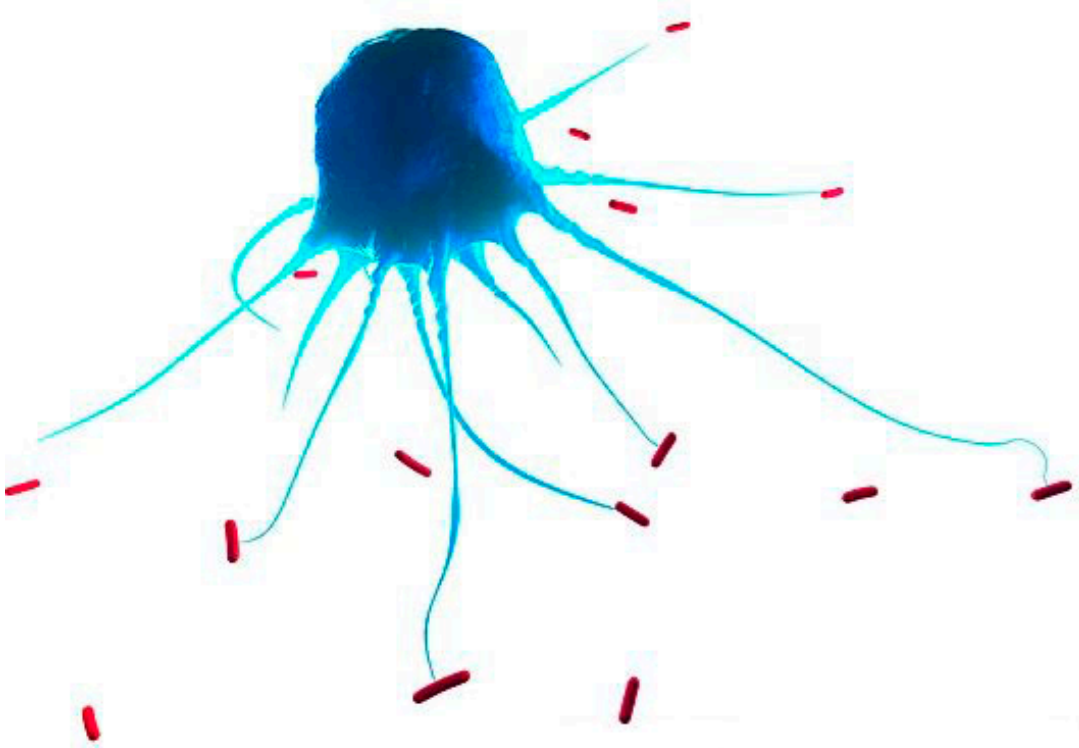
Master Thesis

Intracellular recognition and clearance of microbes



Ella Koppelaar – 3553701
Supervised by Dr. Jovanka Bestebroer and Prof. Dr. Jos van Strijp
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Abbreviations

ATG	Autophagy related gene
BIR	Baculovirus inhibitor of apoptosis repeat
CMA	Chaperone-mediated autophagy
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DED	Death effector domain
dsRNA	Double stranded RNA
ER	Endoplasmic reticulum
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
iE-DAP	ϒ-D-glutamyl-meso-diaminopimelic acid
IFN	Interferon
IL	Interleukin
LRR	Leucine-rich repeat
LPS	Lipopolysaccharide
MCMV	Murine cytomegalo virus
MDP	Muramyl dipeptide
mTor	Mammalian target of rapamycin
NLR	Nod-like receptor
NOD	Nucleotide binding oligomerization domain
PE	Phosphatidylethanolamine
Poly(I:C)	Polyinosinic-polycytidylic acid
PAMP	Pathogen-associated molecular pattern
PRR	Pathogen-recognition receptor
PYD	Pyrin domain
RLR	RIG-I like receptor
Tb	Tuberculosis
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
VSV	Vesicular stomatitis virus

Abstract

Microbial recognition is mediated by pattern recognition receptors (PRRs). These germline-encoded receptors recognizing highly conserved microbial structures are essential for survival and are therefore not subjective to high frequent mutations. The recognition of these so-called pathogen-associated molecular patterns (PAMPs) enables the immune system to distinguish between self and non-self and induce the appropriate immune responses upon infection.

PRRs are located on extracellular surfaces, on intracellular membranes or in the cytosol. However, there will be specifically focused on receptors enabling intracellular recognition of microbes. Furthermore, a newly identified intracellular killing mechanism by autophagy will be discussed. This process is important in the metabolic homeostasis of cells, and it was recently found to be important in the clearance of bacteria as well. Interestingly, PRRs are able to influence autophagy, contributing to the newly established link between innate immunity and the evolutionary highly conserved process of autophagy.

1. Introduction

Innate immunity is a highly conserved immunologic process and is found in plants and all metazoans. Adaptive immunity on the contrary is only present in vertebrates. [Saleh, 2011] The innate immune system is the first line of defense against microbes and makes use of professional phagocytes like neutrophils, dendritic cells and macrophages, the complement system and antimicrobial peptides. [Bardoel and Strijp, 2011][Akira, Uematsu, Takeuchi, 2006] The innate immune system is of great importance in the survival of an individual, and of fundamental aspect is its capacity to discriminate between self and non-self.

The innate immune system recognizes a wide range of microbes, like bacteria, viruses, fungi and parasites. Recognition is for a large part mediated through a limited number of germline-encoded pattern recognition receptors (PRRs), which are expressed by a diverse range of cell types, including monocytes, macrophages, dendritic cells (DCs), neutrophils and epithelial cells. [Martinon, 2009] These PRRs are evolved to distinguish between self and non-self via the recognition of pathogen-associated molecular patterns (PAMPs). [Kawai and Akira, 2010] PAMPs are highly conserved structures, which are essential for survival of pathogens and therefore not subjective to mutations. [Akira, Uematsu, Takeuchi, 2006] Upon recognition, direct killing of microbes is initiated via phagocytosis. In addition, other appropriate immune responses are induced that lead to the maturation of dendritic cells (DCs) and to the induction of pathogen-specific adaptive immunity that relies on long-lasting B and T cell immunity. [Kawai and Akira, 2011][Kumar, 2011]

Microbes can be recognized by PRRs prior to their entrance into a host cell. At this stage, recognition by cell surface PRRs can induce immune responses to eliminate microbes with an appropriate inflammatory and immune response. The extracellular recognition of microbes is discussed elsewhere in recent reviews (Kawai and Akira, 2010; Kumar, 2011; Bardoel and Strijp, 2011). Furthermore, microbes can also be recognized by PRRs intracellularly in the phagosome, upon entrance into the cytosol or escape from the phagosome.

The main focus of this thesis is the intracellular recognition of microbes, both in the phagosome as in the cytosol. Several intracellular PRR families have been identified, which all consist of a wide variety of members. These receptors are capable of recognizing microbial PAMPs, danger-associated molecular patterns (DAMPs), toxins and extracellular ATP. Upon recognition the appropriate immune responses are initiated, which subsequently leads to microbial clearance. PRR families are also known to interact or respond to the same ligands, thereby influencing the induced immune responses. Both the receptor families as the induced responses will be discussed. Furthermore, this thesis will focus on the newly identified intracellular killing mechanism by autophagy. It will discuss its initiation upon infection, its role in immunity and in clearance of invading bacteria. An interesting aspect is the link between the recognition of microbes by specific PRRs and the subsequent induction of autophagy. It has been reported that interaction of both can lead to an enhanced efficiency of microbial clearance.

2. Microbial uptake mechanisms by professional phagocytes

Professional phagocytes survey the body for invading microbes. Upon recognition, phagocytes are able to internalize and process microbes via endocytic pathways, thereby inducing the right pro-inflammatory immune response and clearing the microbe. The uptake is mediated via phagocytosis, specific endocytosis or nonspecific fluid phase endocytosis. [Blasius and Beutler, 2010] **Figure 1** depicts various processes of internalization of microbes or ligands.

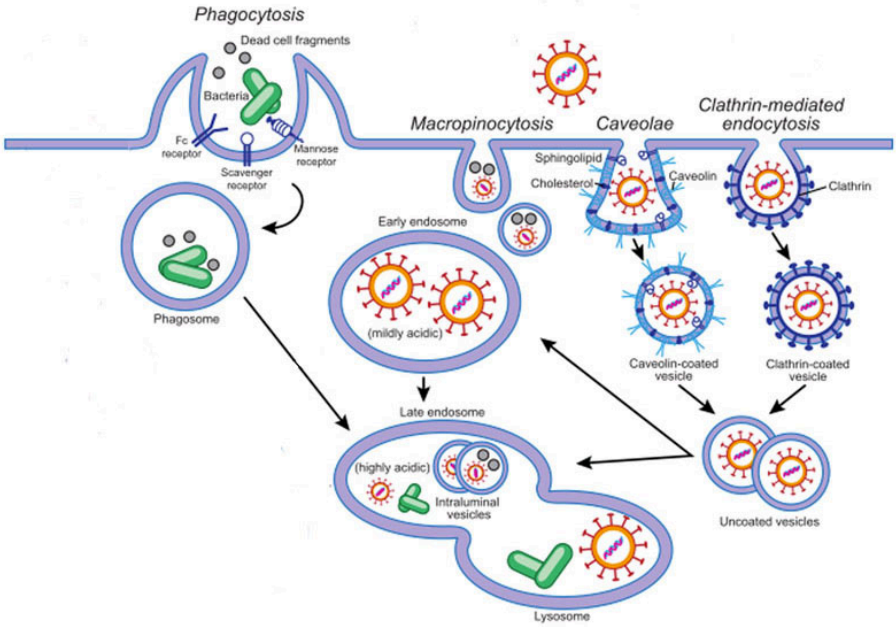

Figure 1. Phagocytosis, macropinocytosis and caveolae- and clathrin- mediated endocytosis.
Phagocytosis is triggered by specific receptors, including Fc receptors, scavenger receptors and mannose receptors. Phagosomes can immediately fuse with highly acidic late endosomes or lysosomes. Macropinocytosis mediates the uptake of suspended or cell-adherent particles and receptor mediated endocytosis via caveolin or clathrin allows the uptake of a variety of particles, like nutrients and microbes. [Cell, Alberts][Brock][Blasius and Beutler, 2010] [Kuby] These vesicles can fuse with mildly acidic early endosomes that subsequently fuse with late endosomes or they can directly fuse with late endosomes or lysosomes. [Blasius and Beutler, 2010] Adapted from Blasius and Beutler (2010)

Phagocytosis enables the engulfment of entire bacteria or dead cell fragments and is triggered by specific receptors on the cell surface. These receptors, i.e. the mannose receptor, complement receptor, Fc receptors, phosphatidylserine receptor and scavenger receptors, recognize extracellular microbes and mediate their uptake into a phagosome. [Cell, Alberts][Brock][Blasius and Beutler, 2010] This organelle fuses with highly acidic lysosomes or late endosomes forming a phagolysosome. This ensures the delivery of hydrolytic enzymes and microbicidal proteases to the phagolysosome, which simultaneously digests the microbe. The digested microbes are then eliminated in a process called exocytosis. [Kuby][Blasius and Beutler, 2010]

Endocytosis is mediated via clatherin- or caveolae-coated pits. It is similar to phagocytosis as it is also receptor-mediated. Here the class A macrophage scavenger receptor (SR-A), TLR2, TLR4 and the Fc receptors play a role. [Andersson, 2008][Zhu et. al., 2011] In addition, cholesterol and sphingolipid are required for caveolin-dependent endocytosis. Upon the uptake of clathrin- or caveolin-coated vesicles in the cytoplasm, the vesicles rapidly uncoat, which allows the fusion of the vesicle with an early or late endosome. [Cell, Alberts][Kuby][Blasius and Beutler, 2010]

Finally, macropinocytosis or fluid phase endocytosis, allows the uptake of suspended or cell-adherent particles. This process is different from phagocytosis and

endocytosis because it mediates the uptake of smaller particles suspended in liquid and is not initiated by specific receptors. [Cell, Alberts][Brock][Blasius and Beutler, 2010]

Upon internalization, microbial recognition determines appropriate immune responses and clearance.

3. Intracellular recognition of microbes by PRRs

Upon entrance and recognition of microbes several immune responses are initiated. A wide variety of PRR mediates the recognition of microbes and determines the appropriate responses. Recent research has unraveled the role of receptors involved in intracellular recognition of microbes and will be reviewed here.

3.1 Intracellular Toll-like receptors

The first identified PRR family, with the ability to distinguish between self and non-self via the recognition of pathogen-associated molecular patterns (PAMPs), is the Toll-like receptor family. [Kawai and Akira, 2010] This family consists of 10 and 12 functional members in humans and mice, respectively. [Kawai and Akira, 2010] TLR1 till TLR9 are conserved in both species, while TLR10 is only functional in humans because of a stop codon in the murine tlr10 gene. TLR11/12/13 are only expressed in mice. [Kumar, 2011]

TLRs are type I transmembrane proteins that consist of ectodomains containing leucine-rich repeats (LRR) **(Figure 2)** that mediate the recognition of PAMPs from a wide range of microbes like bacteria, viruses, parasites and fungi. Furthermore, TLRs consist of a transmembrane domain and intracellular Toll-interleukin 1 receptor (TIR) domains, which is located intracellularly and essential for downstream signal transduction. [Akira, Uematsu, Takeuchi, 2006][Kawai and Akira, 2010]

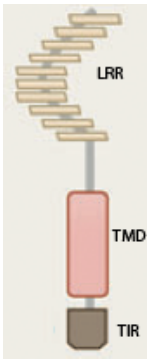

Figure 2. Molecular domain organization of Toll-like receptors. TLRs are made up of several distinct domains. The N-terminal leucine-rich repeat (LRR) is followed by a transmembrane domain (TMD) and a Toll-interleukin 1 receptor domain (TIR) that is found intracellularly. [Akira, Uematsu, Takeuchi, 2006][Kawai and Akira, 2010] Adapted from Martinon (2009)

The expression of the TLRs varies from the extracellular cell surface to intracellular membranes. TLRs found on the cell surface are TLR1/2/4/5/6, and they mainly recognize microbial cell wall components whereas TLR3/7/8/9 are expressed on intracellular membranes like the endoplasmic reticulum (ER), endosomes, lysosomes and endolysosomes. [Kawai and Akira, 2010][Kawai and Akira, 2011] Recently, it has been shown that TLR11 can be found on cell surface and in intracellular compartments. [Kawai and Akira, 2011][Pifer et. al., 2011] The correct localization of TLRs is thought to be important in the maintenance of tolerance to self-molecules and ligand accessibility. Besides the difference in their cellular localization, TLRs are also differentially expressed on various cell types, including immune cells like DCs, macrophages, B cells and specific types of T cells. Non-immune cells such as fibroblasts and epithelial cells, present in the lung and gastro-intestinal tract, also express TLRs. [Akira, Uematsu, Takeuchi, 2006]

The three TLRs first recognized as intracellular PRRs are TLR7/8/9. They are evolutionary conserved and form a cluster within the TLR family. [Heil et. al., 2003]. Later,

TLR3 was also recognized to function intracellularly. These TLRs reside in the intracellular compartments of macrophages and DCs, where they recognize intracellular microbes. They are expressed within the ER, endosomes, multivesicular bodies and lysosomes. [Diebold et. al., 2004][Blasius and Beutler, 2010][Kawai and Akira, 2010][Kawai and Akira, 2011] The TLRs traffic from the ER, via the common secretory pathway, through the Golgi, and eventually end up in the ligand containing endolysosomes **(Figure 3A)**. [Blasius and Beutler, 2010][Kawai and Akira, 2010][Kawai and Akira, 2011] The ER-localizing protein UNC93B1 regulates the translocation of TLR7 and TLR9 towards the endosomes. [Kim et. al., 2008] UNC93B1 binds to the transmembrane region of the receptors localized in the ER. [Kawai and Akira, 2010] Another translocation protein, PRAT4A, is also responsible for the trafficking of TLR9 to the endosomal compartments. [Kawai and Akira, 2010] Due to the acidic conditions in mature endolysosomal compartments the conformation of the TLRs changes. This is mediated by multiple lysosomal proteases, involving cathepsins and asparagine endopeptidase for TLR7 and TLR9. However, this effect is not observed for TLR3. [Diebold et. al., 2004][Blasius and Beutler, 2010] The compartmentalization of ligands and the conformational changes of the receptors increase the specificity of the innate immune system and the ability to distinguish self from non-self.

3.1.1 Toll-like receptor 3

The intracellular TLRs, TLR3/7/8/9 are evolutionary conserved and therefore highly homologous. Though, they recognize different ligands and induce different immune responses depending on the cell type. TLR3 is able to recognize intracellular viral ligands **(Figure 3B)**. This receptor binds the universal viral PAMP, double-stranded RNA (dsRNA), and was first identified to recognize the synthetic analog of dsRNA polyinosinic-polycytidylic acid (poly(I:C)). For example, dsRNA of reoviruses activate TLR3. [Blasius and Beutler, 2010] Single-stranded RNA (ssRNA) and dsDNA viruses, such as influenza A and herpes simplex virus (HSV), respectively, can be recognized during replication when dsRNA is formed. [Kawai and Akira, 2011] TLR3-deficient mice are significantly more susceptible to infection with murine cytomegalovirus and TLR3 deficiency in humans is implicated with higher susceptibility to HSV. [Tabeta et. al., 2004][Zhang et. al., 2007] Upon recognition, antiviral immune responses, such as type 1 interferon (IFN) and inflammatory cytokines, such as interleukin (IL) -6 and IL-12 are induced. [Alexopoulou et. al., 2001][Kawai and Akira, 2010]

TLR3 is expressed in conventional DCs, however not in plasmacytoid DCs. Furthermore, it is commonly expressed in a wide variety of epithelial cells like airway, uterine, corneal, vaginal, cervical, biliary, and intestinal epithelial cells. These specific epithelial cells are potent barriers to infection. [Akira, Uematsu, Takeuchi, 2006]

3.1.2 Toll-like receptor 7 and 8

As TLR3, TLR7 and TLR8 recognize intracellular viral ligands **(Figure 3B)**. TLR7 and human TLR8 recognize uridine-rich or uridine/guanosine-rich ssRNA. The function of the murine TLR8 is still unknown. [Heil et. al. Science, 2004] TLR7 and human TLR8 are able to detect ssRNA viruses [Kawai and Akira, 2010], like human immunodeficiency virus (HIV), influenza and vesicular stomatitis virus (VSV). Also bacteria that are taken up by phagocytosis and are (partly) degraded in endolysosomes can be sensed by TLR7. For instance, degradation of group B *Streptococcus* bacteria delivers RNA ligand to TLR7 located in the endolysosomes. [Mancuso et. al., 2009]

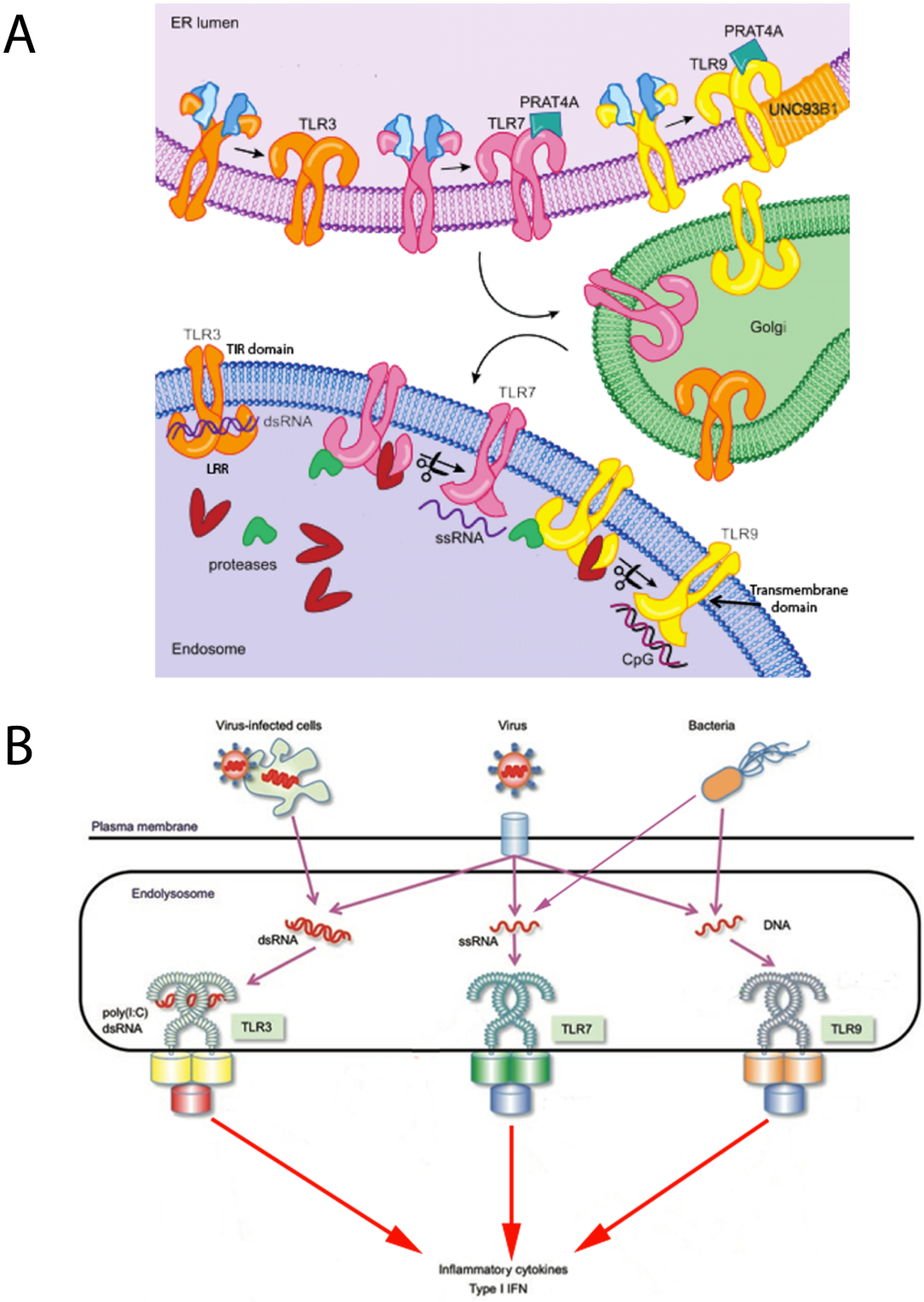

Figure 3. TLR translocation and conformational changes prior to ligand recognition. A) TLR3/7/9 are located in the ER prior to stimulation. PRAT4 and UNC93B1 are required for these TLRs to exit the ER and travel through the Golgi apparatus to the endosomes where they encounter their ligands. [Kim et. al., 2008][Kawai and Akira, 2010] TLR7 and TLR9 are cleaved by proteases present in the endosome. Cleavage of TLR3 has not been observed. [Diebold et. al., 2004][Blasius and Beutler, 2010] Adapted from Blasius and Beutler (2010). **B)** TLR3 recognizes dsRNA originating from viruses or virus-infected cells. Upon recognition the production of inflammatory cytokines and type I IFN is induced. [Alexopoulou et. al., 2001][Kawai and Akira, 2010] TLR7 recognizes ssRNA originating from viruses and from bacteria degraded in the endolysosome. [Heil et. al. Science, 2004] TLR9 recognizes unmethylated CpG rich DNA originating from bacteria and viruses. Activation of TLR7 and TLR9 induce the production of inflammatory cytokines and type I IFN. [Latz et. al., 2004][Kawai and Akira 2010] Adapted from Kawai and Akira (2010).

Interestingly, activation of TLR7 by the same ligand but expressed in different cell types does not necessarily lead to induction of the same immune response. The immune response elicited by activation of TLR7 is found to be cell type dependent. This is illustrated by the expression of TLR7 in conventional DCs, which induces the production of INF-β in response to ligand stimulation. [Mancuso et. al., 2009] However, when TLR7 is expressed by plasmacytoid DCs, inflammatory cytokines are produced such as IFN-α, IL-6 and IL-12. [Kawai and Akira, 2010]

It is crucial that innate immunity has the capacity to discriminate between self and non-self. However, TLR7 can be activated by self-ssRNA but due to the degradation of RNA by extracellular RNases and compartmentalization of self-RNA, it seldom reaches the endosomal compartments, preventing activation of TLR7 by self-ssRNA. [Diebold et. al., 2004][Akira, Uematsu, Takeuchi, 2006]

3.1.3 Toll-like receptor 9

Another TLR that is important in the recognition of intracellular microbes is TLR9. Although highly homologous to TLR7 and TLR8, it recognizes a completely different type of ligand. TLR9 recognizes bacterial genomic DNA containing unmethylated CpG dinucleotides **(Figure 3B)**. CpG DNA is internalized into lysosomal compartments via the clatherin-dependent endocytic pathway, where it directly binds to TLR9. [Latz et. al., 2004] Upon recognition of the CpG motifs, TLR9 induces strong immunomodulatory responses, such as the induction of inflammatory cytokines that directly activate DCs, macrophages and B cells and drive strong Th1 responses. [Blasius and Beutler, 2010][Akira, Uematsu, Takeuchi, 2006] In contrast, mammalian DNA does not stimulate TLR9 as it contains four times less methylated CpG dinucleotides. [Hemmi et. al., 2000][Akira, Uematsu, Takeuchi, 2006] In addition to other receptors, TLR9 recognizes bacteria such as *Salmonella typhimurium* and *Mycobacterium tuberculosis*. Gram-negative and Gram-positive bacteria, respectively, that replicate in macrophages. [Brock][Gerold et. al., 2007] TLR9, together with TLR2, is partly responsible for the induction of inflammatory cytokines during infection with *M. tuberculosis*. [Gerold et. al., 2007][Saiga, 2011]

In addition to the recognition of bacterial DNA, TLR9 can also recognize viral DNA **(Figure 3B)**. DNA of herpes viruses such as HSV-1 and 2, and murine cytomegalo virus (MCMV) have high frequency unmethylated CpG DNA, which is recognized by TLR9. [Krug et. al.,2004][Tabeta et. al., 2004][Akira, Uematsu and Takeuchi, 2006][Kawai and Akira, 2010] It is postulated that encapsulated viruses enter the endosomal compartment and upon internalization, viral DNA is released where it can be recognized by TLR9. Recognition of viral DNA elicits a different type of response as bacterial DNA does. In case of recognition of viral DNA, inflammatory cytokines and type 1 IFN are secreted. Type 1 IFN secretion is absent in the recognition of bacterial DNA. [Krug et. al., 2004][Akira, Uematsu and Takeuchi, 2006][Kawai and Akira, 2010]

TLR9 is expressed in different cell types such as plasmacytoid DCs and B cells and Northern blot analysis revealed that mouse TLR9 transcripts were most abundantly expressed in the spleen **(Figure 4)**. [Hemmi et. al., 2000][Krieg, 2002]

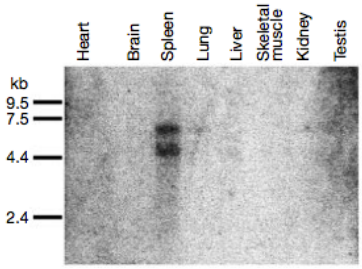

Figure 4. Tissue distribution of TLR9. Different mice tissues containing RNA are analyzed by Northern blot for TLR9 expression using a mouse TRL9 cDNA probe. TLR9 is expressed in the spleen. [Hemmi et. al., 2000]

3.2 Nod-like receptors

Recently, another large family of cytosolic PRRs has been identified, namely the Nod-like receptors (NLR). The NLR family consists of 23 members in humans and over 30 members in mice. The most important and well-described NLRs are the NODS, NALPs and IPAF or NAIP receptors.

While the above-mentioned TLRs are membrane-bound receptors, the NLRs are soluble proteins that survey the cytoplasm for PAMPS and DAMPs. [Martinon, 2009] Like TLRs, NLRs have a C-terminal LRR region that recognizes bacterial ligands or endogenous host molecules and mediates auto-repression **(Figure 5)**. [Akira, Uematsu and Takeuchi, 2006][Kumar, 2011][Elinav, 2011][Delgado, 2009] The N-terminus of NLRs contains a death effector domain (DED), a pyrin (PYD) domain, a caspase activation and recruitment (CARD) domain or baculovirus inhibitor of apoptosis repeat (BIR) domain, which initiates downstream signaling. [Akira, Uematsu and Takeuchi, 2006][Kumar, 2011][Elinav, 2011] The nucleotide-binding oligomerization (NACHT or NOD) domain forms the intermediate domain and binds nucleotides. NACHT is possibly involved in conformational changes and self-oligomerization, which is required for functional NLR proteins. [Kumar, 2011][Elinav, 2011]

The NLRs recognize a wide variety of intracellular PAMPs and DAMPs. [Kawai and Akira, 2010][Kumar, 2011] DAMPs are released during tissue damage and cell lysis; events often associated with ongoing infection. The recognition of DAMPs by NLRs is crucial for the ability of the innate immune system to distinguish between pathogenic microorganisms and commensal or non-pathogenic microorganisms. [Martinon, 2009] In addition to the initiation of an appropriate immune response, sensing of DAMPs can also induce the repair of tissue damage caused by the ongoing infection.

Upon recognition of ligands by the members of the NLR family, NFκB and MAP kinases are activated and inflammatory cytokine production is induced. In addition, multi-protein complexes called inflammasomes are formed. This initiates proteolytic cleavage of the pro-inflammatory cytokines IL-1β and IL-18 and thereby promotes their maturation, or it initiates apoptosis of the cell. [Kumar, 2011]

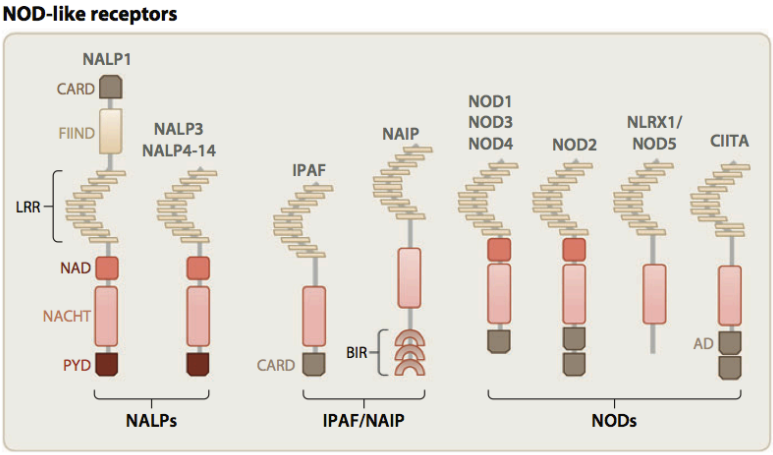

Figure 5. Molecular domain organization of Nod-like receptors: NALPs, IPAF or NAIP and NODs. Three distinct domains characterize NLRs: the ligand sensing Leucine-rich repeat domain, the nucleotide-binding oligomerization (NACHT or NOD) domain that mediates the oligomerization; and an effector domain that can be either a Pyrin (PYD), caspase activation and recruitment (CARD) or baculovirus inhibitor of apoptosis repeat (BIR) domain. Most NLRs also include a NACHT-associated domain (NAD). [Martinon, 2009] Adapted from Martinon (2009).

3.2.1 NOD1 and NOD2

Two members of the large NLR family that play an important role in intracellular innate immunity are NOD1 and NOD2. Various cell types express these receptors in the cytosol and they are highly expressed in professional phagocytes. NOD1 is expressed in epithelial cells, while NOD2 is restricted to expression in more specialized cells in the small intestines, namely the Paneth cells. Recently, this receptor was also identified in other hematopoietic lineages. [Elinav, 2011] NOD1 and NOD2 have one or two N-terminal CARD domains, respectively, and a C-terminal LRR that mediates the recognition of PAMPs. [Kumar, 2011]

Both receptors are able to recognize degradation products of the bacterial cell wall component peptidoglycan **(Figure 6)**. [Elinav, 2011] The breakdown products that NOD1 and NOD2 recognize are Ƴ–D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively. [Akira, Uematsu and Takeuchi, 2006][Kumar, 2011] Gram-negative contain less peptidoglycan in their cell wall than Gram-positive bacteria. MDP is a component present in all peptidoglycan structures of Gram-positive and Gram-negative bacteria and iE-DAP is a structure present in peptidoglycan of mainly Gram-negative bacteria. [Elinav, 2011]

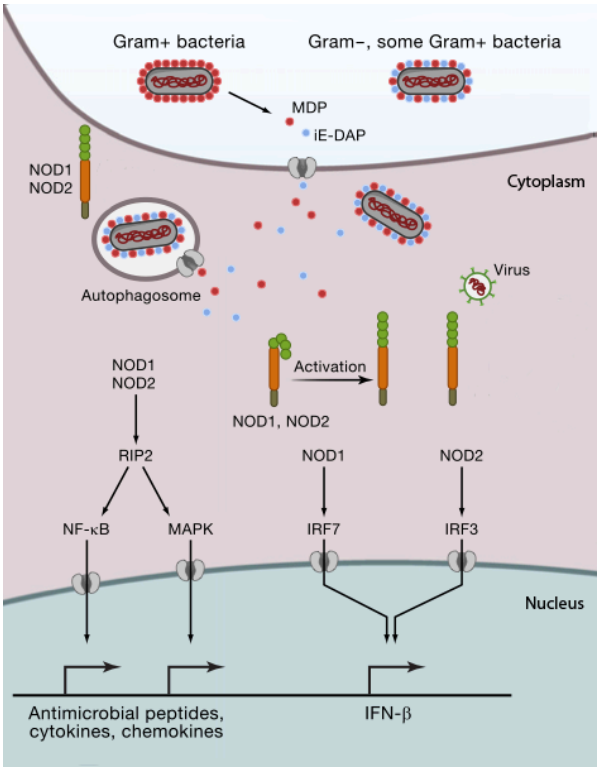

Figure 6. Microbial activation of intracellular NOD1 and NOD2. NOD1 and NOD2, two members of the NLR family, recognize iE-DAP and MDP, respectively. [Akira, Uematsu and Takeuchi, 2006][Kumar, 2011] Upon recognition antimicrobial peptides, cytokines, and chemokine production is upregulated via the NFκB and MAPK pathway. NOD2 induces the transcription of IFN-β upon recognition of viral ssRNA via IRF3. NOD1 is able to recognize *H. pylori* and induces IFN-β production via IRF7. [Elinav, 2011] Adapted from Elinav (2011).

NOD1 and NOD2 recognize the breakdown products of peptidoglycan encountered in the cytoplasm. Therefore, bacteria that replicate in the cytoplasm are easily sensed by these receptors. In addition to the recognition of intracellular bacteria, they also seem to recognize extracellular and phagocytosed bacteria. For these bacteria other means are needed to translocate ligands from the extracellular compartments and the phagosomes towards the vicinity of NOD1 and NOD2. The exact mechanism that facilitates this process is not yet fully understood. However, several transport systems including pannexin, PepT1 and PepT2, endocytosis and the Type IV secretion system of bacteria are potential candidates to transfer hydrophilic MDP across the cell membrane

into the cytoplasm. [Franchi et. al, 2008] The type IV secretion system is exploited by the extracellular pathogen *Helicobacter pylori* to translocate peptidoglycan into the cytoplasm where it is recognized by NOD1. [Elinav, 2011] NOD1 deficient mice (Nod1-/-) are significantly more susceptible to infection with *H. pylori*. [Viala et. al., 2004][Allison et. al., 2009] This implies that NOD1 is an important receptor in the first line of defense in the gastro-intestinal route where it is expressed in the epithelial cells and prevents infection. [Martinon, 2009]

The signaling cascades that are initiated downstream of NOD1 and NOD2 induce immune responses appropriate to eliminate invading microbes **(Figure 6)**. Upon ligand recognition, NOD1 and NOD2 become activated and recruit the kinase RIP2 through CARD-CARD interactions. RIP2 oligomerizes and NOD1 and NOD2 undergo conformational changes thereby activating signaling cascades that lead to NFκB and MAPK activation followed by the secretion of proinflammatory cytokines, chemokines and antimicrobial peptides. [Martinon, 2009][Elinav, 2011][Kawai and Akira, 2010] NOD2 can also sense viral ssRNA and this pathway is independent of a CARD domain and requires a different transcription factor, specifically interferon regulatory factor 7 (IRF7). Activation of this pathway produces IFN-β in response to viral infection. [Elinav, 2011] Furthermore, increasing evidence is found that NLRs and other PRRs synergistically respond to the same ligand, thereby inducing amplified inflammatory responses.

3.2.2 Inflammasomes

NLRs are capable of inducing immune responses via the induction of inflammatory cytokines, chemokines, antimicrobial peptides and type 1 IFNs. In addition, they induce formation of multi-protein complexes called inflammasomes **(Figure 7)**. These inflammasomes are formed by proteases, like caspase-1; an adaptor protein Apoptosis-associated speck-like protein containing a CARD (ASC); and one of the members of the NLR family e.g. NALP1 or NLRP1, NALP3 or NLRP3, IPAF or NLRC4 and AIM2. [Akira, Uematsu and Takeuchi, 2006][Martinon, 2009][Elinav, 2011] Inflammasomes are assembled via homophilic CARD-CARD and PYD-PYD interactions between NLR, ASC proteins and caspase proteases. The activation of inflammasomes is essential for the secretion of mature IL-1β and IL-18, as they cleave inactive pro-IL-1β and pro-IL18 into the functional cytokine IL-1β and IL-18. [Akira, Uematsu and Takeuchi, 2006] These NLRs recognize a variety of PAMPs and DAMPs, such as flagellin, uric acid crystals, extracellular ATP and toxins, which are summarized in table 1.

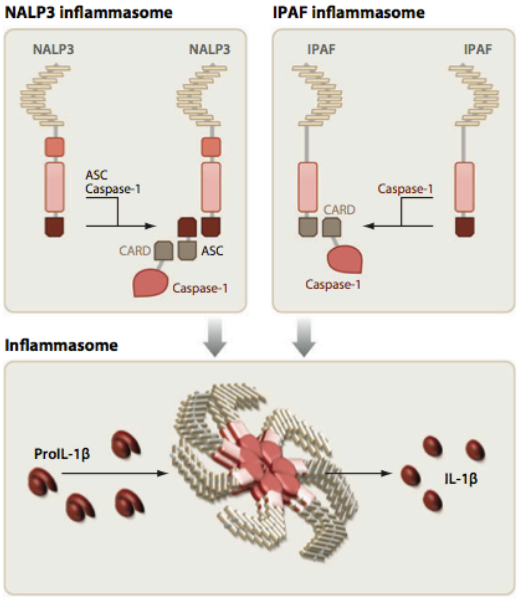

Figure 7. Inflammasome formation by NALP3 and IPAF receptors. The core structure of the NALP3 inflammasome is formed by NALP3, the adaptor protein ASC, and caspase-1 and is formed via Pyrin (PYD)-PYD and caspase activation and recruitment (CARD)-CARD homotypic interactions. IPAF forms an inflammasome via the recruitment of caspase-1 via CARD-CARD interactions. The inflammasome mediates the maturation of pro-IL-1β into IL-1β. [Akira, Uematsu and Takeuchi, 2006][Martinon, 2009][Elinav, 2011]

NLRs	Ligand or pathogen
NOD1	iE-DAP from *L. monocytogenes*, *Shigella flexneri*, *Campylobacter jejuni* and *H. pylori*
NOD2	MDP from *Streptococcus pneumonia*, *M. tuberculosis*, *L. monocytogenes* and *S.flexneri*
NALP3 (NLRP3)	Crystals (uric acid), extracellular ATP, fibrillar amyloid-β peptide, pollutants (asbestos), bacterial and viral RNA, toxins (nigericin and maitotoxin), UV light, bacteria (*Staphylococcus aureus* and *L. monocytogenes*), viruses (Sendai virus, adenovirus and Influenza virus)
NALP1 (NLRP1)	Viral and bacterial PAMPs? Anthrax lethal toxin from *Bacillus anthracis*
IPAF (NLRC4)	Flagellin and possibly other virulence factors secreted through type III (T3SS) or type IV (T4SS) secretion systems, from *S. flexneri, S. thypimurium, Pseudomonas aeruginosa and Legionella pneumophila*
AIM2	DNA

Tabel 1. Nod-like receptor family members are able to recognize ligands and specific pathogens upon which they induce appropriate immune responses. Adapted from Kumar (2011); Martinon (2009); Franchi et. al. (2008).

Species	PAMPs	TLR	Other PRRs involved recognition
Bacteria, mycobacteria	Lipoproteins, peptidoglycan	TLR2/1, TLR2/6	NOD1, NOD2, NALP3, NALP1
	Flagellin	TLR5	IPAF
	DNA	TLR9	AIM2
	RNA	TLR7	NALP3
Viruses	DNA	TLR9	AIM2, DAI
	RNA	TLR3, TLR7, TLR8	RIG-1, MDA5, NALP3

Table 2. Toll-like receptors recognize the same ligands as some other pattern recognition receptors, which positively or negatively influences the outcome of the induced immune responses. Adapted from Kawai and Akira (2011).

3.2.3 Cross-talk between NLRs and TLRs

NLRs and other PRRs families consist of a wide variety of members. All these receptors interact with specific ligands and elicit immune responses. In addition to these 'single' responses, there is increasing evidence that there is crosstalk between NLRs and other PRRs. This positively or negatively influences the induced immune responses via enhancement or inhibition. As an example, redundancies in the recognition of bacterial PAMPs between TLRs and other NLRs are found. Intracellular flagellin activates IPAF, while simultaneously activating TLR5 on the cell surface leading to enhanced production of inflammatory cytokines. [Franchi et. al., 2008][Elinav, 2011] In addition, TLR9 senses intracellular CpG-motif rich DNA and AIM2, not a member of the NLR family but capable of forming inflammasome multi-protein complexes, senses intracellular dsDNA thereby initiating immune responses to *Francisella tularensis* and *Listeria monocytogenes*. [Kawai and Akira, 2011] AIM2 contains a PYD and HIN-200 DNA-binding domain that binds microbial dsDNA. Upon binding of dsDNA, AIM2 is able to form an inflammasome together with ASC and this triggers the maturation of IL-1β. [Kawai and Akira, 2010]. Further overlap in ligand recognition between the two groups of receptors can be found in table 2.

3.3 RIG-I like receptors

A different class of intracellular PRRs that sense viral ligands are the retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs). The RLRs form a family of three cytosolic PRRs: RIG-I, Melanoma differentiation-associated gene 5 (Mda5) and laboratory of genetics and physiology 2 (LGP2) **(Figure 8)**.

RIG-I, the first member of the RLR family identified, has two characteristic CARD domains that are responsible for the downstream signaling at the N-terminus. The helicase domain is located at the C-terminus and is responsible for the specific recognition of intracellular viral RNA. [Yoneyama and Fuijata, 2009] Upon recognition, RIG-I induces an antiviral response via the induction of type 1 IFN. [Loo and Gale, 2011][Yoneyama and Fuijata, 2009][Kawai and Akira, 2010][Kawai and Akira, 2011]

Mda5 is another RLR that shares high homology with the N-terminal domain of RIG-I. [Yoneyama and Fuijata, 2009] The C-terminal domain of Mda5 does not contain similar sequences. Therefore, both receptors detect different types of viruses, which can be explained by the preferential RNA structure recognition. [Yoneyama and Fuijata, 2009]

The third member of the RLR family, LPG2, shows 41% and 31% amino acid similarity to the helicase domain of RIG-I and Mda-5, respectively. [Yoneyama and Fuijata, 2009] Strikingly, LPG2 completely lack the N-terminal CARD. In vitro studies indicate that LPG2 negatively regulates RIG-I and Mda5. [Loo and Gale, 2011] However, a recent study using KO LPG2 in encephalomyocarditis virus infection experiments indicates a positive regulatory function for LPG2. Further studies are needed to elucidate this mechanism. [Yoneyama and Fuijata, 2009]

RLRs are found to respond to the same ligands as TLR3/7/8. These TLRs and RLRs both recognize viral RNA of VSV, influenza A and Newcastle disease virus. However, TLR7 expressed in plasmacytoid DCs induce type 1 IFN secretion. RIG-I receptors, on the other hand, are expressed in multiple cell types including conventional DCs, macrophages and fibroblasts where they induce the production of type 1 IFN upon recognition of viral DNA. This induction of type 1 IFN in these specific cell types is found to be critical for the regulation of the adaptive immune response. [Yoneyama and Fuijata, 2009][Kawai and Akira, 2011] Furthermore, Mda5 is found to respond to the same ligand as TLR3, poly(I:C), and induce antiviral responses. The interplay between RIG-I and TLR3 leads to the recognition of hepatitis C virus by both receptors. [Kawai and Akira, 2011] Besides,

recognizing the same ligands as TLRs, RLRs also interact with NLRC5, which leads to inhibition of type 1 IFN production. [Loo and Gale, 2011]

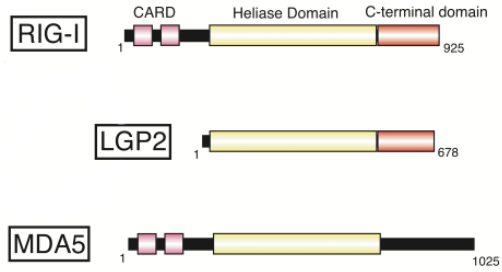

Figure 8. RLRs domains. The RIG-I like receptor family has three members, RIG-I, LPG2 and Mda5. These receptors are assembled of caspase recruitment domains (CARDs) at the N terminal domain, a helicase domain and a C-terminal domain. [Yoneyama and Fuijata, 2009] Adapted from Yoneyama and Fuijata (2009).

3.4 STING and DAI

TLR9 is an intracellular Toll-like receptor that senses CpG rich bacterial DNA and this receptor is located on the endosomal compartments. Other DNA cytosolic DNA sensors have been identified and include the stimulator of interferon genes (STING) and the putative cytosolic DNA-dependent activator of IFN-regulatory factors (DAI). Upon activation by microbial DNA, the receptors STING and DAI induce the secretion of type 1 IFN. [Yoneyama and Fuijata, 2009][Kawai and Akira, 2010][Kawai and Akira, 2011]

STING is critical for the induction of type 1 IFN by non-CpG containing DNA originating from intracellular microbes. [Ishikawa, 2009] Therefore, it can recognize DNA microbial species that cannot be recognized by TLR9. STING is required to induce appropriate innate immune responses via the induction of type 1 IFN in antigen presenting cells and murine embryonic fibroblasts infected with HSV-1 and *L. monocytogenes*. [Ishikawa, 2009]

STING is also found to interact with another intracellular PRR, namely RIG-I. During this crosstalk it functions as an RLR signaling cofactor, and it broadens the innate immune response against RNA and DNA viruses as an essential signaling adaptor protein. [Loo and Gale, 2011]

Another cytosolic dsDNA sensor is DAI. It is found that DAI-deficient mice are still capable of inducing type 1 IFN in response to dsDNA. This suggests redundancy with uncharacterized cytosolic DNA sensors and more research needs to be done to elucidate other possible DNA recognizing receptors. [Yoneyama and Fuijata ,2009][Kawai and Akira, 2010]

4. Intracellular killing through xenophagy

4.1 Autophagy

Autophagy is an essential intracellular degradation process used to breakdown cellular constituents to maintain cellular homeostasis and the quality of organelles and proteins. [Franchi et. al., 2008][Travassos, Philpott, 2010][Levine, Mizushima and Virgin, 2011] Cellular organelles, proteins and macromolecular aggregates that are too large to be degraded by proteasomes, can be degraded via this lysosomal degradation pathway. [Delgado, 2009][Levine, Mizushima and Virgin, 2011] This process is evolutionary conserved and is found in all eukaryotes. [Travassos, 2010] Furthermore, it is observed in a large range of cell types like epithelial cells, murine embryonic fibroblasts and professional phagocytes. It can be induced by physiological stimuli, like starvation or growth factor deprivation that leads to natural inhibition of mammalian target of rapamycin (mTor). In addition, it can also be induced by chemicals, such as rapamycin that inhibits mTor, or by immunological stimuli, like IFN-γ and TNF-α. [Delgado, 2009][Virgin and Levine, 2009] The Tor-ATG1 system transduces growth, nutritional and some stress signals to initiate autophagy. Beclin 1-hVps34 represents another regulatory system that reacts to stress. [Deretic and Levine, 2009]

There are three distinct types of autophagy described in literature: chaperone-mediated autophagy (CMA), micro-autophagy and macro-autophagy **(Figure 9)**. Macro-autophagy, the classical form of autophagy and the main focus of this thesis as it was recently described to be involved in microbial clearance as well, is a process in which typically large portions of the cytoplasm or organelles, such as mitochondria or peroxisomes, are engulfed by a primary isolation membrane or phagophore **(Figure 9A)**. [Delgado, 2009][Levine, Mizushima and Virgin, 2011] This isolation membrane encloses the cargo and gives rise to a double membrane structure called the autophagosome. [Delgado, 2009][Virgin and Levine, 2009] The outer membrane fuses with a lysosome, an endosome or multivesicular body, forming an autolysosomal structure. The inner membrane dissolves and the acidic environment containing lysosomal hydrolases degrades the contents of the autolysosomes. [Virgin and Levine, 2009][Delgado, 2009]

The origin of the paghophore membrane is not known with certainty, but research indicates that the ER has a crucial role in the induction and formation of phagophores and autophagosomes. Recent studies also identified compartments such as the Golgi-apparatus, mitochondria and the plasma and nuclear membrane as a source for phagophore membrane formation. [Deretic and Levine, 2009][Levine, Mizushima and Virgin, 2011]

The induction and maturation of macro-autophagy is regulated by complex signaling cascades. Autophagy-related (ATG) genes, a lipid kinase signaling complex and ubiquitin-like conjugation systems regulate this autophagy pathway **(Figure 9B)**. Over 30 ATG genes have been identified in yeast, and human homologues are still being defined. [Munz, 2009][Levine, Mizushima and Virgin, 2011]

The first step during the initiation of autophagy is the formation of the isolation membrane followed up by the elongation. A lipid kinase signaling complex of class III phosphatidylinositol-3-OH kinase (PI3K) or Vps34 and ATG6/Beclin-1 initiates this step. Beclin-1, an autophagy-inducing factor, and Vsp34 control this process via activating or inhibiting two ubiquitin-like conjugation systems, thereby providing positive and negative regulation of autophagy. [Virgin and Levine, 2009][Munz, 2009]

The ubiquitin-like conjugation systems are important in the elongation of the isolation membrane and the closure of the autophagosome. [Deretic and Levine, 2009][Munz, 2009] This system couples ATG8, its human homologue LC3, to phosphatidylethanolamine (PE) on the outside and inside of the isolation membrane. Prior to this step, ATG8 is processed by the cytosolic protease ATG4. [Delagdo, 2009][Munz, 2009] ATG8 is then activated by the E1-like enzyme ATG7 and conjugated to PE by the E2-like enzyme ATG3. [Munz, 2009][Knodler and

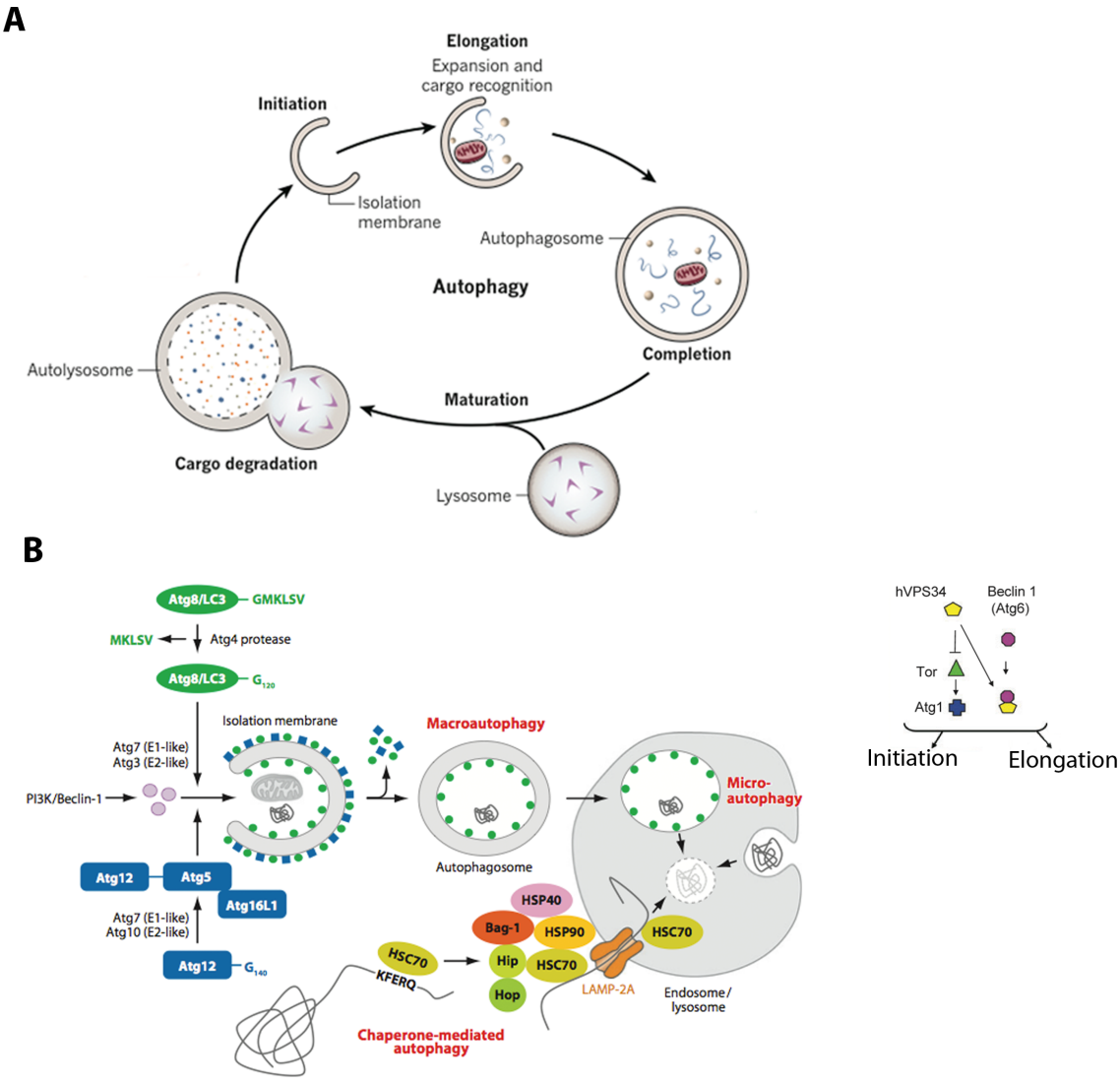

Figure 9. Autophagy delivers cytoplasmic constituents for lysosomal degradation and a schematic overview of the autophagy pathway. A) The first phase of macro-autophagy is the initiation of an isolation membrane or phagophore. This is followed up by elongation and completion of the autophagosome. The autophagosome fuses with a lysosome and the cargo is degraded within the autolysosome. [Delgado, 2009][Virgin and Levine, 2009][Levine, Mizushima and Virgin, 2011] **B)** The delivery is mediated through three distinct types of autophagy: chaperone-mediated autophagy (CMA), micro-autophagy and macro-autophagy. CMA imports cytosolic macromolecules tagged by a signal motif directly into lysosomes. HSC70 chaperones recognize the signal motif and unfold the substrate before it docks it to the membrane of endosomes or lysosomes with help of the co-chaperones HSP90, HSP40, Bag-1, Hip and Hop. The lysosome associated membrane protein (LAMP)-2A functions as a translocation channel to facilitate the entry of the chaperone-bound substrate into endosomes or lysosomes. Micro-autophagy is not well documented in higher eukaryotes. During this process in yeast the lysosomal or vacuolar membrane buds into the lysosomal lumen containing cytoplasmic material in an autophagic body. Upon which the lysosomal hydrolases degrade the content of the autophagic body. [Munz, 2009][Virgin and Levine, 2009][Levine, Mizushima and Virgin, 2011] ATG genes like ATG6/Beclin-1, together with a lipid kinase signaling complex (PI3K or Vps34) and ubiquitin-like conjugation systems regulate macro-autophagy. [Virgin and Levine, 2009][Munz, 2009] Adapted from Münz (2009) and from Levine, Mizushima and Virgin (2011).

Celli, 2011][Levine, Mizushima and Virgin, 2011] Fluorescent protein-tagged ATG8/LC3 are cellular markers used to visualize the autophagosome and these markers are widely used in autophagy research. ATG7 E1-like and ATG10 E2-like enzymes form the second ubiquitin-like conjugation system. These enzymes ligate ATG12 to ATG5, which then forms a complex with ATG16L on the outer membrane. This complex disintegrates from the outer membrane upon closure of the autophagosome. [Munz, 2009][Knodler and Celli, 2011][Levine, Mizushima and Virgin, 2011] The autophagosome is now fully developed and ready to fuse with a lysosome, endosome or multi-vesicular body, thereby degrading its content.

4.2 Xenophagy: autophagy in innate immunity

Autophagy has a crucial role in the homeostasis of the cell. During starvation conditions it can induce non-selective uptake of cytoplasmic material or organelles, ensuring cellular survival by immediate energy generation. Recently, it has been demonstrated that autophagy also has an important role in the elimination of intracellular microbes or their products, in which case it is referred to as xenophagy. Autophagy is both a regulator and an effector of PRR responses against microbes. [Deretic and Levine, 2009] It enables the direct elimination of cytosolic microbes via the uptake by autolysosomes and those microbes that are taken up in a phagosome can be targeted for degradation via enhanced fusion of phagosomes with autophagosomes. [Munz, 2009] Studies have identified that bacteria, parasites and viral microbes are targeted for autophagy. The focus of this thesis will be on the mechanisms of microbial clearance by autophagy and how microbes are recognized for targeting by autophagy. Examples of bacteria that are targeted for autophagy are: group A *Streptococcus*, *M. tuberculosis*, *S. flexneri*, and *S. typhimurium*. [Virgin and Levine, 2009] Autophagy does not only serve to eliminate microbes, but it further acts as an effector mechanism by facilitating the delivery of cytosolic microbial products to PRRs, such as TLRs and NLRs, which induce the activation of the innate immune system and provides appropriate antimicrobial responses. [Delgado, 2009][Virgin and Levine, 2009]

4.3 Clearance of intracellular microbes by autophagy

The initiation of an intracellular killing mechanism, called autophagy, can lead to the direct elimination of intracellular microbes. Intracellular microbes that freely move around the cytosol can be selectively targeted for degradation via engulfment by structures comparable to autophagosomes **(Figure 10A)**. These structures will fuse with lysosomes upon maturation. [Virgin and Levine, 2009][Levine, Mizushima and Virgin, 2011] An example of an intracellular microbe that is targeted for autophagic degradation is Group A *Streptococcus*. This bacterium escapes from the endosome of non-phagocytic cells via the secretion of a hemolytic toxin streptolysin O. [Nakagawa et. al., 2004][Munz, 2009] Once escaped from the endosome, it resides free in the cytosol. The *Streptococcus* bacteria are targeted for degradation via the formation of ATG8/LC3-positive autophagosomes that engulf the bacteria and provide degradation after fusion with lysosomes. [Nakagawa, 2004][Munz, 2009]

In addition to degradation of free cytosolic microbes, microbes located in phagosomes are also targeted for degradation via autophagy **(Figure 10A)**. An example of a pathogen located in phagosomes when targeted for autophagy is *M. tuberculosis*. Macrophages are primarily infected and it is the causative agent of tuberculosis (Tb). [Gerold, 2007][Munz, 2009] Initially, Tb is primarily a local disease and infects the lung. It is acquired by inhalation of *M. tuberculosis* in aerosols and dust particles. [Kuby] After inhalation, alveolar macrophages internalize *M. tuberculosis* via phagocytosis and the bacterium is capable of surviving and replicating within phagosomal structures by inhibiting the fusion with lysosomes. [Parham] By this means, it provides itself a protective niche to replicate and hide from the immune system.

M. tuberculosis is targeted for autophagy by the activation of macrophages with IFN-ϒ. This leads to enhanced fusion of autophagosomes with phagosomes containing the bacterium. [Munz, 2009] Also exposure to ATP induces autophagy in human macrophages and this is associated with a decrease in *Mycobacterium bovis* BCG viability within infected cells **(Figure 10B)**. [Biswas, 2008]

Furthermore, microbes are also able to escape phagosomes and become cytosolic. These microbes are directly sequestered into autophagosomes, and colocalize with the autophagy marker LC3 **(Figure 10A)**. [Gutierrez, 2004][Travassos, 2010][Levine, Mizushima and Virgin, 2011] *S. flexneri* is a bacterium that is capable of escaping the phagosome and once it is cytosolic it is targeted for autophagy. [Delgado, 2009] By secreting an intracellular motility-associated protein IscB, *S. flexneri* is capable of evading autophagy. IcsB competitively binds to VirG thereby preventing the interaction between ATG5 and VirG, which is a bacterial surface protein required for actin-based motility and targeting *S. flexneri* towards the autophagosome. [Levine, Mizushima and Virgin, 2011]

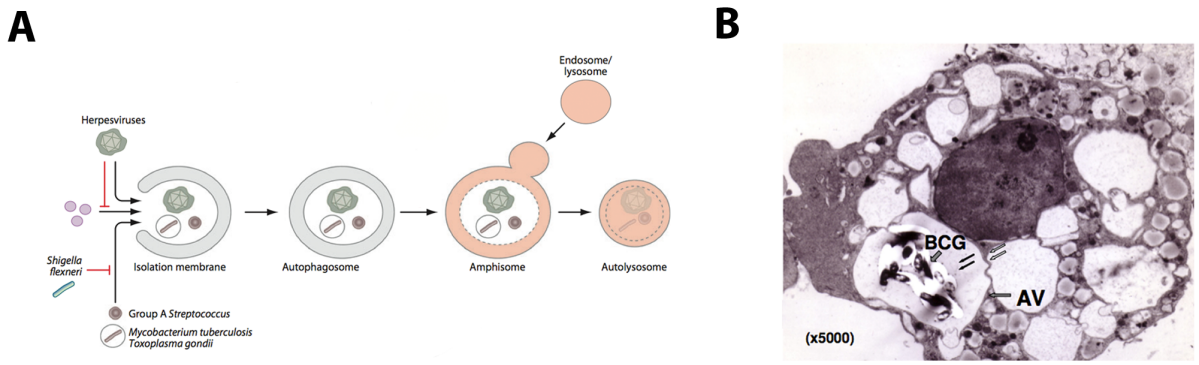

Figure 10. Autophagy mediated microbial uptake. Autophagy can directly mediate the clearance of intracellular microbes via engulfment of free cytosolic microbes and microbial phagosomes by an autophagosome. Autophagy activation during infection depends on the microbe and the route of cell infection. [Virgin and Levine, 2009][Levine, Mizushima and Virgin, 2011] **A)** Both free cytosolic pathogens such as group A *Streptococcus* and microbial phagosomes containing *M. tuberculosis* are delivered to autophagosomes for lysosomal degradation. *S. flexneri* prevents its engulfment and herpesvirus inhibits autophagosome formation. [Nakagawa et. al., 2004][Munz, 2009] **B)** Localization of *M. tuberculosis* and autophagic vacuoles. Upon treatment with ATP the bacteria localize with autophagic vacuoles build up of the inner (double black arrows) and outer membrane (double white arrows). [Biswas, 2008][Munz, 2009] 10A Adapted from Munz (2009) and 10B adapted from Biswas (2009).

4.4 Autophagy induction and recognition of microbes

The initiation of autophagy-mediated killing of intracellular microbes can be induced via a variety of different mechanisms. Protein aggregates originating from microbes, immune-related signalling molecules, or the recognition of PAMPs by PRRs are responsible for the induction of autophagy.

Polyubiquitinated protein aggregates can be recognized and targeted for degradation by autophagy via binding to the polyubiquitin-binding protein p62. p62 is known to serve as an ubiquitin receptor and binds ubiquitinated protein aggregates via its ubiquitin associated domain. [Bjørkøy et. al., 2005][Zheng et. al., 2009][Knodler and Celli, 2011] In addition, p62 directly binds the autophagic marker ATG8/LC3, via its LC3 interaction region and it is observed that p62 and LC3 positive bodies are degraded in autolysosomes. [Pankiv et. al., 2007][Hussey, Travassos and Jones, 2009] Therefore, p62 has been implicated in targeting polyubiquitinated protein aggregates to the autophagosomes. [Munz, 2009] Zheng et. al. (2009) observed the recruitment of p62 to polyubiquitinated protein aggregates on the pathogen *S. typhimurium*. This facilitated the degradation by autophagy via autophagosome formation and p62 expression is required for efficient degradation by

autophagy of this bacterium. Thereby, a novel role for p62 in innate immunity is described. [Zheng et. al., 2009]

Other mechanisms that induce the intracellular killing mechanism autophagy are immune-related signaling molecules, such as IFN-Ɣ and TNF-α, and DAMPs such as ROS and ATP. [Deretic and Levine, 2009][Levine, Mizushima and Virgin, 2011] In turn, DAMPs stimulate NLRs that will form inflammasomes involved in the cleavage of pro-IL1β into active IL-1β that in turn stimulates autophagy. [Deretic and Levine, 2009]

The importance of autophagy and its role in immunity and microbial clearance is underscored by the finding that autophagy is induced by different families of PRRs. [Deretic and Levine, 2009][Levine, Mizushima and Virgin, 2011] It has recently been demonstrated that stimulation and downstream signaling of TLRs activates autophagy and initiates the direct killing of intracellular microbes. The first indications of this process were demonstrated for TLR4. Stimulation of a murine macrophage cell line and primary human macrophages with lipopolysaccharide (LPS), a component of the bacterial cell wall and a TLR4 ligand, induced autophagy via downstream signaling of TLR4. [Xu, 2007] Likewise, the stimulation of TLR7 by ssRNA ligand induced the formation of autophagosomes. [Delgado, 2008] Activation of TLR4 with LPS and activation of TLR7 with ssRNA were observed to induce the co-localization of *M. tuberculosis* with autophagosomes and decreased the survival of the bacterium. [Xu, 2007][Delgado, 2008][Virgin and Levine, 2009]

In addition, the ligand that stimulates TLR3, dsRNA, induces autophagy. [Delgado, 2008] Autophagy induction via the stimulation of TLR9 with CpG remains controversial. Delgado et. al. (2008), described that this TLR9 ligand does not induce autophagy. However, this finding is opposed by Sanjuan et al. (2007) who showed the opposite result. [Sanjuan et. al., 2007] More research is needed to resolve this discrepancy.

In addition to TLRs, the Nod-like receptors were also found to influence intracellular killing by autophagy. As described above, NLRs are cytoplasmic proteins that are ideally located in the cytoplasm to sense cytosolic microbial products. Ipaf is a member of the NLR family activated by flagelling and virulence factors secreted through T3SS and T4SS secretion systems. Ipaf is known to be part of the multi-protein inflammasome complex that mediates caspase-1 activation and IL-1β processing. Observations made by Franchi et. al. (2009) indicate a role for Ipaf in autophagy, which was identified by *S. flexneri* infection studies. [Franchi, 2009] Franchi et. al. (2009) observed that during infection of macrophages with *S. flexneri* autophagy is inhibited, and this was dependent on the presence of Ipaf and caspase-1. Ipaf detects *S. flexneri* in a flagellin-indepent manner, since *S. flexneri* does not express flagellin. [Franchi, 2009] Cell death of *Shigella*-infected macrophages is increased in the presence of Ipaf and caspase-1, leading to inhibition of autophagy. [Suzuki and Nunez, 2008][Franchi, 2008] As mentioned above *S. flexneri* is able to evade autophagy via the secretion of bacterial virulence factors IscB and VirG. [Franchi, 2008][Levine, Mizushima and Virgin, 2011] By preventing autophagy, cell death is induced according to the results from Suzuki and Nunez (2008). However, this is a controversial result as the protective and replicative niche of the bacterium is destroyed due to the induction of cell death. This could be detrimental to the survival of this pathogen and one could argue that a pathogen would prevent this ratter than stimulating the degradation of its protective niche via cell death. More research is needed to resolve this controversy.

Since Ipaf showed to be involved in the recognition of bacteria and the induction of autophagy, research has since extended to other members of the NLR family. This led to the discovery that also NOD1 and NOD2 can induce autophagy **(Figure 11)**. NOD1 and NOD2, activated by iE-DAP and MDP, respectively, recruit the key autophagy regulator protein ATG16L1 to the plasma membrane at the bacterial entry site. In the case of *S. flexneri*, NOD1 and NOD2 recognize the bacterium, upon which the interaction of ATG8 or LC3 is enhanced. [Travassos, 2010]

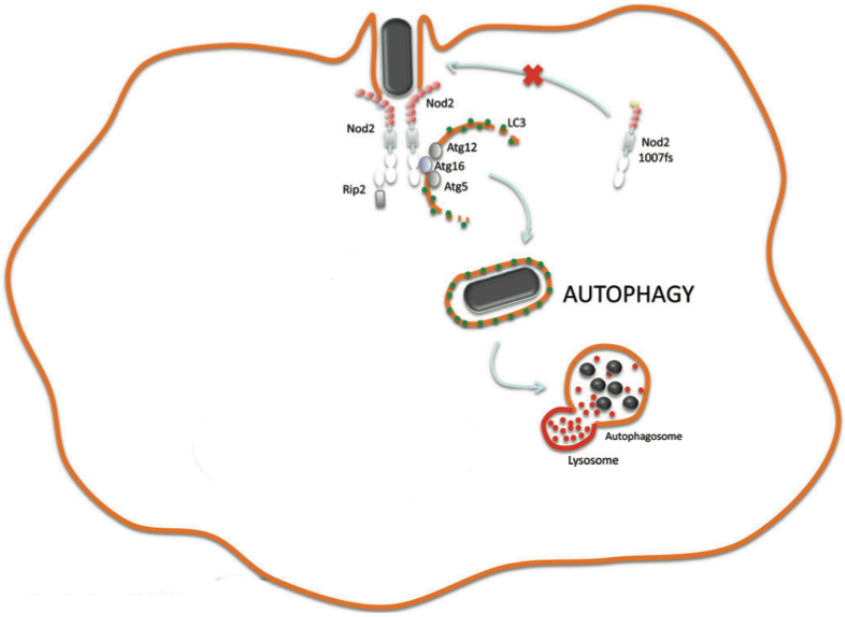

Figure 11. NOD2 induces autophagy via recruiting ATG16L to the plasma membrane. NOD2 is a member of the Nod-like receptor family and is capable of inducing autophagy upon recognition of muramyl dipeptide originating from *S. flexneri*. Upon bacterial invasion, NOD2 is recruited to the entry site to induce an early autophagic response by recruiting ATG16L. [Travassos, 2010] Adapted from Travassos, Philpott (2010).

4.5 Delivery of PAMPs to PRRs via autophagy

As mentioned above, free cytosolic or phagocytosed microbes can be taken up by autolysosomes. In addition, autophagy can also induce the presentation of intracellular PAMPs to PRRs located in endosomal compartments. Autophagosomes containing PAMPs fuse with endosomes upon maturation. This facilitates the presentation of PAMPs to PRRs, like TLR3/7/9. For example, recognition of cytosolic intermediates of replicating VSV by TLR7 is mediated by autophagy, via the delivery of ssRNA to the endosomal compartment. This leads to the secretion of type I IFN in plasmacytoid DCs. [Delgado, 2009][Virgin and Levine, 2009][Deretic and Levine, 2009]

5. Discussion

Innate immune responses against intracellular microbes are initiated via recognition by highly conserved PRRs. These PRRs have the capacity to discriminate between self and non-self, thereby eliciting only antimicrobial responses when needed during microbial infection. Extracellular microbes can be sensed prior to their entrance. However, some microbes can transmigrate and become intracellular. Then, different mechanisms are required that sense the microbe and elicit appropriate immune responses essential for their elimination. Many intracellular PRRs have been identified that recognize microbes that have crossed the cell membrane and escaped the extracellular recognition mechanisms.

The TLRs were the first PRR family to be identified with members located on intracellular surfaces. TLR3/7/8/9 are located on endosomal membranes and recognize a wide range of PAMPs, including dsRNA, ssRNA and DNA containing CpG motifs. The second identified, and largest, family of PRRs are the NLRs. These NLRs differ from TLRs because they are cytosolic soluble proteins that survey the cytoplasm for PAMPs and DAMPs instead of being membrane bound. This could serve as an extra surveillance system for microbes that escape the phagosomal compartments and become cytosolic. Furthermore, the recognition of DAMPs by NLRs is crucial for the ability of the immune system to distinguish between pathogenic microorganism and commensal or non-pathogenic microorganisms. This additional feature of NLRs distinguishes them from TLR-mediated immune responses and contributes to a more effective immune system. Moreover, some members of the NLR family like NALP1, NALP3 and IPAF are able to form multi-protein complexes called inflammasomes. These inflammasomes mediate the maturation of IL-1β and IL-18, cytokines that would otherwise not be upregulated and that are important in the elimination of specific microbes.

In addition to TLRs and NLRs, another class of intracellular PRRs was identified that sense intracellular viral ligands, namely the RLRs. This small family, consisting of three members, are expressed in a broad range of different tissues including non-immune cells. The expression of RLRs in non-immune cells is important in the detection of microbes at the primary site of infection. Like NLRs, the RLRs are able to sense cytosolic viral ligands and induce anti-viral immune responses required for microbial clearance and for the initiation of the adaptive immune response. NLRs and RLRs contribute to the elimination of microbes once they have become cytosolic. Either upon entry of the cytosol or upon escape from the phagosome. In this manner, cells are able to sense microbial infections in both compartments.

STING and DAI are intracellular sensors that sense dsDNA, just as TLR9. Unlike TLR9, that only recognizes DNA containing CpG motifs, STING is capable of recognizing microbial DNA that does not contain CpG motifs. This means, that STING contributes to the recognition of a wider spectrum of microbial species, while still being capable of discriminating between self and non-self. Of DAI it is less clear what the specific function is. Studies with DAI-deficient mice indicate that there is redundancy since the mice were still capable of inducing type 1 IFN. This redundancy could be beneficiary since highly conserved structures are simultaneously sensed by different receptors in different compartments. This increases the efficiency in sensing intracellular microbes and eliciting the right immune responses. More research is needed to identify uncharacterized cytosolic DNA sensors involved in the redundancy with DAI and the characteristic function of this receptor.

Up till now, a large variety of intracellular receptors involved in microbial recognition have been identified. In addition to their specific induction of responses, an increasing amount of evidence demonstrates that PRR families also respond to the same

ligands, which influences the outcome of the induced immune responses via enhancement or inhibition. NLRs and RLRs are found to recognize the same bacterial or viral PAMPs as TLRs. This leads to enhanced or decreased production of inflammatory cytokines and this stimulates or inhibits the induced immune responses. Since it is already known that PRR families can respond to the same ligand, it would be highly interesting to determine whether PRRs can directly or indirectly influence each other. Either via direct binding or via the secretion of signaling proteins that enhances or inhibits the response against a specific ligand. This network of interacting PRRs might be crucial in the regulation of the immune response by enhancing the response during infection and dampening it when the infection is cleared. Furthermore, it would be interesting to identify new members within known PRR families or entirely new classes of PRR families to increase our knowledge on the complex networks making up the innate immune system.

Innate immunity thus has a set of PRRs families that recognize a wide range of microbial ligands. Crosstalk between the different PRRs influences the response outcome, thereby even diversifying the process. Therefore, the innate immune responses elicited upon recognition by these few PRRs are very effective to a large number of microbes. Compared to the adaptive immune system, the innate immune system is not microbe specific since it does not rely on microbe specific B and T cells. However, the innate immune system makes the responses against extracellular and intracellular microbes 'specific' via the recognition of conserved regions or PAMPs. In most cases, the innate immune response is sufficient to eliminate microbes and most infections remain unnoticed.

An interesting and recent discovery with implications on microbial killing by innate immunity was that the intracellular degradation process autophagy is also involved in the elimination of intracellular microbes. Autophagy was already known as an essential intracellular degradation process used to maintain cellular homeostasis via the degradation of cellular constituents like cell organelles, proteins and macromolecular aggregates. Only recently, research has elucidated that this degradation process is also very important in the elimination and breakdown of intracellular microbes. Autophagy is conserved through evolution, and is found in all eukaryotes and in a wide range of cell types. This could implicate that already at the beginning of evolution autophagy played an important role in the elimination of these intracellular microbes. This emphasizes the importance of this intracellular killing mechanism, since it is conserved throughout evolution and preserved in higher eukaryotes.

Interestingly, different families of PRRs are identified to induce autophagy and thereby enhance the clearance of microbes. Therefore, autophagy has a significant role in innate immunity and microbial clearance. Autophagy assists in the elimination of microbes that escaped the initial killing mechanism via phagocytosis. Furthermore, it can enhance the presentation of PAMPs to PRRs thereby inducing the appropriate immune response. More research needs to be done to identify other possible PRRs, thereby finding more links between the innate immune system and autophagy mediated intracellular killing.

As described above, PRRs are able to interact with each other via crosstalk between the receptors. In this manner, PRRs responding to the same ligand can influence the outcome of the immune responses. An example is the recognition of flagellin, which is a TLR5 and an Ipaf ligand. Simultaneous recognition leads to enhanced production of inflammatory cytokines. Autophagy is known to be involved in the clearance of microbial infection and can be initiated via the recognition of microbes by PRRs. Therefore, it would be interesting to investigate whether PRR crosstalk also influences autophagy regulation, induction or outcome. As such, it would be fascinating to stimulate different PRRs known to induce autophagy simultaneously by using a combination of ligands acting on different PRRs. By this means, we could elucidate their effect on each other, either via inhibitory or

enhancing responses, and their effect on autophagy. After all, during infection several PAMPs and DAMPs are present and sensed by PRRs and as some PRRs have been implicated in autophagy, it is likely that other type of immune receptors can perform similar functions. Therefore, more research is needed to identify other receptors capable of inducing autophagy. For example, G protein coupled receptors or C-type lectins.

As mentioned above, autophagy is observed in a large range of cell types like epithelial cells, murine embryonic fibroblasts and professional phagocytes. Non-immune cells are often the site of primary infection and can serve as a protective replicating niche. However, microbes can be sensed by PRRs expressed in these cell types and upon recognition appropriate immune responses and autophagy are induced. Since, non-immune cells are not capable of clearing microbial infection via the induction of phagocytosis and professional phagocytes are not directly present at the site of infection, it is important that autophagy assists in the elimination of intracellular microbes. This emphasizes the significance of this highly conserved killing mechanism in non-phagocytic cell types. Therefore, it is particularly interesting to understand the exact mechanisms of microbial recognition, initiation of autophagy and microbial clearance at the site of primary infection. To understand this process in more detail, more research needs to be done on the role of PRRs in the induction of autophagy in primary non-immune cells.

Nowadays, antimicrobial treatment strategies, like antibiotics, have become increasingly difficult due to the emergence of highly resistant strains. To circumvent this problem, alternative strategies need to be developed to treat microbial infections. As it is now understood that autophagy is also implicated in the clearance of microbes, it could be valuable to investigate its potential as an alternative antimicrobial treatment. In the case of *M. tuberculosis*, it has been observed that the induction of autophagy could be an alternative for the treatment of Tb. Stimulation of autophagy induces an enhanced degradation of *M. tuberculosis*-containing phagosomes. [Biswas, 2008][Munz, 2009] This leads to the destruction of the protective niche of this bacterium, upon which the survival is markedly decreased. Thus, activation of autophagy could lead to better treatment strategies against highly resistant strains of *M. tuberculosis*.

Another concept that needs to be considered here is that although autophagy can be detrimental to pathogens, its induction can sometimes also be beneficial to pathogen survival. Some microbes have evolved mechanisms to evade or even exploit the autophagosomal elimination pathways. For example, they are able to antagonize autophagy initiation or autophagosomal maturation, evade autophagic recognition, or use components of autophagy regulation for their own survival or replication. A hypothesis is that when fusion with a lysosomal compartment is prevented, the autophagosome serves as a protective niche and/or serves as a source of nutrition for the autophagocytosed pathogens. Before regarding autophagy as a valid alternative in the treatment of microbial infections, we need to fully understand its role during specific infections to be able to exclude the possibility that autophagy induction is in favor of the pathogen.

To conclude, the innate immune system is able to recognize extracellular and intracellular microbes and is crucial in the survival of an individual. Its characteristic feature is that it can distinguish between self and non-self via the recognition of highly conserved PAMPs. The recognition of PAMPs is mediated by PRRs, which are either located on extracellular surfaces, intracellular membranes or in the cytosol. Upon recognition immune responses are induced leading to microbial clearance. In addition, PRRs are also capable of inducing the highly conserved intracellular killing mechanism autophagy that leads to autophagy-mediated clearance. Interesting aspects are which mediators recognize intracellular microbes, how this leads to the induction of immune responses, autophagy and microbial clearance.

6. References

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