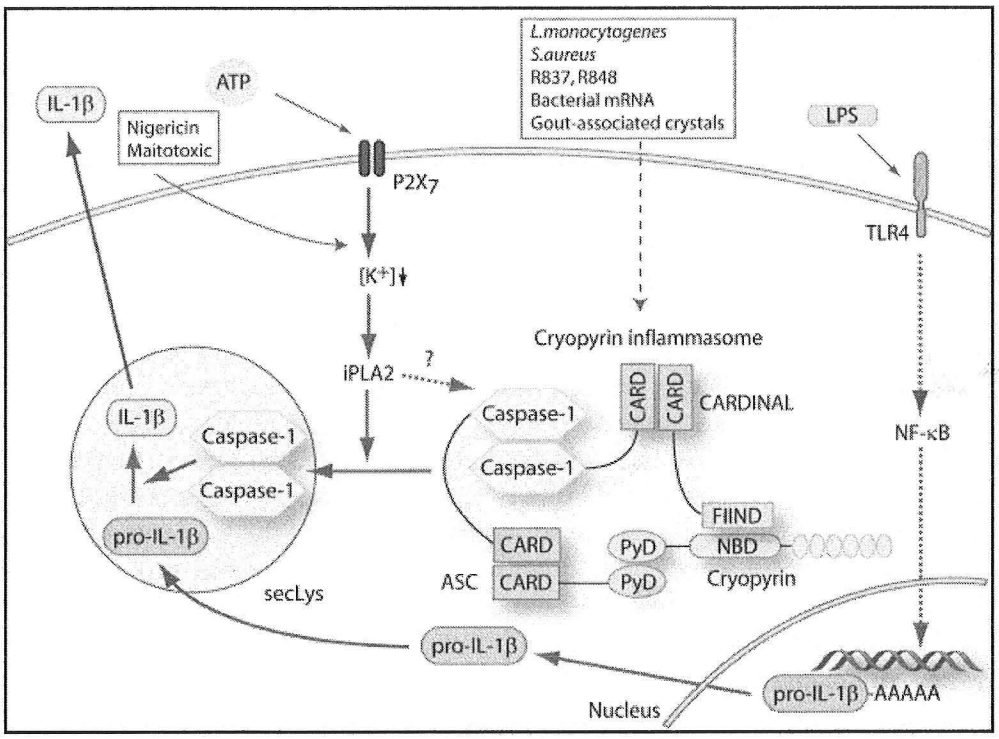
RECENT ADVANCES IN INNATE IMMUNITY



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Internal Medicine Grand Rounds

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This is to acknowledge that Joel D. Taurog, M.D., has not disclosed any financial interests or other relationships with commercial concerns related directly or indirectly to this program. Dr. Taurog will be discussing off-label uses in this presentation.

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Overview:

- Innate immunity refers to germline encoded mechanisms of host defense that respond directly to infection or danger, recognize microbial- or damage-associated molecular patterns, and lack immunologic memory.
- In recent years, several families of phylogenetically conserved molecules have been identified, termed pattern recognition receptors (PRRs), that mediate innate immune responses
- These receptors recognize pathogen-associated molecular patterns (PAMPs) that are essential to the survival of the pathogens and not shared by the host
- Some of these receptors recognize endogenous molecules, termed damage-associated molecular patterns (DAMPs), in the context of cellular stress or damage
- Toll-like receptors (TLRs), a class of membrane-associated PRR, recognize a broad spectrum of pathogenic microorganisms
- NOD-like receptors (NLRs), another class of PRR, recognize intracellular bacterial products and endogenous signals
- The focus of this Grand Rounds will be on the TLRs and on one particular example of NLR termed cryopyrin or NALP3 that is essential to the secretion of IL-1
- A recurring theme in the signal transduction pathways of the TLRs and the intracellular interactions of the NLRs is the modular organization of proteins into domains and protein-protein interactions through the homotypic interaction of like domains
- There is extensive regulation of innate immune pathways, and extensive interaction between innate and adaptive immunity
- Although most of the literature on these recently recognized processes has been published in basic science journals, the emerging clinical relevance of these findings is very great

Legend to Cover Figure: Cryopyrin Is a Key Component of IL-1 β Maturation in Response to Different Stimuli

LPS treatment of macrophages induces the expression of pro-IL-1 β that accumulates in secretory lysosomal vesicles. ATP stimulation of the purine receptor $P2X_7$ leads to rapid efflux of K+. This activates phospholipases such as calcium-independent phospholipase A2 (iPLA2). iPLA2 induces the colocalization of caspase-1 with pro-IL-1 β within secretory lysosomes. The precise mechanism by which ATP and other agonists (boxes) induce the assembly/activation of the cryopyrin inflammasome is not known. The inflammasome consists of cryopyrin and the core components ASC and caspase-1. These proteins assemble via homotypic interactions between their respective pyrin (PyD) and CARD domain. In humans, the adaptor protein CARDINAL is thought to interact with the nucleotide binding domain (NBD) of cryopyrin via its FIIND domain. CARDINAL also binds caspase-1 via CARD-CARD interactions. At present, a CARDINAL homolog has not been identified in mice. The activation of caspase-1 is thought to occur due to the induced proximity of multiple caspase-1 molecules within the inflammasome. In addition to ATP, other stimuli activate the cryopyrin pathway. These include Gram-positive bacteria, R837/R848, bacterial RNA, gout-associated crystals, nigericin, and maitotoxin. (Lich *et al.*, 2006)

Abbreviations are explained in the Appendix on page 21

Two recent clinical trials of the recombinant IL-1 receptor antagonist anakinra in common adult metabolic diseases

In March 2007, a paper from Switzerland was published in *Arthritis Research & Therapy* (So *et al.*, 2007) describing treatment of gouty arthritis with the biological agent anakinra. Anakinra is a slightly modified version of the naturally occurring IL-1 receptor antagonist first identified in the 1980's and cloned in 1990 (Eisenberg *et al.*, 1990). In this study by So *et al.*, 10 patients with recurrent or chronic gouty arthritis in which conventional agents were either ineffective or contraindicated were treated with a 3 day course of anakinra. Pain improvement ranged from 50 to 100% (median 85%). Here is an illustrative case from that paper:

> A 70-year-old man with an 8-year history of chronic tophaceous gout was assessed for hypouricemic treatment. The patient's past medical history included congestive cardiac failure, severe ischemic heart disease, hypertension and renal insufficiency (serum creatinine 3 mg/dl). Previous trials of treatment with allopurinol had to be abandoned because acute gout developed after the first dose and did not respond to small doses of NSAIDs. Higher doses of NSAIDs were contraindicated because of renal failure. Colchicine at low doses (<1 mg/day) provoked rapid onset of diarrhea.
>
> The patient was again started on a low dose of allopurinol (100 mg per day), and after the first dose developed acute arthritis of the right foot and ankle. Anakinra was administered, 100 mg subcutaneously daily for 3 days, with rapid and complete resolution of the signs and symptoms of arthritis. The patient continued on allopurinol 100 mg daily, and at follow-up 2 months later he had no further flare-ups while continuing on the same dose of allopurinol.

In another study (Larsen *et al.*, 2007), published a month later by collaborating groups from Switzerland and Denmark, 70 patients with type 2 diabetes (BMI > 27, HgbA1c > 7.5%) were randomized to 13 weeks of daily anakinra, 100 mg s.c., or placebo. Significant improvement was seen in the anakinra-treated subjects in HgbA1c, C-peptide secretion, ratio of proinsulin to insulin, and in levels of interleukin-6 and C-reactive protein. The only significant adverse events were injection site reactions in half the treated group.

Among the many interesting questions raised by these studies is the following. Monosodium urate crystals triggering of IL-1 release from blood and synovial fluid monocytes, and IL-1 toxicity to pancreatic beta islet cells, were both demonstrated over 20 years ago (Bendtzen *et al.*, 1986; Di Giovine *et al.*, 1987), and the toxic effect of IL-1 on islet cells was the subject of many subsequent studies. Anakinra became available for clinical trials over a decade ago (Campion *et al.*, 1996). The question then is, why is it that only very recently has IL-1 antagonism been attempted in the treatment of these common disorders?

To answer this question, in this Grand Rounds I would like to review some interrelated areas of knowledge that have blossomed in the past decade and that are directly responsible for the current renaissance in IL-1-related therapy.

The concept of innate versus adapative immunity

Although proteins or fragments thereof represent most of the antigens recognized by B lymphocytes and virtually all of the antigens recognized by T lymphocytes, it has been understood for many decades that most proteins by themselves make very poor immunogens. In raising antibodies immunologists have routinely used adjuvants such as complete Freund's adjuvant, which consists of killed mycobacteria suspended in oil. More generally, it can be said that in vivo antigen-specific immune responses require an accompanying inflammatory stimulus. The molecular and cellular basis of this accompanying stimulus has come to be called *innate immunity*, and the antigen-specific response of T cells and B cells has come to be called *adaptive immunity*.

Strangers or Dangers? The concept of innate immunity vs. adaptive immunity which has developed over the past 15-20 years is credited to the late Charles Janeway (Janeway, 1989) whose ideas were influenced by the observation that both T cells and B cells require an antigen-nonspecific second signal for immune activation. Whereas adaptive immune recognition is carried out by somatically rearranged T cell and B cell receptors, Janeway conceptualized innate immune recognition as based on what he termed **pattern recognition receptors (PRRs)**, which are germline encoded and which have broad specificities for conserved microbial structures. These latter structures are now commonly referred to as **pathogen-associated molecular patterns (PAMPs),** although they are also found on non-pathogenic microorganisms. This was a break from the prevailing concept that innate immune mechanisms were largely non-specific. The past decade has seen extensive experimental confirmation of this conceptualization. Basic features distinguishing innate and adaptive immunity are listed in Table 1.

Table 1. Innate vs. adaptive immunity

	Innate	Adaptive
Response time	minutes to hours	$\geq$ 4 days
Memory	no	yes
Specificity	broad	narrow
Self vs. nonself discrimination	yes	no
Heritable specificity	yes	no

Table 2. The concept of innate immunity vs. the use of the term innate immunity

Years	Publications with MeSH Keyword "innate immunity"	Publications containing the term "innate immunity"	Ratio
1950-59	573	2	287
1960-69	3,158	7	451
1970-79	9,864	44	224
1980-89	15,104	175	86
1990-99	12,020	1,032	11.6
2000-07	15,187	10,093	1.5

Source: Medline at PubMed

It should be emphasized that Janeway did not originate the term innate immunity, and that research in the area that is now considered innate immunity had been going on for many decades

before Janeway's ideas appeared. This is illustrated in Table 2, in which the MeSH keyword of innate immunity has been applied, presumably retroactively, to thousands of publications over the past decades. However, it can be seen that the term itself is now much more commonly used.

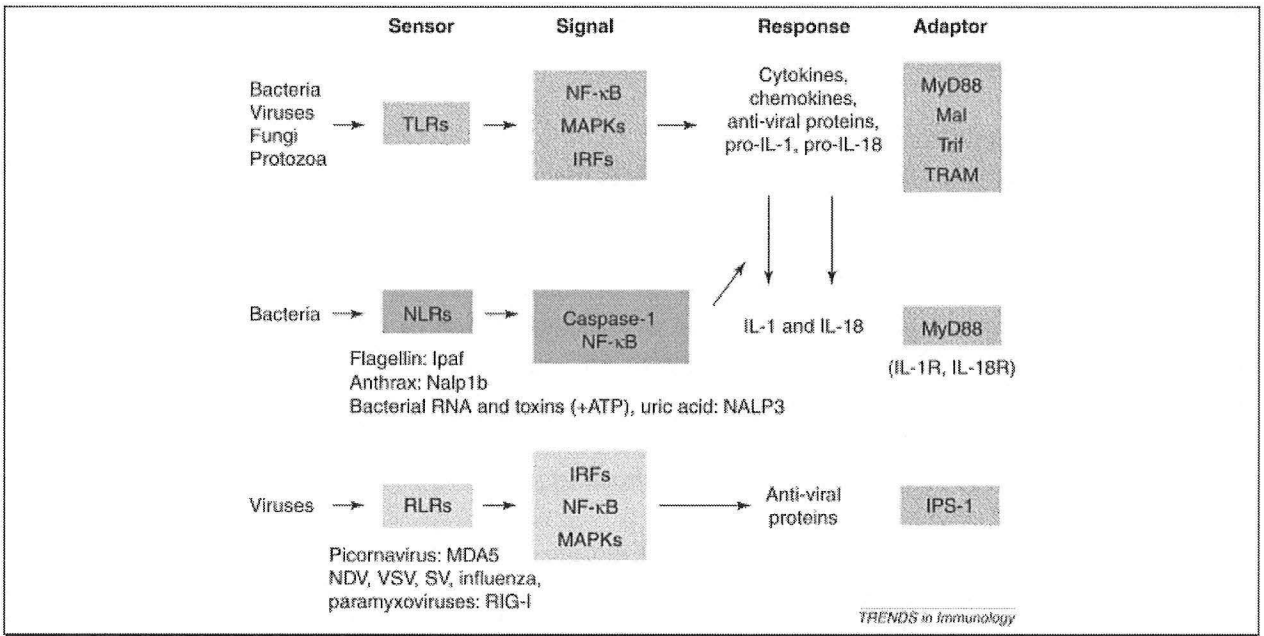

Figure 1. Three families of receptors mediate the recognition of microbes. TLRs are defined by the presence of a TIR domain and LRRs; they recognize multiple pathogens and, by the specific recruitment of the adaptors MyD88, Mal, TRIF and TRAM, result in the induction of immune and inflammatory genes. The signals for this response are the activation of NF- κ B and mitogen activated protein (MAP) kinases, and, in the case of anti-viral TLRs, IRFs. Pro-IL-1 β and pro-IL-18 are important proteins induced by TLRs. NLRs have a tripartite domain structure consisting of a PYD or CARD domain, a NBD and LRRs. NOD1 and NOD2 detect the subcomponents of PGN, mDAP and MDP, respectively, resulting in NF- κ B activation; NALP3 detects uric acid, bacterial RNA and Gram-positive bacterial toxins and ATP; NALP1b detects *Bacillus anthracis*, and Ipaf detects flagellin. NALP3 interacts with NALP2, ASC and Cardinal, which recruit and enable the activation of caspase-1. Caspase-1 acts on pro-IL-1 β and pro-IL-18, resulting in the production of mature bioactive forms whose receptors, in turn, signal through MyD88. The NALP1 inflammasome occurs in a complex with ASC, caspase-5 and caspase-1, and Ipaf is complexed with ASC and caspase-1. TLR signalling is required to prime inflammasome function by an unknown mechanism. RLRs are defined by the presence of a CARD and helicase domain; they detect viral RNA and recruit the adaptor IPS-1 to activate IRFs, NF- κ B and MAPKs, resulting in the induction of anti-viral proteins, complimenting the activity of anti-viral TLRs. (Creagh and O'Neill, 2006)

Janeway's model was criticized by Polly Matzinger, who argued that what the immune system is programmed to respond to is not necessarily microbial molecules, but "danger signals," which potentially could be derived from damaged or distressed cells (Matzinger, 1994). Janeway maintained that infection was the driving force in immune development (Janeway, 1992).

In recent years, great progress has been made in identifying the molecular basis for innate immunity. For most of this time, it appeared that Janeway was right, as whole families of the PRRs and PAMPs that he predicted have been identified, along with the mechanisms through which they operate (Fig. 1). However, in recent years, evidence in support of Matzinger's hypothesis has also been obtained, with the discovery of endogenous DAMPs (danger-associated molecule patterns). In this Grand Rounds, I would like to present a few highlights from the thousands of publications that have appeared on the subject of innate immunity. I will focus

most of the time on recognition of microbial products, but touch on the emerging fascinating story of endogenous danger signals.

The pathogen sensor PRRs that have been identified can be classified into three broad categories, the TLRs (Toll-like receptors), NLRs (NOD-like receptors), and RLRs (Rig-I-like receptors) (Fig. 1). I will focus here on the TLRs, one of the biggest topics of the past decade in immunology, and also on one particular example of NLR, namely NALP3.

The Toll-like receptors

In 1986, the transcription factor called NF-kB was discovered in David Baltimore's lab at MIT (Sen and Baltimore, 1986). At first it appeared to be specific for mature B cells, but it was soon shown to be ubiquitously distributed in the cytoplasm of many other cell types in an inactive form bound to the inhibitor, IκB. In cells of the immune system, a wide variety of stimuli were found to activate signaling pathways leading to the degradation of IκB and translocation of NF-κB to the nucleus, where it in turn was shown to upregulate a host of genes involved in inflammation and the immune response, including B and T cell receptors, adhesion molecules, cytokines, growth factors, acute phase proteins, other transcription factors, enzymes involved in NO production (Baeuerle and Henkel, 1994; Ghosh *et al.*, 1998).

Active NF-κB is a dimer of members of the **rel** family of proteins. Although NF-κB was first described as a heterodimer of p50 and p65 subunits, p65 homodimers and other heterodimeric forms are also transcriptionally active. Rel proteins are named for their homology to the c-rel protooncogene and v-rel viral oncogene identified in turkeys in the early 1980's. Rel proteins contain a conserved N-terminal 300 amino acid region known as the rel homology domain. This region is responsible for DNA-binding, dimerization, and interaction with IκB family members, and it contains a nuclear localization sequence (Ghosh *et al.*, 1998).

Meanwhile, in an apparently completely unrelated area, by the mid-1980's Drosophila workers had characterized a large number of genes involved in Drosophila embryogenesis. Among these was a gene *dorsal*, one of 12 involved in determining dorsal-ventral patterning. Dorsal delineates polarity by specifically activating genes on one side of the embryo and repressing genes on the other. In 1987, this gene was found to have striking sequence similarity to the c-rel and v-rel oncogenes (Steward, 1987), and, as was subsequently shown, to NF-κB. Moreover, the dorsal gene product was found to be activated by dissociation from an inhibitor and to translocate in a manner similar to NF-κB, and the inhibitor of Dorsal, called Cactus, was found to have homology with the IκB subunits.

One of the other genes in this Drosophila pathway, called *Toll*, was found to encode a transmembrane protein with features of a signaling receptor (Hashimoto *et al.*, 1988). Its extracellular domain contains leucine-rich repeats, a motif found in many proteins is related to protein-protein interactions (Kobe and Deisenhofer, 1995), consistent with its known interaction with a ligand called Spätzle. Even more intriguing, the Toll intracellular domain was found to have a 135 amino acid region of homology to the functional IL-1 receptor IL-R1I (Gay and Keith, 1991).

One of the intermediate proteins in the Spätzle-Toll-Cactus-Dorsal pathway was a serine-threonine kinase called Pelle. Because of the emerging similarity of this pathway with the IL-1-signaling pathway, kinase activity associated with IL-1RI was sought, and a critical kinase called IRAK (IL-1-receptor-associated kinase) was identified (Cao *et al.*, 1996) (now known as IRAK-1).

Given the strong homology between the Spätzle/Toll-Pelle-Cactus-Dorsal pathway in Drosophila and the IL-1/IL-1RI-IRAK-I κB-NF-κB pathway in mammals, it was reasonable to ask whether this pathway also played a role in immunity in Drosophila, and not just in embryogenesis. In 1996, a French group showed that Drosophila lacking Toll or any of several other proteins in this pathway were unable to produce the antifungal peptide drosomycin, and were highly susceptible to overwhelming fungal infection (Lemaitre *et al.*, 1996). This pathway also induced several antibacterial peptides, but since these were not under the sole control of the Toll pathway, the mutant flies were still protected from bacterial infections.

Around this time, several human cDNA sequences were identified with homology to Drosophila Toll, containing both LRR and IL-1R-like domains, which were termed TILs (Toll/IL-1R-Like) (Taguchi *et al.*, 1996). However, it was the 1997 paper of Medzhitov and Janeway (Medzhitov *et al.*, 1997) identifying a human homolog of Toll capable of activating NF-κB that convincingly demonstrated what is now called a Toll-like receptor (TLR) in humans. Almost simultaneously, a family of related TLR genes were identified in humans and mice (Chaudhary *et al.*, 1998; Rock *et al.*, 1998). The receptor identified by Medzhitov *et al.*, initially called hToll, turned out to be TLR-4. However, it still was not clear what activated this system in vivo.

The next critical piece of information came from work here at UT Southwestern in the laboratory of Bruce Beutler, at that time a member of our department. The C3H/HeJ mouse was a mutant strain of C3H mouse that arose in the 1960's with a codominant single gene defect in the response to bacterial endotoxin (LPS) (Sultzer, 1968). Subsequently, another mutant mouse strain, C57BL/10ScCr, was found to have a similar phenotype due to a recessive defect mapping to the same locus. These mutant mice are unusually sensitive to gram-negative bacteria infections, but resistant to the effects of in vivo administered LPS. Beutler's group undertook to identify the underlying gene by positional cloning. The C3H/HeJ mouse was found to have a single missense (H->P) mutation in the Tlr-4 gene (mouse homolog of human TLR-4), whereas Tlr-4 mRNA was entirely absent in C57BL/10ScCr mice, proving that the Tlr-4 gene product is essential for LPS signaling (Poltorak *et al.*, 1998a; Poltorak *et al.*, 1998b). Identical findings were obtained independently by a Canadian group and published a few months later (Qureshi *et al.*, 1999), and the same phenotype was later observed in Tlr-4 knockout mice.

This cleared up a mystery regarding the host response to LPS, namely, how does sensing of LPS by the cell signal the downstream activation of NF-kB and other pathways? It was long been known that LPS binds in plasma to LBP (LPS binding protein), a plasma lipid transfer protein that moves LPS monomers from aggregates or bacterial membranes to a binding site on CD14. This molecule is a GPI (glycosylphosphotidylinositol)-linked protein and thus is not capable of signal transduction, but is capable of transferring LPS to other molecules within the cell membrane (Hailman *et al.*, 1994). Although a direct interaction between CD14 and TLR-4 could

not be demonstrated, it was clear that TLR4 was critical to signal transduction. More on TLR signaling later.

LPS was not the only microbial product known to activate NFκB. Numerous viral families, including Sendai virus, HIV, HTLV, HBV, HCV, EBV, and influenza, and also bacterial DNA containing unmethylated CpG and LTA (lipotechoic acid) from gram positive bacterial cell walls, were all known to activate NFκB (reviewed in (Doyle and O'Neill, 2006). Over the past several years in has become clear that the TLRs are central to the action of all of these stimuli on NFκB.

Table 3 lists, and Fig. 2 depicts, some of the many microbial molecules that have been found to activate innate immunity through the Toll-like receptors (discussed below). As will be noted below, some of these microbial PAMPs are also recognized by other PRRs, whereas other PAMPs are evidently recognized only by non-TLR PRRs. These molecules generally fulfil three criteria: (1) they are invariant among microorganisms of a given class, (2) they are unique to microorganisms, and (3) they have essential roles in microbial physiology (Medzhitov, 2007).

Table 3. Some microbial components (PAMPs) recognized by TLRs, the prototypical PRRs

	Component	Species	TLR Usage
Bacteria	Lipopolysaccharide	Gram negative bacteria	TLR4
	Diacyl lipopeptides	Mycoplasma	TLR6/TLR2
	Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
	OspA	*Borrelia burgdorferi*	TLR1/TLR2
	Lipotechoic acid	Group B streptococcus	TLR6/TLR2
	Peptidoglycan	Gram positive bacteria	TLR2
	Porins	Neisseria	TLR2
	Lipoarabinomannan	Mycobacteria	TLR2
	Flagellin	Flagellated bacteria	TLR5
	CpG-DNA	Bacteria and mycobacteria	TLR9
	?	Uropathogenic bacteria	TLR11 (mice)
Fungi	Zymosan	*S. cerevisiae*	TLR6/TLR2
	Phospholipomannan	*C. albicans*	TLR2
	Mannan	*C. albicans*	TLR4
	Glucuronoxylomannan	*Cryptococcus neoformans*	TLR2 and TLR4
Viruses	DNA	viruses	TLR9
	Poly (I-C) dsRNA	viruses	TLR3
	ssRNA	RNA viruses	TLR7 and TLR8
	Envelope proteins	RSV, MMTV	TLR4
	Hemagglutinin protein	Measles virus	TLR2
	?	CMV, HSV1	TLR2
Parasites	tGPI-mutin	*Trypanosoma*	TLR2
	Glycoinositolphospholipids	*Trypanosoma*	TLR4
	Hemozoin (?)	*Plasmodium*	TLR9
	Profilin-like molecule	*Toxoplasma gondii*	TLR11 (mice)

From (Akira *et al.*, 2006; Gay and Gangloff, 2007)

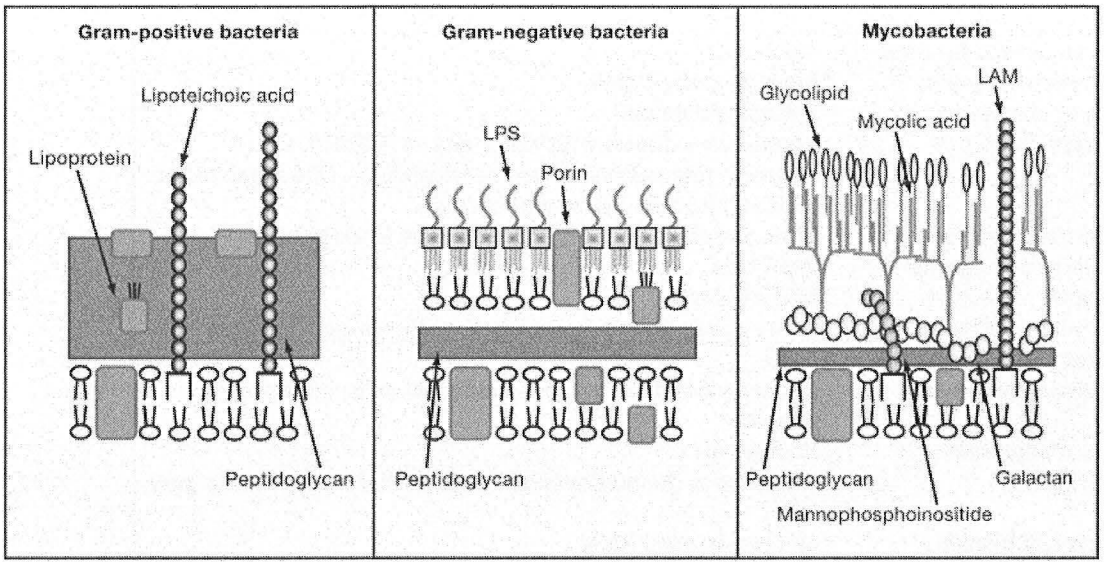

Figure 2. Schematic representation of bacterial cell walls
Gram-positive bacteria have a thick layer of PG. Lipoteichoic acids and lipoproteins are embedded in this cell wall. The cell wall of Gram-negative bacteria is characterized by the presence of LPS. Mycobacteria have a thick hydrophobic layer containing mycolyl arabinogalactan and dimycolate, in addition to a lipid bilayer and a PG layer. Lipoarabinomannan (LAM) is a major cell-wall-associated glycolipid. Lipoproteins are common structures for various types of bacteria. (Akira *et al.*, 2006)

Based on their amino acid sequences, TLRs can also be divided into subfamilies, each of which recognizes related PAMPs. For example, the subfamily of TLR1, TLR2, and TLR6 recognizes lipids, whereas TLR7, TLR8, and TLR9 recognize nucleic acids. However, there is also more promiscuous recognition. For example, TLR4 recognizes LPS, the plant diterpene paclitaxel, the fusion protein of RSV, and also such endogenous ligands as fibronectin and heat-shock proteins, all of which have different structures. TLR2 also binds a wide array of molecules of bacterial, viral, mycobacteria, and fungal origin (Akira *et al.*, 2006; Cristofaro and Opal, 2006).

Another way to group the TLRs is by cellular location. TLRs 1, 2, 4, 5, 6, and 10 are primarily localized to the external plasma membrane, whereas TLRs 3, 7, 8, and 9 are primarily expressed within the endosomal compartment. Other grouping are based on the intracellular signaling apparatus engaged by the various TLRs, to be discussed further below, and on lineages of cells expressing the TLRs.

Table 4. Features of the respective TLRs expressed in humans

TLR1	Chromosomal location	4p14
	Cellular expression	Myeloid cells, T and B cells, NK cells
	Site of expression	Plasma membrane
	Coexpression	Forms heterodimer with TLR2
	Microbial ligands	Triacyl lipopeptides in conjunction with TLR2, Bb Osp
	Endogenous ligands	None defined
	Adapter molecules	MyD88, MAL
	Phenotype of ko mice	Deficient triacyl lipopeptide signalling, poor antibody response to Bb OspA vaccine

TLR2	Chromosomal location	4q32
	Cellular expression	Myeloid cells, T cells
	Site of expression	Plasma membrane
	Coexpression	Forms heterodimers with TLR1 and with TLR6; CD14 enhances response to PG and lipopeptides; CD36 required for TLR2/6 response to diacylglycerides
	Microbial ligands	Wide variety (e.g., LTA, PG, zymosan, hemagglutinin)
	Endogenous ligands	See TLR6
	Adapter molecules	MyD88, MAL
	Phenotype of ko mice	More susceptible to *S. aureus, S. pneumoniae*, high dose Mtb
TLR3	Chromosomal location	4q35
	Cellular expression	Plasmacytoid dendritic cells; epithelial cells; brain, heart, muscle
	Site of expression	Endosomes
	Interactions	Presumed to homodimerize; CD14 critical to response to poly I:C
	Microbial ligands	Double stranded RNA
	Endogenous ligands	mRNA
	Adapter molecules	TRIF
	Phenotype of ko mice	Reduced CNS pathology to WNV
TLR4	Chromosomal location	9q32-q33
	Cellular expression	Myeloid cells
	Site of expression	Plasma membrane
	Interactions	Forms dimerized complex with MD-2; requires CD14
	Microbial ligands	Hexa-acylated LPS, fungal components, taxol, RSV fusion proteins
	Endogenous ligands	fibrinogen, heparan sulfate, heat shock proteins -22, -60
	Adapter molecules	MyD88, MAL, TRAM, TRIF
	Phenotype of ko mice	Susceptible to gram-negative bacterial infection
TLR5	Chromosomal location	1q41-q42
	Cellular expression	Epithelial cells, myeloid cells
	Site of expression	Plasma membrane
	Interactions	Presumed to homodimerize
	Microbial ligands	Flagellin (essential central core structure)
	Endogenous ligands	None defined
	Adapter molecules	MyD88
	Phenotype of ko mice	Susceptible to *E. coli* UTI
TLR6	Chromosomal location	4p14
	Cellular expression	Myeloid cells, dendritic cells
	Site of expression	Plasma membrane
	Interactions	Forms heterodimer with TLR2; CD36 required for TLR2/6 response to diacylglycerides
	Microbial ligands	Diacylated lipopeptides; zymosan; GIPLs, glycolipids
	Endogenous ligands	Heat shock proteins -60, -70, -96 (TLR2/6 heterodimer)
	Adapter molecules	MyD88, MAL
	Phenotype of ko mice	Decreased inflammatory cytokine response to Mtb

TLR7	Chromosomal location	X F5
	Cellular expression	B cells, macrophages, plasmacytoid dendritic cells
	Site of expression	Endosomes
	Interactions	Can dimerize with TLR8
	Microbial ligands	Viral ssRNA (also synthetic antiviral compounds)
	Endogenous ligands	ssRNA
	Adapter molecules	MyD88
	Phenotype of ko mice	Defective IFNα response to Coxsackie B virus; increased autoimmunity in SLE mice
TLR8	Chromosomal location	Xp22
	Cellular expression	Myeloid cells
	Site of expression	Endosomes
	Interations	Can dimerize with TLR7 and TLR9
	Microbial ligands	Viral ssRNA (also synthetic antiviral compounds)
	Endogenous ligands	ssRNA
	Adapter molecules	MyD88
	Phenotype of ko mice	Gene not functional in mice
TLR9	Chromosomal location	3p21.3
	Cellular expression	Epithelial cells, B cells, macrophages, plasmacytoid dendritic cells
	Site of expression	Endosomes
	Interactions	Can dimerize with TLR8
	Microbial ligands	Unmethylated CpG in bacterial and viral DNA
	Endogenous ligands	DNA
	Adapter molecules	MyD88
	Phenotype of ko mice	No cytokine or shock response to CpG DNA; decreased autoimmunity in SLE mice
TLR10	Chromosomal location	4p14
	Cellular expression	B cells, T regulatory cells, plasmacytoid dendritic cells, lung cells
	Site of expression	Plasma membrane
	Coexpression	Possibly forms dimers with TLR1 and/or TLR2
	Microbial ligands	Not determined
	Endogenous ligands	Not determined
	Adapter molecules	Not determined
	Phenotype of ko mice	Gene not functional in mice

(Akira *et al.*, 2006; Cristofaro and Opal, 2006) and other sources

TLR structure and ligand binding

TLRs are type I transmembrane proteins consisting of an ectodomain predominantly consisting of approximately 18-25 LRR (leucine rich repeat) modules flanked by N-terminal and C-terminal regions, a hydrophic transmembrane region, and an intracellular signaling domain homologous to the IL-RI receptor called TIR (Toll/IL-1 receptor homology). It has so far not been possible to crystallize an entire TLR, but crystal structures of the ectodomains of TLR3, TLR4, and the TLR1/2 heterodimer have been published (Choe *et al.*, 2005; Bell *et al.*, 2006; Jin *et al.*, 2007; Kim *et al.*, 2007). The structure of the intracellular TIR domain of TLR4 has also been reported (Xu *et al.*, 2000).

The TLR4 molecule is a LRR protein with a horseshoe-like structure of 22 leucine rich repeat modules with a concave surface formed by parallel beta-strands and a convex surface formed by

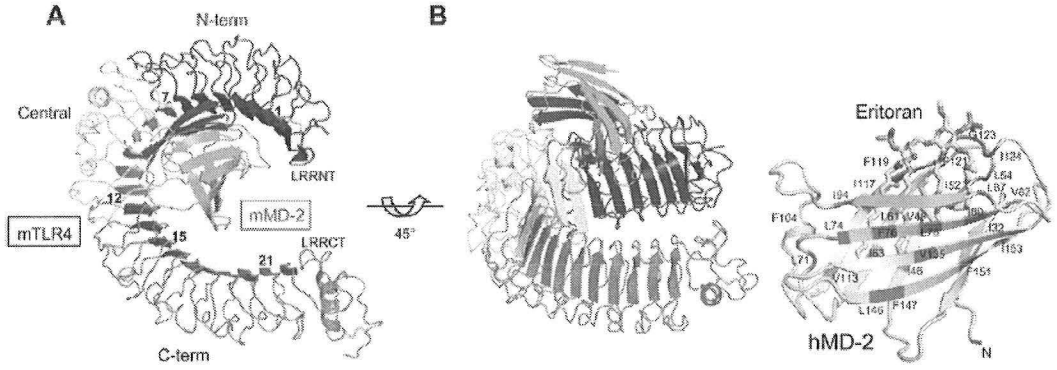

Fig. 3. Structure of TLR4. A and B show mouse TLR4 as a horseshoe-like structure of 22 leucine rich repeat modules with a concave surface formed by parallel beta-strands and a convex surface formed by loops and helices MD-2, comprised predominantly of anti-parallel β sheets, binds to the concave side of TLR4. On the right, the synthetic 4-acyl LPS binds with the hydrophobic pocket of MD-2 (Kim *et al.*, 2007).

loops and helices (Kim *et al.*, 2007). The MD-2 molecule which binds to TLR4 is a beta-cup, comprised of antiparallel beta sheets that form a hydrophobic pocket. The crystal structure of TLR4-bound MD-2 was determined with a bound ligand, Eritoran, a synthetic molecule derived from the lipid A structure of the nonpathogenic LPS of *Rhodobacter spheroides*, which contains 4 acyl chains instead of the 6 acyl chains typical of pathogenic LPS molecules. Eritoran is antagonist of TLR4 signaling and was designed for treatment of sepsis. MD-2 was shown to bind the concave surface of TLR4. Binding of native LPS, but not Eritoran, to TLR4-MD-2 induced apparent dimerization of the TLR4-MD-2 complexes. The authors favored a model in which LPS binding to each of the two MD-2 molecules induces a rearrangement such that each of the MD-2 molecules contacts both TLR4 molecules to form a complex of the 4 molecules. This rearranged complex, containing 2 TLR4 molecules that do not contact each other, presumably induces downstream signalling.

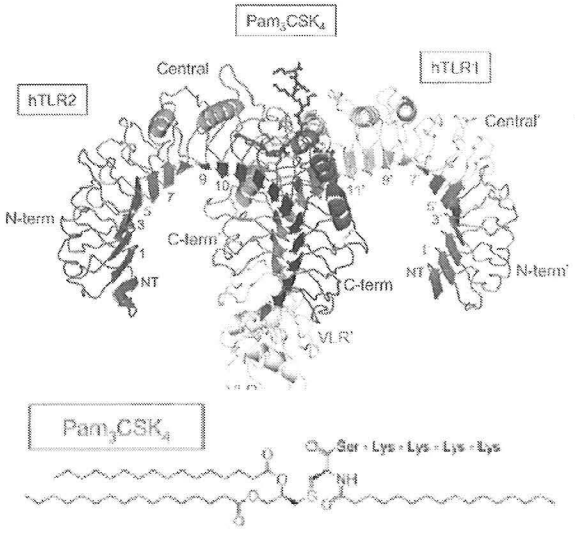

Fig. 4. Structure of TLR1/2 dimer with bound ligand, the synthetic lipopeptide Pam_3CSK_4, binding to the concave sides of the horsehoe-shaped TLR ectodomains. The 2 ester-bound lipid chains insert into a pocket in TLR2 and the amide-linked lipid chain inserts into a hydrophobic channel in TLR1 (Jin *et al.* ,2007).

TLR1 and TLR2 were also found to consist of horseshoe-like structures of 18 and 19 LRR repeats, respectively, together with N- and C-terminal beta strands (Jin *et al.*, 2007). In striking contrast to TLR4, ligand binding is on the convex side of the horseshoe bends, and the TLR ectodomains themselves are in direct contact with the ligand. The ligand used was a synthetic lipopeptide containing three acyl groups, tripalmitoyl-CSK_4 (Fig. 4). The two ester-bound lipid

chains are inserted into the TLR2 pocket, and the amide-bound lipid chain is inserted to a hydrophobic channel in TLR1. It is thought that the lipopeptide ligand causes preexisting weakly associated TLR1-TLR2 dimers to undergo a conformational rearrangement and initiate signaling.

The structure of the TLR3 ectodomain had previously been found also to be a horseshoe-shaped solenoid containing 23 LRRs (Choe *et al.*, 2005; Bell *et al.*, 2006). From sequence and structural analysis, one group of authors postulated that a dsRNA ligand might bind on the convex side of the molecule, inducing dimerization in a manner similar to what was later found for the TLR1/2 complex (Choe *et al.*, 2005). However, the other group used other arguments to postulate ligand binding on the concave side (Bell *et al.*, 2006).

Previously, the structure of the intracellular TIR (Toll/IL-1 receptor) domains of TLR1 and TLR2 had been determined (Xu *et al.*, 2000). This consists of a central 5-stranded parallel beta sheet surrounded by 5 alpha helices. A large conserved surface patch contains the site of the mutation in the Tlr4 mutant C3H/HeJ mice. This residue is conserved among most TLRs and the Pro->His mutation blocks signaling in other TLRs besides TLR4.

TLR Signaling

Ligation of TLRs by their cognate ligands activates signaling cascades. The first step is recruitment of adapter proteins the cytoplasmic domains of the TLR molecules through TIR-TIR homotypic interactions. The adapter molecules are listed in Table 5 and shown schematically in Figure 5.

Table 5. TIR adapter molecules used in TLR signaling

Name(s)	TLRs used by	Downstream pathways activated
MyD88	All except TLR3	NFκB, AP-1, IRF-5, IRF-7
MAL, TIRAP	TLR4, TLR2	
TRAM, TICAM2	TLR4	
TRIF, TICAM1	TLR3, TLR4	IRF-3

There are three common pathways involved in TLR signalling (West *et al.*, 2006; O'Neill and Bowie, 2007; Watters *et al.*, 2007). These are illustrated in Figure 5.

The MyD88-dependent pathway is used by all TLRs except TLR3 and is apparently exclusively used by TLRs 7-9. In the case of TLR4 and apparently also the various TLR2 dimers, the TIR domain of MAL binds to the TIR domain of TLR dimer, and MAL then recruits MyD88. This, through its death domain (DD) binds IRAK4 and IRAK1. IRAK1 is phosphorylated by itself and by IRAK4 and leaves the membrane to activate TRAF6. After TRAF6 is ubiquitinated, it interacts with TAB2 to activate TAK1. TAK1 activates the IKK complex and IκB is phosphorylated, ubiquitinated and degraded allowing NF-κB to translocate to the nucleus to activate its proinflammatory program. TAK1 also activates MKK6, which in turn activates JNK and p38. This leads to activation of the transcription factor AP-1, which also activates a proinflammatory gene program. TRAF6 can also activate IRF5.

The TRIF-dependent pathway. This is the only pathway used by TLR3, which binds the TIR domain of TRIF directly. TRIF is also used by TLR4 through the intermediary adapter TRAM.

One arm of this pathway begins with the activation of TBK1, leading to the activation of IRF3, a transcription factor that translocates to the nucleus to produce IFN-inducible genes. In a second arm, RIP1 is activated by TRIF and this feeds into the MyD88 pathway by activating TRAF6.

The alternative MyD88 pathway. This pathway is thought to be specific for plasmacytoid dendritic cells (pDC), in which activation of TLR7 and -9 leads to the activation of TRAF6 through MyD88, IRAK4 and -1. This results in the activation of IRF7, which translocates to the nucleus to produce IFN-α and IFN-inducible genes. In recent years, it has become clear that plasmacytoid dendritic cells are the major source of the type I interferon response to viral infection (Liu, 2005). They represent <1% of peripheral blood mononuclear cells and populate T cell rich areas of lymphoid tissue. Activation of TLR7 or 9 (possibly in some cases in combination with TLR8) by viral ssRNA or nonmethylated DNA, respectively, in pDC induces a rapid massive IFN- α response that inhibits viral replication and activates NK cells, B and T cells, and myeloid dendritic cells.

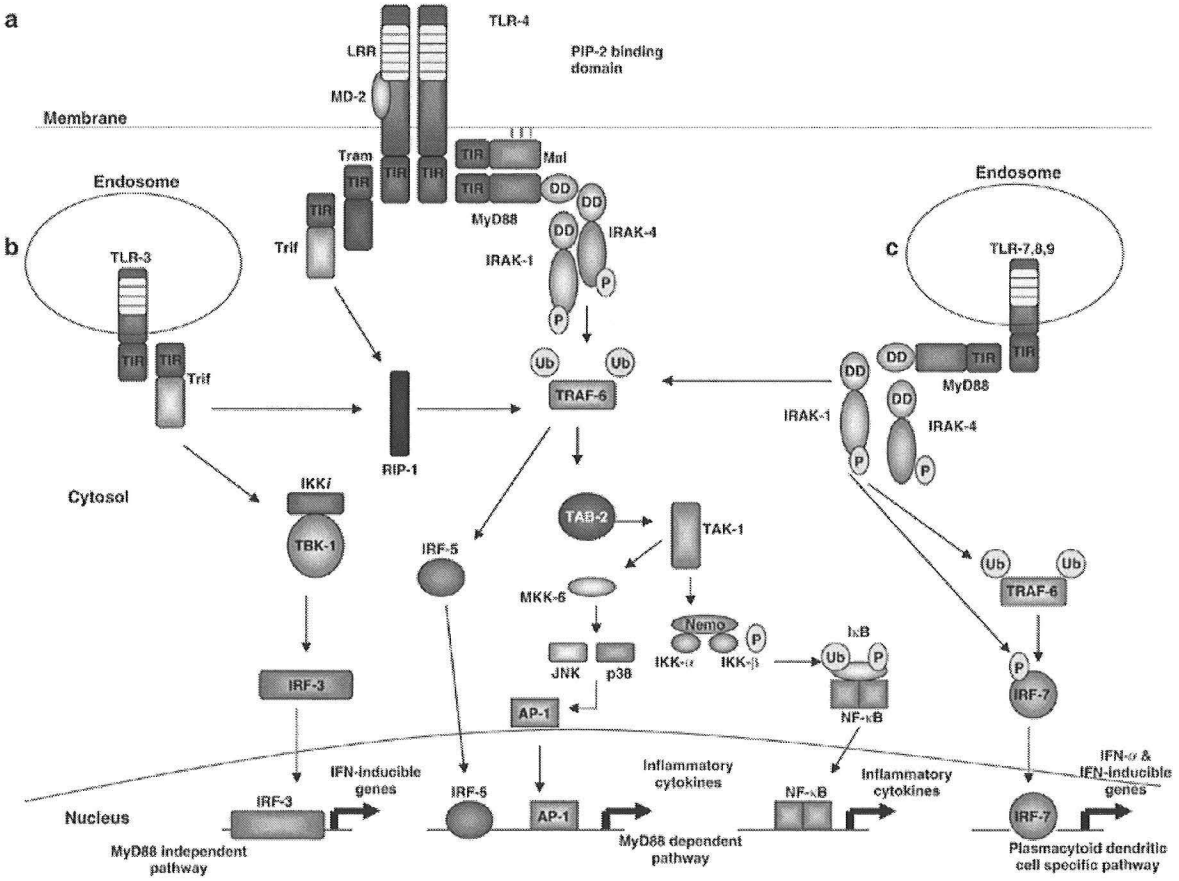

Figure 5. Molecular pathways involved in TLR signalling (i.e., activation by ligands to induce specific transcription factors) (Watters *et al.*, 2007).

The essential role of the TIR-domain containing adapters has been demonstrated in genetically altered mice (reviewed in (O'Neill and Bowie, 2007). MyD88$^{-/-}$ mice shown profoundly defective cytokine responses to a number TLR ligands, and reduced survival to certain infections, including *S. aureus, Listeria monocytogenes*, and *Toxoplasma gondii,* and *VSV.*

$MAL^{-/-}$ mice showed decreased resistance to pulmonary infections with *E. coli* and *K. pneumoniae*. Mice lacking functional TRIF showed decreased resistance to CMV.

It has been known for over 50 years that prior exposure to LPS leads to a transient state of LPS-hyporesponsiveness, both in vivo and in vitro. Subsequently, many other microbial products have similarly been shown to induce a state of hyporesponsiveness, termed "microbial tolerance." This is thought to limit the inflammatory response to infection. A number of regulatory factors have been found to modulate TLR signalling, listed in Table 6, and this is thought to provide at a least a partial molecular explanation for this microbial tolerance. These represent potential sites of pharmacologic intervention. Since most of these involve MyD88, they are presumably also negative regulators of signalling through the IL-1 and IL-18 receptors.

Table 6. Negative regulators of TLR signaling

Factor	Suggested Action
MyD88s (splice variant of MyD88, lacking interdomain region that recruits IRAK4)	Inhibits recruitment of IRAK4 by MyD88
FADD	Inhibits MyD88 activation of IRAKs
IRAK-M	Blocks release of IRAK4 and IRAK1 from MyD88
SOCS-1	Promotes degradation of MAL
ST2/T1	Sequesters MyD88 and MAL
SIGIRR/TIR8	Sequesters IRAK1 and TRAF6
TOLLIP	Blocks activation of IRAK1, plus other actions
SHIP	Unknown
SARM	Upregulated with activation of TLR3 and TLR4, blocks recruitment of effector proteins

(Medvedev *et al.*, 2006; West *et al.*, 2006; O'Neill and Bowie, 2007; Watters *et al.*, 2007)

A dramatic example of the role of TLR signalling in host defense

A remarkable paper published in *Nature Immunology* last year (Montminy *et al.*, 2006) dramatically illustrates the role of TLR4 in host defense against the plague bacillus, *Yersinia pestis*. By way of introduction, I would like to quote from a commentary written on that paper (Dziarski, 2006), which describes in detail the manifold ways that *Y. pestis* subverts immune mechanisms:

> "What makes the plague bacillus so deadly? The strategy of *Y. pestis* is to evade innate immunity and paralyze immune cells. Its type III secretion system (which is a syringe-and-needle–like structure mounted in the bacterial cell wall) injects toxic effector proteins, called Yops (for Yersinia outer proteins), into innate immune cells, mainly macrophages, dendritic cells and granulocytes. YopE, YopT and YopO prevent phagocytosis by targeting Rho GTPases and destroying the cytoskeleton. YopE also inhibits caspase-1–mediated maturation and release of interleukin (IL)-1beta. YopH prevents phagocytosis and release of a chemokine, MCP-1, through its protein tyrosine phosphatase activity, which disrupts several signaling pathways. YopJ shuts down MKK6 and NF-κB signaling and cytokine production, and induces apoptosis of macrophages through the caspase cascade. YopM interacts with protein kinase C–like 2 and ribosomal protein S6 kinase, which are also involved in proinflammatory signaling. These assaults on the immune cells effectively block phagocytosis and induction of proinflammatory cytokines. Another Yop, LcrV, which is the anchoring unit on the tip of the type III secretion needle, is also secreted and induces anti-inflammatory IL-10 through TLR2. And there are other virulence

factors, such as plasminogen activator, murine toxin, and capsular and fimbrial proteins. If that was not enough, Y. pestis has one more trick up its sleeve... *Y. pestis* evades detection by TLR4."

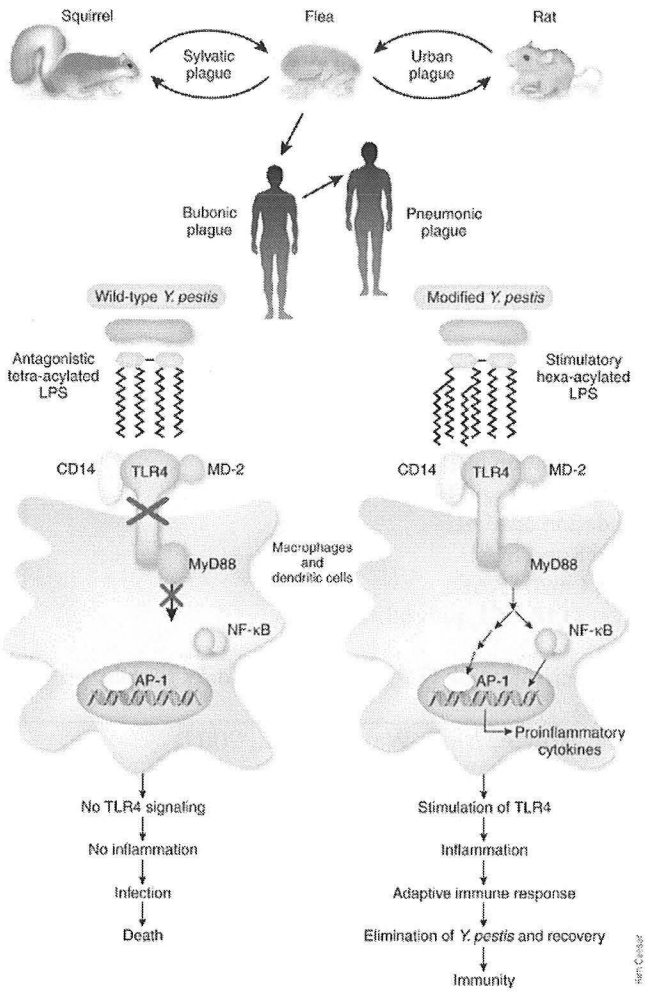

Figure 6. *Y. pestis* life-cycle, showing critical effect of TLR4 inhibition by tetracyl LPS (Montminy *et al.*, 2006; Dziarski, 2006).

Montminy *et al.* found that at 26° *Y. pestis* produces a hexa-acetylated LPS that is a potent stimulator of TLR4, but at 37° it produces a tetra-acetylated LPS that acts as a TLR4 antagonist. They produced a modified strain that produces a hexa-acetylated LPS at all temperatures. They showed that the modified strain lacked virulence in normal mice at doses up to 10^7 CFU, in contrast to the wild type strain that was lethal with s.c. innocula of ≤ 100 CFU. The modified strain was lethal to mice lacking TLR4, MD-2, or MyD88 confirming that signalling through TLR4 was involved in host protection. (Mice lacking CD14 or MAL showed some protection, mice lacking TRAM or TRIF showed substantial protection, and mice lacking TLR2 showed complete protection against the modified strain). The authors also showed that adaptive immunity was required for host survival against the modified strain, since $RAG^{-/-}$ mice that lack T and B cell immunity all succumbed to an innoculum of 1,000 CFU of the modified strain. Consistent with this, wild type mice infected with the modified strain were resistant to a subsequent challenge with 10^6 CFU of the wild type virulent strain, i.e., they were successfully vaccinated. These results highlight the central role of LPS signalling through TLR4 and indicate some of the complexity of the subsequent signalling mechanisms.

Cryopyrin, the NALP3 inflammasome, and autoinflammatory syndromes

The rest of this Grand Rounds will be devoted to a discussion of one subset of one of these modules, namely the cyropyrin/NALP3 inflammasome, the function of which was partially discovered by clinical investigation.

IL-1 is activated by a caspase. Historically, because of its diversity of actions, IL-1 was discovered by a number of different investigators in different experimental settings. These activities included, among others, endogenous pyrogen, co-mitogen for thymocyte proliferation, stimulation of the acute-phase response, cartilage resorption, and muscle wasting (Durum *et al.*, 1985). Upon cloning of the IL-1α and IL-1β genes in 1984 and subsequent experiments with recominant IL-1, it became clear that one cytokine could indeed be responsible for the manifold effects (Pizarro and Cominelli, 2007). It also became clear that IL-1β, the predominant physiologic IL-1 cytokine, was produced as an inactive 35 kD precursor, pro-IL-1β, that required proteolytic cleavage to release the active 17 kD cytokine (Dinarello and Mier, 1986). The enzyme that peforms this cleavage was cloned in 1992. Originally termed ICE (IL-1-converting enzyme), it was later called caspase-1 when it was recognized as the first of a family of cysteine proteases that cleave at unique sites bearing aspartic acid (Alnemri *et al.*, 1996).

IL-1 production is normally very tightly regulated. It is now known that TLRs signaling through the pathways that converge on TRAF6 initate synthesis (transcription and translation) of the inactive IL-1β precursor (see cover figure and Fig. 5). Most of these remains the cytosol, but a fraction moves to specialized secretory lysosomes and co-localizes with the zymogen pro-caspase-1. Activation of pro-caspase-1 leads to autocatalytic activation to caspase-1 and processing of IL-1β precursor to mature p17 IL-1β, which remains ready for secretion. This in turn is thought to be activated by an efflux of intracellular K^+, triggered by ATP binding to the purinergic $P2X_7$ receptor (Dinarello, 2005). More on this below.

Based on homologies to proteins expressed in *C. elegans*, caspase-3 and caspase-9 were found to be essential for apoptosis, and to interact with a protein Apaf-1 (Li *et al.*, 1997). Both Apaf-1 and the caspases contained domains called CARDs (caspase recruitment domains), and it is through homotypic CARD-CARD interaction that caspase-9 is recruited to Apaf-1 when cytochrome C released from mitochondria bind to the C-terminal WD-40 domain of Apaf-1. In addition to the N-terminal CARD domain and the C-terminal WD-40 domain, Apaf-1 also contains a NBS (nucleotide binding site). Through these domains, Apaf-1 assembles a multiprotein complex called the apoptosome that effects the apoptotic program through successive caspase cleavages (Yu *et al.*, 2005). More about this below.

Discovery of cryopyrin mutations in autoinflammatory disorders. Meanwhile, in 1997 two groups identified the gene for Familial Mediterranean Fever, which was named by the NIH-based group as pyrin (Consortium, 1997a; Consortium, 1997b). (This story was the subject of the Grand Rounds given by the Morris Ziff Distinguished Lecturer Dr. D. L. Kastner, in Nov 2004). It was not originally clear how pyrin played a role in inflammation. However, over the next few years, a number of groups identified molecules carrying CARD, NOD, and/or LRR domains, and some of these were found to carrying pyrin domains (PYD). The proteins in this family have been variously called NOD-LRR, NACHT-LRR, NALP, and CATERPILLER proteins (Koonin and Aravind, 2000; Ting *et al.*, 2006), and most recently NOD-like receptors (NLRs) (Creagh and O'Neill, 2006; Martinon *et al.*, 2007). Apaf-1 is included in this family by virtue of its CARD and NOD domains.

Meanwhile, investigation of rare autosomal dominant pediatric periodic inflammatory syndromes led to the positional cloning of a disease gene for two such syndromes, familial cold urticaria and

Muckle-Wells syndrome (Hoffman *et al.*, 2001). The former, first described in 1940 and also called familial cold autoinflammatory syndrome (FCAS), is characterized by episodes of urticaria-like rash, myalgias, arthralgias, arthritis, chills, fever, and conjunctivitis that begin by 6 months of age and last less than 24 hours beginning a few hours after exposure to cold. Amyloidosis is an uncommon sequelae. Muckle-Wells syndrome, first described in the 1970's, has similar episodes, but they last 24-48 hours, there is associated nerve deafness, and 25% of the patients develop renal SAA amyloidosis. The gene, called *CIAS1*, was found on chromosome 1q44. Intriguingly, the encoded protein contained a pyrin domain, a NACHT domain, and a LRR domain. That is, it was member of the NACHT-LRR protein family. The authors called the protein **cryopyrin**.

The following year, a closely related autosomal dominant syndrome called CINCA (chronic infantile neurologic cutaneous and articular) syndrome, and also called NOMID (neonatal-onset multisystem inflammatory disease) was also found to be linked to mutations in *CIAS1* (Feldmann et al., 2002). This syndrome is almost continuous, rather than episodic, although there are periods of exacerbation. It is characterized by chronic generalized rash in first days to weeks of life, relapsing fever, lymphadenopathy, anemia, optic atrophy, optic nerve swelling, arthropathy leading to contractures, gradual onset of osteoporosis with swelling at ends of long bones, large head, saddle-back nose, and a characteristic appearance with frontal bossing a protruding eyes. CNS involvement is severe, with mental retardation, aseptic meningitis, brain atrophy, seizures, migraines, and hearing loss. *CIAS1* mutations were also found in additional patients with FCAS, MWS, and an overlapping phenotype (Aganna *et al.*, 2002). These three conditions are now considered part of a spectrum of disease called CAPS (cryopyrin associated periodic syndromes).

In all of these conditions, the mutations found were missense mutations in exon 3 of *CIAS1*, resulting in amino acid substitutions either in the NACHT domain or in the regions flanking it. The clinical syndromes, the autosomal dominant inheritance, and the nature of the mutations suggested that these were gain of function mutations. More on this below.

Cryopyrin (NALP3) is associated with IL-1 processing, which is excessive in CAPS patients. Meanwhile, investigation of the interactions of CARD-carrying proteins other than Apaf-1with caspases led to the discovery that certain NALP proteins bind and activate caspase-1, the protease that cleaves pro-IL-1β. This process was found to require a complex assembly including NALP1, and another protein called ASC (PYCARD) that consists of a pyrin domain and a CARD domain. This complex, reminiscent of the Apaf-1 complex that activates caspase-9 in apoptosis, was called the **inflammasome** (Martinon *et al.*, 2002). It is now thought caspases in general are activated by platforms of assembled protein complexes, that inflammasomes are one class of these platforms, and that three subfamilies of NLRs are involved in the formation of caspase-1 activating inflammasomes, namely, NALPs, IPAF, and NAIPs (Martinon *et al.*, 2007).

In the inflammasome of NALP-3 (cryopryrin), NALP3 activates caspase-1 indirectly through a complex containing ASC and another protein called CARDINAL. Moreover, this process was found to be more active in macrophages from a MWS patient with a cryopyrin missense mutation (Agostini *et al.*, 2004), with patient macrophages showing significantly more IL-1β cleavage and release both constitutively and following LPS stimulation (Fig 7).

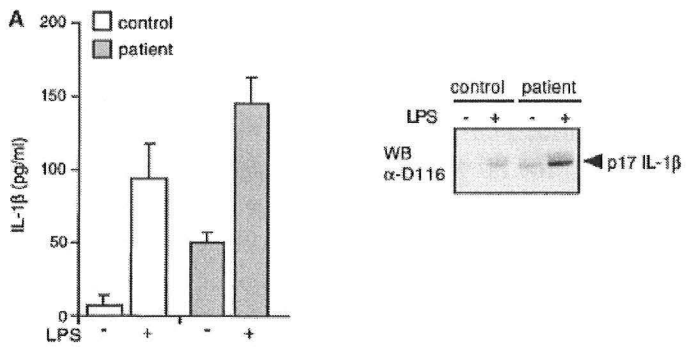

Fig. 7. Monocytes from a Muckle-Wells patient show increased processing and release of IL-1β, both constitutively and following LPS stimulation (from Agostini *et al.,* 2004).

Soon thereafter, administration of the IL-1R antagonist anakinra was shown to produce dramatic improvement in symptoms and laboratory markers, and prevention of further attacks, in patients with all three syndromes (Hawkins *et al.*, 2004a; Hawkins *et al.*, 2004b; Hoffman *et al.*, 2004; Goldbach-Mansky *et al.*, 2006) . More recent investigation has shown that in CINCA and MWS macrophages, unlike normals, ATP was not necessary for IL-1β release, providing further insight into the constitutive caspase-1 activation in these patients (Dinarello, 2007b; Gattorno *et al.*, 2007) .

In a recent report of North American subjects, 56 separate *CIAS1* disease-associated mutations have been identified (Aksentijevich *et al.*, 2007). No mutation in *CIAS1* or any other gene was found in 19 of 39 NOMID patients, 3 of 12 MWS patients, and 2 of 137 FCAS patients. Modeling of cryopyrin based on crystallographic data from other PYRIN, NACHT, and LRR proteins suggested that (1) the molecule can assemble as a hexameric assembly, and (2) most of the CAPS mutations cluster in one area, suggesting a constitutively "open" conformation to the inflammasome.

Both "stranger" and "danger" stimuli promote cryopyrin/NALP3 inflammasome assembly and caspase-1 activiation. Recent experiments with knockout mice lacking NALP3 or other components of the inflammasome have begun to identify some of the stimuli that use this pathway to activate IL-1. Consistent with both the "stranger" and "danger" hypotheses, both microbial and endogenous signals have been found. Two of the microbial components that require NALP3 for caspase-1 activation are bacterial RNA and MDP (Kanneganti *et al.*, 2006; Pan *et al.*, 2007). The later is known to be a major stimulus of NOD2, the gene that is mutated in many cases of Crohn's disease. In one set of mouse experiments, both NOD2 and NALP3 were required for IL-1β activation and secretion in response to MDP (Pan *et al.*, 2007). $NALP3^{-/-}$ macrophages failed to secrete IL-1β in response to *S. aureus* and *L. monocytogenes*, but behaved normally in response to *S. typhimurium* and *Francisella tularensis,* whereas $ASC^{-/-}$ macrophages failed to respond to all four bacteria (Mariathasan *et al.*, 2006; Sutterwala *et al.*, 2006). The data suggested that another NLR, probably IPAF which responds to flagellin, is part of the inflammasome assembled in response to *S. typhimurium.*

NALP3/cryopryrin was also found to be essential for IL-1β release by ATP, certain toxins known to deplete intracellular K^+, and certain antiviral imidazoquinolone compounds that activate TLR7 (Kanneganti *et al.*, 2006; Mariathasan *et al.*, 2006; Sutterwala *et al.*, 2006). (See cover figure and legend). It appears that bacteria and/or bacterial products can enter the cell independent of TLRs via pannexin-1, a recently described hemichannel that associates with the $P2X_7$ receptor upon ATP stimulation and induces a large nonselective pore (Dinarello, 2007a;

Kanneganti *et al.*, 2007). These products activate caspase-1 in a NALP3/cryopyrin dependent manner.

Now we come to the remarkable story of crystal-induced caspase-1 activation (Pope and Tschopp, 2007). As noted above, it has been known for 20 years that monocytes exposed to MSU crystals release cytokines. However, much more recently, the amazing finding was made that uric acid is a principal endogenous danger signal released from dying cells, stimulating dendritic cell maturation and subsequent T cell responses, that uric acid acts as an endogenous adjuvant (Shi *et al.*, 2003). Subsequently, it was shown that endocytosed MSU and CPPD crystals directly activate the NALP3 inflammasome (Martinon *et al.*, 2006). IL-1β processing and release by these crystals was observed in LPS-stimulated peritoneal macrophages from normal mouse, but not from knockout mice lacking caspase-1, ASC, or NALP3. This was confirmed in vivo by experiments of cystal-induced neutrophil accumulation in the peritoneal cavity. Colchicine was shown to block crystal-induced activation of the inflammasome, although only at high concentrations. Both MyD88 and IL-1R were required for in vivo inflammation (Chen *et al.*, 2006; Martinon *et al.*, 2006). In one study, none of the TLRs was essential (Chen *et al.*, 2006), whereas in another study the in vitro and in vivo inflammatory responses to MSU were blunted in $TLR2^{-/-}$ and $TLR4^{-/-}$ mice (Liu-Bryan *et al.*, 2005). Bone marrow chimera experiments showed that in vivo inflammation required IL-1R expression was essential in non-bone marrow derived cells, but not bone marrow derived cells, suggesting that it is the downstream effects of IL-1 signaling that promote crystal-induced inflammation (Chen *et al.*, 2006).

In vivo experiments with anti-IL-1R and with the IL-1R antagonist anakinra demonstrated inhibition of MSU-induced peritoneal inflammation in mice, whereas anti-TNF treatment augmented inflammation (So *et al.*, 2007). This led directly the dramatic open-label clinical trial of anakinra described at the beginning of this Grand Rounds.

Still's Disease. Systemic onset juvenile idiopathic arthritis (SoJIA), also known as Still's disease, shares some of the features of the episodic CAPS syndromes, including fever, rash, systemic inflammation, and arthritis. Major differences include its chronicity and lack of familial association. Most SoJIA patients do not respond to anti-TNF therapy and often require high doses of corticosteroids and other medications for prolonged periods. Our colleagues in pediatric rheumatology investigated the role of IL-1 in SoJIA (Pascual *et al.*, 2005). Sera from SoJIA induced expression of several IL-1 pathway genes, including IL-1β, in healthy peripheral blood mononuclear cells, and IL-1β was excessively induced in SoJIA PBMC. Based on these findings, anakinra was administered to 9 patients with severe chronic SoJIA. Significant clinical responses were seen in all 9, with remittence of fever, leukocytosis, and arthritis. Subsequent experience has led to their adopting anakinra as the standard of care for SoJIA refractory to NSAID therapy. The mechanism of dysregulation of IL-1β in this disorder remains to be discovered.

Appendix: Abbreviations

APAF-1	Apoptosis activating factor 1
ASC	Apoptosis-associated speck-like protein containing a CARD
CAPS	Cryopyrin associated periodic syndromes
CARD	Caspase recruitment domain
CASPASE	Cysteine protease cleaving after aspartic acid
CATERPILLER	CARD transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats
CIAS1	Gene involved in CAPS (encodes cryopyrin/NALP3)
CINCA	chronic infantile neurologic cutaneous and articular syndrome
CPPD	Calcium pyrophosphate dihydrate
DAMP	Danger-associated molecular pattern
DD	Death domain
FADD	Fas-associated via death domain
FCAS	Familial cold autoinflammatory syndrome (familial cold urticaria)
IRAK	IL-1R- associated kinase
IRF	Interferon regulatory factor
JUK	JUN N-terminal kinase
LRR	Leucine rich repeat
MAL	MyD88 adapter-like
MSU	Monosodium urate
MWS	Muckle-Wells syndrome
MyD88	Myeloid differentiation gene 88
NACHT	Domain present in NAIP, CIITA, HET-E, and TP1
NALP	NACHT, LRR, and PYD containing proteins
NBD	Nucleotide binding domain
NBS	Nucleotide binding site
NLR	Nucleotide binding domain leucine rich repeat containing family; NOD-like receptor
NOD	Nuclear oligomerization domain
NOMID	neonatal-onset multisystem inflammatory disease
PAMP	Pathogen-associated molecular pattern
PYD	Pyrin domain
SHIP	SH2-containing inositol phosphatase
SIGIRR	Single immunoglobulin IL-1R-related molecule
SOCS-1	Suppressor of cytokine signalling 1
TAB2	TAK1-binding protein 2
TAK1	TGFβ-activated kinase 1
TOLLIP	Toll-interacting protein
TRAF	TNF-receptor associated factor 6
TRAM	TRIP-related adaptor molecule
TRIF	TIR domain-containing factor inducing IFN-β

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