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INHIBITION OF TOLL LIKE RECEPTOR IMMUNE RESPONSES BY MICROBIAL PATHOGENS

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1. ABSTRACT

Toll Like Receptors (TLRs) are pathogen recognition receptors (PRRs) that respond to specific pathogen associated molecular patterns (PAMPs) during microbial invasion. After TLR stimulation a series of cellular responses initiate an inflammatory response and influence specific adaptive immunity that ultimately destroy the pathogen. But the immune response is not always able to control the infection. Pathogens have developed mechanisms to overcome and evade distinct arms of vertebrate immunity. Many of these strategies have been extensively described, but with the recent discovery of TLRs additional means to manipulate the innate immune response are currently being studied. Pathogens generally inhibit TLR mediated immunity by either blocking signals that stimulate further host defense mechanisms or by down-regulating their expression. These inhibitory mechanisms have been mainly elucidated in bacterial systems, whereas in other microorganisms they remain to be identified. Here the strategies that pathogenic microbes use to subvert TLR mediated immune responses are reviewed.

2. INTRODUCTION

In vertebrates the immune system can be categorized as innate immunity and adaptive immunity. Adaptive immune responses are based in the recognition of non-self small peptides by receptors expressed on the surface of T and B cells. After antigen recognition these cells experience clonal expansion and receptor rearrangement. These features make this response highly specific and able to develop immunological memory. But its main limitation is the 4 to 7 days required for maturation of effector cells before they are able to participate in host defense. However the adaptive immune system does not work independently and almost every aspect of adaptive immunity is linked to the primary control exerted by the evolutionarily ancient and more universal innate immune system. Innate immunity detects the presence and nature of the infection and by different mechanisms (phagocytosis, opsonization, complement activation, activation of proinflammatory signaling cascades and apoptosis) provides the first line of defense, and controls the initiation and determination of effector mechanisms of the adaptive immunity.

Innate immunity uses non-clonal sets of recognition molecules called pattern recognition receptors (PRRs). There are various groups of PRRs, which can be secreted; expressed on the cell surface or reside in the intracellular compartments. They bind to conserved and constitutively expressed microbial molecules named pathogen-associated molecular patterns (PAMPs) that are essential for the survival of the microorganism. The toll-like receptors (TLRs) are one of the most important PRRs and their involvement in innate immunity was first described in *Drosophila.sp* (2). A year later a mammalian homologue was identified and subsequently other TLRs were found (3). The mammalian TLR family consists of 11 members (TLR1-TLR11), and each seems to have a distinct function in microbial immune recognition. Many of the ligands and intracellular pathways activated in TLR immunity have been described (2), but there is limited information about the mechanisms utilized by different microorganisms to overcome this first line of defense and they are the main topic of this review.

3. STRUCTURE AND FUNCTION OF TLRS

TLRs are involved in the recognition of a wide spectrum of pathogens by binding to (PAMPs). These molecules represent broad groups of microbial families rather than a single specific species and their chemical structure is diverse. Microbial products such as saccharides, proteins, lipopolysaccharides (LPSs), peptidoglycans, lipopeptides, glycolipids, lipids and nucleic acids are among the TLR ligands described so far (2).

TLRs are type 1 transmembrane proteins that cross the membrane once and share similar extracellular domains, which include 18 to 31 leucine-rich repeats (LRRs), and similar cytoplasmic domains of approximately 200 amino acids, which are also similar to the intracellular Toll-Interleukin-1 receptor (TIR) domain (2, 4, 5). Deletion of most of the LRR region or mutation of one of the four

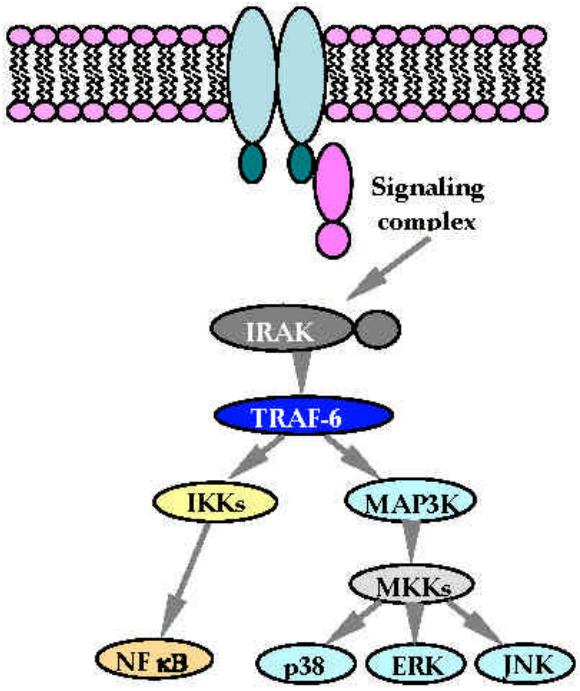

Figure 1. TLR signaling on innate immune cells. Upon interactions with PAMPs, TLR signals are transmitted through two major signaling pathways, MAPKs and NF-kB.

cysteine residues just outside the transmembrane domain leads to constitutive activity of the receptors (3, 6). In addition several cytoplasmic adaptor proteins, including MyD88, TIRAP and TRIF contain the TIR domain and play important roles in TLR signaling (shown in figure 1 as signaling complex). The extracellular domains of the Toll family are quite divergent, for instance the extracellular domains of TLR2 and TLR4 are only 24% identical. This feature makes it possible for different ligands activate different receptors. The divergence of extracellular domains is striking even between homologous genes in mice and humans; for example, the extracellular domains of human TLR4 and mouse TLR4 are only 53% identical (4). Comparison between strains has revealed that the LRRs are responsible for specificity of host–pathogen interaction (7) and suggest that polymorphism between individuals generates sequence diversity that could aid animals in the recognition of rapidly evolving pathogens (8).

Upon interaction with its ligand, the TLR recruits adaptor proteins in the TIR domain. The best characterized signaling pathway is the MyD88-dependant. MyD88 contains a death domain (dd) in the N-terminus and a TIR domain in the C-terminus. When ligand binds to TLR, MyD88 C-terminus interacts with the TIR domain of the receptor and the dd of the N-terminus recruits IL-1 receptor-associated kinases (IRAKs) (figure 1). IRAKs are activated by phosphorylation and then associate with tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) (figure 1) leading to activation of NF-kappa beta (NF-kB) and mitogen-activated protein kinase (MAPK) inflammatory pathways (figure 1). In the NF-kB pathway the IkB kinase (IKK) complex induces phosphorylation of the inhibitor of NF-kB (IkB), which triggers nuclear translocation of NF-kB and induction of inflammatory gene products (2).

After activation by TRAF6, the MAP kinase kinase (MKK) activates MAPKs, which are divided into the extracellular-regulated kinase (ERK), p38 and c-jun amino terminal protein kinase (JNK) (figure 1). These proteins have been implicated in induction of multiple cellular events such as inflammation, proliferation and differentiation (1).

The subsequent inflammatory response and the dominant cytokine profile induced by these inflammatory pathways lead to T-helper and T-cytotoxic development and they are crucial in the defense against different kinds of microbial infections.

The discovery of more TLRs and PAMPs has evolved the recognition system into one in which immune cells use many different TLRs to detect several features of an organism simultaneously. But even this system may be fooled by different pathogens. The ability to evade the harmful effect of a primary immune response may facilitate entrance, establishment and dissemination of the microorganism.

By the other side, when TLR induced immunity is deregulated the control exerted over adaptive immune responses will be abnormal and the final outcome for the host is detrimental.

Some of the mechanisms utilized by several pathogens to evade or alter TLR induced immunity will be described for each microbial group in the next section.

4. BACTERIA

Most of the recent innate immunity research has been focused on bacterial PAMPs and their respective TLRs. Thus, strategies to overcome TLR immune recognition can be classified in two main groups. The first is characterized by changes in the structure of the PAMPs and the second involves manipulation of the host system by the bacteria to overcome the TLR action.

The first is one of the most effective strategies to avoid PRR recognition and involves steric shielding or modification of exposed PAMPs, for instance bacterial capsules and LPS have been recognized as important virulence factors (9). However, PAMPs are essential for microbial survival and major modifications or mutations may be lethal or decrease the viability and virulence of pathogenic bacteria (10), thus placing some limitations on this strategy. Bacterial flagellin, which is recognized by TLR5, is probably the only exception due to the fact that its expression is the result of phase and antigenic variation (11). Although flagellin is an important

virulance factor for many bacteria, it seems that flagellar expression is not essential contributor to the pathogenicity of the enteric pathogen Salmonella enterica serovar Typhimurium (12).

Within the second group there are two main categories. The first is characterized by microorganisms able to downregulate TLR or block its interaction with adaptor proteins. In this category LPS and other endotoxins have been implicated as the downregulators, but the mechanisms involved in the process remain unknown. For instance, the LPS of oral pathogen *Porphyromonas gingivalis* (*P. gingivalis*), is able to downregulate the expression of TLR-4 in human gingival fibroblasts (13). Fimbrillin A is a *P. gingivalis* adhesion protein able to bind TLR4 and induce downregulation of CD18 and the adaptor protein CD14. Moreover, induction of cross tolerance between fimbrillin A and LPS correlated with downregulation of TLR4 (14). *Ehrlichia chaffeensis*, an obligatory intramonocytic bacterium caused downregulation in the expression of CD14, TLR2 and TLR4 as demonstrated by *E. chaffeensis* infected human monocytes becoming progressively less responsive to *E. coli* LPS (15). Treponema spirochetes express small-sized glycolipids that exhibit immunostimulating activities. A *Treponema medium* glycolipid preparation blocked the binding of LPS to immobilized CD14 and LPS binding protein and inhibited nitric oxide (NO) production by a murine macrophage cell line, whereas NO production in response to poly (I:C) RNA and CpG DNA remained unaffected (16) Modulation of TLR1, 2 and 4 have been also studied in experimental human endotoxaemia; stimulation with LPS (2ng/kg) showed TLR4 downregulation on neutrophils, whereas monocytes presented TLR1, 2 and 4 upregulation (17). Thus, endotoxaemia in humans differentially regulated TLR expression in neutrophils and monocytes.

The second group can be subdivided in alteration of TLR intracellular signaling and induction of changes in further immune mechanisms through TLRs. Intracellular signaling by TLRs leads to induction of various genes essential in host defense, including inflammatory cytokines, chemokines, major histocompatibility complex (MHC) and co-stimulatory molecules (2). Mammalian TLRs also induce multiple effector molecules such as inducible nitric oxide synthase (iNOs) and antimicrobial peptides, which can directly destroy microbial pathogens (18). Alterations in the signaling after TLR-ligand interaction are targeted by bacterial products, although for most of them the mechanisms of action are unknown. *Bacillus anthracis*, the causative agent of anthrax uses some strategies to avoid detection by the host immune system. This is likely because of the extensive bacterial spread without evidence of immune response (19). Anthrax lethal toxin is a binary complex composed of lethal factor and protective antigen, lethal factor is a metalloproteinase with unique specificity for MKKs, cleaving between their amino-terminal extension and the catalytic domain. The amino-terminal domain of MKKs is essential for the interaction between MKKs and MAPKs; the cleavage of this domain impairs the activation of MAPKs and inhibits all the innate immune response dependant of this pathway (20). Down regulation of more than one factor involved in intracellular signaling has been also described. *Francisella tularensis* upregulates the expression of a 23 kDa protein during intracellular infection and it is able to block degradation of IkB. Moreover, after LPS or bacterial lipopeptide stimulation the phosphorylation of MAPK p38 and transcription factor c-Jun was inhibited by *F. tularensis* LVS, but not by the 23 kDa protein mutant. Thus *F. tularensis* seems to be capable to abrogate TNF-alpha and IL-1 responses in macrophages (21). Macrophages exposed to CpG DNA experience a hyporesponsive state after subsequent challenge with CpG DNA. Down regulation of the MAPKs JNK, ERK and p38 and the transcription factors AP-1, NF-kB, CREB and STAT1 is associated with limited cytokine release by murine macrophages exposed to CpG DNA. Whereas expression and function of TLR4 and 9, Toll/IL-1R domain containing adaptor protein (Tollip) and TRAF 6 are not downregulated by CpG DNA (22).

Regarding alteration of immune functions after TLR stimulation the majority of studies have been performed with *Mycobacterium.sp* and *Yersina.sp. M. tuberculosis* (MTB) induces vigorous immune responses, yet persists inside macrophages evading host immunity. MTB produces a 19 kDa lipoprotein able to inhibit MHC-II antigen processing and this phenomenon was dependant on TLR2 and independent of TLR4 (23). Further research has shown that MTB, through 19-kDa lipoprotein activation of TLR-2, inhibits IFN-gamma receptor signaling in human macrophages, resulting in decreased MHC-II antigen processing and recognition by MHC-II-restricted CD4 T cells (24). Cytotoxic responses are also important to control MTB infection, but the bacterium has developed strategies to overcome this arm of the immune system. MTB 19 kDa lipoprotein, CpG DNA and LPS are able to inhibit alternate MHC-I antigen processing, but not MHC-I expression through TLR2. MTB 19 kDa lipoprotein and other PAMPs inhibit phagosome maturation and phagosome antigen degradation in a MyD88-dependant manner (25). Thus, MTB is able to silence macrophages and evade immune mechanisms controlled by CD4 and CD8 T cells, promoting chronic infection.

A common characteristic of the three human pathogenic *Yersinia sp*. is the expression of the virulence (V)-antigen (LcrV). LcrV induces immunosupression by upregulation of IL-10 expression (26). To confirm these findings IL-10 knockout mice were utilized; these animals exhibit generic inflammatory responses and marked resistance to infection (27). Further studies demonstrated that LcrV is able to induce IL-10 immunosupression in a CD14 and TLR2 dependant manner (28). *Yersinia sp* is also capable of disabling professional phagocytes by translocation of virulence effectors, termed Yersinia outer proteins (Yops), via type III secretion system. YopJ/YopP down regulates multiple inflammatory molecules and this inhibition mainly involves blocking of the MAP kinase signaling pathway (29). Although Yops have not been described as natural TLR ligands their suppressive functions may serve in the short term, whereas LcrV may

function as the long term systemic immunosupressor in *Yersinia sp* induce pathogenesis.

5. PARASITES

Protozoa and multicellular parasites are known as a major cause of global infectious disease. These microorganisms have complex life cycles and they have evolved with the host immune system, typically producing long lasting chronic infections. In order to be successful in the host harmful environment, parasites depend on highly evolved adaptations that enable them to elude destruction by the immune system.

TLR research in the parasitology field is very limited; *Leishmania.sp, Toxoplasma.sp, Plasmodium.sp, Schistosoma.sp* are among the few parasites known to stimulate innate immune responses through TLRs and the glycoinositolphospholipids (GPIs) from *Trypanosoma cruzi* are the only natural ligand reported so far (2). Mechanisms employed by these microorganisms to inhibit TLRs or immune responses through them are even more limited and they will be described next.

Trypanosoma sp utilized multiple virulence factors to overcome host immunity, GPI anchors are the only parasitic PAMPs known to interact with TLR and they can suppress immune responses by inhibiting maturation of human dendritic cells (DCs), as well as production of TNF-alpha, IL-10 and IL-12p40 in DCs and macrophages (30). *Entamoeba histolytica* express lipophosphopeptidoglycan (LPPG) that is probably recognized by TLR2 and it seems to induce immunosuppression by promoting IL-10 expression and downregulate TLR2 gene expression (31). Other protozoa, specifically, *Toxoplasma gondii* and *Leishmania.sp* are able to interfere with the NF-kB activation pathways in macrophages (32), but it is unknown if such pro-parasite survival strategies are mediated through TLR interaction with protozoan PAMPs.

Parasites evade innate host defenses and some of the strategies utilized by them have been well described. Although some parasites are known to stimulate innate immune responses through TLRs, the parasitic PAMPs involved in this process remain unknown and very few studies have dealt with the inhibition of TLR induce immunity by parasitic molecules.

6. VIRUSES

Viral infection outcome varies from imperceptible to fatal and the clinical picture may be very diverse. This is largely due to the virus-cell interactions occurring in the infected host. Rapidly after infection, cells initiate a first line of defense against the invader, but viruses have developed the ability to modulate different aspects of the cellular physiology and down modulation of the innate immune response is one of them. Amazingly just a couple of viral PAMPs have been described so far. Information about the mechanisms of inhibition of TLR mediated immunity due to viral products is scarce.

Human immunodeficiency virus 1 (HIV-1) expresses several accessory proteins able to manipulate several host-cell processes to achieve optimum life cycle. Viral protein U (Vpu) is one of them and has been showed to interfere with the cellular degradation machinery. Due to the conservation of the Toll/NF-kB signaling pathways between flies and mammals, the *Drosophila melanogaster* system was used to study the effect of Vpu *in vivo.* Vpu was able to inhibit the host NF-kB mediated immune defenses in *D. melanogaster* fat-body cells (33). Vaccinia virus (VV) is a poxvirus also able to downregulate TLR induced immunity; VV encodes proteins that antagonize important components of host antiviral response. For instance VV proteins A46R and A52R share amino acid sequence similarity with the TIR domain and interfere specifically with IL-1 signal transduction inhibiting TLR4 mediated NF-kB activation (34). As parasites, viruses can overcome the host immune defense mechanisms modulating the physiology of the infected cell. MAPK and NK-kB pathways are inhibited during infection, but to date there is no association of these observations with TLR stimulation by viral PAMPs. Recently it was found that TLR7 and TLR8 are able to recognize single stranded RNA (35). This finding may provide more clues to understand the physiology of innate immune responses against viruses and possibly other microbes.

The TLR field is growing fast; most of the research has been focused on the involvement of TLR-2 and TLR-4 in bacterial pathogenesis. Although some inhibitory actions have been discovered, the exact mechanism to downregulate the immune response or the PAMPs involved in these processes remain to be determined. The knowledge of inhibition of TLR induced immunity in parasite and virus pathologies is even more limited and not studied at all in fungal diseases. All this evidence shows a field full of opportunities for researchers interested in TLR dependant immunity.

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