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*Role of endosomal
Toll-like receptors
in pneumococcal pneumonitis*

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1. Introduction

One of the most fundamental questions in immunology concerns self-recognition, which for the most part means microbes. As we initially realize we have been infected with the microbes, and how is the immune response turned on?¹ Host defense against invading microbial pathogens is caused by the immune system, which consists of two components: **innate immunity** and **acquired immunity**.

In *acquired immunity*, B and T lymphocytes use antigen receptors such as immunoglobulins and T cell receptors to recognize non-self. Acquired immunity is characterized by specificity and is developed by clonal selection from a vast repertoire of lymphocytes that carry specific antigen receptors generated through a mechanism generally known as gene rearrangement².

The *innate immune* response is not completely nonspecific, as was originally thought, but is able to discriminate between self and a variety of pathogens. The innate immune system recognizes microorganisms through a limited number of germline-encoded **pattern-recognition receptors** (PRRs). This is in contrast to the great repertoire of reconstituted receptors used by the acquired system. PRRs possess common features. First, PRRs

recognize microbial components, known as "*pathogen associated molecular patterns* (PAMPs)" essential for the survival of the microorganism and are therefore difficult to alter the microorganism. Secondly, PRRs are expressively expressed in host and detect pathogens regardless of their stage of life. Third, PRRs are germline encoded, nonclonal, expressed on all cells of a given type, and independent of the immune memory. Several PRR respond with PAMP specifications, show distinct expression patterns, trigger specific signaling pathways, and lead to distinctive anti-pathogen responses. The basic machinery underlying innate immune recognition is highly conserved among species, plants and fruit flies to mammals³. PRRs include Toll-like receptors (TLRs), retinoid acid-inducible gene (RIG)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs)⁴⁻⁶.

At the end of the twentieth century, Toll has been shown to be an essential receptor for host defense against fungal infection in *Drosophila*, which has only innate immunity⁷. A year later, a Toll receptor mammalian homologue (now defined as TLR4) has been shown to induce the expression of genes involved in inflammatory reactions⁸. Ten TLRs have been identified in humans so far, and twelve in mice. Both humans and mice express TLR1-9. In addition humans but no mice express TLR10, mice also have TLR11, 12 and 13 that human beings are missing.

1.1. TLRs structure

TLRs are expressed in innate immune cells such as dendritic cells (DCs) and macrophages, as well as non-immune cells such as fibroblast cells and epithelial cells. TLR are found on cell surfaces or within endosomes and have important roles in the host defense against pathogenic organisms throughout the animal kingdom. The TLRs are type I integral membrane receptors, each having an N-terminal ligand recognition domain, a single transmembrane helix, and a C-terminal cytoplasmic signaling domain⁹. TLRs signaling domains are known as Toll IL-1 Receptor (TIR) domains because they share homology with the signaling domains of IL-1R family members¹⁰. TIR domains are also found in many adaptive proteins that interact homologously with the TIR domains of TLRs and IL-1 receptors as the first step in the signaling cascade. Significantly, homologues of TIR domains are also found in some plant proteins that confer resistance to pathogens¹¹, suggesting that TIR domain are a very ancient motif that served an immune function before the divergence of plants and animals. TIR domains have a common fold with a β -sheet (β A- β E) parallel to five central wires that is surrounded by a total of five α -helixes (α A- α E) on both sides (Fig. 1)^{12, 13}. The BB loop, connecting strand β B and helix α B, containing three highly conserved residues, arginine BB3, aspartic acid BB4, and glycine BB8, plays a major role in TIR dimerization and/or adaptor recruitment¹⁴⁻¹⁵.

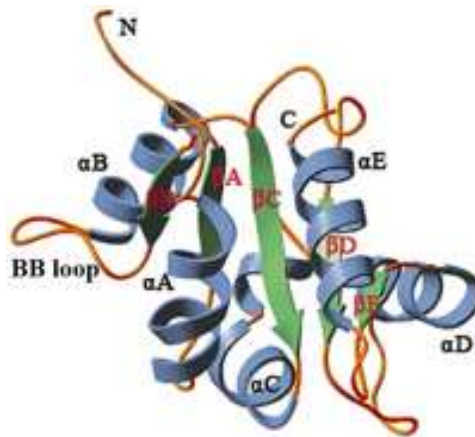


Fig. 1: Schematic drawing of the structure of the TIR domain of human TLR2. The common fold of TIR contains a central five-stranded parallel β -sheet (green) surrounded by a total of five α -helices (blue) on both sides linked by loops (gold).

The TLR transmembrane domains each contain a typical trait of approximately 20 non-loaded, predominantly hydrophobic residues. TLR N-terminal ectodomains (ECD) are glycoproteins with 550-800 residues of amino acids⁹ and meet and recognize the molecules released by invasion of pathogens. All TLR ECDs are constructed of tandem copies of a motif known as the leucine-rich repeat (LRR), which is typically 22–29 residues in length and contains hydrophobic residues spaced at distinctive intervals. LRRs that do not form a complete circle, but form a “horseshoe” structure (Figure 2).

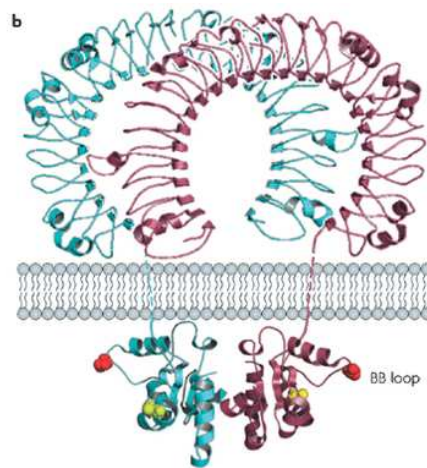


Fig. 2: *The complete structure of a TLR, in which the "horseshoe" structure of the leucine-rich motif is well visible.*

1.2 Positioning and trafficking of the TLRs.

All TLRs are synthesized in ER, traffic to Golgi and recruited to cell surfaces or intracellular compartments such as endosomes. The TLRs belonging to the first group are: TLR1, TLR2, TLR4, TLR5, TLR6. Usually these recognize PAMP on extracellular microbes. TLRs belonging to the second group are: TLR3, TLR7, TLR8, TLR9, TLR11, TLR13 (Fig. 3). These recognize PAMP on intracellular microbes and their nucleic acids after degradation. The multi-pass transmembrane protein UNC93B1 controls intracellular TLR trafficking from the ER to endosomes. TLR trafficking is also controlled by the ER-resident protein PRAT4A, which regulates the output of TLR1, TLR2, TLR4,

TLR7, and TLR9 from the ER and their trafficking to the plasma membrane and endosomes¹⁶.

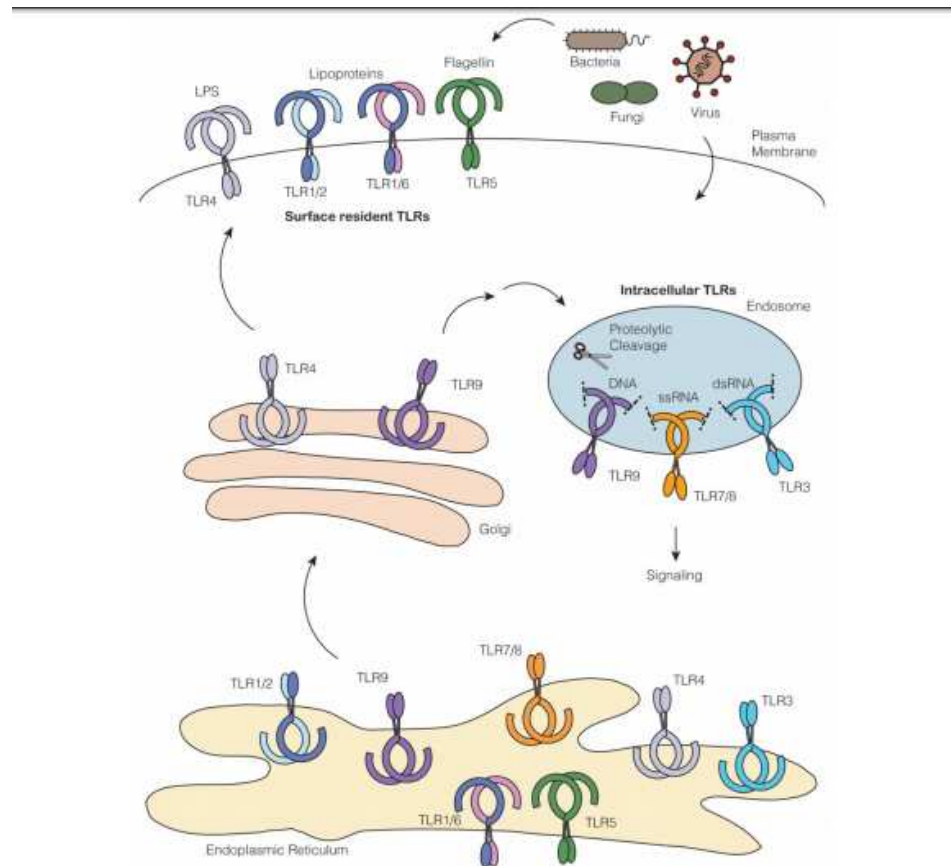


Fig. 3: Overview of Toll-like Receptor Trafficking

Unc93b1 was originally identified through a forward genetic screen in mice ("3d mice") where a mutation (H412R) caused a defect in the signaling of nucleic acid sensing TLR3, 7, and 9 but not surface localized TLRs¹⁷. Subsequent studies focused on the mechanical details of UNC93B1 function, indicating that this ER-resident protein is needed for a proper

trafficking of multiple TLRs^{18, 19}. UNC93B1 facilitates the incorporation of TLR9 into COPII vesicles, carrying protein loads from the ER to Golgi¹⁹ (Figure 4). This finding opens the possibility that active transport mechanisms such as the selection by Sec24 protein loads (part of the COPII machinery) may be involved in TLR export ER regulation. TLRs 3, 7, 11, 12 and 13 also require UNC93B1 to exit the ER¹⁹, presumably by loading into the COPII vesicles, even though this mechanism has not been formally demonstrated for these TLRs. Chemical TLR analyzes implicated TLR transmembrane domains as critical for the UNC93B120 association²⁰. In fact, the recent study has demonstrated the importance of acid residues (D812 and E813) superimposed on the TLR9 transmembrane region for the association with UNC93B1²¹. These residues alone are not sufficient to mediate interaction with UNC93B1, but are required for binding and subsequent UNC93B1-dependent trafficking. Similar residues were identified in TLR3²¹. Previous studies have also described roles for other regions within TLRs for a proper trafficking. For example, the linker region, a short sequence of aminoacids between transmembrane and TIR domains, is important for proper TLR3 traffic, while the TLR7 transmembrane domain is predominantly responsible for its proper trafficking^{22, 23}. In addition, TLR9 trafficking has been shown to depend on both its transmembrane and cytosolic regions^{24, 25, 26}. However, these studies did not examine in detail whether these regions still have a

functional relationship with UNC93B1. A number of additional factors regulate TLR output from the ER as: *Glycoprotein 96 (gp96)*²⁷, and *Protein associated with TLR4 A (PRAT4A)*, also known as *CNPY3*¹⁶. Gp96 and CNPY3 seem to work together to coordinate the folding of all TLRs²⁸. After export from ER, TLR nucleic acid sensors pass through Golgi before being ordered into endosomal compartments^{29, 30, 31}. Surprisingly, the UNC93B1 remains associated with TLRs after ER output and plays a role in these subsequent traffick events^{18, 19}. Additionally, the hepatocyte growth factor-regulated tyrosine kinase substrate (HRS) has been identified as a factor key in TLR7 and TLR9 trafficking³² (Fig 4).

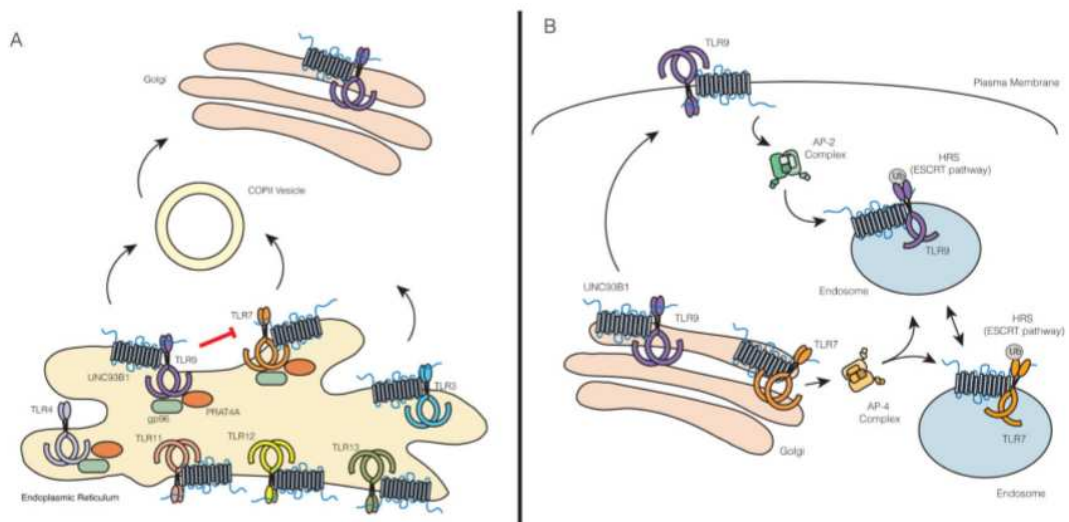


Fig. 4: Trafficking of TLRs from ER to Golgi (A) and from Golgi to endosome (B).

UNC93B1 mediates TLR7 and TLR9 differential traffic, potentially at multiple levels. Infact, point mutation (D34A) in terminal N of UNC93B1

enhanced TLR7 responses, while overlapping TLR9 responses simultaneously³³. Further examination of the cells expressing UNC93B1 D34A revealed an increase in interaction with TLR7 and simultaneous decrease with TLR9 resulting in increased TLR7 endosomal and decreased TLR9. Therefore, the N-terminal portion of UNC93B1 seems to be involved in the selectivity of this chaperone for distinct TLR family members. Additional, TLR9 uses UNC93B1 to interact with AP-2, TLR7 trafficking is unexpectedly independent of this pathway. Instead, TLR7 appears to recruit directly AP-4, a distinct sorting system known to mediate direct transport between the Golgi's network and endosomes¹⁹.

1.3 PAMPs recognized by TLRs.

Each TLR extracellular domains is responsible for recognizing specific PAMPs (Table 1)²⁰.

TLR	Ligands	Origin
TLR1–TLR2	Lipoproteins/triacylated lipopeptides: Pam ₃ CSK ₄ , JBT3002, OspA	Bacteria, Mycobacteria
	Soluble Lipoproteins	<i>Neisseria meningitides</i>
TLR2	Bacterial Lipoproteins (BLPs)	Bacteria
	Lipoarabinomannan (LAM)	Mycobacteria
	MALP-2 (Mycoplasma, Macrophage-activating Lipopeptide-2)	Mycoplasma
	Glycosylphosphatidylinositol (GPI)	<i>Trypanosoma cruzi</i>
	Glycolipids	<i>Treponema maltophilum</i>
	Porins	<i>Neisseria sp.</i>

TLR2	Zymosan	Fungi
TLR3	Double-stranded RNA (dsRNA)	Viral
	Polyinosine-polycytidylic Acid (poly(I:C))	Synthetic
	MRNA	Host
	TRNA	Host/fungi
TLR4	S-Lipopolysaccharides (LPS) (smooth) wild-type (wt), LPS (contains repeated O-polysaccharide units)	Gram-negative bacteria (e.g. <i>E. coli</i> , <i>Salmonella</i>)
	R-Lipopolysaccharides (LPS) (rough) mutant (Ra, Rb: extended core-polysaccharide) LPS (S-LPS-like)	Gram-negative bacteria (e.g. <i>E. coli</i> , <i>Salmonella</i>)
	R-Lipopolysaccharides (LPS) (rough) mutant (Rc, Rd1, Rd2, Re: short core-polysaccharide) LPS (Lipid A-like)	Gram-negative bacteria (e.g. <i>E. coli</i> , <i>Salmonella</i>)
	Flavolipin	<i>Flavobacterium meningosepticum</i>
	Paclitaxel (Taxol®) (recognizes mouse TLR4)	Plant
	Acyclic lipid A-like analog (R-112022)	Synthetic
	Type III Repeat Extra Domain A (EDA)	Host
	LMW Oligosaccharides of Hyaluronic acid (sHA)	Host
	Polysaccharide Fragments of Heparan sulfate	Host (only mouse tested)
	Fibrinogen	Host
	Fusion Protein of RSV	Respiratory syncytial virus
	Envelope Proteins of MMTV	Mouse mammary tumor virus
	Glycoinositolphospholipids (GIPLs)	<i>Trypanosoma cruzi</i>
	Heat Shock Proteins (HSPs)	TLR2 vs TLR4
TLR5	Flagellin	Gram-negative bacteria
TLR6–TLR2	MALP-2 (Mycoplasmal Macrophage-activating Lipopeptide-2)/Diacylated Macrophage-activating Lipopeptide-2	Mycoplasma
	Diacylated Lipopeptide FSL-1	Part of the lipoprotein LP44 of <i>Mycoplasma salivarium</i>
	Diacylated Lipopeptide Pam ₂ CSK ₄	Synthetic
	Soluble tuberculosis factor (STF)	Mycobacteria
TLR7	Imiquimod (R-837)/Gardiquimod	Synthetic
	Resiquimod (R-848)	Synthetic
	S-27610	Synthetic
	Loxoribine/TOG	Synthetic
	3M-13	Synthetic
	Bropiramine	Synthetic

	Single-stranded RNA (ssRNA)/Polyuridylic acid. K (Poly(U). K)	Non-viral and viral ssRNA
	U1snRNA	Host
	siRNA	Synthetic
TLR8	3M-2	Synthetic
	R-848 (Resiquimod)	Synthetic
	Single-stranded RNA (ssRNA)/Polyuridylic acid. K (Poly(U).K)	Non-viral and viral ssRNA
TLR9	Unmethylated CpG DNA	Bacteria/Protozoa/viral
	CpG ODNs	Synthetic
	AT-ODNs	Synthetic
	Hemozoin	Protozoa (<i>Malaria piment</i>)
TLR11	Profilin	Protozoa (<i>Toxoplasma gondii</i>)
TLR13	Single-stranded RNA (ssRNA)	Bacteria

Tab. 1: TRL ligand⁽³⁴⁾

1.4 TLRs signaling pathway

Individual TLRs recruit members of a set of adapters containing the TIR domain such as MyD88, TRIF, TIRAP/MAL or TRAM. Collectively, depending on the use of the adapter, the TLR signaling is largely divided into two pathways: the MyD88-dependent and TRIF-dependent pathways. MyD88 is used by all TLRs and activates NF-κB and MAPK for the induction of inflammatory cytokine genes. TLRs but also IL-1 and IL-18 proinflammatory cytokine receptors share a common TIR domain in their intracellular region and belong to the TLR/IL1-R superfamily^{2, 35}. MyD88 is critical for signaling

responses of IL-1, IL-18, and all TLRs except TLR3^{3, 10, 36, 37}. In addition to its C-terminal TIR domain, MyD88 contains an N-terminal death domain (DD) and a short intermediate domain (ID). Through DD, MyD88 interacts with IRAKs, including IRAK1, IRAK2, IRAK4 and IRAK-M, characterized by an N-terminal DD and a C-terminal Ser/Thr kinase or kinase-like domain^{3, 38, 39}. In this way MyD88 forms a complex with members of the IRAK kinase family, called Myddosome⁴⁰. During Myddosome formation, IRAK4 activates IRAK1, which is then autophosphorylated at several sites⁴¹ and released from MyD88⁴². IRAK1 associates with TRAF6. TRAF6 promotes the polyubiquitination of both TRAF6 and the complex protein kinase complex TAK1. TAK1 is a member of the MAPKKK family and forms a complex with the TAB1, TAB2, and TAB3 regulatory subunits^{43, 44}. TAK1 then activates two different pathways leading to activation of the IKK complex-NF- κ B pathway and -MAPK pathway. The IKK complex is composed of the IKK α and IKK β catalytic subunits and the NEMO regulatory subunit (also called IKK γ). The IKK complex phosphorylates the NF- κ B which translocate into the nucleus to induce proinflammatory gene expression. TAK1 activation also triggers activation of MAPK family members such as ERK1/2, p38 and JNK that mediate the activation of AP-1 transcription factors or mRNA stabilization to regulate inflammatory responses^{3, 45} (Fig. 5).

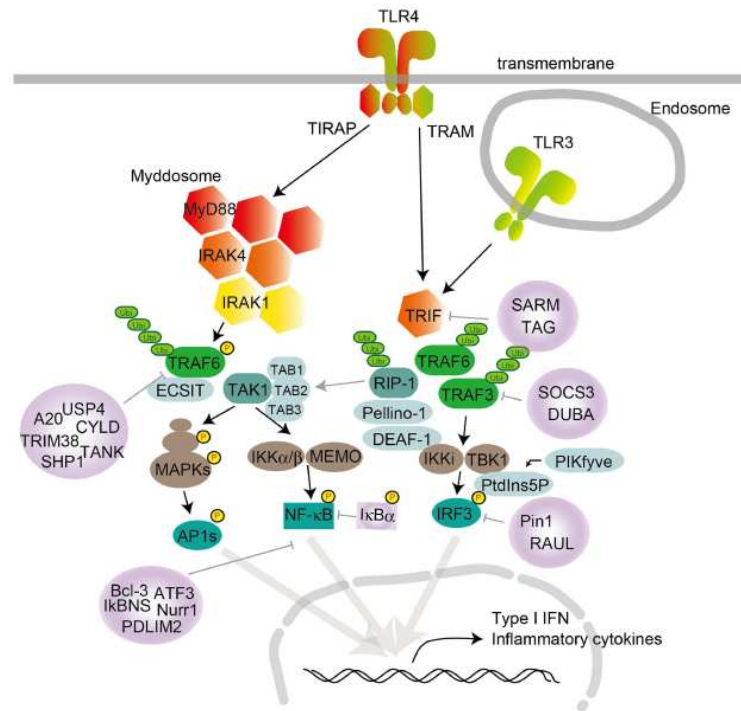


Fig. 5: TLR signaling: MyD88 and TRIF dependent pathway.

TRIF is recruited to TLR3 and TLR4. TRAM is selectively recruited to TLR4 but not TLR3 to connect between TRIF and TLR4. TLR3, indeed, directly interacts with TRIF. TLR3, in fact, interacts directly with the TRIF. TRIF interacts with TRAF6 and TRAF3. Conversely, TRAF3 recruits the IKK-related kinases TBK1 and IKKi together with NEMO for IRF3 phosphorylation. TRAF6 recruits the RIP-1 kinase, which in turn interacts with and activates the TAK1 complex, leading to the activation of NF- κ B and MAPK and the induction of inflammatory cytokines. Subsequently, IRF3 forms a dimer and translocates into the nucleus from the cytoplasm, where it induces the expression of type I IFN genes^{3, 45}. Recently, IRF3

activation has been shown to be regulated by an inositol lipid, PtdIns5P. PtdIns5P binds to both IRF3 and TBK1, and thus facilitates complex formation between TBK1 and IRF3⁴⁶.

It should also be added that the induction of inflammatory genes by endosomal TLRs may follow a more detailed mechanism. In fact, nucleic acids meet TLRs in endosomes after uptake from the extracellular milieu. Depending on the delivery method in the cell (eg in an immune complex, such as oligonucleotides or encapsulated in a viral particle), nucleic acids can reach distinct endosomes with unique signaling properties. This possibility has been first suggested by the finding that several classes of synthetic oligodynucleotides (ODNs) produce very different results of downstream signaling in pDCs^{47, 48, 49, 50}. For example, CpG-A ODN, which aggregate to form large complexes, strongly induce type I IFN, while CpG-B ODN, which remain monomeric, instead favor a pro-inflammatory response characterized by TNF α , IL-6 and IL-12p40 production. It is remarkable that this phenomenon is the result of differential ODN traffic in endosomal compartments, where TLR9 signaling leads to the activation of various transcription factors⁴⁷. CpG-A preferentially traffics to early endosomes where TLR9 signaling recruits the transcription factor IRF7 and leads to a type I IFN response⁴⁷. In contrast, CpG-B preferentially traffics to late endosomes where TLR9 signaling activates transcription factor NF- κ B,

resulting in a proinflammatory cytokine response⁴⁷. Two other compartments capable of type I IFN induction have been described: LROs (lysosome related organelles) induced by AP-3 complex⁵¹, and a compartment for DNA-immune complex (DNA-IC) which requires autophagy pathway components through a mechanism termed LC3 associated phagocytosis (LAP)⁵².

2. Endosomal TLRs

2.1 TLR3

In *Homo Sapiens*, TLR3 is made up of 903 amino acids and coded by a gene on the chromosome 4. It plays a significant role in the modulation of virus-mediated innate immune response. TLR3 (also known as CD283 or IIAE2) is a nucleic acid-sensing receptor that recognizes the viral replication product (dsRNA) and synthetic ligand polyinosinic–polycytidylic acid [poly(I:C)]⁵³. TLR3 is evolutionarily conserved from insects to vertebrates. TLR3 is expressed on the endosomes of B cells, T cells, macrophages, natural killer (NK) cells, dendritic cells, neurons. The astrocytes and microglia cells express TLR3 on its endosome as well as cell surface^{54, 55}. TLR3 triggers several intracellular signaling pathways leading to the activation of

mitogen-activated protein kinases (MAPKs), epidermal keratins, c-Jun, p38, N-terminal kinase, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), which induces the expression of proinflammatory cytokines and IFN-1²⁵. TLR3 recognizes dsRNA that have anti-viral responses as different virus groups are having ds RNA genomes or give rise to dsRNA during replication. TLR3 signals through the TRIF-mediated pathway and induces IFN- α and IFN- β gene expression. To date, 136 SNPs have been identified in TLR3. About seven SNPs are within the protein-coding sequence; however, only four (N284I, Y307D, L412F, and S737T) have led to amino acid substitutions. SNPs, Y307D and S737T, are localized in relatively less conserved residues and do not affect TLR3 activity, whereas N284I and L412F were associated with impaired receptor signaling and functioning in an "in vitro" model. Replacement of amino acids, Leu412Phe, located within a ligand binding surface adjacent to the glycosylated asparagine at position 413, is required for receptor activation⁵⁶. The crystal structure of TLR-3 identifies histidine 539 and asparagine 541 as critical for dsRNA binding and this may justify the importance of proline 554, very close to the RNA binding site; trasformation into a serine could either prohibit ligand-induced dimerization or prevent conformational modifications required for downstream signaling. Different levels of the TLR3 signaling pathway may be affected (e.g., reduced TLR3 binding sensitivity, down-signal alteration,

decreased activation of dendritic cells, which are the link between the innate immunity and adaptive, decreased activity of reactive oxygen species, and diminished production of inflammatory mediators)⁵⁷ and are responsible for disease progression in viral infections. Several studies have shown that TLR3 is involved in many viral infections such as: HSV-1^{58, 59}, HSV-2⁶⁰, Japanese encephalitis virus (JEV)⁶¹, Tick borne encephalitis^{62, 63, 64}, Dengue virus⁶⁵, Chikungunya virus⁶⁶, EV71⁶⁷, Influenza A virus⁶⁸, HIV⁶⁹, Measles Virus⁷⁰, HCV⁷¹.

2.2 TLR7/TLR8

In *Homo Sapiens*, TLR7 (1049 AA) and TLR8 (1059 AA) are coded by a gene on the chromosome X. TLR7 and TLR8 are phylogenetically similar⁷² and both are capable of recognizing single-stranded RNA and short double-stranded RNA (GU-rich)⁷³, hence in sensing different viral pathogens⁷⁴. TLR8 is known to be primarily expressed in monocytes/macrophages and myeloid dendritic cells (DCs)^{75, 76}, while plasmacytoid dendritic cells constitutively express high levels of TLR7 and are the primary source of interferon alpha (IFN- α) following TLR7 activation⁷⁷. Monocytes⁷⁸, eosinophils⁷⁹, NK cells⁸⁰, CD8 + T cells⁸¹, and CD4 + T cells⁸² also express TLR7. Activation of TLR7 in leukocytes initiates MyD88-NF κ B signaling cascade and robust production of type-1 T helper cell (Th1)-related

antiviral and pro-inflammatory cytokines, including IFN- α , tumor necrosis factor alpha (TNF- α), interleukin 12 (IL-12), and IL-6^{83, 84}. TLR7 is also expressed by airway epithelial cells and neuronal cells. In addition to viral RNA, Hornung et al.⁸⁵ showed that some siRNAs activate TLR7, which may complicate the interpretation of studies using siRNA. Endogenous miRNAs such as Let7b⁸⁶, Let7c and miR21⁸⁷ also activate TLR7. The ability to distinguish self-versus non-self-RNAs may hinge on endosomal localization of TLR7⁸⁸ as well as structural features of the specific RNA^{89, 90, 91}. The presence of the most frequent TLR-7 polymorphism, TLR-7 Gln11Leu (rs179008), was associated with higher viral loads and accelerated progression to advanced immune suppression in HIV patients⁹². Conversely, presence of the most frequent TLR-8 polymorphism, TLR-8 1A>G, Met1Val (rs3764880) was shown to confer a significantly protective effect regarding progression of the disease⁹³. These polymorphisms are also related to chronic hepatitis C virus (HCV) infection. Three relatively recent studies suggested that bacterial nucleic acids could induce TLR8 activation. In the first study, TLR8 was upregulated following phagocytosis of *Mycobacterium bovis* by THP-1 cells⁹⁴. In the second study, phagocytosis of *Helicobacter pylori* by THP-1 cells induced TLR8 activation⁹⁵. Third study shows that phagocytosis of live *Borrelia burgdorferi*, the spirochetal bacterial agent of Lyme disease, induced transcription of IFN- β 29 and that this phenomenon was entirely dependent on the availability of TLR8⁹⁶.

Though mouse and human TLR8 are highly related, they have shown differential receptor specificity both to natural/physiological and to synthetic TLR ligands^{97, 98}. Historically, murine TLR8 was thought to be non-functional as it was initially observed that TLR7^{-/-} mice did not respond to the TLR7/8 agonist R848⁹⁷ or TLR8 RNA ligands⁹⁹. However, when murine peripheral blood mononuclear cells were treated simultaneously with TLR8 selective imidazoquinoline 3M-002 and polyT oligonucleotides, enhanced TLR8 activation and suppression of TLR7 was observed¹⁰⁰.

2.3 TLR9

In *Homo Sapiens*, TLR9 is made up of 1032 amino acids and coded by a gene on the chromosome 3. TLR-9 is activated by non-methylated CG motifs from nucleotide sequences. Non-methylated CG motifs are common in bacterial and viral genomes, but occur infrequently in mammalian DNA^{45, 101}. Where CG motifs do occur in mammalian DNA, they tend to be methylated¹⁰¹. The exception is mitochondrial DNA (mtDNA), since mitochondria are evolutionary endosymbionts that were derived from bacteria¹⁰². Injury/trauma releases mitochondrial DNA – i.e., non-methylated CG motifs – into the circulation. Consequently, the non-methylated CG motifs cause a strong immune response, which is mediated primarily by TLR-9¹⁰¹⁻¹⁰⁵. TLR-9 is located in the endoplasmic reticulum,

primarily in plasmacytoid DC (pDC), where the functional role of this receptor has been proven. TLR-9 is also expressed on the majority of innate and adaptive (CD4+, CD8+, NKT and T) effector cells¹⁰⁶, where its function is less clear, and in B cells^{86, 107, 108}. Following this binding, TLR-9 engages with MyD88, which triggers a distinct signaling pathway^{107, 109}. Transduction molecules activated in this cascade include interleukin-1R-associated kinases and tumor necrosis factor receptor-associated factor 6¹¹⁰. In turn, this stimulates both the nuclear factor kappa B and IFN regulatory factor-7 pathways, resulting in production of pro-inflammatory cytokines and type I IFN, respectively^{45, 110}. TLR-9 has also been shown to be associated with certain other tumors^{111, 112}. As a consequence, TLR-9 has become a target of investigation for various malignancies^{113, 114}. In this regard, development of synthetic ODN containing non-methylated CG motifs has been the cornerstone of research into TLR-9 agonists for tumor immunotherapy¹¹³⁻¹²².

2.4 TLR11 and TLR13

TLR11 is known to recognize *Toxoplasma gondii* (T. gondii) profilin (TPRF)¹²⁴ and flagellin (FlaC) from *E. coli* and *Salmonella typhimurium*¹²⁵. In particular, TLR11 recognizes the unconventional apicomplexan actin-binding protein profilin¹²⁵, which regulates parasite motility and host cell

invasion¹²⁶. TLR11 is not expressed in humans¹²⁷, but its investigation informs our understanding of the human immune response against bacterial TLR11 regulates immune responses which are shared with other TLRs^{123, 124, 127, 128}.

TLR13 is expressed in mice and recognizes a highly conserved bacterial 23S rRNA sequence containing the sequence 5'-CGGAAAGACC-3'^{129, 130}. Compared to other nucleic acid-sensing TLRs, TLR13 recognizes RNA in a highly sequence-specific manner. TLR13 is expressed in myeloid dendritic cells (mDCs) and bone marrow-derived macrophages (BMDM) in mice and recognizes a highly conserved bacterial 23S rRNA sequence containing the sequence 5'-CGGAAAGACC-3'^{129, 130}. Compared to other nucleic acid-sensing TLRs, TLR13 recognizes RNA in a highly sequence-specific manner. Binding of the conserved 10-nucleotide pathogenic bacterial rRNA to the ecto-LRR domain of TLR13 induces LRR domain dimerization, which may result in dimerization of the cytoplasmic TIR domain. The TIR domain then recruits MyD88 for its signaling cascade. TLR13 does not adopt a canonical horseshoe-shaped structure as observed for other TLRs, but assumes an almost closed oval-shaped structure (Fig. 6).

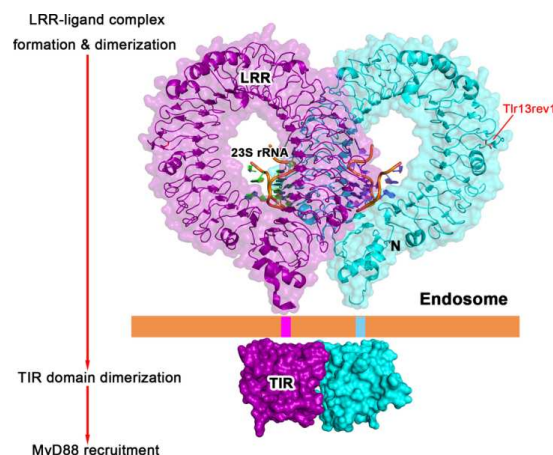


Fig. 6: TLR13 structure

Specific recognition of the ssRNA is through the splayed-out nucleotides that mainly contact the concave surface of TLR13. Hydrogen bonds between TLR13 and the ssRNA appear to be important for their interaction. RNA-specific interactions are important for TLR13 to distinguish RNA from DNA. Structural and biochemical data indicate that ssRNA-induced TLR13 dimerization is mediated by both RNA–protein and protein–protein interactions. The TLR13-bound ssRNA forms a stem-loop-like structure that is completely different from that in the bacterial ribosome. This structure is important for TLR13 recognition. For these reasons we can say that disruption of the secondary structure of 23S rRNA in the ribosome is required prior to its recognition by TLR13¹³¹. The evolutionary loss of TLR13 in several mammalian species, including humans, has been explained in various ways. Oldenburg *et al.* favored the hypothesis that widespread ancient antibiotic resistance^{129, 132} may have

obviated the need for TLR13-mediated antibacterial defenses. This hypothesis appears, however, to contrast with evidence that clinical isolates predating the antibiotic era are highly susceptible to antibiotics¹³³. Li and Chen suggested that TLR13 was lost in humans to avoid the autoimmune threats posed by two mRNAs that display exactly the same sequence as the immunostimulatory sequence in bacterial rRNA¹³⁴. Signorino G. *et al.* suppose that TLR13 (and perhaps other TLR11 family receptors, such as TLR11 and TLR12) might have been lost because other TLRs can compensate for its function and abrogate any significant selective pressure for conservation of this receptor¹³⁵. This thesis is also supported by other studies, which see in the TLR8 the human homologue of the TLR13. Kruger *et al.* have shown that human TLR8 recognizes not only SA19 (typical TLR13 agonist) but also variants of it including mitochondrial (mt) 16S rRNA¹³⁶. Nishibayashi *et al.* have shown that the recognition of SsRNA of EC-12 (*Enterococcus faecalis*) is mediated by: TLR8 in humans and TLR7 and TLR13 in mice¹³⁷. Eigenbrod *et al.* have shown that TLR8 is the receptor for bacterial RNA in human monocytes¹³⁸.

3. Streptococcus pneumoniae

Streptococcus pneumoniae is a Gram-positive, encapsulated diplococcus. The polysaccharide capsule of this bacterium is an essential virulence factor and there are determined >90 distinct types of pneumococcal serotypes based on differences in the composition of this capsule. Generally, the immune system after infection is specific to the serotype, but cross-protection may occur between related serotypes. Pneumococcal diseases range from mild respiratory tract infections such as otitis media and sinusitis to more serious illnesses such as pneumonia, septicemia and meningitis. Although *pneumococcus* can cause lethal diseases, it is more commonly a quiescent colonizer of the upper respiratory tract¹³⁹. Pneumococcal infections are thought to spread from person to person through droplets/aerosols and nasopharyngeal colonization is a prerequisite for pneumococcal disease. The bacteria enter the nasal cavity and stick to the nasopharyngeal epithelial cells and may therefore remain as a colonizer or further spread to other organs, such as the ears, sinuses, or the bronchi to the lungs and then potentially penetrate the mucous barrier to enter the bloodstream and/or cross the blood–brain barrier to cause meningitis (Figure 7). In these last cases we talk about IPD (Invasive Pneumococcal Disease). IPD is commonly defined as morbidity associated

with the isolation of pneumococci from a normally sterile body site, such as the bloodstream, or secondary blood flow diffusion, e.g. meningitis or septic arthritis; it does not include sites such as the middle ear that are infected by contiguous diffusion from nasopharynx.

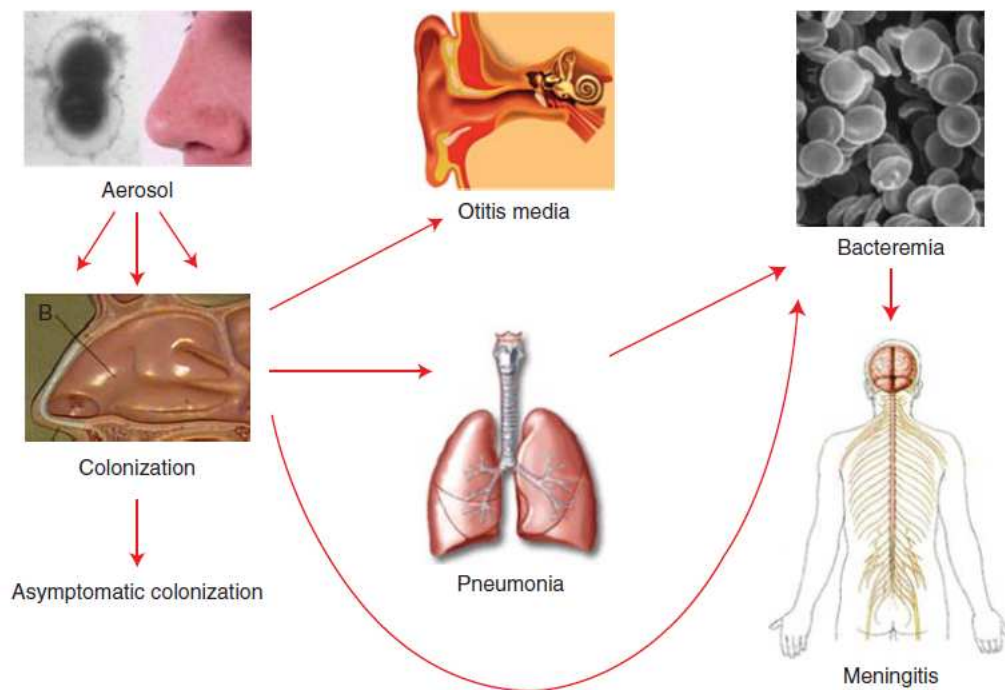


Fig. 7: Colonization of *S. pneumoniae*

3.1 Epidemiology

The distribution of serotypes that cause disease varies by age, disease syndrome, disease severity, geographic region, and over time. In young children and immunocompromised hosts, typically less immunogenic serotypes (e.g. 6, 14, 19 and 23) predominate. More invasive serotypes

(e.g. 1, 5, and 7) tend to infect individuals without co-morbidities¹⁴⁰. Globally, seven serotypes (1, 5, 6A, 6B, 14, 19F and 23F) are the predominant strains causing IPD in most countries^{141, 142} (Figure 8), particularly in developing nations.

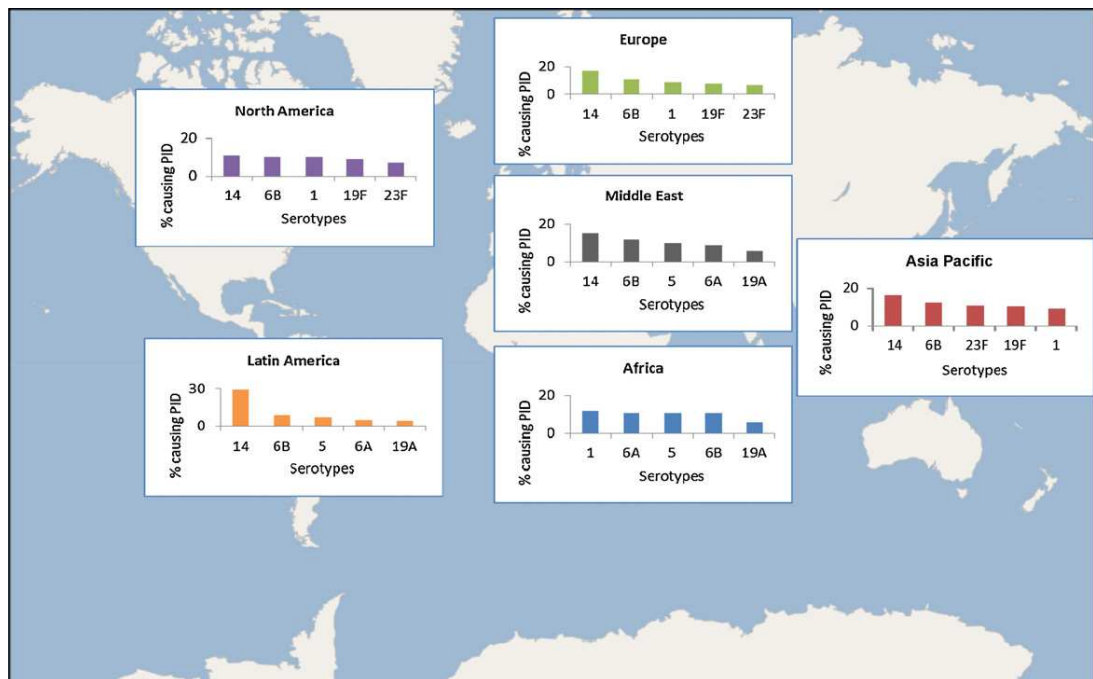


Fig. 8: Distribution of *S. pneumoniae* serotypes.

Pneumococcal infection is a major cause of morbidity and mortality worldwide. In 2005, WHO estimated that 1.6 million deaths were caused by this agent annually; this estimate included the deaths of 0.7–1 million children aged under 5 years. Disease rates and mortality are higher in developing than in industrialized settings, with the majority of deaths

occurring in Africa and Asia. It interests above all children >2 years. Case fatality rates (CFR) can be high for IPD, ranging up to 20% for septicaemia and 50% for meningitis in developing countries. Even in industrialized countries, the overall CFR for pneumococcal bacteraemia may reach 15%–20% among adults and 30%–40% among elderly patients, despite appropriate antibiotic therapy and intensive care. Among meningitis survivors, long-term neurological sequelae such as hearing loss, mental retardation, motor abnormalities and seizures have been observed in frequencies as high as 58% of cases. In Europe and the USA, *S. pneumoniae* is the most common cause of community-acquired bacterial pneumonia in adults. In these regions, the annual incidence of invasive pneumococcal disease ranges from 10 to 100 cases per 100000 population. In Europe and the United States, *S. pneumoniae* is estimated to cause approximately 30–50% of communityacquired pneumonia (CAP) requiring hospitalization in adults. Some of the differences could be explained by differences in case ascertainment and surveillance sensitivity, though incidence in Africa did appear to be generally higher than in Europe or North America. Pneumococcal middle-ear infection and sinusitis are less severe, but they are considerably more common health problems worldwide.

3.2 MICROBIOLOGY

3.2.1 CAPSULE

The pneumococcal surface is covered by a polysaccharide capsule that overlays the cell wall comprised of peptidoglycan and teichoic acid. Although the peptidoglycan has the classical gram-positive structure of N-acetylglucosamine, N-acetylmuramic acid, and a lysine-containing stem peptide, the teichoic acid is unusual in containing a ribitol phosphate backbone and covalently attached phosphorylcholine (PCho). The polysaccharide capsule is antiphagocytic and sterically hinders the access of leukocytes to complement fixed on the underlying cell wall.

3.2.2 PILI

Pili are multimeric filamentous surface structures composed of subunit proteins with LPxTG motifs recognized by sortases and attached to the cell wall. Two pathogenicity islets encoding pili, PI-1 and PI-2, are involved in adhesion^{143, 144}. In particular, PI-1, encoded by the *rlrA* accessory region, has been shown to influence colonization, virulence, and the inflammatory response in mouse challenge models. This pilus is composed of three covalently attached structural proteins: RrgA, RrgB and RrgC.

3.2.3 SURFACE PROTEIN

Surface-exposed pneumococcal proteins are marked by one of three sequence motifs: the LPxTG cell wall anchor, a choline-binding domain, or a lipoprotein domain. Among the most important surface proteins we remember choline binding protein (Cbp) which play important roles in adherence. Between these, pneumococcal surface protein A (PspA) inhibits complement deposition and activation, binds lactoferrin, and interferes with uptake into phagocytes.

3.2.4 GENOME

The complete annotated 2.16 Mbps genome of *S. pneumoniae* encoding 2236 predicted proteins was first published in 2001¹⁴⁵. Onethird of the predicted coding regions have no known function and a third of the genome varies sufficiently between strains to be considered to encode non core functions. With the ability to naturally take up DNA and undergo transformation, the genome is exceptionally highly variable. Besides, next generation sequencing has shown that pneumococci carried in the nasopharynx progressively accumulate recombination events indicative of the sequential transfer of DNA in vivo¹⁴⁶.

3.3 COLONIZATION

When the bacteria enter the nasal cavity, the negatively charged capsule protects the bacteria from being trapped in the mucous and the bacteria can reach the epithelial surface. Many surface proteins affect nasopharyngeal colonization. A prominent example is CbpA that binds to the polymeric immunoglobulin receptor (pIgR) and promotes adherence and uptake of bacteria into nasopharyngeal cells^{147, 148, 149}. Other adhesins include CbpG¹⁵⁰, CbpD¹⁵¹, and LPxTG proteins such as the pilus proteins and IgA protease¹⁵². The formation of a biofilm is promoted by PsrP which mediates bacterial aggregation and intraspecies adherence. Enzymes, including NanA (neuraminidase), BgaA (b-glucosidase), and SrtH (b-N-glucosaminidase), affect colonization by cleaving terminal sugars from human glycoconjugates, thereby potentially exposing receptors for adherence. Many respiratory pathogens have converged on a common strategy to advance mucosal disease to bacteremia. This process is termed “innate invasion” and is counteracted by the very early participants induced in innate immunity^{153, 154}. Control of bacterial multiplication in the lung requires innate immune recognition of the pneumococcal pathogen-associated molecular patterns (PAMPs) that initiate recruitment of neutrophils and the accumulation of hemorrhagic debris as the hallmark of pulmonary consolidation. Pneumococcal peptidoglycan components

induce signaling by TLR2 and LPS binding protein^{155, 156}, PCho-bearing teichoic acid binds to PAFr¹⁵⁷, and small intracellular peptidoglycan fragments are recognized by Nod2^{158, 159}. Pneumolysin, a potent cytotoxin which plays an important role in the respiratory tract, lysing host cells but also by inhibiting the mucociliary beat of respiratory cells and separating epithelial cell tight junction, is detected by TLR4¹⁶⁰. DNA is sensed by TLR9¹⁶¹. NF-κB signaling leads to the release of cytokines, particularly IL-6 and IL-1β, which then induces neutrophil recruitment and macrophage activation in the lung. Although required for clearance of bacteria, the intense inflammation induced by neutrophils contributes to lung damage; neutropenia (although not a complete absence of neutrophils) may attenuate damage and improve outcome¹⁶².

3.4 PNEUMOCOCCAL VACCINES

A 23-valent pneumococcal polysaccharide vaccine (PPSV23) was developed later, in 1983, to provide protection against 80 to 90% of the pneumococcal capsular serotypes causing disease. The current PPSV23 formulation contains the following capsular serotypes: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F. This vaccine's efficacy against pneumococcal infections with serotypes

contained in the vaccine in immunocompetent persons was 65%. Although the rate of invasive pneumococcal disease was reduced in PPSV23-immunized adults, the overall pneumococcal carriage rate was not reduced¹⁶³. Unfortunately, the vaccine did not generate an immune response in the group with the highest rate of pneumococcal disease burden, children younger than 2 years of age. The antibody response generated by PPSV23 is T-cell independent due to the fact that the repeating subunits of the capsular polysaccharide can stimulate an immune response in B-cells independent of T-cell help¹⁶⁴. The theory is that their inability to mount a T-cell-independent immune response until this age prevented the vaccine from generating protective antibodies¹⁶⁵. In order to elicit a protective immune response in children under the age of 2, vaccines with the capsular polysaccharide conjugated to diphtheria toxin were developed. These conjugated antigens generated a T-cell-dependent antibody response that was effective in children under 2 years of age. The first 7-valent pneumococcal polysaccharide conjugate vaccine (PCV7) developed in 2002 greatly reduced the rate of infections in children under 2 years of age and in unimmunized individuals in the same community¹⁶⁶⁻¹⁶⁸. The second conjugate vaccine that was developed, the 13-valent pneumococcal conjugate vaccine (PCV13), contained the 7 serotypes in PCV7, 5 serotypes found in PPSV23, and 1 unique serotype found in neither PPSV23 nor PCV7, serotype 6A.

Despite the many benefits of the new vaccination methodologies, let us not forget that *S. pneumonie*, as mentioned above, still affects millions today. In fact, approximately 3 million cases of pneumonia per year are estimated in Europe, of which 1 million hospital admissions. In 2013, there were more than 120,000 (9,000 in Italy only) pneumonia deaths (of which 86% in the age range of 55-94 years), four times more than those caused by road accidents. Nevertheless, most people at risk do not know or do not care about how to prevent the disease. Besides, pneumococcal vaccination, however, may be a two-edge sword. The initial benefits in disease reduction may be followed by a steady increase in IPD incidence caused by non-included strains. All of these reasons lead us to stress the importance to study the immune mechanisms involved in the etiopathogenesis of these infections. This is still a key objective of public health and this is what we have set ourselves to focus our attention on the study of the role of endosomal TLR, still not well-known in *S. pneumiae* infections.

4. TLRs in *S. pneumoniae* infections

TLR2 involvement in *S. pneumoniae* infections is known in the literature as it seems to be responsible for the recognition of peptidoglycan components and LPS binding protein. TLR2 is responsible for most CW inflammatory signaling¹⁶⁹ and its interaction with *S. Pneumoniae* results in platelet activation that is likely to contribute to the thrombotic complications of sepsis¹⁷⁰. It is so important the function performed by this TLR during this infection that has been shown to be lacking in it results in higher mortality of bacterial meningitis by impaired host resistance¹⁷¹. TLR2 signaling is critical for bacterial clearance and macrophage recruitment during *S. pneumoniae* infection¹⁷².

TLR4 it is known to recognize pneumolysin, a cholesterol-dependent cytolysin released by *S. pneumoniae*. Indeed, mice lacking functional TLR4 are significantly more susceptible to invasive disease after colonization with a virulent, pneumolysin-producing type-3 strain of *S. pneumoniae*¹⁷³. It was shown that induction of endotoxin tolerance, despite reducing cytokine production, improves host defense against infection with a virulent strain of *S. pneumoniae*. Besides, TLR4 induces CCL2 and CCL5 production¹⁷⁴. For these reasons, TLR4 agonist activity can therefore be

potentially exploited to provide short-term resistance to infectious challenge. More recently, however, it has been shown that pneumolysin can activate the NLRP3 inflammasome and can promotes proinflammatory cytokines independently of TLR4¹⁷⁵ and that, although purified PLN is recognized by TLR4 *in vitro*, PLN elicits lung inflammation *in vivo* by mechanisms that may involve multiple TLRs¹⁷⁶.

Regarding the role of endosomal TLRs in *S. pneumoniae* infections, little is known. For TLR3 is known that the released pneumococcal RNA activates TLR3 and TRIF, which subsequently leads to IL-12 expression and secretion¹⁷⁷. Furthermore, it has been shown that Poly IC:LC, a potent agonist of TLR3, significantly increases the survival of mice infected with *S. pneumoniae*¹⁷⁸. It was shown also that TLR3 mRNA expression levels are increased in murine neuronal cells during infection with *S. pneumoniae*¹⁷⁹.

Almost nothing is known about the role of TLR7 in *S. pneumoniae* infections. It is known instead of it phagosomal bacteria such as *group B streptococcus*, potently induced interferon in conventional dendritic cells by a mechanism that required Toll-like receptor 7, the adaptor MyD88 and the transcription factor IRF1, all of which localized together with bacterial products in degradative vacuoles bearing lysosomal markers. Thus, this cell type-specific recognition pathway links lysosomal recognition of bacterial RNA with a robust, host-protective interferon response¹⁸⁰. This may lead

us to think that a very similar mechanism of bacterial RNA recognition by TLR7 can also be carried out during *S. pneumoniae* infections.

It is known that *S. pneumoniae* meningitis led to an enhanced expression of TLR9 mRNA¹⁷⁹. Besides, it's shown that TLR9^{-/-} mice are more susceptible to pneumococcal infection and that TLR9 is not involved in the control of nasopharyngeal colonization but is crucial for bacterial clearance from the lower respiratory tract. Furthermore, TLR9 is not required for local cytokine production and immune cell infiltration during pneumococcal infection¹⁶¹. It also seems to cooperate synergistically with TLR2 and TLR4 in response to *S. pneumoniae*¹⁸¹. It's known also that if a TLR9 agonist is added to the PCV (polysaccharide conjugate vaccine) of *S. Pneumoniae*, this greatly improves the immune response. Finally, it appears that TLR9 is responsible for cardiac inflammation during *S. Pneumoniae* infection and, specifically, as a result of sepsis. ROS produced during the inflammation process would cause mitochondrial DNA damage, these mtDNA fragments would be captured by the TLR9 and trigger the signal transduction pathway¹⁸².

TLR13 receptor, is the major expressed TLR in mouse coinfection models (*Human metapneumovirus* + *S. pneumoniae* and *Influenza A Virus* + *S. pneumoniae*)¹⁸³. In murine macrophages and DCs, the TLR13 endosomal receptor has recently been identified as a sensor for bacterial RNA that

specifically recognizes a conserved region within bacterial 23S rRNA. Recognition by TLR13 can be masked by N6-dimethylation at a critical adenosine residue (A2058, *E. coli*) within the target sequence¹²⁹. This modification also renders bacteria resistant to macrolide antibiotics^{184, 185}. On the basis of these assumptions, it was shown that a naturally occurring A2058G point mutation in *S. pneumoniae* 23S rRNA abolishes TLR13 stimulatory capacity as efficiently as N6-dimethylation at this residue. Despite the striking effect of 23S rRNA A2058 modification on immunostimulation in murine cells, there is a clear evidence that these alterations do not affect the activation of human monocyte¹³⁸. But this function could be done by the human homologue TLR8. Besides, we can say that the role of TLR13 has been more studied in other types of streptococcal infections. Indeed, it was shown that TLR13 is the main macrophage receptor involved in the recognition of streptococci in resident tissue macrophages¹⁸⁶. Besides, TLR13 participates in GBS recognition, although blockade of the function of this receptor can be compensated by other endosomal TLRs (in particular TLR2, TLR7 and TLR9)¹³⁵.

5. MATERIALS AND METHODS

5.1 Mice

C57BL/6 wild-type (WT), used as controls, and IL-1R^{-/-} mice were purchased from Charles River Laboratories. Mice lacking MyD88 or TLR2, TLR7 and TLR9 were originally obtained from S. Akira (Osaka University, Japan). Heterozygous TLR13^{+/-} mice were provided by the KOMP Repository (www.komp.org) and the Mouse Biology Program (www.mousebiology.org) at the University of California Davis. Subsequently, TLR13^{-/-} mice were generated in our laboratory for carrying out research project¹⁸⁹. 3D mutant mice, bearing the H412R mutation of UNC93B1, were obtained from Bruce Beutler (University of Texas Southwestern Medical Center, TX). TLR7, 9, 13^{-/-} triple-knockout mice were generated in our animal facility by crossing TLR9^{-/-} with TLR7^{-/-} and TLR13^{-/-} mice using the genotyping methods described below. All KO mice, bred on a C57BL/6J background, were born and developed normally in the animal facilities of the Department of Human Pathology of the University of Messina. All mice used in the present study were housed under specific pathogen-free conditions in individually ventilated cages at these animal facilities.

5.2 Genotyping TLR7/9/13^{-/-} mice

As already mentioned, TLR7, 9, 13^{-/-} triple-knockout mice were generated in our animal facility by crossing TLR9^{-/-} with TLR7^{-/-} and TLR13^{-/-} mice. To create these triple KO we had to genotypize every single generation of mice. DNA was extracted from mouse tail with "DNeasy Blood & Tissue Kits" (Qiagen).

The protocols and sequences of primers used for genotyping were as follows:

TLR13				
WT primer (WT band lenght: 202 bp)		P_R : TTGTGGCACC GTTATT TCCCATC		
		P_F : AGGAACATTGCATGTGGG		
KO primer (KO band lenght 400 bp)		P_R : TACACAAGTGCAGTCTCCCATGACC		
		P_F : GCAGCCTCTGTTCCACATACACTTCA		
THERMAL PROTOCOL			MIX (TOTAL VOLUME: 25 µL)	
94 °C	5 min.	10 cycles	Buffer (1X) ¹	2,5 µL
94 °C	15 sec.		dNTPs ¹	0,2 µL
65 °C	30 sec.		P _R ²	1 µL
72 °C	40 sec.		P _F ²	1 µL
94 °C	15 sec.	30 cycles	Taq polymerase ¹	0,5 µL
55 °C	30 sec.		dH ₂ O	17,8 µL
72 °C	40 sec.		DNA	2 µL
72 °C	5 min.		TOTAL VOLUME	25 µL

TLR7				
WT primer (WT band lenght: 1200 bp)		P_F : ACGTGATTGTGGCGGTCAGAGGATAAC		
		P_{KO} : ATCGCCTTCTATCGCCTTCTTGACGAG		
KO primer (KO band lenght 1200 bp)		P_R : CCAGATACATCGCCTACCTACTAGACC		
		P_{KO} : ATCGCCTTCTATCGCCTTCTTGACGAG		
THERMAL PROTOCOL			MIX (TOTAL VOLUME: 25 µL)	
94 °C	5 min.		Buffer (1X) ¹	2,5 µL
94 °C	45 sec.	35 cycles	dNTPs ¹	0,2 µL
67 °C	1min and 30 sec.		P _R ²	0,5 µL
72 °C	1 min.		P _F ²	0,5 µL
72 °C	10 min.		Taq polymerase ¹	0,5 µL
			dH ₂ O	18,8 µL
			DNA	2 µL
			TOTAL VOLUME	25 µL

TLR9				
WT primer (WT band lenght: 1200 bp)		P_F : GAAGGTTCTGGGCTCAATGGTCATGTG		
		P_{KO} : GCAATGGAAAGGACTGTCCACTTTGTG		
KO primer (KO band lenght 1200 bp)		P_R : ATCGCCTTCTATCGCCTTCTTGACGAG		
		P_{KO} : GCAATGGAAAGGACTGTCCACTTTGTG		
THERMAL PROTOCOL			MIX (TOTAL VOLUME: 25 µL)	
94 °C	5 min.		Buffer (1X) ¹	2,5 µL
94 °C	30 sec.	35 cycles	dNTPs ¹	0,2 µL
67 °C	1min and 30 sec.		P _R ²	0,5 µL

72 ° C	1 min and 30 sec.		P _F ²	0,5 µL
72° C	10 min.		Taq polymerase ¹	0,5 µL
			dH ₂ O	18,8 µL
			DNA	2 µL
			TOTAL VOLUME	25 µL

PCR products were then passed on agarose gel at the appropriate percentage.

1. VWR International

2. Thermo Fisher Scientific.

5.3 *S. pneumoniae* strain and murine infection models

All studies were performed in strict accordance with the European Union guidelines for the use of laboratory animals. The procedures were approved by the Ethics Committee of the University of Messina (OPBA) and by the Ministero della Salute of Italy.

S. pneumoniae strain D39 was grown to the mid-log phase in Todd-Hewitt broth (Oxoid) with 1% FCS, washed three times in nonpyrogenic PBS [0.01 M phosphate and 0.15 M NaCl (pH 7.2)], and diluted to the appropriate concentration. The number of viable bacteria used in each experiment was carefully determined by plate counting.

Six-week-old female mice were anesthetized intraperitoneally with zolazepam + tiletamina (0,1 mg/mouse) and xilazina (0,16 mg/mouse) and injected intranasal with 7.5×10^7 CFU of *S. pneumoniae*. In lethality

experiments mice were observed for 10 days every 12 hours. For cytokine measurement mice were sacrificed at different times after infection and organ target have been taken, homogenized in 2 ml sterile PBS and centrifuged. The cytokines were then dosed on the supernatants.

5.4 Cytokine measurement

IL-1 β , TNF- α , CXCL1 and CXCL2 levels were determined, according to the manufacturer's recommendations, using the following ELISA kit: mouse IL-1 β /IL-1F2, mouse TNF- α , mouse CXCL1/KC and mouse CXCL2/MIP-2. All kits were purchased from R&D system. The lower detection limits of all these assays were 16 pg/ml.

5.5 Bone-marrow derived cells

Bone marrow-derived cells were obtained as described previously¹⁸⁰. Briefly, after flushing murine femurs and tibiae of C57BL/6J and 3D mice, marrow cells were cultured for 6 to 7 days in RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin (50 IU/ml), and streptomycin (50 μ g/ml). Medium was supplemented with either 100 ng/ml macrophage colony-stimulating factor (M-CSF) or 20 ng/ml granulocyte-macrophage colony-

stimulating factor (GM-CSF) (both from PeproTech) to obtain bone marrow-derived macrophages (BMDMs) or conventional dendritic cells (BMDCs), respectively. On day 3, 10 ml fresh cytokine-supplemented culture medium was added to each petri dish.

BMDCs and BMDMs were seeded in 96-well plates at a concentration of 5×10^5 cells/well in 200 μ l of RPMI 1640 supplemented with 10% heat-inactivated FCS. Cells were then stimulated with different MOI (multiplicity of infection) of *S. pneumoniae* ranging from 5 to 20 and centrifugated for 10 min at $400 \times g$ in order to facilitate bacterial adherence. After incubation at 37°C in 5% CO₂ for 30 min, cells were incubated for 24 h in the presence of penicillin (250 IU/ml) and streptomycin (250 μ g/ml) in order to limit the growth of residual extracellular bacteria. At the end of the incubation, supernatants were then collected and stored at -20°C for ELISAs.

5.6 Neutrophil isolation

Neutrophils were obtained as described previously¹⁹⁰ from the bone marrow of C57BL/6J and 3D mouse using Percoll density gradient centrifugation (Figure 9). Briefly, after removing the femurs and the tibias, a RPMI 1640 containing 10% FBS solution was forced through the bone with a syringe. For neutrophilic isolation, bone marrow cells were layered on the top of 62 and

81% at two layer of discontinuous gradient Percoll (GE Healthcare Life Sciences). After centrifugation at 1060 x g for 30 min at room temperature, PMN cells were collected from the two-layer interface, washed extensively in PBS to remove Percoll, and suspended in RPMI containing 10% (v/v) fetal calf serum (FCS). The viability of the PMN cells obtained through this procedure was normally > 95% as evaluated by trypan blue exclusion assay. Isolated cells ($5 \pm 0.6 \times 10^6$ cells/mouse) were stained with May/Grünwald/Giemsa and 94 % of them were morphologically mature neutrophils (bands and segmented). Cells were stimulated with *S. pneumoniae* strain D39 for 24 and 48 h before supernatants were collected for cytokines determination.

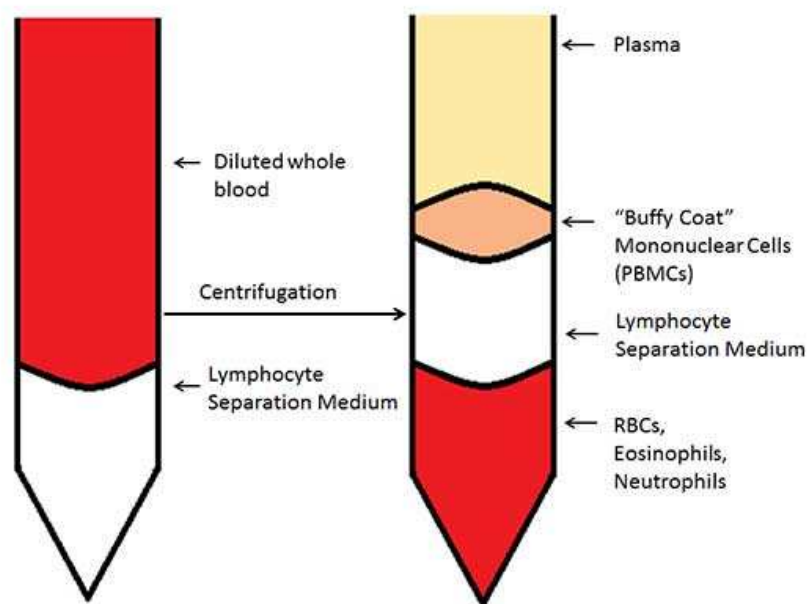


Fig. 9: Neutrophils isolation with Percoll gradient

5.7 Peritoneal macrophages

Resident mouse peritoneal macrophages were isolated from the peritoneal cavity by washing with ice PBS as previously described¹⁹¹. Briefly, after centrifugation, cells were resuspended in RPMI 1640 (Euroclone) supplemented with 5% heat-inactivated fetal calf serum, 50 IU of penicillin/ml, and 50 µg/ml of streptomycin. Cells were then seeded in wells of 96-well plates at a density of 5×10^5 /well and incubated at 37°C in a 5% humidified CO₂ environment. After 24 h, the nonadherent cells were removed by washing with medium. Adherent cells were stimulated with increasing multiplicities of infection (MOI, 1, 5, and 10 µg/ml) of *S. pneumoniae*. All infections were performed by centrifuging bacteria on cell monolayers for 10 min to $400 \times g$ to facilitate bacterial adhesion. The number of viable bacteria used in each experiment was carefully determined by the plate count. After incubation at 37°C in 5% CO₂ for 25 minutes, monolayers were incubated for 18 h in the presence of penicillin (250 IU/ml) and streptomycin (250 µg/ml) to limit the growth of extracellular residual bacteria. Cell culture supernatants were collected at 18 h after stimulation to measure cytokine levels.

For **elicited macrophages**, the isolation procedure is the same, with the only difference that 500 µl of thioglycolated to 4% are administered to the mouse

i.p. 3 days before the peritoneal washing to pre-activate peritoneal macrophages.

5.8 IFN- β mRNA analysis by quantitative real-time PCR

IFN- β mRNA was measured in 3D and C57BL/6J lung mice after different time of *S. pneumoniae* infection (1, 3 and 24 h). The mRNA was extracted with the RNeasy mini kit (Qiagen), following the manufacturer's instructions, from a little piece of lung (10 ng). For the quantification of IFN- β mRNA, real-time quantitative RT-PCR assays were conducted, in duplicate, with an Applied Biosystems 7500 (Applied Biosystems), as described¹⁹². The RNA was retroscribed in cDNA with the cDNA iScript kit (Bio-Rad). PCR conditions were as follows: 50°C, 30 min; 95°C, 10 min; (95°C, 15 s; 60°C, 1 min) X 40 cycles. Real-time PCR data were normalized in each individual sample by the level of β -actin expression. Gene expression was measured by the comparative cycle threshold method ($\Delta\Delta CT$) and was reported as the n-fold difference relative to the normalized expression of C57BL/6J and 3D. Primers and TaqMan MGB probes for β -actin and IFN- β have been previously described^{193, 194} and were purchased from Applied Biosystems.

5.9 Data expression and statistical significance

Differences in cytokine levels and organ CFUs were assessed by two-way analysis of variance and the Bonferroni test. Survival data were analyzed with Kaplan–Meier survival plots followed by the log rank test (JMP Software; SAS Institute). When p values were lower than 0.05, differences were considered statistically significant.

6. Results

6.1 MyD88^{-/-} mice are extremely susceptible to *S. pneumoniae* infections

To investigate the role played by TLRs during pneumococcal pneumonia, we initially used a lung infection experimental model. We used genetically-deficient mice for MyD88, IL-1R, TLR-2, and 3D mice. As mentioned before, MyD88 is an adapter that is recruited both by IL-1R and TLRs following recognition of a PAMP. This makes these mice, from a phenotypic point of view, particularly susceptible to infections. In the initial phase of the experiments we tried to find out if Myd88-defective mice were more susceptible to pneumococcal infection and whether this increased sensitivity could be due to the inactivation of the TLR transduction pathway or to the involvement of inflammasoma (and hence of IL-1R route). TLR2^{-/-} mice have been included in this experiment since the literature has already established the involvement of this receptor in *S. pneumoniae* infections as well as 3D mice that exhibit a mutation at the gene level encoding for UNC93B, a chaperon protein essential for the translocation of endosomal TLR at the endoplasmic reticulum level.

The mice were anesthetized intraperitoneally with zolazepam +tiletamina (0,1 mg/mouse) and xilazina (0,16 mg/mouse) and infected intranasally with 7.5×10^7 CFU of *S. pneumoniae*, a sublethal dose. Mice were observed for 10 days every 12 hours and lethality was recorded. Two experiments of this type were performed with 8 mice per strain per experiment (Figure 10). The data obtained were then put together and provided the following results:

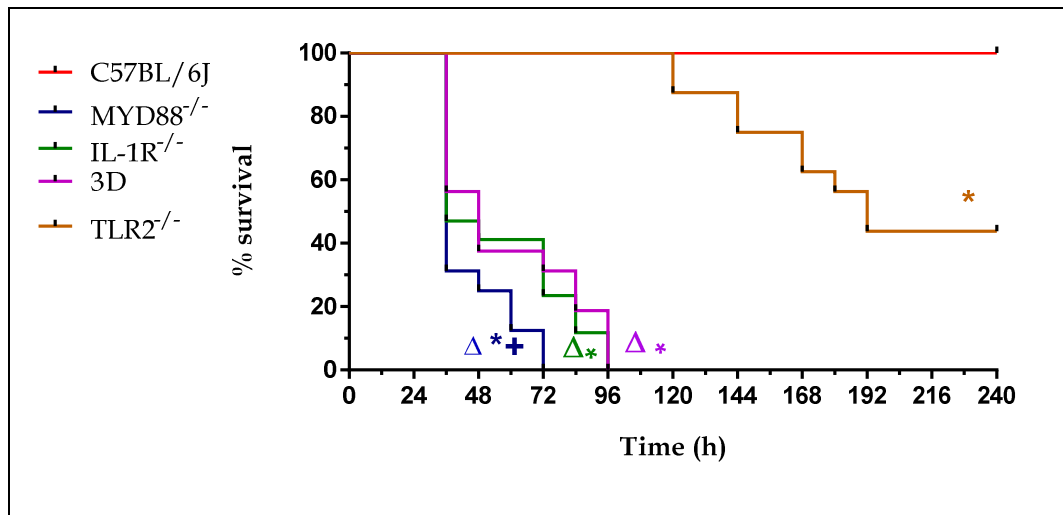


Fig. 10: Lethality of C57BL/6J, MyD88^{-/-}, TLR2^{-/-}, IL-1R^{-/-} and 3D mice. Mice were infected intranasally with 7.5×10^7 CFU of *S. pneumoniae* strain D39. Mice were observed twice a day for 10 days.

*, P < 0.05 versus WT mice as determined with Kaplan-Meier survival plots.

+, P < 0.05 versus 3D, IL-1R^{-/-} and TLR2^{-/-} mice as determined with Kaplan-Meier survival plots.

Δ, P < 0.05 versus TLR2^{-/-} mice as determined with Kaplan-Meier survival plots

As evidenced by Kaplan-Meier's lethality curves, we have a 100% mortality rate for Myd88^{-/-} mice within 72 h of infection while the same percentage of mortality was observed for 3D and IL-1R mice within 96 h. These results suggest that the severe phenotype of Myd88^{-/-} mice was due not only to

inflammasome inactivation and to a lack of recruitment by IL-1R (because Myd88^{-/-} mice tend to die earlier than IL-1R^{-/-}) but also to the inactivation of the TLR pathway. In particular, the severe phenotype reported in 3D mice suggested possible involvement of endosomal TLRs in pneumococcal infection. A 60% mortality was also obtained at 192 h for TLR2^{-/-} mice, confirming the data already reported in literature on TLR2 receptor.

To verify that this high lethality was associated with a defect in the antimicrobial defenses, in further experiments we determine the amount of bacteria in the blood, lungs and brain (Figure 11, 12 and 13). We hypothesized that the severe phenotype observed in 3D mice may be related to a compromised immune response and that therefore the blood and the target organs may be most colonized by *S. pneumoniae*. The results obtained were as follows:

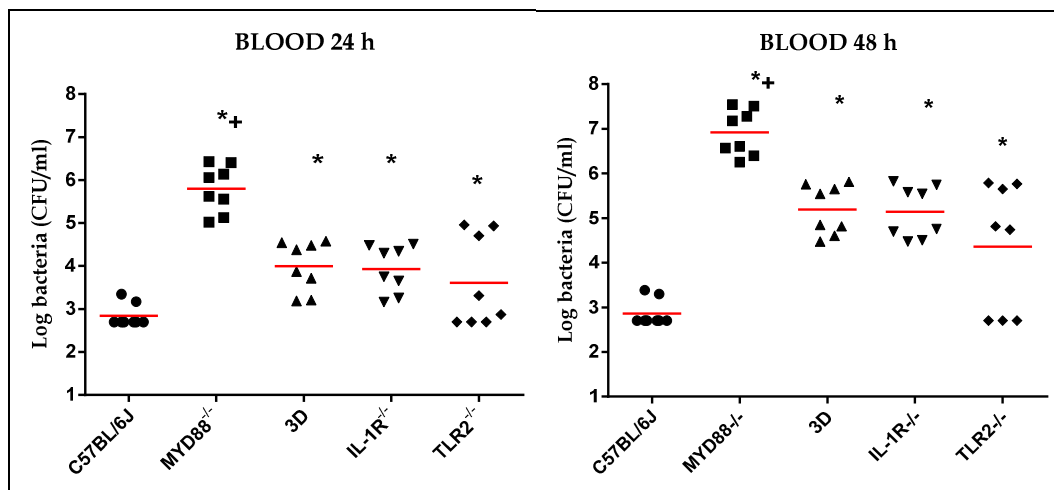


Fig. 11: CFU determination in blood of C57BL/6J, MyD88^{-/-}, TLR2^{-/-}, IL-1R^{-/-} and 3D mice 24 and 48 hours after intranasal infection (7.5×10^7 CFU) with *S. pneumoniae* strain D39.

*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

+, $P < 0.05$ versus 3D, IL-1R^{-/-} and TLR2^{-/-} mice by two-way analysis of variance and the Bonferroni test.

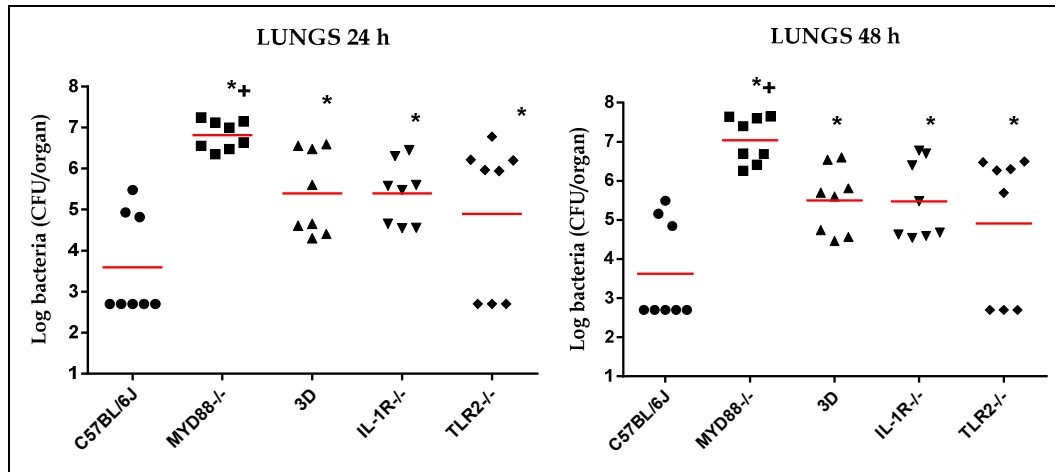


Fig. 12: CFU determination in lungs of C57BL/6J, MyD88^{-/-}, TLR2^{-/-}, IL-1R^{-/-} and 3D mice 24 and 48 hours after intranasal infection (7.5×10^7 CFU) with *S. pneumoniae* strain D39.

*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

+, $P < 0.05$ versus 3D, IL-1R^{-/-} and TLR2^{-/-} mice by two-way analysis of variance and the Bonferroni test.

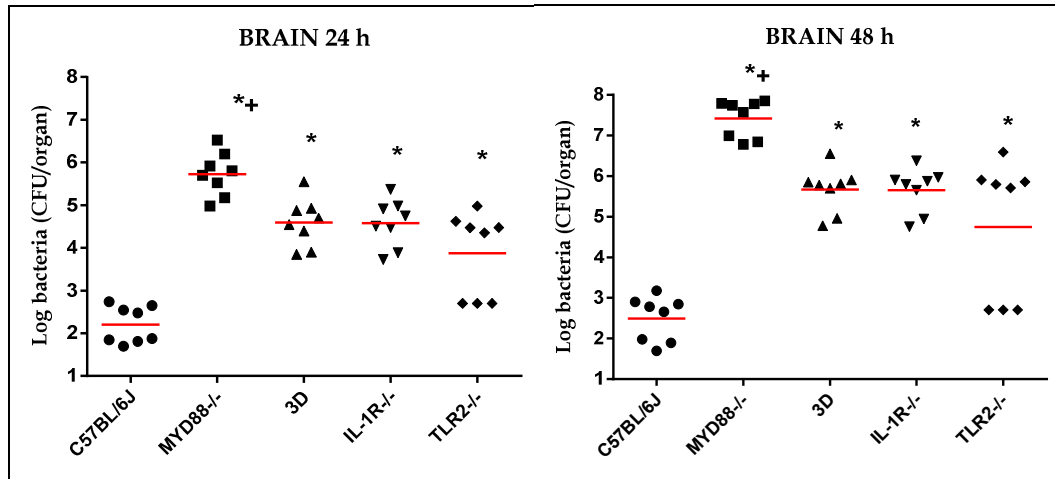


Fig. 13: CFU determination in brain of C57BL/6J, MyD88^{-/-}, TLR2^{-/-}, IL-1R^{-/-} and 3D mice 24 and 48 hours after intranasal infection (7.5×10^7 CFU) with *S. pneumoniae* strain D39.

*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

+, $P < 0.05$ versus 3D, IL-1R^{-/-} and TLR2^{-/-} mice by two-way analysis of variance and the Bonferroni test.

As shown in the figures, bacterial counts in MyD88^{-/-} mice were significantly higher at both 24 and 48 h in the blood and organs than those found in mice wild type C57BL/6J and also respect those found in IL-1R^{-/-} and 3D mice. This confirmed that the severe phenotype of MyD88^{-/-} infected mice was based not only on the lack of recruitment of this molecule by IL-1R, but also by some TLRs receptors.

6.2 The combined absence of TLR7/9/13 recapitulates severe phenotype of 3D mice.

At this point, we went to the second step of our research project: to understand what the endosomal TLR was involved in this type of infection. We then carried another experiment of lethality in which we inserted in addition to the C57BL/6J and 3D mice, including TLR7^{-/-}, TLR9^{-/-}, TLR13^{-/-} and TLR7/9^{-/-} mice. Mice were always intranasally infected with a dose of 7.5×10^7 CFU of *S. pneumoniae* and lethality was observed every 12 h for 10 days (Figure 14). The results of the two experiments were then put together and were the following:

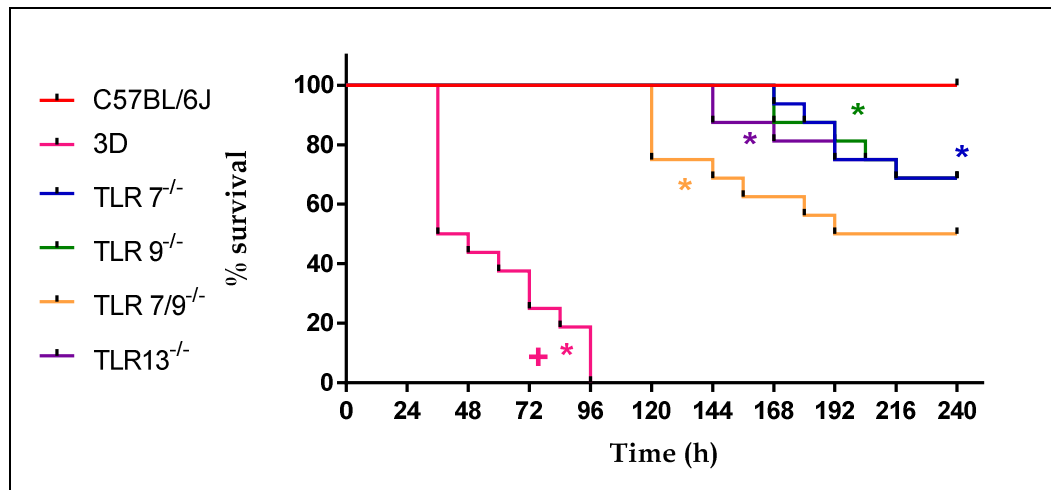


Fig. 14: Lethality of C57BL/6J, TLR7^{-/-}, TLR9^{-/-} TLR13^{-/-}, TLR7/9^{-/-} and 3D mice. Mice were infected intranasally with 7.5×10^7 CFU of *S. pneumoniae* strain D39. Mice were observed twice a day for 10 days.

*, $P < 0.05$ versus WT mice as determined with Kaplan-Meier survival plots.

+, $P < 0.05$ versus TLR7^{-/-}, TLR9^{-/-} TLR13^{-/-} and TLR7/9^{-/-} mice as determined with Kaplan-Meier survival plots.

As evidenced by Kaplan-Meier's lethality curves, we obtained a 100% mortality rate at 96 h in 3D mice, 30% at 216 h for TLR7^{-/-}, TLR9^{-/-} and TLR13^{-/-} mice and 50 % to 192 h in double knockouts (TLR7/9^{-/-}). Therefore, 3D mice showed a most severe phenotype that was accompanied from elevated number of CFUs in the blood, lungs and brain at 24 and 48 h following intranasal infection by *S. pneumoniae*. The results (Figure 15, 16 and 17) confirmed that TLR7^{-/-} and TLR9^{-/-} mice were moderately susceptible to pneumococcal infection and the effect of these two receptors appeared to be additive when both receptors were missing (see double KO). However, the absence of each of these receptors did not seem sufficient to justify the strong susceptibility of 3D mice alone.

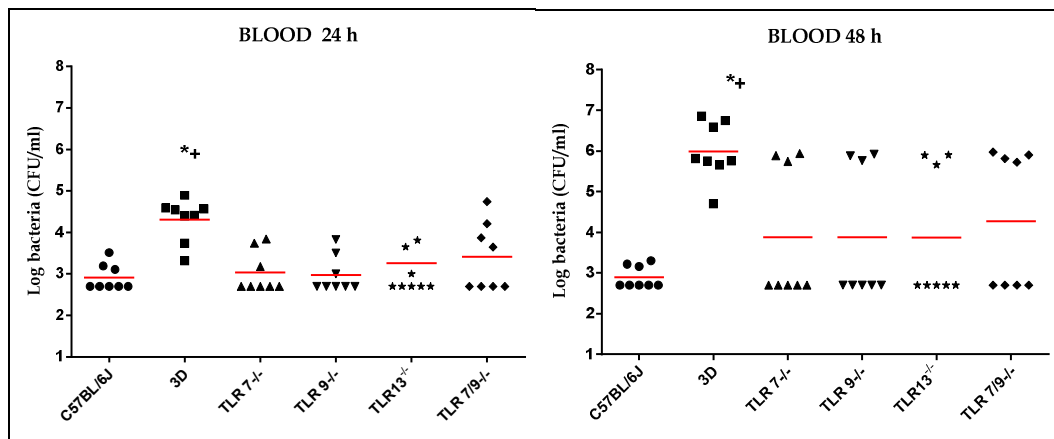


Fig. 15: CFU determination in blood of C57BL/6J, TLR7^{-/-}, TLR9^{-/-} TLR13^{-/-}, TLR7/9^{-/-} and 3D mice 24 and 48 hours after intranasal infection (7.5×10^7 CFU) with *S. pneumoniae* strain D39.

*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

+, $P < 0.05$ versus TLR7^{-/-}, TLR9^{-/-}, TLR13^{-/-}, TLR7/9^{-/-} mice by two-way analysis of variance and the Bonferroni test.

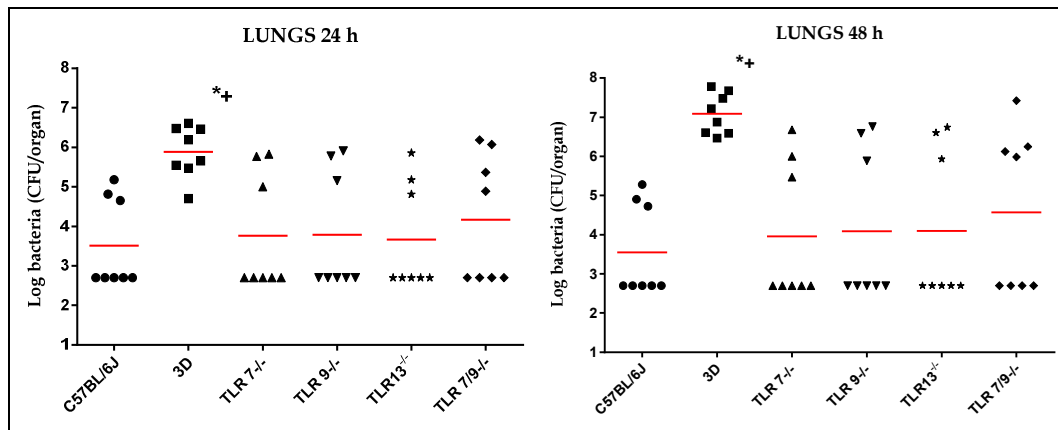


Fig. 16: CFU determination in lungs of C57BL/6J, TLR7^{-/-}, TLR9^{-/-} TLR13^{-/-}, TLR7/9^{-/-} and 3D mice 24 and 48 hours after intranasal infection (7.5×10^7 CFU) with *S. pneumoniae* strain D39.

*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

+, $P < 0.05$ versus TLR7^{-/-}, TLR9^{-/-}, TLR13^{-/-}, TLR7/9^{-/-} mice by two-way analysis of variance and the Bonferroni test.

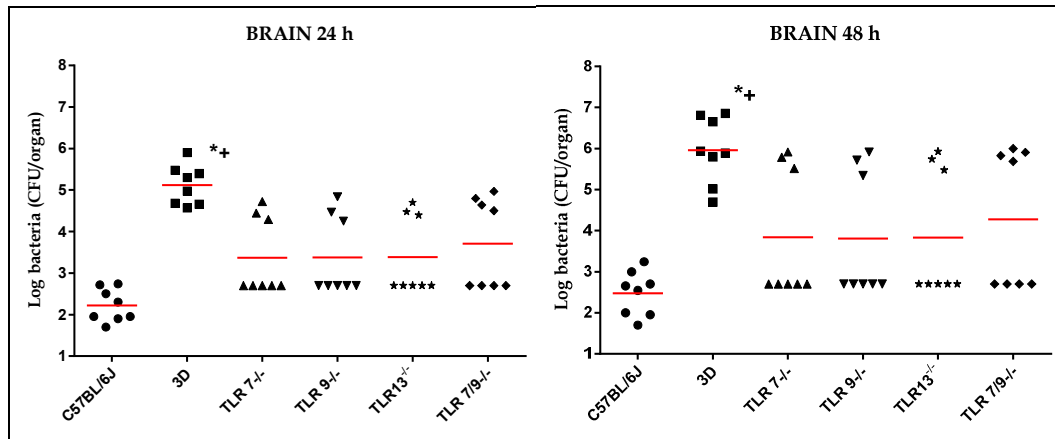


Fig. 17: CFU determination in brain of C57BL/6J, TLR7^{-/-}, TLR9^{-/-}, TLR13^{-/-}, TLR7/9^{-/-} and 3D mice 24 and 48 hours after intranasal infection (7.5×10^7 CFU) with *S. pneumoniae* strain D39.
 *, P < 0.05 versus WT mice by two-way analysis of variance and the Bonferroni test.
 +, P < 0.05 versus TLR7^{-/-}, TLR9^{-/-}, TLR13^{-/-}, TLR7/9^{-/-} mice by two-way analysis of variance and the Bonferroni test.

The results of the number of bacteria in the blood and organs at 24 and 48 hours confirmed what was said earlier for experiments of lethality.

Thanks to these data we come to the conclusion that the absence of endosomal TLRs alone can not reproduce the phenotype of 3D. Although the effects of simultaneous lack of TLR7 and 9 seem to be additives, this is not enough to explain the behavior of 3D mice.

We therefore asked whether the simultaneous absence of TLR7, 9, 13 could justify the phenotype of 3D mice during *S. pneumoniae* infection. For this reason, we have thought to perform the same experiments described above on mice KO for TLR7, 9 and 13 generated by ourselves (see "Materials and Methods"). C57BL/6J, TLR7/9/13^{-/-} and 3D mice were always intranasally infected with a dose of 7.5×10^7 CFU of *S. pneumoniae*

and lethality was observed every 12 h for 10 days (Figure 18). The results of the two experiments were then put together and were the following:

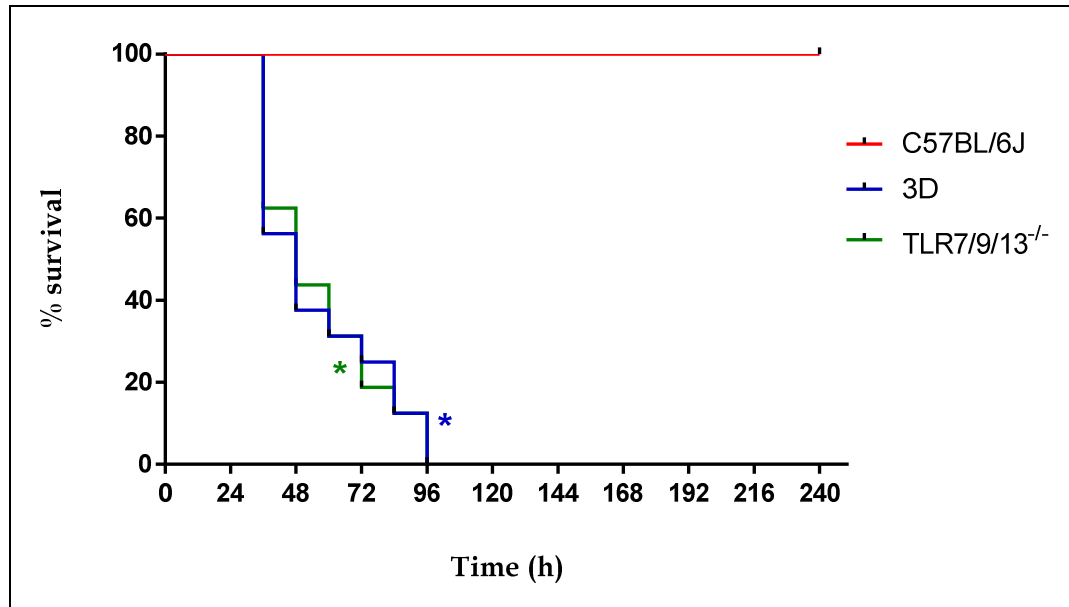


Fig. 18: Lethality of C57BL/6J, TLR7/9/13^{-/-} and 3D mice. Mice were infected intranasally with 7.5×10^7 CFU of *S. pneumoniae* strain D39. Mice were observed twice a day for 10 days.
*, $P < 0.05$ versus WT mice as determined with Kaplan-Meier survival plots.

This experiment was also followed by a further study of the number of CFUs in the blood, lungs and brain at 24 and 48 h following intranasal infection by *S. pneumoniae* (Figure 19, 20 and 21). The data obtained confirmed the results of the lethality experiments.

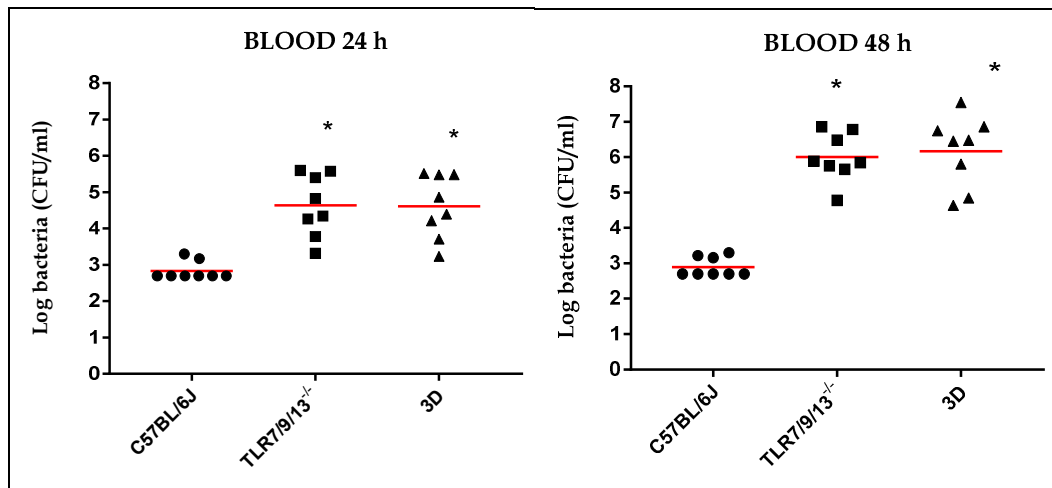


Fig. 19: CFU determination in blood of C57BL/6J, TLR7/9/13^{-/-} and 3D mice 24 and 48 hours after intranasal infection (7.5×10^7 CFU) with *S. pneumoniae* strain D39.
*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

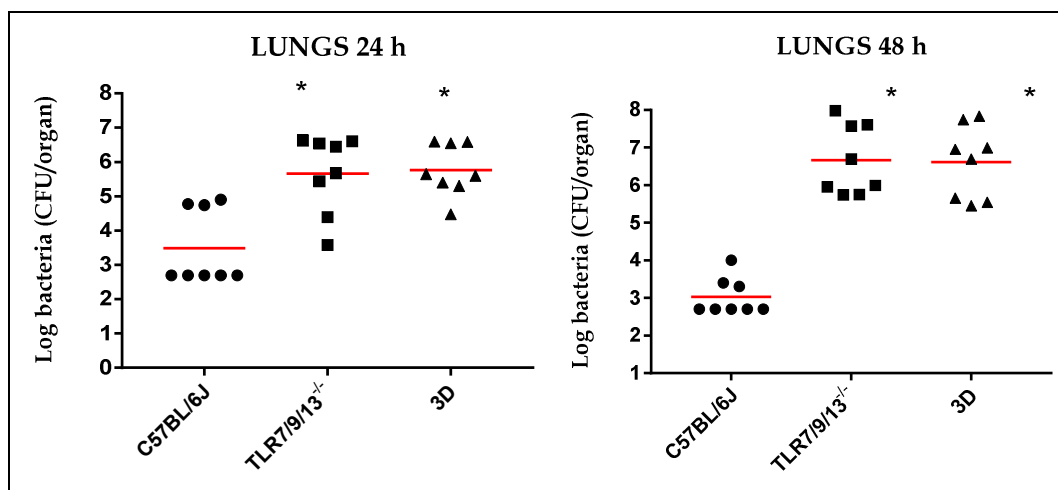


Fig. 20: CFU determination in lungs of C57BL/6J, TLR7/9/13^{-/-} and 3D mice 24 and 48 hours after intranasal infection (7.5×10^7 CFU) with *S. pneumoniae* strain D39.
*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

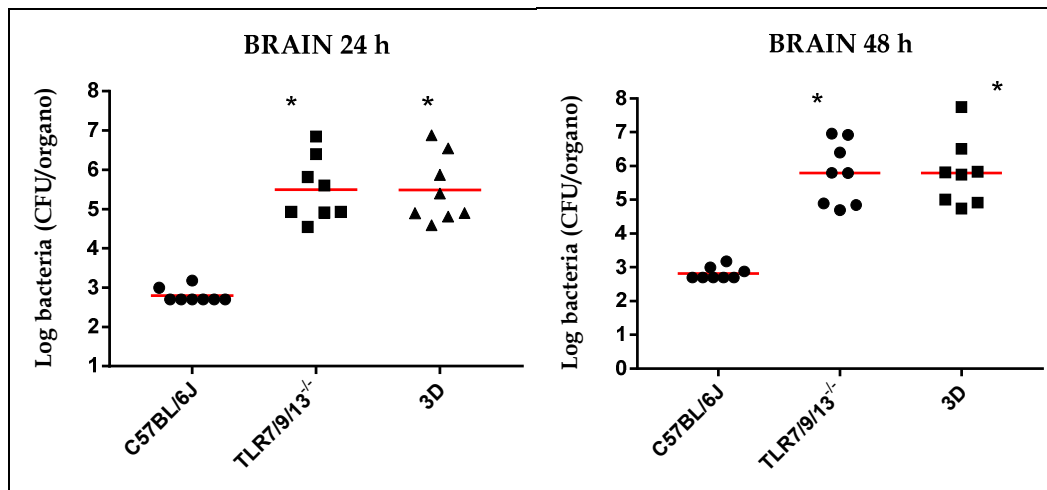


Fig. 21: CFU determination in brain of C57BL/6J, TLR7/9/13^{-/-} and 3D mice 24 and 48 hours after intranasal infection (7.5×10^7 CFU) with *S. pneumoniae* strain D39.

*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

As shown in the figures, the bacterial counts found in the blood and in the organs at 24 and 48 h of TLR7/9/13^{-/-} and 3D mice were similar. Therefore, our results demonstrated that in *S. pneumoniae* infections, the simultaneous absence of TLR7, 9 and 13 has the same effect as the absence of the protein UNC93B1.

6.3 Reduced cytokine/chemokine levels in 3D mice.

At this point, we wondered what the immune mechanisms underlying this involvement of endosomal TLRs in *S. pneumoniae* infections and which was the cause of such severe phenotype in 3D mice. To this end, we have measured in lungs (the first site of infection in our model) of 3D mice, the

levels of the most important cytokines and chemokines involved in the inflammatory process.

The mice were anesthetized intraperitoneally and infected intranasally with a dose of 7.5×10^7 CFU of *S. pneumoniae*. At 3, 24 and 48 hours after infection, lungs were taken and MIP-2, KC, IL-1 β and TNF- α were respectively dosed with appropriate ELISA kits (Figure 22 and 23).

MIP-2 and KC are two chemokines mainly involved in the recruitment of neutrophils. They are capable of inducing the degranulation of polymorphonucleated leukocytes with release of lysozyme and β -glucuronidase. IL-1 β and TNF- α belong to the category of primary cytokines. They are the first to be produced and released at the infection site and have the task of inducing the production and release of secondary cytokines.

The results obtained in these experiments were as follows:

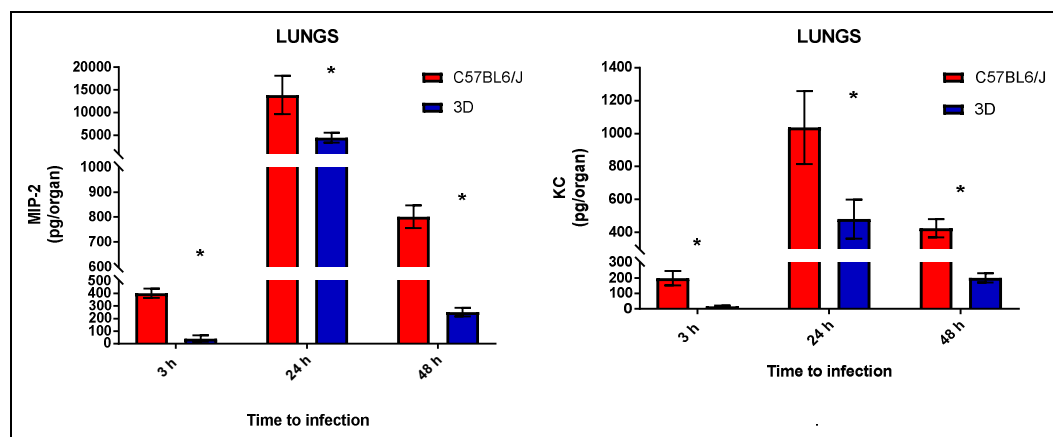


Fig. 22: Levels of MIP-2 and KC in C57BL/6J and 3D mouse lungs at different time after intranasal infection with *S. pneumoniae* (7.5×10^7 CFU).

*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

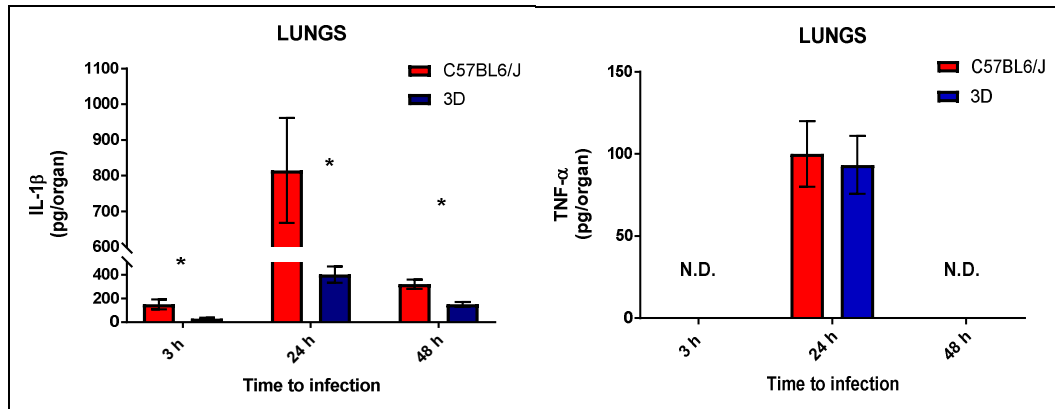


Fig. 23: Levels of IL-1 β and TNF- α in C57BL/6J and 3D mouse lungs at different time after intranasal infection with *S. pneumoniae* (7.5×10^7 CFU).
*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

As it has been previously demonstrated by *Mancuso et al.* that IFN- β plays an important role in defense against extracellular and not only viral pathogens¹⁸⁷, we wanted to see if the severe phenotype of 3D mice could possibly also depend on a deficiency of this cytokine. For this, we measured IFN- β mRNA production with quantitative Real Time PCR in lungs of 3D and C57BL/6J mice at different time after pneumococcal infection (1, 3 and 24 h) (Figure 24).

The data obtained were as follows:

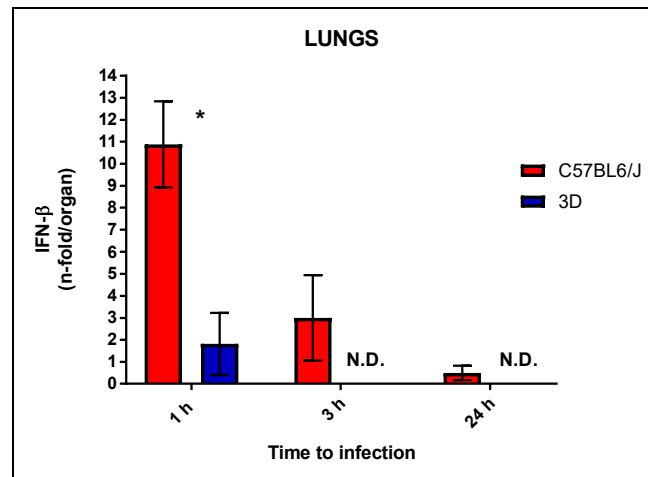


Fig. 24: Levels of IFN- β in C57BL/6J and 3D mouse lungs at different time after intranasal infection with *S. pneumoniae* (7.5×10^7 CFU).
*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

Subsequently, we considered it appropriate to dosage MIP-2 and KC after infection with *S. pneumoniae* also in the brain. Levels of MIP-2 and KC were measured at 24 and 48 h after infection, in order to give streptococci time to reach and colonize the brain (Figure 25).

The results obtained were as follows:

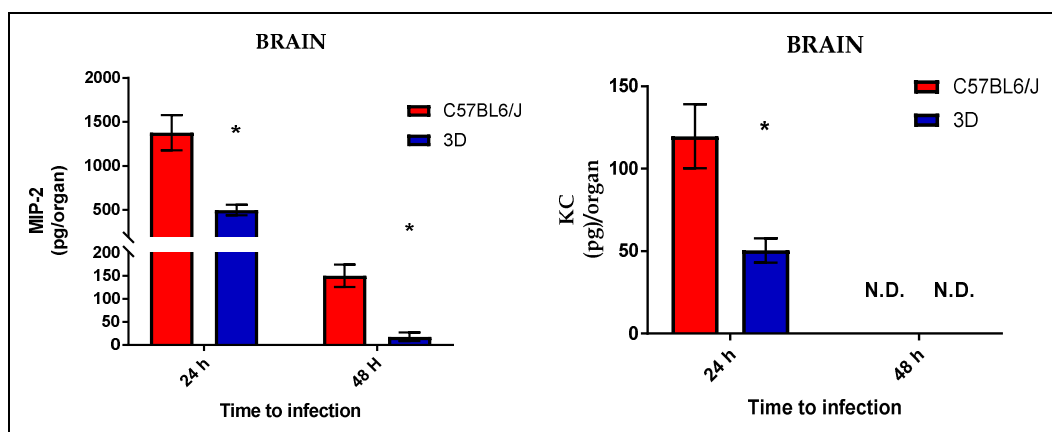


Fig. 25: Levels of MIP-2 and KC in C57BL/6J and 3D mouse brain at different time after intranasal infection with *S. pneumoniae* (7.5×10^7 CFU).
*, $P < 0.05$ versus WT mice by two-way analysis of variance and the Bonferroni test.

The results obtained suggests that IFN- β play a very important role in the early stages of infections. In the later stages of the infection, other cytokines, in particular MIP-2 (significantly less in 3D mice) become important in the defense against pneumococci.

6.4 Differential effects of endosomal TLR in resident vs elicited MØ

We finally tried to identify the cellular populations involved in the production of these chemokines. To this end, we conducted in vitro experiments on the innate immunity cells most involved in host defenses against infections. Hence, we isolated bone marrow cells from WT and 3D mice, which were then differentiated in vitro (via GM-CSF and M-CSF growth factors), respectively, in dendritic and macrophage cells and neutrophils. The cells were stimulated with a MOI 10 of *S. pneumoniae*. After 24 hours, the cell supernatants were harvested for the chemokine assay with ELISA assays (Figure 26).

The results obtained were as follows:

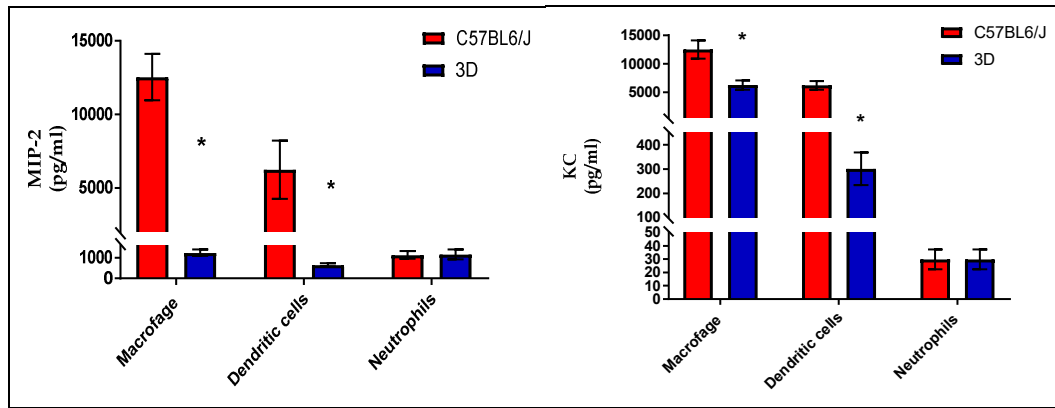


Fig. 26: Levels of MIP-2 and KC in C57BL/6J and 3D mouse in different cellular population infected with *S. pneumoniae* (MOI10) for 24 hours.
*, P < 0.05 versus WT mice by two-way analysis of variance and the Bonferroni test.

As shown in the figure, dendritic cells derived from the bone marrow of 3D mice produce amounts of MIP-2 and KC significantly lower than dendritic cells derived from the C57BL/6J mice bone marrow. Same thing, but less obvious, happens to macrophages. In the neutrophils, there was no significant difference in the production of MIP-2 and KC between cells derived from 3D mice and cells derived from C57BL/6J mice.

We also wanted to measure the two chemokines after stimulation with the same method not only in macrophages derived from the marrow, but also in the resident and elicited ones (Figure 27).

The results obtained were as follows:

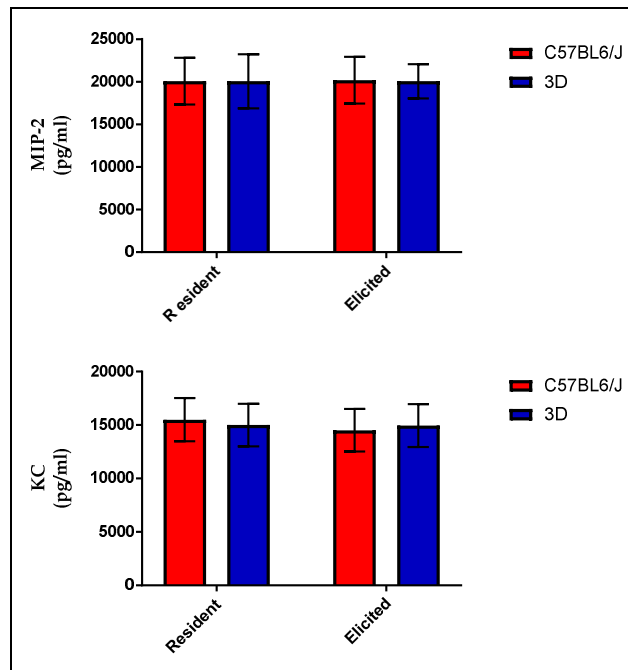


Fig. 27: Levels of shown cytokines in C57BL/6J and 3D mouse in resident and elicited peritoneal macrophages infected with *S. pneumoniae* (MOI10) for 24 hours.

As can be seen, there are no significant differences in the production of these two chemokines between resident and elicited macrophages of 3D mice and mice C57BL/6J.

7. DISCUSSION

In this study we wanted to evaluate the role of TLR endosomes in a pneumococcal pneumonia model. There were already present some data in the literature suggesting the possible involvement of these TLRs in *S. pneumoniae* infections. It is known that the released pneumococcal RNA activates TLR3 and TRIF¹⁷⁷ such as the Poly IC:LC, a potent agonist of TLR3, significantly increases the survival of mice infected with *S. pneumoniae*¹⁷⁸ and that TLR3 mRNA expression levels have increased in murine neuronal cells during infection with *S. pneumoniae*¹⁷⁹. Other studies have demonstrated that *S. pneumoniae* meningitis has led to a greater expression of TLR9 mRNA¹⁷⁹ and that the TLR9 receptor is not involved in the control of nasopharyngeal colonization, but is critical to the lower respiratory tract bacterial clearance. It has also been seen that TLR9¹⁶¹ was responsible for cardiomy inflammation during infection by *S. Pneumoniae*¹⁶¹.

The role of TLR13, has been studied in mouse coinfection models (*human metapneumovirus* + *S. pneumoniae* and *Influenza A Virus* + *S. pneumoniae*)¹⁸³ and it has been shown that in murine macrophages and DCs, the TLR13 recognize bacterial RNA and in particular a conserved region within bacterial 23S rRNA¹²⁹.

As you can see, the data in the literature are therefore only partial and very often applied to different types of infection. The originality of our work was to investigate the involvement of these receptors and to activate immune mechanisms in a pattern of intranasal infection with consequent development of pneumonia, the typical model of colonization of this microorganism in daily life. An infection mechanism that today brings hundreds of thousands of deaths around the world today.

With our study we have initially demonstrated that in infections by *S. pneumoniae*, MyD88 is activated not only by the signal transduction cascade from IL-1R, but also by the TLR, and in particular by TLRs endosomal. This was a first demonstration of a hypothetical role played by these TLRs in this model of infection.

At this point, we wanted to analyze TLR7, TLR9 and TLR13 singly in this model and we showed that all three are involved in this type of infection because knockout mice for each of these TLRs died early on C57BL/6J mice. In particular, we have shown that the simultaneous absence of all three receptors resulted in a particularly severe phenotype in mice infected with *S. pneumoniae*; phenotype similar to that of 3D mice, knockout mice for the UN93B1 protein that allows the translocation of endosomal TLRs on the endoplasmic reticulum. From the colony counts in the various organs, we also found that, compared to the C57BL/6J, in

knockout mice for endosomal TLRs, *S. pneumoniae* is able to better escape immune defenses, enter the bloodstream and colonize the brain within the 48 h. This leads us to think that the premature death of these mice is due to the development of encephalitis/meningitis. We therefore asked what the immune mechanisms underlying this involvement of TLR in lung infection by *S. pneumoniae*. Therefore, we wanted to measure the major proinflammatory cytokines and chemokines (MIP-2, KC, IL-1 β , TNF- α , IFN- β) in lungs and brain at 3, 24 and 48 h from infection in 3D and C57BL/6J mice. From the data obtained from the assays, we found that the absence of endosomal TLR seems to cause both the lungs and the brain to produce less production of MIP-2 and KC especially at 24 and 48 hours after infection. This leads us to believe that the signal transduction cascade from the activation of endosomal TLRs is essential for the transcriptional activation of CXCL-1 and CXCL-2 genes. We also observed lower IFN- β production in 3D mice versus C57BL/6J, specially in the early stages of infection. The particularly severe phenotype of 3D mice may therefore be due to a deficit in the immune response caused by the decreased production of these cytokines. No significant difference was found for the other dosed cytokines. To understand which of these cell populations was specifically responsible for lower MIP-2 and KC production in 3D mice we then conducted in vitro experiments on innate immunity cells. We have shown that in particular are the macrophages and dendritic cells derived

from the marrow to produce significantly smaller amounts of MIP-2 and KC in 3D mice than the C57BL/6J. Therefore, it appears that the endosomal receptors present in these cellular populations are more likely to be involved and play a more important role in *S. pneumoniae* infection.

With our study we have been able to explain in detail the role of endosomal TLRs in lung infections by *S. pneumoniae* and how they function to defend an organism from the attack of this bacterium. The endosomal TLRs we have studied are also present in humans, so we can say that the results obtained may have important implications in the human clinical field. Only TLR13 is not present in humans. However, it is important to know the molecular mechanisms that regulate it during infections as recent studies have seen in TLR8 the human homologue of TLR13^{136, 137, 138}.

Considering how much this organism represents a clinical and hospital emergency for humanity, our data can be used to better understand the immune mechanisms that trigger during infection with *S. pneumoniae* and to search for new pharmaceutical targets aimed at the treatment of pathologies caused by this microorganism (for example, endosomal TLRs agonists).

8. BIBLIOGRAPHY

- 1) Bruce A. Beutler. *Review Article TLRs and innate immunity*. Blood. 2009 Feb 12; 113(7): 1399–1407. Prepublished online 2008 Aug 29. doi: 10.1182/blood-2008-07-019307 PMID: PMC2644070. [✉](#)
- 2) Kiyoshi Takeda Shizuo Akira *Toll-like receptors in innate immunity*. Int Immunol (2005) 17 (1): 1-14. 01 January 2005
- 3) Shizuo Akira, Satoshi Uematsu, and Osamu Takeuchi, Yamada-oka, Suita. *Pathogen Recognition and Innate Immunity*. DOI 10.1016/j.cell.2006.02.015
- 4) Costa A., Gupta R., Signorino G., Malara A., Cardile F., Biondo C., Midiri A., Galbo R., Trieu-Cuot P., Papasergi S., Teti G., Henneke P., Mancuso G., Golenbock Dt., Beninati C. *Activation Of The Nlrp3 Inflammasome By Group B Streptococci*. J Immunol 2012; 188: 1953-1960.
- 5) Barbalat R., Ewald Se, Mouchess MI, Barton Gm. *Nucleic acid recognition by the innate immune system*. Annu Rev Immunol 2011; 29: 185-214.
- 6) Sharma S., Fitzgerald Ka. *Innate immune sensing of DNA*. PLoS Pathog 2011; 7: e1001310.
- 7) Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.-M. and Hoffmann, J. A. 1996. *The dorsoventral regulatory gene cassette spa^{tz}le/Toll/cactus controls the potent antifungal response in Drosophila adults*. Cell 86:973.
- 8) Medzhitov, R., Preston-Hurlburt, P. and Janeway, C. A. Jr. 1997. *A human homologue of the Drosophila Toll protein signals activation of adaptive immunity*. Nature 388:394.
- 9) Bell JK, Mullen GE, Leifer CA, Mazzoni A, Davies DR, Segal DM. *Leucine-rich repeats and pathogen recognition in Toll-like receptors*. Trends Immunol. 2003; 24:528–533. [PubMed: 14552836]
- 10) O'Neill LA, Bowie AG. *The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling*. Nat. Rev. Immunol. 2007; 7:353–364. [PubMed: 17457343]
- 11) Burch-Smith TM, Nesh-Kumar SP. *The functions of plant TIR domains*. Sci. STKE. 2007; 2007:e46.
- 12) Nyman T, Stenmark P, Flodin S, Johansson I, Hammarstrom M, Nordlund P. *The crystal structure of the human toll-like receptor 10 cytoplasmic domain reveals a putative signaling dimer*. J Biol Chem 2008;283:11861–11865.
- 13) Xu YW, Tao X, Shen BH, Horng T, Medzhitov R, Manley JL, Tong L. *Structural basis for signal transduction by the Toll/interleukin-1 receptor domains*. Nature 2000;408:111–115.
- 14) Underhill DM, Ozinsky A, Hajjar AM, Stevens A, Wilson CB, Bassetti M, Aderem A. *The toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens*. Nature 1999;401:811–815.
- 15) Tao X, Xu YW, Zheng Y, Beg AA, Tong L. *An extensively associated dimer in the structure of the C713S mutant of the TIR domain of human TLR2*. Biochem Biophys Res Commun 2002;299:216–221.
- 16) Takahashi K, Shibata T, Akashi-Takamura S, Kiyokawa T, Wakabayashi Y, Tanimura N, et al. *A protein associated with toll-like receptor (TLR) 4 (PRAT4A) is required for TLR-dependent immune responses*. JExpMed (2007)204:2963–76. doi:10.1084/jem.20071132.
- 17) Tabeta K, et al. *The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9*. Nature Immunology. 2006; 7(2):156–164. [PubMed: 16415873]
- 18) Kim YM, et al. *UNC93B1 delivers nucleotide-sensing toll-like receptors to endolysosomes*. Nature. 2008; 452(7184):234–238. [PubMed: 18305481].
- 19) Lee BL, et al. *UNC93B1 mediates differential trafficking of endosomal TLRs*. eLife. 2013;2.
- 20) Brinkmann MM, et al. *The interaction between the ER membrane protein UNC93B and TLR3, 7, and 9 is crucial for TLR signaling*. The Journal of Cell Biology. 2007; 177(2):265–275. [PubMed: 17452530].
- 21) Kim J, et al. *Acidic Amino Acid Residues in the Juxtamembrane Region of the Nucleotide-Sensing TLRs Are Important for UNC93B1 Binding and Signaling*. Journal of immunology. 2013; 190(10):5287–95.
- 22) Nishiya T, et al. *TLR3 and TLR7 are targeted to the same intracellular compartments by distinct regulatory elements*. The Journal of biological chemistry. 2005; 280(44):37107–37117. [PubMed: 16105838].
- 23) Funami K, et al. *The cytoplasmic 'linker region' in Toll-like receptor 3 controls receptor localization and signaling*. International Immunology. 2004; 16(8):1143–54. [PubMed: 15226270].
- 24) Kajita E, Nishiya T, Miwa S. *The transmembrane domain directs TLR9 to intracellular compartments that contain TLR3*. Biochemical and Biophysical Research Communications. 2006; 343(2):578–584. [PubMed: 16554027].
- 25) Nishiya T, DeFranco AL. *Ligand-regulated chimeric receptor approach reveals distinctive subcellular localization and signaling properties of the Toll-like receptors*. The Journal of biological chemistry. 2004; 279(18):19008–17. [PubMed: 14976215].

- 26) Leifer CA, et al. *Cytoplasmic targeting motifs control localization of toll-like receptor 9. The Journal of biological chemistry.* 2006; 281(46):35585–35592. [PubMed: 16990271].
- 27) Yang Y, et al. *Heat Shock Protein gp96 Is a Master Chaperone for Toll-like Receptors and Is Important in the Innate Function of Macrophages.* Immunity. 2007; 26(2):215–226. [PubMed: 17275357].
- 28) Liu B, et al. *Folding of Toll-like receptors by the HSP90 paralogue gp96 requires a substratespecific cochaperone.* Nature Communications. 2010; 1(6):1–10.
- 29) Ewald SE, et al. *The ectodomain of Toll-like receptor 9 is cleaved to generate a functional receptor.* Nature. 2008; 456(7222):658–662. [PubMed: 18820679].
- 30) Ewald SE, et al. *Nucleic acid recognition by Toll-like receptors is coupled to stepwise processing by cathepsins and asparagine endopeptidase.* Journal of Experimental Medicine. 2011; 208(4): 643–651. [PubMed: 21402738].
- 31) Chockalingam A, et al. *TLR9 traffics through the Golgi complex to localize to endolysosomes and respond to CpG DNA.* Immunology and cell biology. 2009; 87(3):209–17. [PubMed: 19079358].
- 32) Chiang, C-y, et al. *Cofactors Required for TLR7- and TLR9-Dependent Innate Immune Responses.* Cell Host and Microbe. 2012; 11(3):306–318. [PubMed: 22423970].
- 33) Fukui R, et al. *Unc93B1 biases Toll-like receptor responses to nucleic acid in dendritic cells toward DNA- but against RNA-sensing.* Journal of Experimental Medicine. 2009; 206(6):1339– 1350. [PubMed: 19451267].
- 34) Gao D¹, Li W1. *Structures and recognition modes of toll-like receptors.* Proteins. 2017 Jan;85(1):3-9. doi: 10.1002/prot.25179. Epub 2016 Oct 24.
- 35) O'Neill LA. *The interleukin-1 receptor/Toll-like receptor superfamily: 10 years of progress.* Immunol Rev. 2008; 226:10–18. [PubMed: 19161412].
- 36) Dinarello CA. *Interleukin-1, interleukin-1 receptors and interleukin-1 receptor antagonist.* Int Rev Immunol. 1998; 16:457–99. [PubMed: 9646173]
- 37) Kawai T, et al. *Unresponsiveness of MyD88-deficient mice to endotoxin.* Immunity. 1999; 11:115–122. [PubMed: 10435584].
- 38) Dunne A, O'Neill LA. *The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense.* Sci STKE. 2003; 2003:re3. [PubMed: 12606705]
- 39) Bowie A, O'Neill LA. *The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products.* J Leukoc Biol. 2000; 67:508–514. [PubMed: 10770283].
- 40) Lin SC, Lo YC, Wu H. *Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling.* Nature (2010) 465:885–90. doi:10.1038/nature09121.
- 41) KollweC, MackensenAC, NeumannD, KnopJ, CaoP, LiS ,et al. *Sequential autophosphorylation steps in the interleukin-1receptor-associated kinase-1regulate its availability as an adapter in interleukin-1 signaling.* J BiolChem (2004) 279:5227–36.doi:10.1074/jbc.M309251200
- 42) Jiang Z, Ninomiya-Tsuji J, Qian Y, Matsumoto K, Li X. *Interleukin-1 (IL1) receptor-associated kinase-dependent IL-1-induced signaling complexes phosphorylate TAK1 and TAB2 at the plasma membrane and activate TAK1 in the cytosol.* Mol Cell Biol (2002) 22:7158–67. doi:10.1128/MCB.22.20.71587167.2002
- 43) Chen ZJ. *Ubiquitination in signaling to and activation of IKK.* Immunol Rev (2012) 246:95–106. doi:10.1111/j.1600-065X.2012.01108.x
- 44) Ajibade AA, Wang HY, Wang RF. *Cell type-specific function of TAK1 in innate immune signaling.* Trends Immunol (2013) 34:307–16. doi:10.1016/j.it. 2013.03.07
- 45) Kawai T, Akira S. *The role of pattern-recognition receptors in innate immunity: update on toll-like receptors.* NatImmunol (2010)11:373–84.doi:10.1038/ ni.1863
- 46) Kawasaki T, Takemura N, Standley DM, Akira S, Kawai T. *The second messenger phosphatidylinositol-5-phosphate facilitates antiviral innate immune signaling.* Cell Host Microbe (2013) 14:148–58. doi:10.1016/j.chom.2013.07.011
- 47) Honda K, et al. *Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction.* Nature. 2005; 434(7036):1035–1040. [PubMed: 15815647].
- 48) Kerkmann M, et al. *Activation with CpG-A and CpG-B oligonucleotides reveals two distinct regulatory pathways of type I IFN synthesis in human plasmacytoid dendritic cells.* Journal of immunology. 2003; 170(9):4465–74. 92.
- 49) Guiducci C. *Properties regulating the nature of the plasmacytoid dendritic cell response to Toll-like receptor 9 activation.* Journal of Experimental Medicine. 2006; 203(8):1999–2008. [PubMed: 16864658]
- 50) Vollmer J, et al. *Characterization of three CpG oligodeoxynucleotide classes with distinct immunostimulatory activities.* European Journal of Immunology. 2004; 34(1):251–262. [PubMed: 14971051]


- 51) Sasai M, Linehan MM, Iwasaki A. *Bifurcation of Toll-Like Receptor 9 Signaling by Adaptor Protein 3*. Science. 2010; 329(5998):1530–1534. [PubMed: 20847273].
- 52) Henault J, et al. *Noncanonical autophagy is required for type I interferon secretion in response to DNA-immune complexes*. Immunity. 2012; 37(6):986–97. [PubMed: 23219390].
- 53) L. Alexopoulou, A.C. Holt, R. Medzhitov, R.A. Flavell, *Recognition of double-stranded RNA and activation of NF-kappaB by toll-like receptor 3*, Nature 413 (6857) (2001) 732–738.
- 54) M. Lafon, F. Megret, S.G. Meuth, O. Simon, M.L. Velandia Romero, M. Lafage, et al., *Detrimental contribution of the immuno-inhibitor B7-H1 to rabies virus encephalitis*, J. Immunol. 180 (11) (2008) 7506–7515.
- 55) M. Bsibsi, R. Ravid, D. Gveric, J.M. van Noort, *Broad expression of toll-like receptors in the human central nervous system*, J. Neuropathol. Exp. Neurol. 61 (11) (2002) 1013–1021.
- 56) J.K. Bell, I. Botos, P.R. Hall, J. Askins, J. Shiloach, D.M. Segal, et al., *The molecular structure of the toll-like receptor 3 ligand-binding domain*, Proc. Natl. Acad. Sci. U. S. A. 102 (31) (2005) 10976–10980.
- 57) S. Biyani, R.K. Garg, A. Jain, H.S. Malhotra, R. Kumar, S. Prakash, et al., *Toll-like receptor- 3 gene polymorphism in patients with Japanese encephalitis*, J. Neuroimmunol. 286 (2015) 71–76.
- 58) Y. Guo, M. Audry, M. Ciancanelli, L. Alsina, J. Azevedo, M. Herman, et al., *Herpes simplex virus encephalitis in a patient with complete TLR3 deficiency: TLR3 is otherwise redundant in protective immunity*, J. Exp. Med. 208 (10) (2011) 2083–2098.
- 59) L.S. Reinert, L. Harder, C.K. Holm, M.B. Iversen, K.A. Horan, F. Dagnaes-Hansen, et al., *TLR3 deficiency renders astrocytes permissive to herpes simplex virus infection and facilitates establishment of CNS infection in mice*, J. Clin. Invest. 122 (4) (2012) 1368–1376.
- 60) O. Willmann, P. Ahmad-Nejad, M. Neumaier, M.G. Hennerici, M. Fatar, *Toll-like receptor 3 immune deficiency may be causative for HSV-2-associated mollaret meningitis*, Eur. Neurol. 63 (4) (2010) 249–251.
- 61) R.L. DeBiasi, K.L. Tyler, *West Nile virus meningoencephalitis*, Nat. Clin. Pract. Neurol. 2 (5) (2006) 264–275.
- 62) S. Daffis, M.A. Samuel, M.S. Suthar, M. Gale Jr., M.S. Diamond, *Toll-like receptor 3 has a protective role against West Nile virus infection*, J. Virol. 82 (21) (2008) 10349–10358.
- 63) K.F. Kong, K. Delroux, X. Wang, F. Qian, A. Arjona, S.E. Malawista, et al., *Dysregulation of TLR3 impairs the innate immune response to West Nile virus in the elderly*, J. Virol. 82 (15) (2008) 7613–7623.
- 64) J.S. Mackenzie, D.J. Gubler, L.R. Petersen, *Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses*, Nat. Med. 10 (12 Suppl) (2004) S98–109.
- 65) U.K. Misra, J. Kalita, U.K. Syam, T.N. Dhole, *Neurological manifestations of dengue virus infection*, J. Neurol. Sci. 244 (1–2) (2006) 117–122.
- 66) S. Torres, J.C. Hernandez, D. Giraldo, M. Arboleda, M. Rojas, J.M. Smit, et al., *Differential expression of toll-like receptors in dendritic cells of patients with dengue during early and late acute phases of the disease*, PLoS Negl. Trop. Dis. 7 (2) (2013), e2060.
- 67) L. Pang, X. Gong, N. Liu, G. Xie, W. Gao, G. Kong, et al., *A polymorphism in melanoma differentiation-associated gene 5 may be a risk factor for enterovirus 71 infection*, Clinical microbiology and infection: the official publication of the European Society of Clinical Microbiology and Infectious Diseases 20 (10) (2014) O711–O717.
- 68) F. Hidaka, S. Matsuo, T. Muta, K. Takeshige, T. Mizukami, H. Nunoi, *A missense mutation of the toll-like receptor 3 gene in a patient with influenza-associated encephalopathy*, Clin. Immunol. 119 (2) (2006) 188–194.
- 69) M. Sironi, M. Biasin, R. Cagliani, D. Forni, M. De Luca, I. Saulle, S. Lo Caputo, F. Mazzotta, J. Macías, J.A. Pineda, A. Caruz, M. Clerici, *A common polymorphism in TLR3 confers natural resistance to HIV-1 infection*, J. Immunol. 15 (188(2)) (2012) 818–823.
- 70) Y. Ishizaki, M. Takemoto, R. Kira, K. Kusuhara, H. Torisu, Y. Sakai, et al., *Association of toll-like receptor 3 gene polymorphism with subacute sclerosing panencephalitis*, J. Neurovirol. 14 (6) (2008) 486–491.
- 71) S. Medhi, M. Deka, P. Deka, S.S. Swargiary, R.K. Hazam, M.P. Sharma, et al., *Promoter region polymorphism & expression profile of toll like receptor-3 (TLR-3) gene in chronic hepatitis C virus (HCV) patients from India*, Indian J. Med. Res. 134 (2011) 200–207.
- 72) Gantier MP, Tong S, Behlke MA, Xu D, Phipps S, Foster PS et al. *TLR7 is involved in sequence-specific sensing of single-stranded RNAs in human macrophages*. J Immunol 2008; 180: 2117–2124.
- 73) Blasius AL, Beutler B. *Intracellular toll-like receptors*. Immunity 2010; 32:305–315
- 74) Gringhuis SI, van der Vlist M, van den Berg LM, den Dunnen J, Litjens M, Geijtenbeek TB. *HIV-1 exploits innate signaling by TLR8 and DC-SIGN for productive infection of dendritic cells*. Nat Immunol 2010; 11: 419–426
- 75) Alexopoulou L, Desnues B, Demaria O. *Toll-like receptor 8: the awkward TLR*. Med Sci (Paris) 2012; 28: 96–102.

- 76) Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T et al. *Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides*. J Immunol 2002; 168: 4531–4537
- 77) Ito T, Amakawa R, Kaisho T, Hemmi H, Tajima K, Uehira K, Ozaki Y, Tomizawa H, Akira S, Fukuhara S: *Interferon-alpha and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets*. J Exp Med 2002; 195: 1507–1512.
- 78) Applequist SE, Wallin RP, Ljunggren HG: *Variable expression of Toll-like receptor in murine innate and adaptive immune cell lines*. Int Immunol 2002; 14: 1065–1074.
- 79) Nagase H, Okugawa S, Ota Y, Yamaguchi M, Tomizawa H, Matsushima K, Ohta K, Yamamoto K, Hirai K: *Expression and function of Toll-like receptors in eosinophils: activation by Toll-like receptor 7 ligand*. J Immunol 2003; 171: 3977–3982.
- 80) Hart OM, Athie-Morales V, O'Connor GM, Gardiner CM: *TLR7/8-mediated activation of human NK cells results in accessory cell-dependent IFN-gamma production*. J Immunol 2005; 175: 1636–1642.
- 81) Song Y, Zhuang Y, Zhai S, Huang D, Zhang Y, Kang W, Li X, Liu Q, Yu Q, Sun Y: *Increased expression of TLR7 in CD8(+) T cells leads to TLR7-mediated activation and accessory cell-dependent IFN-gamma production in HIV type 1 infection*. AIDS Res Hum Retroviruses 2009; 25: 1287–1295.
- 82) Dominguez-Villar M, Gautron AS, de Marcken M, Keller MJ, Hafler DA: *TLR7 induces anergy in human CD4(+) T cells*. Nat Immunol 2015; 16: 118–128.
- 83) Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S: *Species-specific recognition of single-stranded RNA via Toll-like receptor 7 and 8*. Science 2004; 303: 1526–1529.
- 84) Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C: *Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA*. Science 2004; 303: 1529–1531
- 85) Hornung V, Guenther-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, Noronha A, Manoharan M, Akira S, de Fougères A, Endres S, Hartmann G: *Sequence-specific potent induction of IFN α by short interfering RNA in plasmacytoid dendritic cells through TLR7*. Nat Med 2005; 11: 263–270.
- 86) Park CK, Xu ZZ, Berta T, Han Q, Chen G, Liu XJ, Ji RR: *Extracellular microRNAs activate nociceptor neurons to elicit pain via TLR7 and TRPA1*. Neuron 2014; 82: 47–54.
- 87) Liu HY, Huang CM, Hung YF, Hsueh YP: *The microRNAs Let7c and miR21 are recognized by neuronal Toll-like receptor 7 to restrict dendritic growth of neurons*. Exp Neurol 2015; 269: 202–212.
- 88) Barton GM, Kagan JC, Medzhitov R: *Intracellular localization of Toll-like receptor 9 prevents recognition of self DNA but facilitates access to viral DNA*. Nat Immunol 2006; 7: 49–56.
- 89) Gantier MP, Tong S, Behlke MA, Irving AT, Lappas M, Nilsson UW, Latz E, McMillan NA, Williams BR: *Rational design of immunostimulatory siRNAs*. Mol Ther 2010; 18: 785–795.
- 90) Sioud M, Furset G, Cekaite L: *Suppression of immunostimulatory siRNA-driven innate immune activation by 2'-modified RNAs*. Biochem Biophys Res Commun 2007; 361: 122–126.
- 91) Kariko K, Buckstein M, Ni H, Weissman D: *Suppression of RNA recognition by Toll-like receptors: the impact of nucleoside modification and the evolutionary origin of RNA*. Immunity 2005; 23: 165–175.
- 92) Oh DY, Baumann K, Hamouda O et al. *A frequent functional Toll-like receptor 7 polymorphism is associated with accelerated HIV-1 disease progression*. AIDS 2009; 23:297–307
- 93) Oh DY, Taube S, Hamouda O et al. *A functional Toll-like receptor 8 variant is associated with HIV disease restriction*. J Infect Dis 2008; 198:701–9.
- 94) Davila S, Hibberd ML, Hari Dass R, Wong HE, Sahiratmadja E, Bonnard C et al. *Genetic association and expression studies indicate a role of Toll-like receptor 8 in pulmonary tuberculosis*. PLoS Genet 2008; 4: e1000218
- 95) Gantier M. P., Irving A. T., Kaparakis-Liaskos M., Xu D., Evans V. A., Cameron P. U., Bourne J. A., Ferrero R. L., John M., Behlke M. A., Williams B. R. (2010) *Genetic modulation of TLR8 response following bacterial phagocytosis*. Hum. Mutat. 31, 1069–1079
- 96) Cervantes JL, Dunham-Ems SM, la Vake CJ, Petzke MM, Sahay B, Sellati TJ et al. *Phagosomal signaling by Borrelia burgdorferi in human monocytes involves Toll-like receptor (TLR) 2 and TLR8 cooperativity and TLR8-mediated induction of IFN-beta*. Proc Natl Acad Sci USA 2011; 108: 3683–3688.
- 97) Jurk M, Heil F, Vollmer J, Schetter C, Krieg AM, Wagner H et al. *Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848*. Nat Immunol 2002; 3: 499.
- 98) Zhu J, Lai K, Brownile R, Babiuk LA, Mutwiri GK. *Porcine TLR8 and TLR7 are both activated by a selective TLR7 ligand, imiquimod*. Mol Immunol 2008; 45: 3238–3243.
- 99) Forsbach A, Nemorin JG, Montino C, Muller C, Samulowicz U, Vicari AP et al. *Identification of RNA sequence motifs stimulating sequence-specific TLR8dependent immune responses*. J Immunol 2008; 180: 3729–3738.

- 100) Gorden KK, Qiu XX, Binsfeld CC, Vasilakos JP, Alkan SS. *Cutting edge: activation of murine TLR8 by a combination of imidazoquinoline immune response modifiers and polyT oligodeoxynucleotides*. J Immunol 2006; 177: 6584–6587.
- 101) Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. *A Toll-like receptor recognizes bacterial DNA*. Nature 2000;408(6813):740–5.
- 102) Collins L, Hajizadeh S, Holme E, Jonsson I, Tarkowski A. *Endogenously oxidized mitochondrial DNA induces in vivo and in vitro inflammatory responses*. J Leukoc Biol 2004;75:995–1000.
- 103) Zhang Q, Raoof M, Chen Y, Sumi Y, Sursal T, Junger W, et al. *Circulating mitochondrial DAMPs cause inflammatory responses to injury*. Nature 2010;464(7285):104–7.
- 104) Oka T, Hikoso S, Yamaguchi O, Taneike M, Takeda T, Tamai T. *Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure*. Nature 2012;485:251–5.
- 105) Yamada H, Gursel I, Takeshita F, Conover J, Ishii KJ, Gursel M, et al. *Effect of suppressive DNA on CpG-induced immune activation*. J Immunol 2002;169(10):5590–4.
- 106) Reynolds JM, Dong C. *Toll-like receptor regulation of effector T lymphocyte function*. Trend Immunol 2013;34(10):511–9.
- 107) Kanzler H, Barrat FJ, Hessel EM, Coffman RL. *Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists*. Nat Med 2007;13(May (5)):552–9.
- 108) Iwasaki A, Medzhitov R. *Toll-like receptor control of the adaptive immune responses*. Nat Immunol 2004;5(10):987–95.
- 109) Ahmad-Nejad P, Häcker H, Rutz M, Bauer S, Vabulas RM, Wagner H. *Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments*. Eur J Immunol 2002;32(7):1958–68.
- 110) Guiducci C, Coffman RL, Barrat FJ. *Signalling pathways leading to IFN- α production in human plasmacytoid dendritic cell and the possible use of agonists or antagonists of TLR7 and TLR9 in clinical indications*. J Intern Med 2009;265(1):43–57.
- 111) Holtick U, Scheulen ME, von Bergwelt-Baildon MS, Weihrauch MR. *Toll-like receptor 9 agonists as cancer therapeutics*. Expert Opin Invest Drugs 2011;20(3):361–72.
- 112) Eiró N, González L, González LO, Andicoechea A, Fernández-Díaz M, Altadill A, et al. *Study of the expression of toll-like receptors in different histological types of colorectal polyps and their relationship with colorectal cancer*. J Clin Immunol 2012;32(4):848–54.
- 113) Connolly DJ, O'Neill LAJ. *New developments in Toll-like receptor targeted therapeutics*. Curr Opin Pharmacol 2012;12(4):510–8.
- 114) Galluzzi L, Vacchelli E, Eggermont A, Fridman W, Galon J, Sautès-Fridman C. *Trial watch: experimental toll-like receptor agonists for cancer therapy*. Oncoimmunology 2012;1:699–716.
- 115) Krieg AM. *Development of TLR9 agonists for cancer therapy*. J Clin Invest 2007;117(5):1184–94.
- 116) Krieg AM. *Therapeutic potential of Toll-like receptor 9 activation*. Nat Rev Drug Discov 2006;5(6):471–84.
- 117) Liu Y, Luo X, Yang C, Yu S, Xu H. *Three CpG oligodeoxynucleotide classes differentially enhance antigen-specific humoral and cellular immune responses in mice*. Vaccine 2011;29(34):5778–84.
- 118) Kim Y, Girardi M, Duvic M, Kuzel T, Link B, Pinter-Brown L, et al. *Phase I trial of a Toll-like receptor 9 agonist, PF-3512676 (CPG 7909), in patients with treatment-refractory, cutaneous T-cell lymphoma*. J Am Acad Dermatol 2010;63:975–83.
- 119) Link BK, Ballas Z, Weisdorf D, Wooldridge J, Bossler A, Shannon M. *Oligodeoxynucleotide CpG 7909 delivered as intravenous infusion demonstrates immunologic modulation in patients with previously treated non-Hodgkin lymphoma*. J Immunother 2006;29:558–68.
- 120) Pashenkov M, Goess G, Wagner C, Hormann M, Jandl T, Moser A. *Phase II trial of a Toll-like receptor 9 – activating oligonucleotide in patients with metastatic melanoma*. J Clin Oncol 2006;24:5716–24.
- 121) Thompson J, Kuzel T, Drucker B, Urban W, Bukowski R. *Safety and efficacy of PF-3512676 for the treatment of stage IV renal cell carcinoma: an open-label, multicenter phase I/II study*. Clin Genitourin Cancer 2009;7:E58–65.
- 122) Zent C, Smith B, Ballas Z, Wooldridge J, Link B, Call T. *A phase I clinical trial of CpG oligonucleotide 7909 (PF-03512676) in patients with previously treated chronic lymphocytic leukemia*. Leuk Lymphoma 2012;53:211–7.
- 123) Yarovinsky F, Zhang D, Andersen JF, Bannenberg GL, Serhan CN, et al. (2005) *TLR11 activation of dendritic cells by a protozoan profilin-like protein*. Science 308: 1626–1629. PMID: 15860593
- 124) Mathur R, Oh H, Zhang D, Park SG, Seo J, et al. (2012) *A mouse model of Salmonella typhi infection*. Cell 151: 590–602. doi: 10.1016/j.cell.2012.08.042 PMID: 23101627

- 125) Yarovinsky F (2014) *Innate immunity to Toxoplasma gondii infection*. Nat Rev Immunol 14: 109–121. doi: 10.1038/nri3598 PMID: 24457485
- 126) Plattner F, Yarovinsky F, Romero S, Didry D, Carlier MF, et al. (2008) *Toxoplasma profilin is essential for host cell invasion and TLR11-dependent induction of an interleukin-12 response*. Cell Host Microbe 3: 77–87. doi: 10.1016/j.chom.2008.01.001 PMID: 18312842
- 127) Zhang D, Zhang G, Hayden MS, Greenblatt MB, Bussey C, et al. (2004) *A toll-like receptor that prevents infection by uropathogenic bacteria*. Science 303: 1522–1526. PMID: 15001781
- 128) Shi Z, Cai Z, Yu J, Zhang T, Zhao S, et al. (2012) *Toll-like receptor 11 (TLR11) prevents Salmonella penetration into the murine Peyer patches*. J Biol Chem 287: 43417–43423. doi: 10.1074/jbc.M112. 411009 PMID: 23135279
- 129) Oldenburg M, Kr uger A, Ferstl R, Kaufmann A, Nees G, Sigmund A, Bathke B, Lauterbach H, Suter M & Dreher S (2012) *TLR13 recognizes bacterial 23S rRNA devoid of erythromycin resistance-forming modification*. Science 337, 1111–1115.
- 130) Li X-D & Chen ZJ (2012) *Sequence specific detection of bacterial 23S ribosomal RNA by TLR13*. eLife 1, e00102.
- 131) STRUCTURAL SNAPSHOT. *Structure of the mouse Toll-like receptor 13 ectodomain in complex with a conserved sequence from bacterial 23S ribosomal RNA* Jiawei Wang^{1,2}, Jijie Chai^{2,3,4} and Hongwei Wang^{2,3,4}
- 132) D’Costa VM, King CE, Kalan L, Morar M, Sung WW, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD. 2011. *Antibiotic resistance is ancient*. Nature 477:457–461. <http://dx.doi.org/10.1038/nature10388>.
- 133) Hughes VM, Datta N. 1983. *Conjugative plasmids in bacteria of the ‘preantibiotic’ era*. Nature 302:725–726. <http://dx.doi.org/10.1038/302725a0>.
- 134) Li XD, Chen ZJ. 2012. *Sequence specific detection of bacterial 23S ribosomal RNA by TLR13*. eLife 1:e00102. <http://dx.doi.org/10.7554/eLife.00102>.
- 135) Signorino G, Mohammadi N, Patan  F, Buscetta M, Venza M, Venza I, Mancuso G, Midiri A, Alexopoulou L, Teti G, Biondo C, Beninati C. *Role of Toll-like receptor 13 in innate immune recognition of group B streptococci*. Infect Immun. 2014 Dec;82(12):5013–22. doi: 10.1128/IAI.02282–14. Epub 2014 Sep 15.
- 136) Kr ger A, Oldenburg M, Chebrolu C, Beisser D, Kolter J, Sigmund AM, Steinmann J, Sch fer S, Hochrein H, Rahmann S, Wagner H, Henneke P, Hornung V, Buer J, Kirschning CJ. *Human TLR8 senses UR/URR motifs in bacterial and mitochondrial RNA*. EMBO Rep. 2015 Dec;16(12):1656–63. doi: 10.15252/embr.201540861. Epub 2015 Nov 6.
- 137) Nishibayashi R, Inoue R, Harada Y, Watanabe T, Makioka Y, Ushida K. *RNA of Enterococcus faecalis Strain EC-12 Is a Major Component Inducing Interleukin-12 Production from Human Monocytic Cells*. PLoS One. 2015 Jun 17;10(6):e0129806. doi: 10.1371/journal.pone.0129806. eCollection 2015.
- 138) Eigenbrod T, Pelka K, Latz E, Kreikemeyer B, Dalpke AH. *TLR8 Senses Bacterial RNA in Human Monocytes and Plays a Nonredundant Role for Recognition of Streptococcus pyogenes*. J Immunol. 2015 Aug 1;195(3):1092–9. doi: 10.4049/jimmunol.1403173. Epub 2015 Jun 22.
- 139) Nunes T, Sa-Leao R. 2005. *Trends in drug resistance, serotypes, and molecular types of Streptococcus pneumoniae colonizing preschool-age children attending day care centers in Lisbon, Portugal: A summary of 4 years of annual surveillance*. J Clin Microbiol 43: 1285–1293.
- 140) Jansen AG, Rodenburg GD, van der Ende A, et al. *Invasive pneumococcal disease among adults: associations among serotypes, disease characteristics, and outcome*. Clin Infect Dis 2009;49:e23–9.
- 141) Reinert RR, Paradiso P, Fritzell B. *Advances in pneumococcal vaccines: the 13-valent pneumococcal conjugate vaccine received market authorization in Europe*. Expert Rev Vaccines 2010;9:229–36.
- 142) Johnson HL, Deloria-Knoll M, Levine OS, et al. *Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project*. PLoS Med 2010;7:e1000348.
- 143) Bagnoli F, Moschioni M, Donati C, Dimitrovska V, Ferlenghi I, Facciotti C, Muzzi A, Giusti F, Emolo C, Sinisi A, et al. 2008. *A second pilus type in Streptococcus pneumoniae is prevalent in emerging serotypes and mediates adhesion to host cells*. J Bacteriol 190: 5480–5492.
- 144) Barocchi M, Ries J, Zogaj X, Albiger B, Kanth A, Dahlberg S, Moschioni M, Massignani V, Hultenby K, von Euler A, et al. 2006. *A pneumococcal pilus influences virulence and host inflammatory responses*. Proc Natl Acad Sci 103: 2857–2862.
- 145) Tettelin H, Nelson KE, Paulsen IT, Eisen JA, Read TD, Peterson S, Heidelberg J, DeBoy RT, Haft DH, Dodson RJ, et al. 2001. *Complete genome sequence of a virulent isolate of Streptococcus pneumoniae*. Science 293: 498–506.

- 146) Hiller NLAA, Powell E, Martin DP, Eutsey R, Earl J, Janto B, Boissy RJ, Hogg J, Barbadora K, Sampath R, et al. 2010. *Generation of genic diversity among Streptococcus pneumoniae strains via horizontal gene transfer during a chronic polyclonal pediatric infection.* PLoS Pathog 6: e1001108.
- 147) Hammerschmidt S, Talay SR, Brandtzaeg P, Chhatwal GS. 1997. *SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component.* Mol Microbiol 25: 1113–1124.
- 148) Zhang JR, Mostov KE, Lamm ME, Nanno M, Shimida S, Ohwaki M, Tuomanen E. 2000. *The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells.* Cell 102: 827–837.
- 149) Braathen R, Sandvik A, Berntzen G, Hammerschmidt S, Fleckenstein B, Sandlie I, Brandtzaeg P, Johansen F, Lauvrak V. 2006. *Identification of a polymeric Ig receptor binding phage-displayed peptide that exploits epithelial transcytosis without dimeric IgA competition.* J Biol Chem 281: 7075–7081.
- 150) Mann B, Orihuela C, Antikainen J, Gao G, Sublett J, Korhonen TET. 2006. *Multifunctional role of choline binding The Pneumococcus Cite this article as Cold Spring Harb Perspect Med 2013;3:a010215 13 protein G in pneumococcal pathogenesis.* Infect Immun 74: 821–829.
- 151) Gosink KK, Mann ER, Guglielmo C, Tuomanen EI, Masure H. 2000. *Role of novel choline binding proteins in virulence of Streptococcus pneumoniae.* Infect Immun 68: 5690–5695.
- 152) Weiser JN, Bae D, Fasching C, Scamurra RW, Ratner AJ, Janoff EN. 2003. *Antibody-enhanced pneumococcal adherence requires IgA1 protease.* Proc Natl Acad Sci 100: 4215–4220.
- 153) Gould JM, Weiser JN. 2002. *The inhibitory effect of C-reactive protein on bacterial phosphorylcholine platelet activating factor receptor-mediated adherence is blocked by surfactant.* J Infect Dis 186: 361–371.
- 154) Thornton JA, Durick-Eder K, Tuomanen EI. 2010. *Pneumococcal pathogenesis: “Innate invasion” yet organ-specific damage.* J Mol Med (Berl) 88: 103–107.
- 155) Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D. 1999. *Cutting edge: Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2.* J Immunol 163: 1–5.
- 156) Weber JR, Freyer D, Alexander C, Schroder NW, Reiss A, Kuster C, Pfeil D, Tuomanen EI, Schumann RR. 2003. *Recognition of pneumococcal peptidoglycan: An expanded, pivotal role for LPS binding protein.* Immunity 19: 269–279.
- 157) Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI. 1995. *Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor.* Nature 377: 435–438.
- 158) Girardin S, Boneca I, Viala J, Chamaillard M, Labigne A, Thomas G, Philpott D, Sansonetti P. 2003. *Nod2 is a general sensor of peptidoglycan through muramyl dipeptide (MDP) detection.* J Biol Chem 278: 8869–8872.
- 159) Moreira LO, ElKasbi KC, Smith AM, Finkelstein D, Fillon S, Kim YG, Nunez G, Tuomanen E, Murray PJ. 2008. *The TLR2-MyD88-NOD2-RIPK2 signalling axis regulates a balanced pro-inflammatory and IL-10-mediated anti-inflammatory cytokine response to Gram-positive cell walls.* Cell Microbiol 10: 2067–2077.
- 160) Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, Kurt-Jones E, Paton JC, Wessels MR, Golenbock DT. 2003. *Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection.* Proc Natl Acad Sci 100: 1966–1971.
- 161) Albiger B, Dahlberg S, Sandgren A, Wartha F, Beiter K, Katsuragi H, Akira S, Normark S, Henriques-Normark B. 2007. *Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection.* Cell Microbiol 9: 633–644.
- 162) Marks M, Burns T, Abadi M, Seyoum B, Thornton J, Tuomanen E, Pirofski LA. 2007. *Influence of neutropenia on the course of serotype 8 pneumococcal pneumonia in mice.* Infect Immun 75: 1586–1597.
- 163) Hamborsky J, Kroger A, Wolfe S, eds. Centers for Disease Control and Prevention. *Epidemiology and Prevention of Vaccine-Preventable Diseases.* 13th ed. Washington DC; Public Health Foundation; 2015.
- 164) Heilmann C. *Human B and T lymphocyte responses to vaccination with pneumococcal polysaccharides.* APMIS Suppl. 1990;15:1-23.
- 165) Bridy-Pappas AE, Margolis MB, Center KJ, Isaacman DJ. *Streptococcus pneumoniae: description of the pathogen, disease epidemiology, treatment, and prevention.* Pharmacotherapy. 2005;25(9):1193-1212.
- 166) Hammit LL, Bruden DL, Butler JC, et al. *Indirect effect of conjugate vaccine on adult carriage of Streptococcus pneumoniae: an explanation of trends in invasive pneumococcal disease.* J Infect Dis. 2006;193(11):1487-1494.
- 167) Whitney CG, Farley MM, Hadler J, et al. *Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine.* N Engl J Med. 2003;348(18):1737-1746.
- 168) Richter SS, Heilmann KP, Dohrn CL, et al. *Pneumococcal serotypes before and after introduction of conjugate vaccines, United States, 1999–2011.* Emerg Infect Dis. 2013;19(7):1074-1083.

- 169) Lip Nam Loh,  Geli Gao, and Elaine I. Tuomanen. *Dissecting Bacterial Cell Wall Entry and Signaling in Eukaryotic Cells: an Actin-Dependent Pathway Parallels Platelet-Activating Factor Receptor-Mediated Endocytosis*. mBio. 2017 Jan-Feb; 8(1): e02030-16. Published online 2017 Jan 3. doi: 10.1128/mBio.02030-16
- 170) Keane C¹, Tilley D, Cunningham A, Smolenski A, Kadioglu A, Cox D, Jenkinson HF, Kerrigan SW. *J Invasive Streptococcus pneumoniae trigger platelet activation via Toll-like receptor 2*. Thromb Haemost. 2010 Dec;8(12):2757-65. doi: 10.1111/j.1538-7836.2010.04093.x.
- 171) Böhland M, Kress E, Stope MB, Pufe T, Tauber SC, Brandenburg LO. *Lack of Toll-like receptor 2 results in higher mortality of bacterial meningitis by impaired host resistance*. J Neuroimmunol. 2016 Oct 15;299:90-97. doi: 10.1016/j.jneuroim.2016.09.003. Epub 2016 Sep 6.
- 172) Huang Y, Wang Z, Jin C, Wang L, Zhang X, Xu W, Xiang Y, Wang W, He X, Yin Y, He Y. *TLR2 promotes macrophage recruitment and Streptococcus pneumoniae clearance during mouse otitis media*. Pediatr Res. 2016 Dec;80(6):886-893. doi: 10.1038/pr.2016.154. Epub 2016 Jul 27
- 173) Malley R, Henneke P, Morse SC, Cieslewicz MJ, Lipsitch M, Thompson CM, Kurt-Jones E, Paton JC, Wessels MR, Golenbock DT. *Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection*. Proc Natl Acad Sci U S A. 2003 Feb 18;100(4):1966-71. Epub 2003 Feb 4.
- 174) Bernatoniene J, Zhang Q, Dogan S, Mitchell TJ, Paton JC, Finn A. *Induction of CC and CXC chemokines in human antigen-presenting dendritic cells by the pneumococcal proteins pneumolysin and CbpA, and the role played by toll-like receptor 4, NF-kappaB, and mitogen-activated protein kinases*. J Infect Dis. 2008 Dec 15;198(12):1823-33. doi: 10.1086/593177.
- 175) Edel A, McNeela, Áine Burke, Daniel R. Neill, Cathy Baxter, Vitor E. Fernandes, Daniela Ferreira, Sarah Smeaton, Rana El-Rachkidy, Rachel M. McLoughlin, Andres Mori, Barry Moran, Katherine A. Fitzgerald, Jurg Tschopp, Virginie Pétrilli, Peter W. Andrew, Aras Kadioglu, and Ed C. Lavelle. *Pneumolysin Activates the NLRP3 Inflammasome and Promotes Proinflammatory Cytokines Independently of TLR4*. PLoS Pathog. 2010 Nov; 6(11): e1001191. Published online 2010 Nov 11. doi: 10.1371/journal.ppat.1001191
- 176) Mark C. Dessing, Robert A. Hirst, Alex F. de Vos, and Tom van der Poll. *Role of Toll-Like Receptors 2 and 4 in Pulmonary Inflammation and Injury Induced by Pneumolysin in Mice*. PLoS One. 2009; 4(11): e7993. Published online 2009 Nov 24. doi: 10.1371/journal.pone.0007993
- 177) Spelmink L, Sender V, Hentrich K, Kuri T, Plant L, Henriques-Normark B. *Toll-Like Receptor 3/TRIF-Dependent IL-12p70 Secretion Mediated by Streptococcus pneumoniae RNA and Its Priming by Influenza A Virus Coinfection in Human Dendritic Cells*. MBio. 2016 Mar 8;7(2):e00168-16. doi: 10.1128/mBio.00168-16.
- 178) Pennini ME, Perkins DJ, Salazar AM, Lipsky M, Vogel SN. *Complete dependence on IRAK4 kinase activity in TLR2, but not TLR4, signaling pathways underlies decreased cytokine production and increased susceptibility to Streptococcus pneumoniae infection in IRAK4 kinase-inactive mice*. J Immunol. 2013 Jan 1;190(1):307-16. doi: 10.4049/jimmunol.1201644. Epub 2012 Dec 3.
- 179) Böttcher T, von Mering M, Ebert S, Meyding-Lamadé U, Kuhnt U, Gerber J, Nau R. *Differential regulation of Toll-like receptor mRNAs in experimental murine central nervous system infections*. Neurosci Lett. 2003 Jun 19;344(1):17-20.
- 180) Mancuso G, Gambuzza M, Midiri A, Biondo C, Papasergi S, Akira S, Teti G, Beninati C. *Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells*. Nat Immunol. 2009 Jun;10(6):587-94. doi: 10.1038/ni.1733.
- 181) Lee KS, Scanga CA, Bachelder EM, Chen Q, Snapper CM. *TLR2 synergizes with both TLR4 and TLR9 for induction of the MyD88-dependent splenic cytokine and chemokine response to Streptococcus pneumoniae*. Cell Immunol. 2007 Feb;245(2):103-10. Epub 2007 May 22.
- 182) Xiao Yao, Deborah Carlson, Yuxiao Sun, Lisha Ma, Steven E. Wolf, Joseph P. Minei, and Qun S. Zang *Mitochondrial ROS Induces Cardiac Inflammation via a Pathway through mtDNA Damage in a Pneumonia-Related Sepsis Model*. PLoS One. 2015; 10(10): e0139416. Published online 2015 Oct 8. doi: 10.1371/journal.pone.0139416
- 183) Irena Kukavica-Ibrulj, Marie-Ève Hamelin, Gregory A. Prince, Constance Gagnon, Yves Bergeron, Michel G. Bergeron, and Guy Boivin. *Infection with Human Metapneumovirus Predisposes Mice to Severe Pneumococcal Pneumonia* J Virol. 2009 Feb; 83(3): 1341–1349. Published online 2008 Nov 19. doi: 10.1128/JVI.01123-08
- 184) Lai, C. J., J. E. Dahlberg, and B. Weisblum. 1973. *Structure of an inducibly methylatable nucleotide sequence in 23S ribosomal ribonucleic acid from erythromycin-resistant Staphylococcus aureus*. Biochemistry 12: 457–460.
- 185) Weisblum, B., C. Siddhikol, C. J. Lai, and V. Demohn. 1971. *Erythromycin-inducible resistance in Staphylococcus aureus: requirements for induction*. J. Bacteriol. 106: 835–847.

- 186) Kolter J, Feuerstein R, Spoeri E, Gharun K, Elling R, Trieu-Cuot P, Goldmann T, Waskow C, Chen ZJ, Kirschning CJ, Deshmukh SD, Henneke. *Streptococci Engage TLR13 on Myeloid Cells in a Site-Specific Fashion*. J Immunol. 2016 Mar 15;196(6):2733-41. doi: 10.4049/jimmunol.1501014. Epub 2016 Feb 12.
- 187) Mancuso G, Midiri A, Biondo C, Beninati C, Zummo S, Galbo R, Tomasello F, Gambuzza M, Macrì G, Ruggeri A, Leanderson T, Teti G. *Type I IFN signaling is crucial for host resistance against different species of pathogenic bacteria*. J Immunol. 2007 Mar 1;178(5):3126-33.
- 189) Mohammadi N, Midiri A, Mancuso G, Patanè F, Venza M, Venza I, Passantino A, Galbo R, Teti G, Beninati C, Biondo C. *Neutrophils Directly Recognize Group B Streptococci and Contribute to Interleukin-1 β Production during Infection*. PLoS One. 2016 Aug 10;11(8):e0160249. doi: 10.1371/journal.pone.0160249. eCollection 2016.
- 190) Mocsai A, Ligeti E, Lowell CA, Berton G. *Adhesion-dependent degranulation of neutrophils requires the Src family kinases Fgr and Hck*. Journal of immunology (Baltimore, Md: 1950). 1999;162(2):1120–6. .[PubMed]
- 191) Mancuso G, Midiri A, Beninati C, Biondo C, Galbo R, Akira S, Henneke P, Golenbock D, Teti G. 2004. *Dual role of TLR2 and myeloid differentiation factor 88 in a mouse model of invasive group B streptococcal disease*. J. Immunol. 172:6324–6329. 10.4049/jimmunol.172.10.6324 [PubMed] [Cross Ref]
- 192) Biondo, C., A. Midiri, L. Messina, F. Tomasello, G. Garufi, M. R. Catania, M. Bombaci, C. Beninati, G. Teti, and G. Mancuso. 2005. *MyD88 and TLR2, but not TLR4, are required for host defense against Cryptococcus neoformans*. Eur. J. Immunol. 35: 870–878.
- 193) Karaghiosoff, M., R. Steinborn, P. Kovarik, G. Kriegshauser, M. Baccarini, B. Donabauer, U. Reichart, T. Kolbe, C. Bogdan, T. Leanderson, et al. 2003. *Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock*. Nat. Immunol. 4: 471–477. 39.
- 194) Nagarajan, U. M., D. M. Ojcius, L. Stahl, R. G. Rank, and T. Darville. 2005. *Chlamydia trachomatis induces expression of IFN-inducible protein 10 and IFNindependent of TLR2 and TLR4, but largely dependent on MyD88*. J. Immunol. 175: 450–460.