment that allows T cells to be less activated and more susceptible to the effects of costimulatory blockade. Indeed, costimulatory blockade in combination with a lack of MyD88 signaling is sufficient to significantly increase allograft survival time when both donor and recipient are deficient of MyD88 (versus costimulatory blockade with donor and recipient both being wild-type) (61). Furthermore, we demonstrated that this acceptance was donor-specific, as long-term acceptors rechallenged with a graft from the same donor strain did not acutely reject (in contrast to a third-party allograft) (61).

The lack of MyD88 signaling impaired both DC-inflammatory responses and T cell priming (61). As mentioned previously, normal MyD88 signaling allows for the production of a variety of inflammatory cytokines by DCs, augmenting T effector proliferation and decreasing the suppressive activity of CD4+CD25+ Tregs (56, 61). The importance of this Treg subset was demonstrated when MyD88-/- recipients of MyD88-/- grafts and costimulatory molecule blockade were given anti-CD25 treatment; these mice lost their tolerance phenotype and rejected their skin grafts (56, 61). It is important to note that mechanisms other than Treg activity (such as clonal deletion and anergy) may also play a role in the tolerance induction in our model (61).

Work by Thornley and colleagues demonstrated roles for specific TLRs in decreasing transplant tolerance (62). Their fully allogeneic mouse skin graft model coupled with a tolerance-inducing protocol of donor-specific transfusion (before transplantation) along with anti-CD154 treatment (before and after transplantation) results in the activation, proliferation and subsequent apoptosis of alloreactive CD8+ T cells, prolonging allograft survival. Activation and proliferation are tied to apoptosis, with increased proliferation being tied to increased apoptosis (62). Interestingly, when TLR agonists were administered at the same time as costimulation blockade, alloreactive CD8+ T cell apoptosis was prevented, causing a decrease in allograft survival. In fact, Thorley and colleagues found that TLR activation is as effective as LCMV infection in diminishing skin allograft survival, although viruses do not always act through TLRs. The authors believe that TLR activation results in mature APCs with the capacity to circumvent the signal 2 inhibition caused by anti-CD154; alteration of pro-apoptotic and anti-apoptotic molecule concentration in activated T cells may be a result of TLR4 stimulation.

A study by Chen and colleagues used a fully allogeneic murine heart transplant model to examine the effects of TLR stimulation on the abrogation of tolerance (30). Cardiac transplant recipients that receive anti-CD154 and DST accept their fully allogeneic grafts over 100 days; treatment with CpG or Pam3CysK4 (a TLR2 agonist) abrogates this effect and causes swift rejection in an allograft-specific manner (syngeneic grafts are not rejected). In contrast to Thorley’s work, Chen and colleagues found that both subsets of T cells participate in the allosresponse, as accumulation of both CD4+ and CD8+ infiltrating cells is seen in grafts 30 days post-transplant and lack of CD8+ T cells does not prevent CpG-promoted rejection in their model; CD4+ but not CD8+ cells are necessary for CpG-mediated rejection. There are a variety of reasons why these studies differ; the use of a skin vs. heart transplant model, differing dosage schedules for anti-CD154 and different CpG sequences are a few of the possible culprits. In addition, the Thorley paper gave a
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single dose of agonist at the same time as donor skin transfusion, while the Chen paper gave multiple doses at the time of anti-CD154 administration. Mechanistic studies demonstrated that anti-CD154 treatment (in the absence of CpG) causes an increased Treg/CD4+CD25- ratio in the allograft; this effect is abrogated in CpG treated mice, implying TLR engagement prevents Treg expansion and/or migration to the graft (30).

Finally, it should be noted that although the above studies that used TLR agonists to examine the importance of innate immunity on transplant tolerance induction provided useful fundamental information, the relevance of such an approach is debatable. First, systemic administration of a TLR agonist activates many cells of the immune system. Furthermore, TLRs are expressed on non-immune cells such as epithelial and myocardial cells. Thus, systemic administration of TLR agonists likely activates a wide array of cells. Second, the administration of TLR agonists neither mimics the presence of commensal organisms (which actually may be important for dampening immune responses (63)) nor mimics the presence of an infection, which likely activates many components of the innate immune system both TLR-dependent and independent.

9.6. Toll-like receptors and xenograft responses

Only one paper has been published in regards to this subject; the paper by Schmidt and colleagues investigated the role of MyD88-dependent TLR signaling and fetal islet xenograft rejection (64). The authors previous work demonstrates that deficiencies in single cytokines are not sufficient to prevent xenograft rejection, and inhibition of multiple cytokines only produces transplant tolerance in a minority of subjects (64). Therefore, the authors wished to examine MyD88 knockouts in their model in order to examine the contributions of innate immune and generalized immune deficiency (Th1) to xenografts (note, Th1 responses by the immune system are normally directed towards intracellular pathogens and bacteria while Th2 responses are directed toward multi-cellular eukaryotic pathogens and allergens) (64). Although the authors show that MyD88 knockout mice demonstrate a lower level of IFN-gamma and interleukin (IL-12) mRNA production (Th1 cytokines) during the period of acute rejection, this is insufficient to prevent xenograft rejection (64). In fact, MyD88 deficient mice are able to reject their xenografts in a similar tempo to wild type mice (64). This finding may be due in part to the fact that the mRNA levels of two representative Th2 cytokines (IL-4 and IL-10) are equivalent between wild type and knockout mice during the period of acute rejection and are only higher in knockout mice once rejection has completed (64).

Clearly, future studies are warranted to study the role of TLRs in xenotransplantation. It is possible that TLRs play a more significant role in solid organ transplantation (as opposed to cellular transplantation) or xenotransplantation may rely on a MyD88-independent pathway. Additionally, it is possible that the highly divergent immunological nature of xenografts (as opposed to allografts) may reduce the activation requirements for cellular immunity against xenoad antigens. Indeed, our own work using allograftic mice (mice without lymph nodes and Peyer’s patches; rendered devoid of all secondary lymphoid organs via splenectomy) demonstrates that while these mice are able to accept fully allogeneic skin grafts, porcine xenografts are rapidly rejected (65, 66).

10. PERSPECTIVE

TLRs are an important facet of the innate immune system that detect the presence of a staggering array of ligands and initiate adaptive immune responses. The future of TLR discoveries is as varied and multifaceted as the TLRs themselves. Much work still needs to be done to fully understand the intricate details of TLR structure, dimerization and subsequent signaling pathways. Discovery of additional TLR ligands is an ongoing process, and much work needs to be done to elucidate the role of endogenous ligands and transplantation, as there are many conflicting results in this area, as well as the continual issue of possible contamination with exogenous ligands.

Although it has been known for some time that TLRs are present on APCs and some other tissues, the discovery of TLRs on T cells further obscures the already fine line between innate and adaptive immunity and provides numerous avenues for future research. Only time will tell what other cells of the body may possess TLRs.

Notwithstanding their contributions to transplantation, the understanding of TLR contribution to fields of study as diverse as vaccination, aging and alloimmunity are also of critical importance. In sum, TLRs play an important role in many biological settings and are potential clinical targets for modulating these responses.

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12. REFERENCES

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**Abbreviations:** APCs, antigen presenting cells; BOS, bronchiolitis obliterans syndrome; CpG, cytidyl phosphate guanosine oligodeoxynucleotide; DCs, dendritic cells; fHA, hyaluronan fragments; GdCl3, ionic gadolinium; HSP, heat shock protein; IFN, interferon; IL, interleukin; IRF, interferon regulatory factor; KC, keratinocyte chemoattractant; LRR, leucine-rich repeat; MCP-1, monocyte chemoattractant protein 1; MHC, major histocompatibility complex; MyD88, myeloid differentiation primary response gene 88; NF, nuclear factor; NK, natural killer cells; pALT, plasma alanine aminotransferase; pAST, plasma aspartate aminotransferase; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; ROS, reactive oxygen species; TCR, T cell receptor; TEC, tubular epithelial cells; TGF beta, transforming growth factor beta; TIR, Toll/IL-1 receptor domain; TIRAP, toll-interleukin 1 receptor domain containing adaptor protein; Treg, regulatory T cell; TRIF, TIR-domain containing adaptor inducing interferon beta

**Key Words:** Allograft, Innate Immunity, Ischemia, Mouse, MyD88, Regulatory T Cell, Rejection, Reperfusion, Review, Tolerance, Toll-like receptor, Transplantation, Xenograft, Review

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