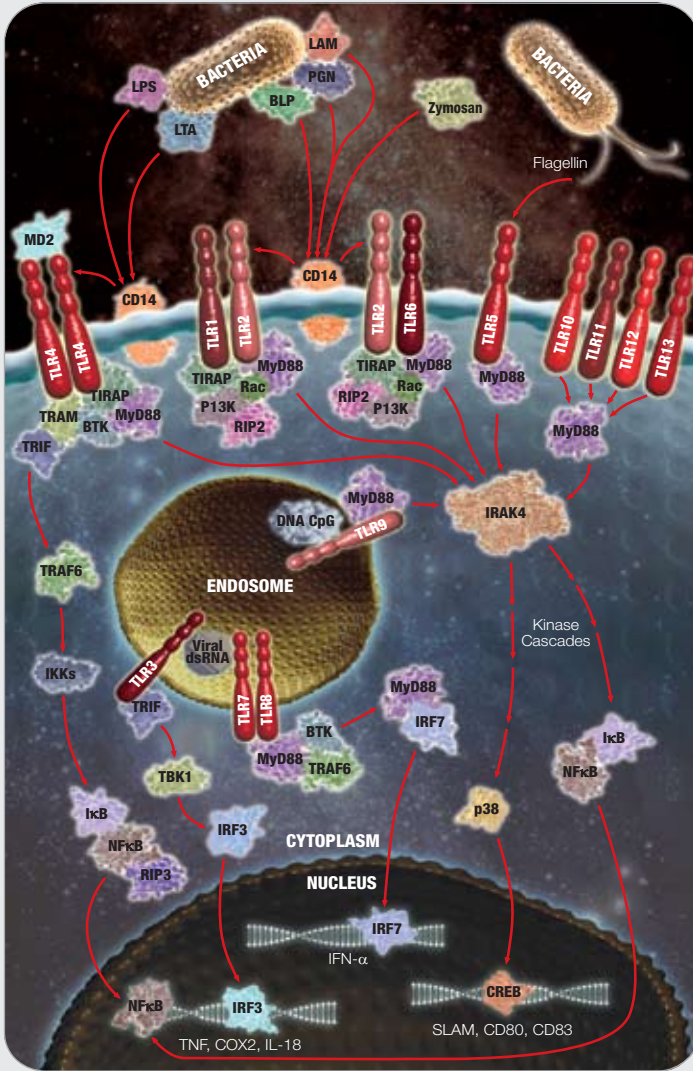
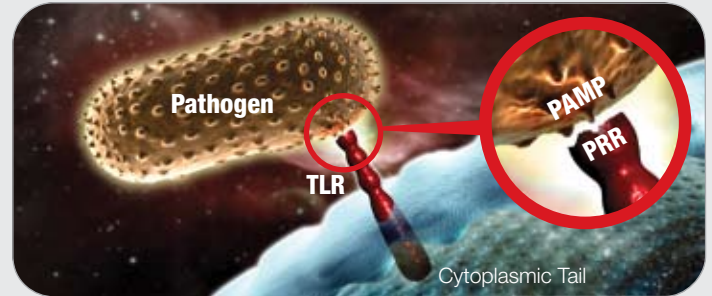


# TOLL-LIKE RECEPTORS AND INNATE IMMUNITY



Toll-like Receptor Signaling Pathway

These highly conserved soluble and membrane bound proteins are collectively called Pattern-Recognition Receptors (PRRs), and it is the PAMP/PRR interaction that triggers the innate immune system. While the history of TLR-dependent observations goes back 100 years, most of the definitive work started about fifteen years ago. A tremendous amount of work has been done during this time, including the discovery of other PRR pathways. The cytosolic NOD (nucleotide oligomerization domain) proteins have been shown to be important innate immune response components.



**Figure 1: Binding of a Pathogen via Its PAMP (Pathogen Associated Molecular Pattern) to a TLR's PRR (Pattern Recognition Receptor) Domain. The Extracellular Leucine-Rich Repeats of the TLR, constitute the PRR Region.**

## Toll-like Receptors (TLR)

TLRs are transmembrane proteins expressed by cells of the innate immune system, which recognize invading microbes and activate signaling pathways that launch immune and inflammatory responses to destroy the invaders. Toll receptors were first identified in *Drosophila*. In mammals, the TLR family includes eleven proteins (TLR1–TLR11). Recently, two new members, TLR12 and TLR13 have been discovered in murine cells, but not much information is known about them. Mammalian TLRs consist of an extracellular portion containing leucine-rich repeats, a transmembrane region and a cytoplasmic tail, called the TIR (Toll-IL-1R (Interleukin-1-Receptor)) homology domain. Different TLRs serve as receptors for diverse ligands, including bacterial cell wall components, viral double-stranded RNA and small-molecule anti-viral or immunomodulatory compounds (Table 1).

Activation of TLRs occurs after binding of the cognate ligand to the extracellular leucine-rich repeats portion of the TLR. In humans, TLR1, 2, 4, 5 and 6 are outer membrane associated, and respond primarily to bacterial surface associated PAMPs. The second group, TLR3, 7, 8 and 9 are found on the surface of endosomes, where they respond primarily to nucleic acid based PAMPs from viruses and bacteria. Upon binding with their cognates, TLRs activate two major signaling pathways. The core pathway utilized by most TLRs leads to activation of the transcription factor NF-κB (Nuclear Factor-κB) and the MAPKs (Mitogen-Activated Protein Kinases) p38 and JNK (c-Jun N-terminal Kinase).

The second pathway involves TLR3 and TLR4 and leads to activation of both NF-κB and another transcription factor IRF3 (Interferon Regulatory Factor-3), allowing for an additional set of genes to be induced, including anti-viral genes such as IFN-β (Interferon-Beta) and others (1). The innate immune response is a complex set of interactions that have evolved to optimize the response to pathogens. While the structure of the TLRs has been highly conserved, the innate immune response for each organism has selectively been driven to best protect against the pathogens found in the host's environment.

## Innate and Adaptive Immunity

The immune system is divided into two parts, the innate and adaptive systems. The adaptive immune response depends on B and T lymphocytes which are specific for particular antigens. This system involves clonal selection of antibody producing B cells to respond to foreign antigens, and works well, but has a major limitation in that it takes from 4 to 7 days to ramp up. In that time period, pathogens could overwhelm the organism.

In contrast, the innate immune system is immediately available to combat threats. There is no complicated method of selecting cells that react to foreign substances from those that react to self. There is no memory to change how the system responds to the same threat upon the second or third exposure. Instead, the innate immune system responds to common structures shared by a vast majority of threats. These common structures are called pathogen associated molecular patterns, or PAMPs, and are recognized by the toll-like receptors, or TLRs. In addition to the cellular TLRs, an important part of the innate immune system is the humoral complement system that opsonizes and kills pathogens through the PAMP recognition mechanism.

**Table 1: The TLRs and Their Pathogen Derived Activators (2).**

PAMP	PRR	Pathogen
Pam3CSK4, PGN, Zymosan et al	TLR1,2,6	Gram Positive Bacteria (eg <i>S. aureus</i> )
LPS, Lipid A	TLR4	Gram Negative Bacteria (eg <i>E. coli</i> )
Flagellin	TLR5	Bacteria, Flagellum
dsRNA	TLR3	Virus
ssRNA	TLR7,8	Virus
CpG DNA	TLR9	Bacteria, DNA

## Innate Defense Against Bacteria

As shown in Table 1, the defense against bacteria involves all the TLRs except for 3,7 and 8 which are virus specific. Gram positive and negative bacteria differ in their surface PAMPs and bind to different TLRs. The specificity of the TLRs for bacterial cell wall fragments has just recently been established, as early reports were later found to be due to trace contaminants.

The signal transduction pathway for TLR4 activation by LPS (Lipopolysaccharide) serves as a representative example of the surface bound TLRs (see the opening illustration of the pathway).

LPS first binds to the CD14 (Cluster of Differentiation-14) receptor, which then transfers it to TLR4. TLR4 homodimerizes and forms a complex with the protein MD2. Cells need both MD2 and TLR4 in order to recognize LPS. TLR4 activation engages a set of MyD88 (Myeloid Differentiation Primary-Response Protein-88) adaptor family members, including TIRAP, TRIF, TRAM (all three are TIR domains containing adapter proteins) and MyD88. This pattern of activation is general for cell surface TLRs, but the subsequent intracellular signal cascades, which include a number of transcription factor activations, are unique for each TLR. This results in a response that is appropriate to each threat (1).

TLR2 is activated by bacterial LAM (Lipoarabinomannan), BLP (Bacterial Lipoprotein), and PGN (Peptidoglycans). LAM and PGN act on TLR2 through the CD14 receptor, similar to the process followed by the TLR4 with a similar downstream effect. BLP mediates both apoptosis and NF- $\kappa$ B activation through TLR2. TLR2 is also responsible for the recognition of the Yeast cell-wall particle Zymosan. Zymosan acts through the CD14 receptor to influence TLR2. The phagocytosed TLR2 vesicle signals the production of TNF (Tumor Necrosis Factor), through the NF- $\kappa$ B pathway. TLR6 associates with TLR2 and recognizes diacylated MALP2 (Mycoplasmal macrophage-Activating Lipopeptide-2 kD). Like TLR4, they also signal through MyD88 and TIRAP. PI3K (Phosphatidylinositol-3 Kinase), RIP2 (Receptor-Interacting Protein-2) and Rac (Ras-Related C3 Botulinum Toxin Substrate) are also involved in TLR6-TLR2 mediated signaling. TLR1 also associates with TLR2 and recognizes the native mycobacterial 19-kDa lipoprotein along with TLR2. TLR1-TLR2 also signals through MyD88, TIRAP, PI3K, RIP2 and Rac. TLR1 and TLR6 may participate in the activation of macrophages by gram positive bacteria. TLR5 is a signaling mediator of bacterial flagellin, thus activating NF- $\kappa$ B and may play a role in resistance to Salmonella infection (3). Human TLR10 is an orphan member of the TLR family. Genomic studies indicate that TLR10 is in a locus that also contains TLR1 and TLR6, two receptors known to function as coreceptors for TLR2. TLR10 not only homodimerizes but also heterodimerizes with TLRs 1 and 2. It has been found to activate gene transcription through MyD88. TLR9 is responsible for the recognition of CpG islands of bacterial DNA. The extracellular CpG fragment may activate TLR9, thus inducing the endocytosis of the DNA along with TLR9, or perhaps the bacteria is phagocytosed and TLR9, which has separately formed on the phagosome, is activated by the CpG islands; whatever the exact method, TLR9 activates the NF- $\kappa$ B pathway from the endocytosed vesicle. Recently IRF8 (Interferon Regulatory Factor-8) has been shown to be activated by TLR9 through MyD88 (3).

Co-receptors on TLR-bearing cells play a critical role in the inflammatory response. In monocytes for example, the CD36 and CD14 co-receptors are necessary for the TLR2 response to gram positive bacteria.

The continued arms race between bacteria and immune defense mechanisms is demonstrated by pyloric bacteria, which have evolved a modified flagellum that evades detection by TLR5, helping this pathogen to establish residency in the mammalian digestive system.



**Figure 2: The Flagella of *H. pylori* (*Helicobacter pylori*) Does Not Activate TLR5 due to Sequence Changes in the Flagellar Protein That Prevent Detection by TLR5.**

In addition to the TLRs, two NOD (nucleotide oligomerization domain) proteins in the cytoplasm, have recently been found to play an important role in the innate defense against *E. coli* & *S. aureus*. The NOD proteins contain leucine-rich repeats very similar to those in TLRs that recognize specific components of these bacteria (diaminopimelic acid) and form a cytoplasmic signaling platform with other proteins known as the inflammasome. This signaling leads to IL-1 & IL-8 production.

Recently it has been argued that while it is well established that a strong innate defense response to bacteria is essential for survival, the most important role of this TLR activation in the long term, may be in the induction of the adaptive immune response. This is discussed further in the three sentinel cells section.

## Innate Defense Against Viruses

Viral nucleic acids contain PAMPs that are recognized by intracellular TLRs. These TLRs are located on the intracellular endosome membranes. The TLRs found on endosomes are TLR3, TLR7, TLR8 and TLR9. TLR3 activates immune cells in response to double-stranded viral RNA. The stimulation of the TLR3 triggers TRIF activation that ultimately activates the IRF3 transcription factor through TBK1, independent of MyD88. This leads to the secretion of IFN- $\beta$ . TRIF also activates RIP1 (Receptor-Interacting Protein-1) and TRAF6, which may further activate the NF- $\kappa$ B pathway. Small anti-viral compounds activate immune cells via the TLR7/MyD88-dependent signaling pathway. TLR7 binds MyD88 and activates IRAF and TRAF6. TRAF6 then activates TANK (also known as I-TRAF). TANK interacts with TBK1 and IKK- $\epsilon$  to activate IRF3. TLR7 or TLR8 may also activate IRF7 through interaction of MyD88, BTK and TRAF6, thus inducing anti-viral responses by producing IFN- $\alpha$  (Interferon-Alpha). Recently, Mouse TLR11 has been identified as a participant in defense against uropathogenic bacteria. The ligands for Mouse TLR12 and TLR13 are currently unknown.

It should be noted that only plasmacytoid cells use the TLR pathway for viral defense. Other cells use RIG1 (retinoic acid inducible gene1)-like helicases (RLHs) to recognize viral PAMPs which results in primarily an IFN response (4,5). The fact that plasma cells utilize TLRs suggests that the TLR-dependent response to viral infection is both important for immediate viral protection, as well as the activation of adaptive immunity via the inflammatory cytokines. A number of studies have shown that a weakened TLR response to particular viruses leads to poor antibody & Th1 responses, and the combination leads to persistent viral infections.

## Innate Defense Against Parasites

The study of TLR activation in parasitic diseases is just beginning, but these early results indicate a significant role. Polymorphisms in TLRs have been linked with the severity of systemic malarial infections. In contrast, an intact TLR signaling system has been shown to contribute to the severe cerebral malarial infection that is often lethal (6). This is an example of how a vigorous TLR response to a parasite can lead to a more severe disease.

In leishmaniasis, which affects 10 million people, TLR2 and TLR4 are required for proper parasite control, due to the activity of inducible nitric oxide (iNOS). A second factor induced by TLR4 activation, neutrophil elastase, is also important for the leishmanicidal activity of macrophages (7).

*Toxoplasma gondii*, the common parasite causing toxoplasmosis in humans, binds to the newly discovered TLR11, which has no other known ligands in humans.

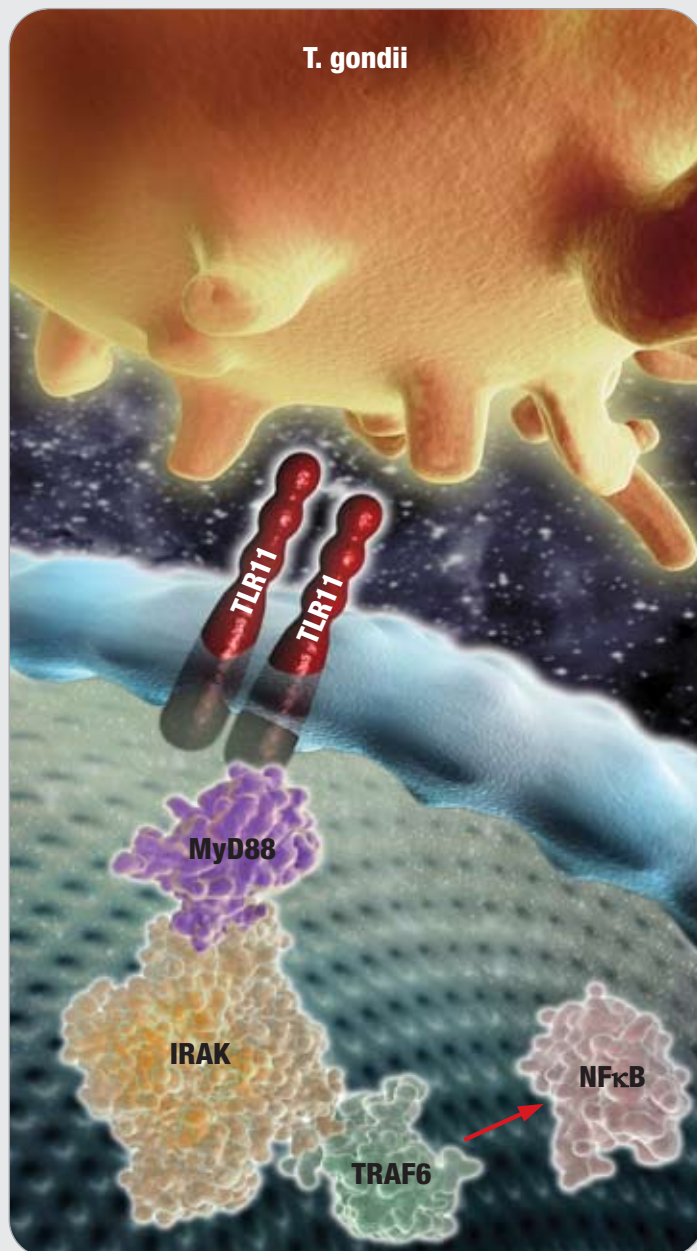


Figure 3: TLR11 Recognizes and Is Activated by the *T. Gondii* Parasite.

## The Three Sentinal Cells of Innate Immunity and TLRs

TLRs are primarily found on macrophages, mast cells and dendritic cells, the three sentinal cells of the innate immune response. It is interesting that the surface expression of TLRs in humans is highly variable and this has been linked to susceptibility to infections (8). It should be noted that the activation of TLRs on these cells begins a complex set of signaling cascades that are not yet completely understood. These interactions are not restricted to the innate immune response, but they also play an important role in adaptive immunity.

We are at an exciting point in immunological research, where our knowledge of the innate immune response, and how it guides adaptive immunity, may lead to more effective treatments of immunological diseases.

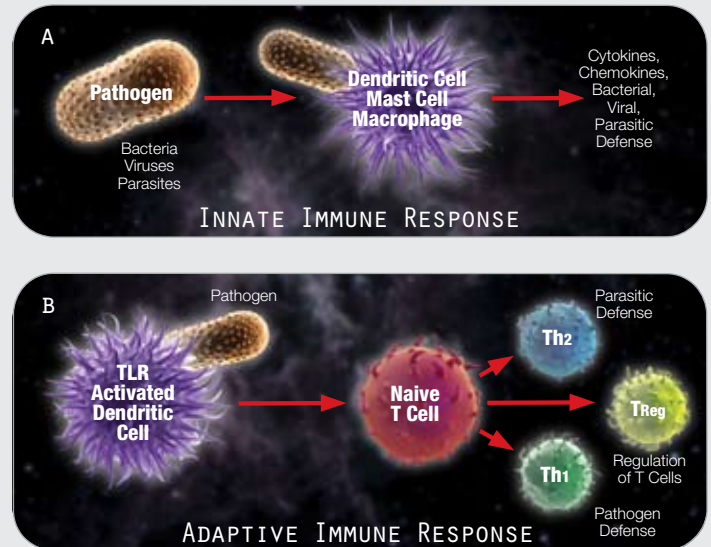


Figure 4: The 3 Sentinal Cells, Dendritic, Mast & Macrophages Protect Against Pathogens. Dendritic Cells Also Are Critical in the Adaptive Immune Response.

## Fine Tuning TLR Activation

Unchecked TLR activation by pathogens can lead to serious medical consequences, such as sepsis and autoimmune diseases. In the last few years, negative modulators of TLR activation have been identified, and their important role in reducing the inflammatory response has been demonstrated in animal models (9-11). The TAM family members are one example.

The TAM family, has been found to be central to the fine tuning of the TLR response. Loss of function of the three members of this family (Tyro3/Axl/Mer) in a triple knockout mouse results in a profound dysregulation of the immune response (10). This includes massive splenomegaly and lymphadenopathy, lymphocyte infiltration into all tissues, and high levels of autoimmunity. Even a single knockout of just Mer is sufficient to elevate susceptibility to LPS induced shock via the TLR4 signaling. These mice have elevated levels of dendritic cells, and the cells express elevated levels of activation markers, including MHC class II antigens. This effect was not restricted to TLR4, as hypersensitivity to the TLR3 activator polyIC was also observed. While the details of the mechanisms for this modulation of the innate immune response are not yet known, the TAM receptor ligands Gas6 and ProS, play an important role. The inhibitory effect requires the synthesis of SOCS1 (suppressor of cytokine signaling 1) which had been previously identified in the cytokine response. Further downstream, the transcription of STAT1 was shown to be essential, as was the IFN receptor IFNAR1.

## Summary

Innate immunity is recognized to play an important role in the response to challenge by pathogens.

The immune functions in which toll-like receptors play important roles include:

- **Orchestration of the immediate tissue specific and global response of the innate immune system to pathogens.** This orchestration is driven primarily by cytokine and chemokine production (TNF, Interferons, IL-1, IL-2, IL-6, IL-8 and IL-12 among others). Perhaps the most important of these early signals are the chemokines that draw the phagocytes to the site of infection.
- **Transition from innate to adaptive immunity.** In addition to the role in the innate immune response, TLRs have an important role in adaptive immunity by activating antigen presenting cells. The cytokine signaling cascade stimulated by TLR activation, begins a complex series of interactions that has evolved in each organism to maximize the odds for survival. Among the more important of these signals is T cell differentiation and regulation. TLRs on dendritic cells in particular, are essential in the T-helper-1 (Th1) versus Th2 pathways (12). An important early component of the Th1 response is the activation of cytotoxic T cells that helps to control the infection (Fig 4).

Our knowledge of the complex innate immune response is rapidly increasing. An organism's survival depends on a prompt response to pathogens, but it is equally important to avoid unregulated inflammation that can lead to dangerous pathologies such as sepsis and autoimmune disease. It is this fine balance between protection and self-damage that drives the complexity of the innate immune response.

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## SABiosciences Toll-Like Receptor Research Products

SABiosciences offers a number of research tools for the study of toll-like receptors and their signaling pathways:

Technology	Product	Catalog #
PCR Array	Toll-like Receptor Signaling PCR Array	Human PAHS-018
		Mouse PAMM-018
		Rat PARN-018
PCR Array	Interferons (IFN) & Receptors PCR Array	Human PAHS-064
		Mouse PAMM-064
PCR Array	Chemokines & Receptors PCR Array	Human PAHS-022
		Mouse PAMM-022
		Rat PARN-022
PCR Array	Inflammatory Cytokines PCR Array	Human PAHS-011
		Mouse PAMM-011
		Rat PARN-011
Oligo-Microarray	Inflammatory Cytokines & Receptors GEArray®	Human OHS-011
		Mouse OMM-011
		Rat ORN-011.2
siRNA Array	NFκB Signaling Pathway siRNA Array	Human SAH-025A
ELISArray	Th1, Th2, Th17 Cytokines Multi-Analyte Kits	Human MEH-003A
		Mouse MEM-003A
ELISArray	Common Cytokines	Human MEH-006A
ELISArray	Inflammatory Cytokines	Human MEH-004A
		Mouse MEM-004A
Cell-Based Assay	Signal NFκB Reporter Assay Kit ( <i>Luciferase</i> )	Human CCS-013L
Cell-Based Assay	Signal ISRE Reporter Assay Kit ( <i>Luciferase</i> )	Human CCS-008L
Cell-Based Assay	Signal GAS Reporter Assay Kit ( <i>Luciferase</i> )	Human CCS-009L
Cell-Based Assay	Signal SMAD Reporter Assay Kit ( <i>Luciferase</i> )	Human CCS-017L
Cell-Based Assay	Signal NFκB Reporter Assay Kit ( <i>GFP</i> )	Human CCS-013G
Cell-Based Assay	Signal SMAD Reporter Assay Kit ( <i>GFP</i> )	Human CCS-017G
Cell-Based Assay Array	Signal Finder™ Immune Response 10-Pathway Reporter Array ( <i>tube</i> )	Human CCA-002L
Cell-Based Assay Array	Signal Finder Immune Response 10-Pathway Reporter Array ( <i>plate</i> )	Human CCA-102L

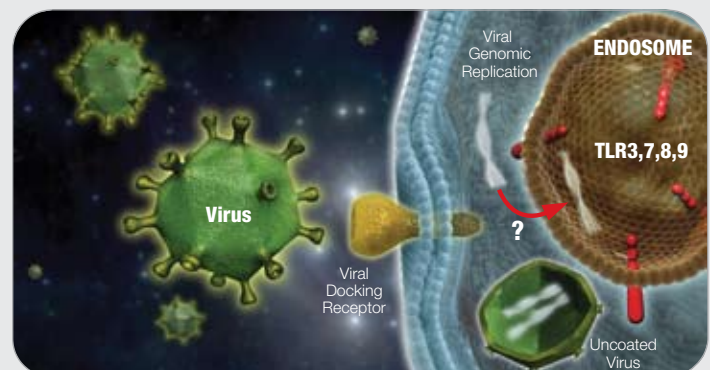


Figure 5: Viral Nucleic Acids Activate Endosomal TLR3,7,8,9.