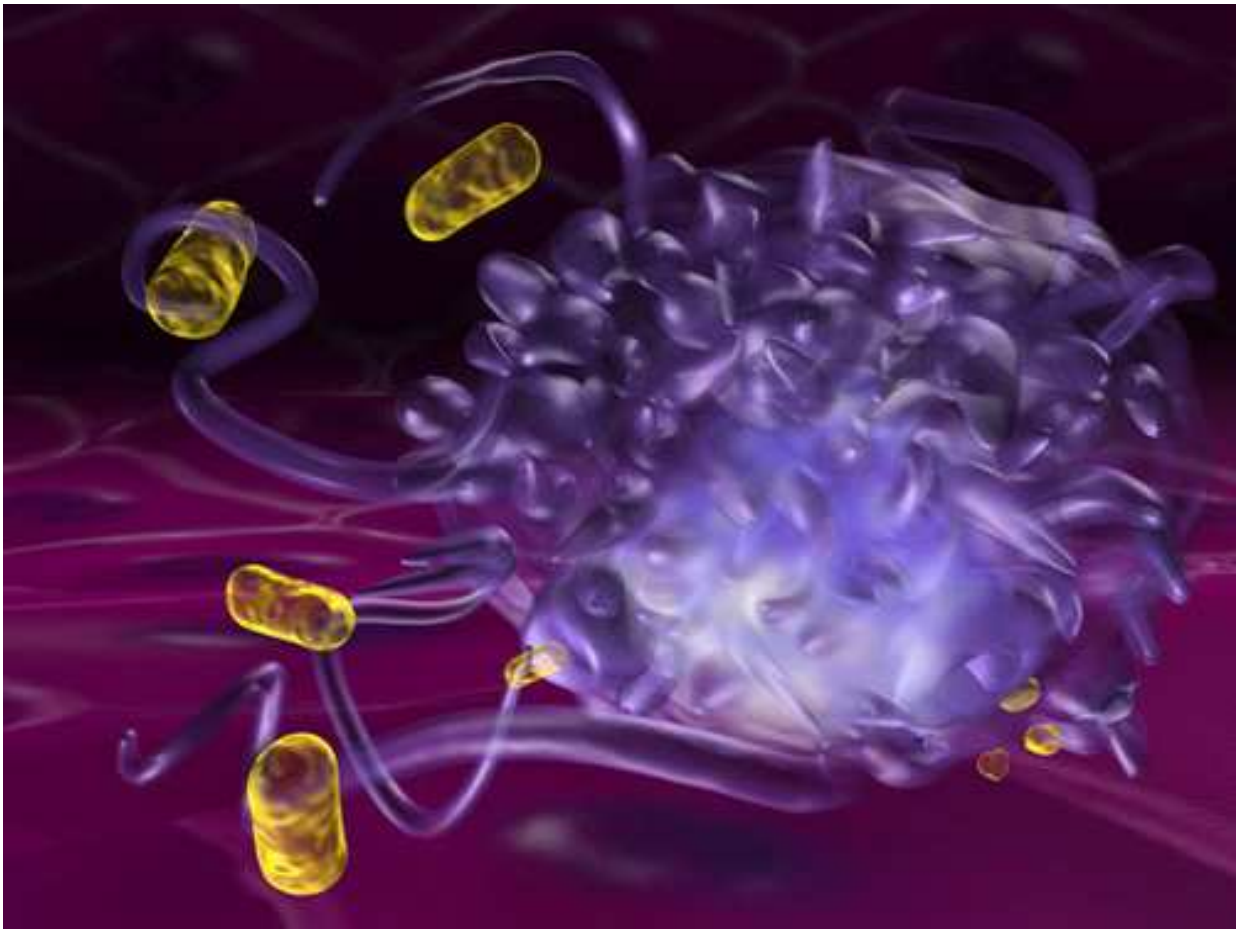
Antiphagocytosis

A bacterial mechanism to inhibit phagocytosis



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Outline

Abbreviations

ABC transporter	ATP binding cassette transporter
CR3	Complement receptor 3
DC	Dendritic cell
ECM	Extracellular matrix
EPEC	Enteropathogenic *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
GAP	GTPase activating protein
GEF	Guanine exchange factor
IgG	Immunoglobulin G
ITAM	Immunoreceptor tyrosine-based activation motif
MBL	Mannose binding lectin
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
PAI	Pathogenicity island
PAMP	Pathogen associated molecular pattern
PIP_2	Phosphatidylinositol-4,5-biphosphate
PIP_3	Phosphatidylinositol-3,4,5-biphosphate
PRR	Pattern recognition receptors
STEC	Shiga toxin-producing *Escherichia coli*
T1SS	Type I secretion system
T2SS	Type II secretion system
T3SS	Type III secretion system
T4SS	Type IV secretion system
T5SS	Type V secretion system
T6SS	Type VI secretion system
T7SS	Type VII secretion system
TLR	Toll-like receptor
UPEC	Uropathogenic *Escherichia coli*
Yop	*Yersinia* outer protein

Chapter 1: Phagocytosis

Elie Metchnikoff (1845-1916) received the nobel price (1908) for describing and naming the process of phagocytosis,[1,2,3] after the Greek words “phages” to eat and “cite” cell.[2] Phagocytosis is the internalization of cell material or whole pathogenic microbes from the environment[3] and is only fully effective when the microbes are degraded after the internalization.[4]

Professional phagocytes possess many different surface receptors, signaling molecules and anti-bacterial systems. They are specialized in recognition, capture, phagocytosis, and killing of microbes. Conversely, other cells or non-professional phagocytes are not specialized for microbe phagocytosis and killing. Some bacteria are able to trigger these cells to start the phagocytic process to enter the cells.[5]

Professional phagocytosis

Macrophages, neutrophils and dendritic cells (DCs) are professional phagocytes[4,5] and are able to take up particles up to 0.5 µm.[4,6] Phagocytosis can be triggered by direct recognition or indirect recognition of the microbes.[4]

Direct recognition

Direct recognition of the microbe surface triggers many processes of the phagocytes of the innate immune system, DCs and macrophages.[7] By direct recognition the pattern recognition receptors (PRRs) of the phagocytes recognize the pathogen-associated molecular patterns (PAMPs) on the bacteria surface.[3,7] Although, it is strongly believed that lipid raft also plays an important role in the direct recognition.[6] Thus PAMP recognition by the PRRs results in the activation of the Rho family of small GTPases, RhoA, Rac, and Cdc42, which leads to actin remodeling, cytoskeletal rearrangements and phagocytosis. Besides the phagocytosis, proinflammatory cytokines and chemokines are secreted and the microbal killing mechanisms becomes activated.[1,7]

Indirect recognition

Indirect microbe recognition involves opsonins. Opsonins can be part of the innate immune system, like components of the complement system,[3,4] mannose-binding lectin (MBL) and the C-reactive protein (CRP),[3] or part of the acquired immune system, like immunoglobulin G (IgG).[3,4] Opsonins of the innate immune system can, besides their function as opsonins, also kill the microbes directly by inducing cell lysis.[3] Phagocyte receptors recognize specific opsonins, for example the Fcγ receptor (RcγR) recognize the tail of IgG and complement receptors (e.g. CR3) by components of the complement system.[4] Once the RcγRs are activated, they cluster together. The intracellular part of the RcγR contains an immunoreceptor tyrosine-based activation motifs (ITAMs), which are cross phosphorylated upon clustering. ITAM phosphorylation provides a binding and activation place for the protein tyrosine kinase (Syk), which phosphorylates various other substrates.[4,6] Syk is required to trigger the phagocytosis after RcγR activation.[6]

Syc activates a guanine exchange factor (GEF) which activates members of the Rho family of small GTPases, like Rac1 and/or Rac2, and Cdc42 which are regulators of the rapid F-actin remodeling process.[4,6] Other adaptor proteins which are activated by Syc, like LAT, SLP-76, BLNK, Crkl, Nck and Fyb/SLAP, are likely to be involved in the activation process of the receptor complex. Syc also activates ARF6, a member if the ARF family of small GTPases. ARF6 targets Rac present near the plasma membrane. Rac1 and Cdc42 are activated during FcγR-mediated phagocytosis via the Syc activated pathway. Rho, which is also a member of the same family is activated during complement receptor 3 (CR3)-mediated phagoctytosis. Although, these pathways are not strictly separated. ROCK, a Rho-effector, activates LIM kinase 2, which phosphorylates together with LIM kinase 1, activated by Rac-effectors, cofilin. Cofilin leads also to actin remodeling and is important during phagocytosis.[6]

Phosphatidylinositol 3-kinase (PI3K), an enzyme which is involved in the membrane traffic events, is also activated by receptor activation. PI3K generates phosphoinositides at the side of the phagosomal cup;[6] phosphatidylinositol-4,5-biphosphate (PIP_2), which is involved in the initiation of the actin polymerization process, and phosphatidylinositol-3,4,5-biphosphate (PIP_3), regulates the pseudopod extension and the closure of the phagosome.[4] PIP_2 target phospholipase C, which is involved in the generation of diacylglycerol (DAG) during phagocytosis. DAG recruites and activates protein kinase C to the phagosomal side. Other kinases, involved in the FcγR-mediates phagocytosis, activate MEK1, ERK and protein kinase A. Phospholipase A_2 and D are most probably involved in vesicle trafficking during phagocytosis.[6]

Macrophages and dendritic cells kill the microbe after phagocytosis by leading it towards the death pathway ending at the lysosomes. The phagosomes contain soluble NSF attachment protein receptor (SNARE)-proteins. The vesicle SNARE-proteins recognize the target SNARE-proteins, and thereby they can lead the vesicles toward the lysosomes. After degradation, bacterial antigens are presented via MHC class II to activate the acquired immune system.[6] Neutrophils, however, kill bacteria by fusing the phagosome with antimicrobial enzymes.[8]

Macrophages

Macrophage precursors, small monocytes, are derived from the bone marrow and present in the blood. In the blood vessels the monocytes start to grow and mature. Then they migrate into the tissue and become large tissue specific macrophages,[3] or mononuclear phagocytes.[9] Macrophages of the gut are intestinal macrophages, alveolar macrophages are located in the lungs, histiocytes in the connective tissue, kupffer cells in the liver, mesangial cells in the kidney, microglial cells in the brain and osteoclasts are macrophages specific for the bone tissue.[3]

Macrophages are activated by the direct recognition of antigens or recognition of opsonins.[3] Receptors that trigger the phagocytosis are CR1, CR3, or CR4 (3), integrin β1 receptor,[10] Toll-like receptors (TLRs),[3] PRRs (recognize PAMPs), scavenger receptors, or mannose receptors,[5] and cytokine receptors.[3,9] Macrophages contain a CD14 receptor. This receptor is a co-receptor of TLR-4 for

activation by lipoplysaccharide (LPS), and triggers phagocytosis and the inflammatory process. CD14 is able to distinguish self from non-self, for the phagocytic triggering of CD14 by apoptotic cells does not lead into a cytokine storm.[11] The adhesion macrophage activation is further stimulated by T_H-activated excreted cytokines.[3]

Adhesion of microbial products results in the formation of pseudopodia. Fusion of these pseusdopodia,[3] leads to the characteristic zipper-like phagocytosis mechanism of macrophages (Figure 1A).[10] During phagocytosis the membrane surface of macrophages increases. Therefore, macrophages are able to engulf over 100% of ifs surface area.[6]

Classic macrophage (M1) activation is caused[9] by microbial products, like LPS and INFγ[12] and results in the release of reactive nitrogen and oxygen and the production of IL-12,[9] IL-1, 6, TNFα through the NF-κB pathway.[12] Alternative macrophage (M2) activation is the result of IL-4/IL-13 recognition, binding of immune complexes/MyD88, IL-10, IL-21, CSF-1; TGF-b/Activin; y Rays; bacteria, chronic virus, parasites, cancer. M2-activated macrophages have many scavenger, mannose, and galactose receptors on the surface. M1 and M2 macrophages correspondent with T_H1 and T_H2 cells activation and INFγ and IL-4 respectively.[9]

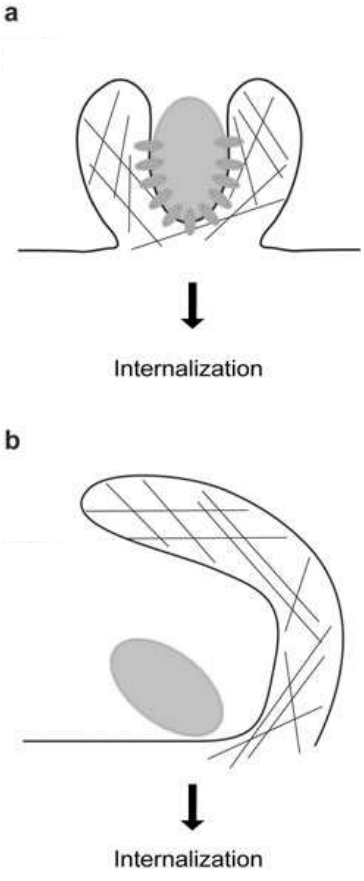

Figure 1 Phagocytosis mechanisms. **A.** Zipper-like mechanism of macrophages and neutrophils. **B.** ruffle like mechanism of dendritic cells.[10]

Neutrophils

Neutrophils, or polymorphnuclear leukocytes,[3,9] are members of the granulocytes, just as eosinophils and basophils. Neutrophils represent 50-70% of all the circulating white blood cells. Upon inflammation, neutrophils are the first cells migrating towards the tissue by extravasation,[3,8] attracted by the chemotactic factors, like components of the complement system or cytokines. Neutrophils are capable of direct recognition of the bacteria, however, they have far more efficient phagocytosis of opsonized bacteria.[3] The bacteria are phagocytosed with a zipper-like mechanism (Figure 1A).[10] The bacteria contaning phagosomes are fused with granules containing antimicrobial enzymes, like lactoferrin, lysozyme, and metalloproteinases, or with peroxidase positive azurophilic granules, containg small antimicrobial peptides, like α-defensin and antibiotic proteases, resulting in degradation of the phagocytosed bacteria.[8]

Dentritic cells

Four major categories of DCs are known; Langerhans cells are located in epithelial tissue; interstitial DCs are present in interstitial spaces of all organs except the brain; monocyte-derived DCs travel through the lymph nodes and back into the bloodstream;

and plasmacytoid-derived DCs, which play a role in the innate immune defense and function as an antigen presenting cell. DCs kill bacteria in the same way, using the same agents as macrophages. DCs express both MHC class I and class II receptors and are therefore able to activate both T_H-cells and T_C-cells.[3]

Unlike macrophages and neutrophils, adherence to the microbe is not required for DCs to initiate phagocytosis. DCs use a ruffle-like mechanism for phagocytosis resulting in more spacious phagosomes (Figure 1B).[10] Pathogens are recognized and captured by immature DCs. After phagocytosis, the DC maturates and migrates towards the lymph nodes. Once the DC is mature it becomes an antigen-presenting cell in stead of an antigen-capturing cell. In the lymph node, the antigen is presented to the T-cells, which is essential to activate naïve T-cells.[3]

Non-Professional phagocytosis

Epithelial cells

Naturally, epithelial cells are unable to phagocytose pathogens. Some pathogens, like *Salmonella, Shigella, Yersinia,* and *Listeria monocytogenes* can induce epithelial cells to phagocytose the bacteria and thereby entering the body.[5]

M-cells

M-cells are specialized flattened epithelial cells, which lack the microvilli and contain a deep invagination at the basolateral site. M-cells are located in the follicle-associated epithelium (FAE) of the peyer's patches, where they actively transcytose microorganisms and antigens to the invagination. This invagination is filled up with B-cells, T-cells, and macrophages. The B-cells are activated by antigen recognition, differentiate and start to secrete IgA into the intestinal lumen.[3]

Phagosomal maturation

After, of sometimes even before, the closure of the phagosome, phagsomal maturation starts. The phagosome fuses with early endosomes, late endosomes, and lysosomes,[4] directed by SNARE-proteins.[6] Early endosomes are mildly acidic (pH 6.1-6.5) and contains only a poor hydrolytic activity. Late endosome becomes more acidic (pH 5.5-6.0) and contains proteases and lysosomal-associated membrane protreins. The phagolysosome is highly acidic (pH up to 4.5) and is the ultimate microbicidal organelle.[4]

Chapter 2: Bacterial secretion systems

Pathogens contains different strategies to evade, escape, or inhibit phagocytosis. They can hide from the immune system, for example with a polysaccharide capsule. However, this is not completely effective, for the bacteria might still be phagocytosed. More effective is the inhibition of the phagocytotic machinery, the inhibition of the phagosomal maturation or to induce apoptosis.[8] However, in order to achieve this, bacteria must secrete effector proteins across the bacterial membranes[13] and mostly even directly into the host cell.[14] Nowadays, seven different secretion systems are known (Figure 2).[13] These secretion systems are not only use as a defence mechanism for the human immune system, but protein secretion is used to communicate with other bacteria or their direct environment in order to survive.[13,15]

The two most simple secretion systems are the general secretory (Sec) pathway and the Tat pathway, which are found on both gram positive as gram negative bacteria.[13,15] The sec pathway secretes proteins with a hydrophobic N terminal leader sequence. The proteins are translocated either in the extracellular matrix (ECM) (gram positive bacteria) or in the periplasmic membrane (gram negative bacteria) in a unfolded state. The Tat pathway, or the two-arginine pathway, excretes proteins with a motif on the N-terminal region which is rich in basic amino acid residues. These proteins are translocated in the ECM or in the periplasmic space remaining in a folded state.[15] Besides the Sec and Tat pathway, gram positive and negative bacteria share also the type I and IV secretion systems.[13] The type II, III, V and VI are solely found on gram negative bacteria, whereas the type VII secretion system is specialized to secrete the protein across the mycomembrane of gram positive bacteria.[15]

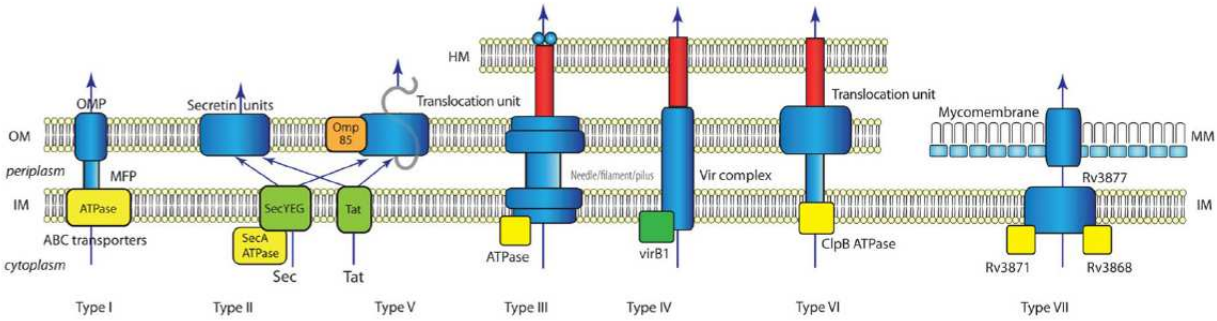

Figure 2. A schematic overview of the different secretion systems present on bacterial membranes. HM - host membrane; OM – outer membrane; IM – inner membrane; MM – mycomembrane; OMP – outer membrane protein; MFP – membrane fusion protein.[15]

Type I secretion system

The Type I secretion system (T1SS) is present on both gram positive and gram negative bacteria.[13,15] The proteins in gram negative bacteria are mostly directly secreted across both the inner and outer membrane. However, T1SS is also capable of protein secretion from the periplasmic space, acting as a second step after the Sec or Tat pathway secretion.[15] T1SS secretes different kind of proteins, like, cytotoxins that belong to the RTX (repeats in toxin) protein family, proteins for the cell surface layer,

proteases, bacteriocins, and heam-acquisition proteins.[16] Besides proteins, T1SS secretes also non-protein substrates,[13] for example exo-polysaccharides for biofilm formation.[15]

T1SS is a simple system consisting of three major components. The first component is the ATP binding cassette (ABC) transporter or a proton antiporter. Both facilitate the energy required for the secretion. Second, there are membrane fusion proteins (Figure 1) that bridge the inner and outer membrane, consisting of outer membrane factors. This part of the T1SS makes up a channel the periplasmic space. Lastly the membrane fusion proteins form the outer membrane pore, which also connect the channel and the ABC transporter. The fusion proteins are found in both gram positive as gram negative bacteria.[15,16] The proteins of the ABC transporter determine whether large proteins are secreted or small proteins and peptides.[15]

Type II secretion system

Type II secretion system (T2SS) is found widespread on the outer membrane of gram negative bacteria[13,15] and is a multicomponent of the two-step machinery for translocation.[16] T2SS, also called the main terminal branch,[13] transports proteins from the periplasmic space into the ECM. The effector proteins were first transported by the Sec or Tat pathway into the periplasmic space.[15,16] Before the substrates are translocated by T2SS across the outer membrane, the effector proteins secreted by the Sec pathway must first be correctly folded,[13] because the Sec pathway unfolds proteins before translocation.[15]

T2SS is made up of 12 to 16 protein components, which are found on both the inner and outer membranes, in the cytoplasm and in the periplasm.[16] The T2SS includes an inner membrane component, however, this does not transport the effector proteins into the periplasm across the inner membrane.[15] The outer membrane component proteins are member of the secretin family. Secretins are also found in T3SS and in type IV pillus assembly.[13]

Bacteria may express more than a single T2SS. The T2SS is required for the virulence of many pathogens, and is a specialized system for the interaction between bacteria with the biotic or abiotic environment. T2SS is found on many gram negative bacteria, from obligate symbionts (mutalistic, commensal or pathogenetic) to free-living bacteria.[15]

Type III secretion system

Type III secretion system (T3SS) is only found on gram negative bacteria[13,15,17] and translocates effector proteins directly across the inner membrane, the peptidoglycan layer, and the outer membrane of the bacteria right into the cytoplasm of the target host cell.[15-19] T3SS is present on several plant and animal pathogens,[16,17] that interact with animal and plants host cells, other pathogens or mutualists.[15] However, T3SS is also found on some endosymbiotic bacteria.[17]

T3SS is the most complex secretion system found on bacteria.[13,20] The structure is composed of more than 20 proteins,[13,16,17,20,21] which form a large structure across the entire bacterial envelope.[16] T3SS is genetically related to the flagellum, which probably share a common ancestor.[15-17,20] Especially the structure, but not the sequence, of the needle complex is comparable to the flagellum.[17] T3SS secretion is completely Sec-independent, although the T3SS assembly starts with a Sec-dependent phase. The components that forms the base of the needle complex are secreted in a Sec-dependent into the periplasmic space. Once in the periplasmic space, these proteins localizes and olimerizes into the bacterial membrane.[13,18]

The T3SS macromolecule (Figure 3 and 4) contains a base structure, consisting of rings in the inner and outer membrane. The periplasmic space is traversed by the neck structure. The inner rod traverse the whole base structure and transfer into the needle structure.[15,17,20] The tip of the needle is connected to the translocase complex, which forms a pore in the host cell membrane.[17,18,20] T3SS actually injects effector proteins upon contact between the bacteria directly into the host cell cytoplasm. T3SS is in fact no secretion system, but a specialized delivery system of the virulence factors,[5,13] therefore, T3SS is also called the injectosome.[15]

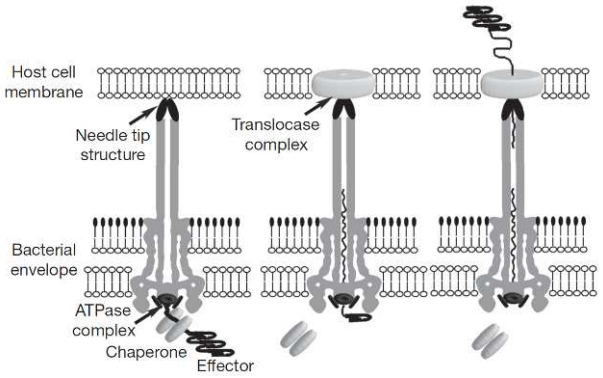

Figure 3 T3SS consist of a base complex in the inner and outer bacterial membrane. The needle complex inject the effector proteins through the translocase complex into the host cell. The chaperone protein leads the effector protein towards the T3SS, where it is translocated in an unfolded state.[20]

Bacteria must take care T3SS is only activated, and thus the effector proteins are only secreted, upon bacterial contact with the host cell. A conserved multiprotein complex senses this contact. Without stimulatory signals this complex prevents effector protein secretion. Effector proteins are highly selected before secretion, to ensure that the right effector proteins are secreted at the right time and in the correct functional order.[20,21] Therefore the substrate recognition is very complex and involves multiple signals.[20]

Most T3SS effector proteins contain a secretion signal within the first 15-30 amino acids.[17,20] The secretion signals are not cleaved off after secretion, as is the case after Sec-dependent secretion. In addition, the secretion signals are not conserved.[20] Very recently, a new computer program, EffectiveT3, was developed. This program is able to detect effectors with a secretion signal in the N-terminus with a sensitivity of ~71% and a selectivity of ~85%.[22]

In addition to the secretion signale, chaperones may also play an important role in effector protein selection and hierarchical secretion.[20,21] These T3SS specific chaperones are homodimeric proteins which, unlike other chaperones, lack the ATP-binding or ATP-hydrolysing activity.[17,20] Chaperones

show only 10% sequence identity between different bacteria but have a highly conserved αβα sandwich fold structure, which is unique for T3SS specific chaperones.[17]

Base structure

The assembly of the base structure is Sec-dependent. The proteins are translocated by the Sec secretion system into the periplasmic space. The base is composed of three proteins. One protein, member of the secretin superfamily, forms the outer ring. The other two proteins form the inner rings, neck, cup, and socket. Once this part of the base is fully assembled, it starts to function as a secretion system. The first secreted proteins form the inner rod and the needle structure.[20]

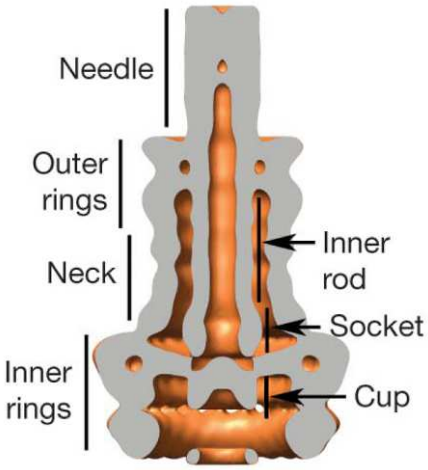

Figure 4 The T3SS structure. The base structure spans both the inner and the outer membrane, containing inner and outer rings connected by the neck. The base is traversed by the inner rod, which is connected to the needle structure.[20]

The cylindrical inner rod traverses the entire length of the base structure. It connects the basal side to the needle structure.[19,20] Once the assembly of the inner rod is finished, conformational changes in the cyplasmic side of the base occur. This is the signal which stops the secretion of needle proteins. Therefore it can be stated that the inner rod assembly determines the length of the needle.[19] The cup and the inner ring undergo a downward movement once the assembly of the needle structure is fully finished. Thereby, the inner ring also shows clumping. These physiological changes of the base structure result in the final shape of the T3SS. These changes are probably required for the effector protein and chaperone recognition of the final T3SS.[20]

Needle complex

The needle complex, composed of a single protein structured in a multi ring base, is a straight hollow structure (Figure 3).[17,19,20] The needle contains a narrow channel with an average inner diameter of about 20 Å. Because folded proteins have a diameter of 20-30 Å, the proteins must be translocated in an unfolded manner.[17] The length of the needle is about 60-80 nm. The needle must extend all other bacterial surface structures, otherwise these structures will interfere with the T3SS secretion.[17,20]

Once the needle is fully assembled, the needle will be close to the host cell membrane but will not perforate the membrane itself. T3SS starts to secrete proteins to form the translocase complex in the host membrane, forming a pore through which the effector proteins enter the host cytoplasm. The needle complex docks to the translocase complex and forms a very efficient translocation complex together. Less then 0.1% of the secreted effector proteins will accidently ends up in the ECM.[20,23]

T3SS families

Seven different families of T3SS are known, widely distributed by horizontal gene transfer among gram negative bacteria.[15,24] Both genes and genetic organisation of T3SS loci are highly conserved. Some bacteria may have multiple T3SS belonging to different families. This is always caused by horizontal gene transfer and never the result of gene duplication. The effector proteins of the different T3SS families interfere always with host cell pathways or cellular functions, e.g. with cytoskeleton dynamics, vascular trafficking, gene expression, apoptosis pathway, cell cycle progression,[20] adhesion, invasion or cytotoxity of the bacterium.[13] Although the different T3SS translocate effector proteins targeting differerent host cell pathways, due to the horizontal gene transfere, the structure of the different T3SS family is comparable.[17,20] The main T3SS families are: Ysc family, Inv-Mxi-Spa family, Ssa-Esc family, Hrc-Hrp1 family, Hrc-Hrp 2 family, Rizobiales family, and Chlamidiales family.[24]

The Ysc family is named after the Yop secretion (Ysc) of *Yersinia* spp. The presence of T3SS of the Ysc family indicates that the bacterium probably is an extracellular pathogen. Bacteria which express a T3SS of the Ysc family are: *Pseudomonas aeruginosa*, *Photorhabdus luminescens*, *Aeromonas* spp., *Vibrio parahaemolyticus*, *Desulfovibrio vulgaris*, and *Bordetalla* spp.. *P. luminescens* survives in the hemocel of insects and blocks phagocytosis of insects macrophages when containing T3SS. A-proteobacteria, e.g. *Bordetella* spp. are protected for phagocytosis and trigger apoptosis in macrophages with a T3SS.[24]

The Inv-Mxi-Spa family includes Inv-Spa T3SS of *Salmonella* and Inv-Mxi T3SS of *Shigella*. Bacteria containing a T3SS of this family are likely to induce uptake by non-profssional phagocytes by triggering actin proliferation. *Yersinia enterocolitica*, *Yersinia ruckeri, Sodalis glossinidius, Escherichia coli*, *Burkholderia* spp. and *Chromobacterium violaceum* express a T3SS belonging to this family. Although many *E. coli* strains encode for this T3SS, it is only functional in a few strains. However, when an *E. coli* strain contain an intact T3SS of this family and an intact entry locus, the bacterium is most likely invasive.[24]

The SSa-Esc family is named after the Ssa T3SS of *Salmonella enterica* and the Esc T3SS of enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). *Edwardstella tarda, C. violaceum, Y. pestis, Y. pseudotuberculosis, Y. enterocolitica* express T3SS of this family. Effector proteins of this T3SS prevent the endocytic trafficking and phagosome maturation in macrophages. Therefore, these bacteria are most likely be able to survive in macrophages. *S. enterica* express this T3SS only during the early phase of infection. The T3SS of *Y. pestis* might not be functional due to an open reading frame disrupting the gene by frame shifts.[24]

Both the Hrc-Hrp1 and the Hrc-Hrp 2 family are typically for plant pathogens. All plant pathogens express one or more T3SS of these two families. The Rhizobiales T3SS family, found on some *Rhizobium* species, is involved in symbiotic relationships, which require intensive communication between the bacteria and the host. And lastly the Chlamidiales family, found on *Chlamidiales,*

characterize intracellular pathogens. This T3SS is widespread in the Chlamydiae order, including the environmental species which infect amoeba.[24]

Type IV secretion system

Type IV secretion system (T4SS) is found on both gram positive and gram negative bacteria.[13,15,16] The effector proteins are secreted in a single step across both bacterial membranes and directly injected in the host cytoplasm,[15,25] or, less commonly, the effector proteins are first secreted in the periplasm via Sec secretion.[15] T4SS is the most versatile secretion system, which secrete single proteins or DNA into the environment or directly into eukaryotic of prokaryotic cells.[16,25]

T4SS can be categorized into three categories (Figure 5). First, the conjugate system, which transfers DNA between the different bacteria to maintain bacterial fitness during changing conditions. The conjugate system thereby contributes to antibiotic resistance among bacteria. Second, the DNA uptake and release system, which either releases DNA into the ECM or picks DNA up from the ECM, thereby also contributes to the bacterial fitness.[16,26] Lastly, the effector translocator system, which is comparable with T3SS. It injects effector proteins directly into the host cell cytoplasm, which allow bacteria containing T4SS to interfere with intracellular pathway.[16,25,26]

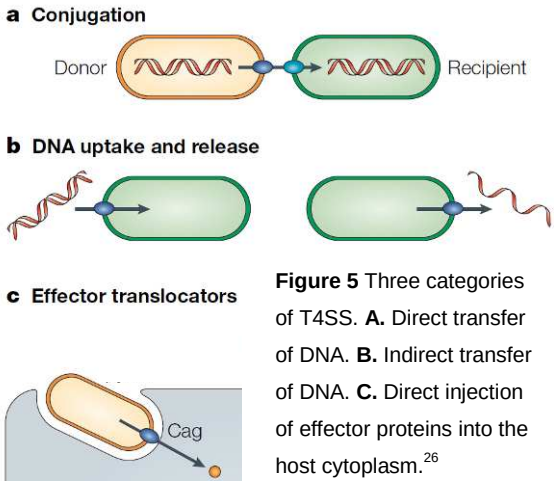

Figure 5 Three categories of T4SS. **A.** Direct transfer of DNA. **B.** Indirect transfer of DNA. **C.** Direct injection of effector proteins into the host cytoplasm.[26]

T4SS consists of 12 components[16] and is related to the ABC transporter system.[13,16] The three ATPases of T4SS providing all the energy required for the structure assembly and substrate secretion.[16] The core complex, consisting of an I and O layer, spans both membranes. The I layer is located in the inner membrane, whereas the O layer forms the body and the cap located in the outer membrane. The structure which traverse the periplasm and connect the both layers, is also the location where Sec-dependent effector proteins enter the T4SS.[25]

Type V secretion system

Type V secretion system (T5SS) is, like T2SS, only found on the outer membrane of gram negative bacteria. T5SS is part of a two step secretion system.[15,16] First the effector proteins are secreted by the Sec-secretion pathway. Second T5SS transfer the effector proteins from the periplasm into the ECM. T5SS secretes over 7000 different effector proteins with a large variety of functions.[16]

Type VI secretion system (T6SS)

T6SS is the third secretion system that secretes the effector proteins directly across all membranes into the host cytoplasm. T6SS was long thought to be only a pathogenicity island (PAI) in gram negative bacteria,[15] located in the IcmF-associated homologous proteins (IAHP) gene cluster.[27] It was

not until 2006 that T6SS was identified as a secretion system with a tail-spike-like injectosome, which is comparable to T3SS and T4SS.[28] T6SS appears to be required for the virulence of many pathogens and may contribute to biofilm formations.[15]

T6SS is composed of 12-25 subunits[16,27] and secretes effector proteins that lack signal peptides. Three classes of T6SS are distinguished using phylogenetic analyses, namely, T6SS A, B, C, and D. Bacteria may contain multiple T6SSs. Although T6SS are mainly present on Proteobacteria, either pathogenetic or symbiotic, it is also found in some non-pathogenic soil bacteria. The secretion system is found on both animal and plant pathogens. The expression of T6SS is highly regulated by temperature or environment, e.g. inside the macrophage. Effector proteins are either secreted in the ECM or injected into the host cell.[27]

Type VII

T7SS is the only secretion system which is only found on gram positive bacteria. Some gram positive bacteria, mycobacteria, protect itself with a almost impermeable cell wall, the mycomembrane. T7SS secretes the effector protein in one step across the inner membrane and the mycomembrane. Nowadays, up to five T7SS gene clusters are known, although the are not functionally complement to each other.[15]

Chapter 3: Phagocytosis evasion strategies

Pathogens have different strategies to evade killing by phagocytes. Bacteria can evade the immune system, inhibit phagocytosis, inhibit phagosomal maturation, endure the lysosomal enzymes, escape out of the phagosome, or induce cell death.[8]

Immune evasion

Some bacteria can avoid the immune system and thereby phagocytosis. In the literature, this immune evasion is often called antiphagocytosis, however these are two different processes. Bacteria evade the immune system by scavenging, inhibiting or degrading opsonic antibodies or components of the complement system.[4,8] Some bacteria surround themselves with a thick polysaccharide capsule,[5,8] a very efficient shield against the host immune system, e.g. *Streptococcus pneumoniae, Neisseria meningitis, Heamophilus influenza, Klebsiella pneumoniae, Staphylococcus aureus, Salmonella typhi*.[5] The uropathogenic *Escherichia coli* (UPEC), for example, avoid the phagocytosis with the K5 capsule and the O75 antigen. Without these two proteins, UPEC is easily recognized and engulfed by macrophages and neutrophils.[8,29] *S. pneumoniae* is another example of immune evasion. The capsular polysaccharide causes only a weak activation of serine/threonine kinases, leading to only a weak phagocytosis response.[30] The M-protein, which causes an impaired complement C3 deposition on the bacterial surface, causes avoiding of the opsonic-dependent phagocytosis,[31] by inhibiting C3 convertase activities.[32] The bacterial capsule only protects the bacteria from opsonization dependent phagocytosis.[33] *S. aureus* also inhibit opsonization dependent phagocytosis. Staphylokinase creates a bacterium-bound protease activity which degradates the IgG and C3b opsonins.[34] The staphylococcus complement inhibitor (SCIN), targets C3 convertases, thereby interfere with all three complement pathways.[8,35] Some bacteria, like *H. influenzae, S. pneumoniae*, and *N. meningitis* secrete also an IgA protease, further evading the phagocytosis.[33]

Antiphagocytosis

Antiphagocytosis is the ability of bacteria to destroy or weaken the phagocytotic machinery within both professional or non-professional phagocytes.[4] Therefore bacteria are able to remain extracellular and avoiding degradation inside the phagolysosome.[10] Antiphagocytosis is characterized by the injection of effector proteins directly into the cytoplasm of the host cells by the bacteria, whereas T3SS is the most commonly used for antiphagocytosis.[14] These effector proteins can directly interfere with specific cellular targets, e.g. disruption of the cytoskeleton, disruption of the NF-κB or MAPK pathways, blocking the production of cytokines or induce apoptosis.[14,33] Effector proteins interfere most common with the cytoskeleton,[8,14] signal transduction pathways and vesicle transport, all resulting in the impairing of the phagocytosis machenery.[14] Antiphagocytotic strategies of some bacteria will be further discussed in chapter 4.

Inhibition of phagosomal maturation

To inhibit the phagosomal maturation, the fusion between the phagosome and the lysosome must be prevented.[8] After phagocytosis of *Legionella pneumophila*, it forms legionella containing vacuoles (LCVs). The LCVs fuse with a COPII coated secretory vesicle in stead of endolysosomes as they should, thereby enhancing bacterial survival.[4] *Mycobacterium tuberculosis* arrest the phagosomal maturation. Coronin 1 is recruited, which suppresses the calcium signaling. This contributes to the evasion of phagosome-lysosome fusion. The fusion of phagsome and lysosome is regulated by SNARE proteins. *Clostridium tetani* and *Clostridium botulinum* secrete the tetanus toxin and botulinum toxin respectively, which cleaves the SNARE proteins and thereby inhibiting the phagosome-lysosome fusion.[6]

Surviving inside the lysosome

Some bacteria are able to survive inside the phagolysosome.[8] *Coxiella burnetii*, causing the Q-fever, creates a large spacious compartments, containing lysosomal proteins. The pH of such a *Coxiella* vacuole is around 4.8. *C. burnetii* needs this low pH to replicate.[4] Other bacteria, like *S. aureus* and *S. pyogenes* regulate genes required for the resistance of the oxidative stress up, for example superoxide dismutases, catalases, and gluthione peroxidase. These genes neutralizes the oxidative attack of the lysosome.[8]

Phagosomal escape

Another strategy to survive phagocytosis is to escape the phagosome before it fuses with the lysosome.[8] *Listeria monocytogenes* is secretes the cholesterol-dependent cytolysin listeriolysin O (LLO). LLO creates a pore in the membrane of the phagosome within 5 minutes after phagocytosis. LLO is activated by the acidification in the phagosome and by the host enzyme INFγ-induceble lysosomal thiol reductase. Beside LLO, *Listeria* also expresses phosphoinositol-specific phospholipase C and a broad-range phospholipase C. Thogether with LLO, provide these enzymes phagosomal membrane breakdown.[4] *Rickettsia* species escape from the phagosome into the cytoplasm where they replicate. *Rickettsia* species perforate the phagosomal membrane using the enzymes phospholipase A_2 and phospholipase D.[36]

Inducing cell death

Bacteria may also induce apoptosis of phagosomes in order to escape bacterial killing. *Yersinia* may induce macrophage apoptosis.[7,33,37] *E. coli* may also leads to cell lysis and necrosis of professional phagocytes.[23] *S. aureus* secretes several toxins leading to cell lysis of neutrophils or other host cells.[8]

Chapter 4: Antiphagocytic strategies of bacteria

Yersinia

Yersinia have three pathogenic subspecies. *Y. pestis* causes the plague or 'the black death'. It spreads via fleabites or aerosols. *Y. enterocolitica* and *Y. pseudotuberculosis* cause gastroenteritis and spread via oral ingestion of contaminated food or water.[7,38] During the initial phase of infection, enteropathogenic *Yersinia* is specialized to penetrate the body through the M-cells of the gut epithelium (18,30,54). *Y. pestis* binds also to host cells and thereby also penetrates the host during the initial phase of infection.[38] After invasion, *Yersinia* avoids phagocytosis and replicates extracellularly.[39] Phagocytosis avoidance is regulated by T3SS dependent secretion of *Yersinia* outer proteins (Yops). Once *Yersinia* adhere to DCs, neutrophils, or macrophages,[14,38,40] the T3SS injectosome is activated and the Yop effector proteins are injected in the host cell.[7,38,41] *Yersinia* primarily targets macrophages and neutrophils, however, DCs are targeted as well, especially those of the spleen.[10,42] T3SS is not activated upon lymphocyte contact.[41]

The *Yersinia* species have three adhesins: Invasin, YadA, and Ail.[40] Besides adhesion and activation of T3SS, adhesins may also have additional functions. Invasin triggers the phagocytosis of *Yersinia* in the absence of opsonins in the early phase of infection, e.g. to enter the M-cells.[7,38,43,44] YadA is also involved in avoiding the phagocytosis by immune evasion. YadA degrades the C3b opsonization component, which reduces bacterial phagocytosis by neutrophils.[43,45] After adhesion to macrophages or neutrophils to integrin β1,[10] the T3SS is activated.[14]

YopB and YopD are first secreted by T3SS. These integrate into the host membrane and form the translocase complex.[5,17] T3SS now can inject the six other Yop effector proteins: YopH, YopE, YopO, YopT, YopJ, and YopM.[7] Many Yops are specialized to disrupt the cytoskeletal assembly to inhibit the phagocytosis.[5] However, the strategies to inhibit phagocytosis may differ upon activation signal. For example, inhibition of NF-κB pathway occurs when macrophages are activated upon LPS recognition.[12]

All Yops have their own target and function, however, they may have synergic effects while inhibiting phagocytosis.[43] YopE, YopO, YopH, and YopT have an important role in the disruption of the actin cytoskeleton. YopJ shows no signs of actin disruption, but inhibits the TNFα production and stimulates macrophage apoptosis and the bacterial release.[5,42] Yop E, YopH, and YopT are able to disrupt the actin fibers alone,[42] however less efficiently then all together. Therefore it can be state that these Yop proteins have no redundancy.[43] When only YopE or YopH are secreted, phagocytosis by DCs was completely blocked, although, strains secrete only YopJ or YopT were phagocytosed in the same rate as non-pathogenic strains.[42] Therefore YopH and YopE are thought to be the main antiphagocytotic factors of the *Yersinia* subtypes.[42,43]

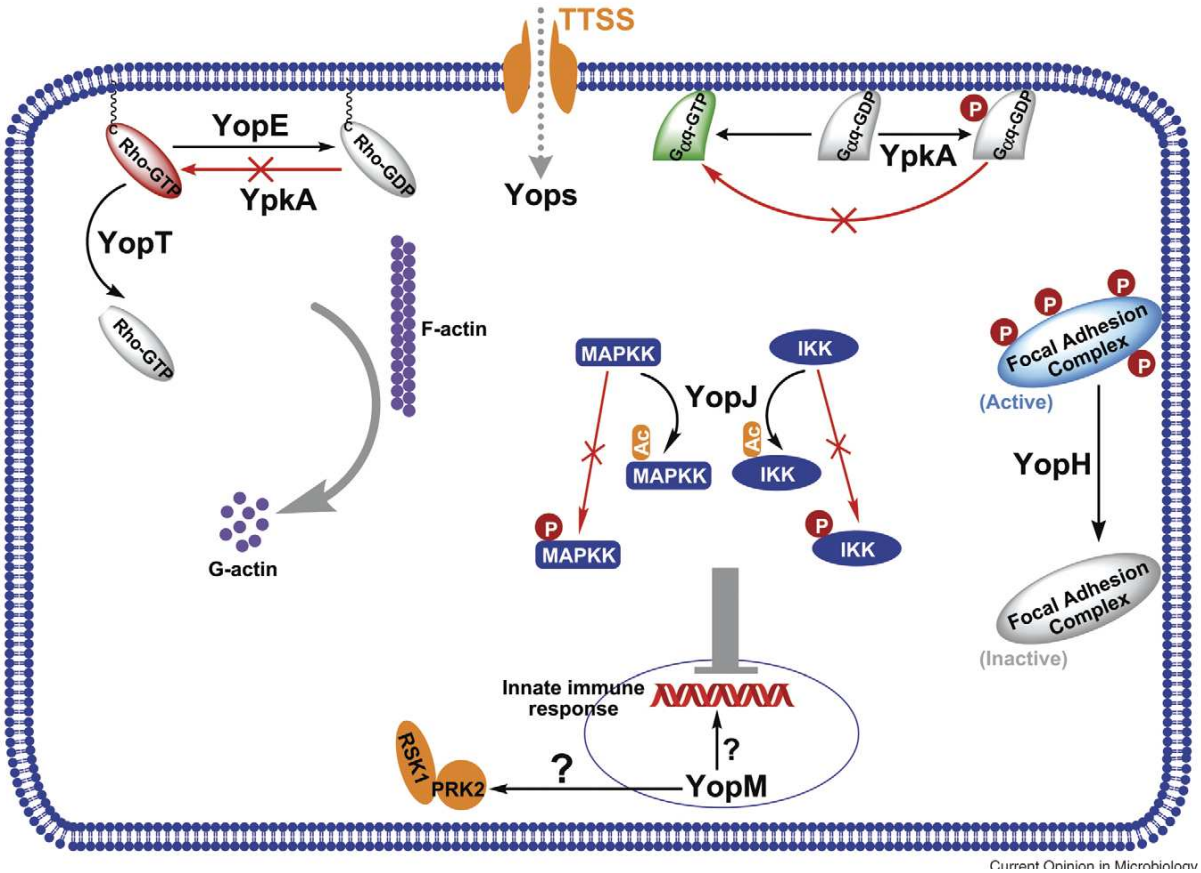

Figure 6 *Yersinia* injects six Yop effector proteins into the host cell. **YopH** dephosphorylates the focal adhesion complex, thereby inhibiting the activation signal of integrin β1 to activate Rac1 and thus the actin polymerization. **YopE** targets Rho-GTPases located on the inner membrane. With its Rho-GAP effector domain it hydrolyzes the GTP and inactivate the RhoGTPases. Resulting in F-actin disruption. **YopO** (or YpkA) phosphorylates the N-terminal conserved diphosphate binding region of Gα, prevents Gαq to be activated. Therefore less RhoA is activated and actin stress fiber formation is inhibited. **YopT** leads to proteolytic disruption of the lipid group of RhoA. RhoA can only regulate the actin cytoskeleton when bound to the membrane. **YopJ** acetylerate proteins of the MAPK and NF-κB pathway, resulting in a decreased TNFα and IL-8 production and in apoptosis of macrophages. **YopM** is the only Yop which is located at the nuclei. It inhibits the IL-15 production, which leads to NK-cell depletion. The further mechanism of YopM is still inknown.[7]

YopH

YopH is a 51 kDa protein, which contain an N-terminal T3SS secretion signal, a chaperone binding region and a C-terminal tyrosine phosphatases (PTPases) catalytic domain.[7] YopH is the most important Yop for the pathogenicity of *Yersinia*.[10,41] It is involved in antiphagocytosis of both opsonization-mediated and non-opsonization-mediated phagocytosis.[43] YopH inhibit integrin β1 induced phagocytosis.[10] Host cells target of YopH are proteins of the focal adhesion complex, e.g. Crk-associated substrate ($p130^{Cas}$), focal adhesion kinase (FAK), paxillin, Fyn-binding protein (Fyb), and SKAP-HOM.[7,10,43,39,46] Normally, the focal adhesion complex becomes activated by integrin β1 receptor activation, which results in Rac1 activation, actin polymerization and phagocytosis.[7,41] The PTPase domain of YopH is highly similar to eukaryotic PTPases, which enables YopH to dephosphorylate and deactivate the focal adhesion complex, thereby resulting in inhibition of phagocytosis (Figure 6).[7,42,46]

DCs express Fyb is in much lower concentration then macrophages and not in the cell periphery like macrophages. Therefore YopH has an antiphagocytotic effect in macrophages, but not in dendritic

cells, although it is injected in both macrophages and DCs in similar levels.[10] Fyb and $p130^{Cas}$ are involved in the integrin β1 signalling, which is an important component of the phagocytosis of macrophages and neutrophils, but not involved in the uptake by DCs, because DCs do not intregrin β1.[10,41]

Besides antiphagocytosis, YopH also impaires the oxidative burst in macrophages and neutrophils to prevent the production of macrophage chemoattractant protein 1 (MCP-1). Therefore, YopH dephosphorylates the host proteins necessary for the MCP-1 production, like Akt and proteins of the phophatidylinositol 3-kinase (PI-3K) pathway. Other targets of YopH are LAT, SLP-76, and Lck, causing suppression of TCR-mediated IL-2 production and the expression of the B-cell costimulatory receptor.[7]

YopE

YopE is a 23 kDa protein, containing an N-terminal T3SS secretion signal, a chaperone binding domain and a C-terminal Rho GTPase activating (Rho-GAP) effector domain, which contains, just like eukaryotic Rho-GAP, an arginine finger domain.[7] The Rho-GAP of YopE targets RhoGTPases located on the inner membrane, like RhoA, Rac, and Cdc42 by accelerating GTP hydrolysis and thereby inhibit the phagocytosis and the IL-1β production (Figure 6).[7,39,43] *In vivo*, YopE appears to target only RhoA and Rac1.[43] Whether YopE targets RhoA or its downstream target Rac1 is not fully understand.[7] Nevertheless, YopE results in actin cytoskeleton disruption,[7,39,42] and plays an important role in the antiphagocytic strategy of *Yersinia*.[7]

YopE has antiphagocytotic effects in all opsonization-mediated and non-opsonization-mediated phagocytosis.[43] YopE is, like YopH, injected in similar levels in both macrophages and DCs.[10] However, YopE also fully inhibits phagocytosis of DCs without YopH, whereas YopH requires YopE for full phagocytosis inhibition in macrophages.[10,42] With the phagocytosis inhibition of neutrophils, also both YopE and YopH are required.[10]

YopO

YopO is a 80 kDa protein, containing an eukaryotic like Ser/Thr kinase domain[7,47] and is called YpkA in *Y. pestis* and *Y. pseudotuberculosis*, however the function is comparable.[43,47] YopO inhibits both opsonization-mediated and non-opsonization-mediated phagocytosis. The autophosphorylating Ser/Thr kinase is activated by phosphorylation due to the contact to filamentous actin, which makes its activity dependent of eukaryotic host factors.[43] The similarity of the Ser/Thr kinase domain of YopO with the one of the PKA family, mediates the binding to the small GTPases RhoA and Rac.[7,47] YopO decreases the levels of activated RhoA[43] by phosphorylating the target protein and inhibit the nucleotide exchange, and therefore causes cytoskeletal disruption, inhibition of phagocytosis and maybe also inhibition of transcription.[7]

Besides the kinase-dependent antiphagocytosis mechanism of YopO, it also contain a kinase-independent mechanism.[47] After translocation YopO is localized at the inner surface of the plasma membrane and impaires efficient the nucleotide exchange of Gαq by phosphorylating the N-terminal conserved diphosphate-binding region of Gα, and thereby the activation of Gαq is efficiently prevented. Gαq can now no longer control the RhoA mediated actin stress fiber formation (Figure 6).[7]

YopT

YopT is a 36 kDa proteins which is the least secreted Yop of *Yersinia*. It is not found in *Y. pseudotuberculosis* so far.[47] YopT contains an N-terminal T3SS secretion signal, a chaperone binding site and a cys-protease site. *In vitro* cleaves YopT the lipid modification of many RhoGTPases, like RhoA, Rac, and Cdc42. *In vivo,* YopT localizes after injection at the host plasma membrane, just like RhoA. Most likely, YopT disrupts the lipid group of RhoA, which results in the dissociation of RhoA from the membrane. Only membrane bound RhoA can regulate the actin cytoskeleton.[7] Therefore, YopT causes indirectly dramatic destruction of the actin cytoskeleton, resulting in antiphagocytosis.[7,39,42,43,48] YopT is involved in the inhibition of phagocytosis of both opsonization-mediated and non-opsonization-mediated phagocytosis.[43]

YopJ

YopJ, or YopP in *Y. enterocolitica*, is a 33 kDa protein.[7] YopJ contains a acetyltransferase activity domain,[37] which places an acetyl group on proteins of the MAPK pathway and the NF-κB pathway, resulting in the inhibition of the TNFα and IL-8 cytokine production and macrophage apoptosis.[7,32,33,37] YopJ is not involved in the disruption of cytoskeleton[48] and is not involved in the inhibition of phagocytosis.[7,10,42,43] YopJ does impair DC maturation,[10] inhibition of antigen uptake,[7] and the stimulation of apoptosis.[7,33,37]

YopM

YopM, a 41 kDa protein, contains 15 leucine rich repeats (LRR). YopM is the only Yop without a catalytic domain, but most likely, interferes with the LRR-domain in protein-protein binding. YopJ is located in the nuclei of HeLa cells after injection and not present in the cytosol like the other Yop effector proteins.[7] YopM is not involved in the inhibition of phagocytosis,[43] but inhibit the IL-15 production and result in the depletion of NK-cells.[7] The further mechanism and function of YopM is still unknown.

Escherichia coli

Escherichia coli is a normal component of the commensal colonies of the gut. Some *E. coli* strains are highly pathogenic. Enteropathogenic *E. coli* (EPEC) causes diarrhea in developing countries. It mainly spreads through drinking water contaminated with feces. Enteroheamorrhagic *E. coli* (EHEC) causes bloody diarrhea in humans. In cattle it is more common and symptomless.[44,49] Uropathogenic *E. coli* (UPEC) causes urinary tract infections. Lastly shiga-toxin-producing *E. coli* (STEC) causes food-borne epidemics in western countries ranging from mild to severe bloody diarrheas. EPEC targets the small

intestine, whereas EHEC and STEC infect mostly the colon.[49] Besides the immune evasion of UPEC, *E. coli* has antiphagocytic mechanisms with the T3SS effector proteins.[50]

The pathogenic strains of *E. coli* inhibit phagocytosis with T3SS effector molecules. To activate the T3SS, *E. coli* must adhere to the host cell.[40] Pathogenic *E. coli* strains contain adhesins that commensal *E. coli* does not express, e.g. type IV pilli structures,[44] or a flagellum that can adhere.[40] *E. coli* adhere to the translocated intimin receptor (Tir), which is secreted by *E.* coli.[49] Tir is after translocation integrated into the plasma membrane in a hairpin loop structure[51] and is absolutely essential for the actin polymerization required for the pedestal formation.[44,51] These pedestals contain both F-actin and intermediate filaments.[51] Both the N- and C-terminus are located into the cytosol, whereas the central domain is remains extracellular.[44] The central domain of Tir functions as a receptor for intimin present on the bacterial surface.[49] Together with Tir, EspG is translocated providing the attaching and effacing (A/E) lesions.[52] These lesions are characterized by bacterial attachment and the effacement of the host cell microvilli.[53] The components of the T3SS of *E. coli* are involved in the A/E leasions, e.g. EspA, a filament of the translocase complex, EscF, which is a component of the needle structure,[44] and the pore forming components of the translocase complex: EspB and EspD.[44,49] T3SS injects several effector proteins into the host cells, e.g. EspB,[50,54] Map, Nck, EspG/EspG2,[18] EspG, EspH[52] and cytotoxic necrotizing factor type 1 (CNF1)[55,56] which influences on several signaling pathways, resulting in actin rearrangements,[50] disruption of cytoskeletal assembly[5,54] and antiphagocytosis.[51] All effector proteins are encoded on the PAI named the locus of enterocyte effacement (LEE), except EspG2, which is the only effector proteins located on a different PAI.[52]

Map, Tir and EspH all target the Cdc42 signalling pathway[26] and the formation of pedestals. Nck causes actin polymerization. None of these effectors appear to be involved in antiphagocytosis. Remarkably, actin polymerization which is required for pedestal formation, is also involved in phagocytosis. However, after pedestal induction, internalization does not follow,[51] possibly EPEC induced pedestal formations and at the same time it avoids macrophage phagocytosis.[50]

EPEC inhibits phagocytosis by macrophages by inhibiting the phosphatidylinositol-3 kinase (PI-3K) dependent pathway.[57] Normally, this pathway leads to the polymerization of F-actin and the rearrangement into a cup formation to start phagocytosis.[14,32,39] PI-3K is activated upon bacterial contact to macrophages.[57] EPEC inject in a T3SS dependent manner an effector protein, the precise effector protein is not yet known,[51] which dephosphorylates PI-3K. This inhibits the formation of the PI-3K product PIP_3, which is required in the phagocytic process,[57] as described in chapter 1. This antiphagocytosis of EPEC blocks the Fc-receptor mediated phagocytosis,[49,57] but not the CR3-mediated phagocytosis[39,57] in an EspF-, Tir-, and intimin-independent manner.[49]

EspB

EspB is a bifunctional protein. First is targeted to the plasma membrane where it is part of the translocase complex. EspB is also injected into the host cytosol where it modulates the cytoskeleton. The three coiled-coil domains of EspB allow it to form a functional translocase complex.[50] When EspB is injected into the cytosol it causes microvilli effacing and inhibition of myosin and actin interactions.[54] EspB is shown to bind all members of the myosin family tested so far.[50] It binds the myosin motor domain of myosin-1a, -2, -5, -6, and -10, all known to be involved in the microvilli formation and phagocytosis. Therefore, EspB is an antiphagocytotic effector protein.[54]

EspG/EspG2

EspG/EspG2 interferes in the cross talk between the microtubules and the actin cytoskeleton during EPEC infection. EspG/EspG2 activates the RhoA-ROCK signaling pathway. It bind and disrupts microtubule cytoskeleton.[51-53] This causes the release of microtubule bound RhoA-specific GEF-H1. Unbound GEF-H1 is the active form, which promotes the nucleotide exchange on RhoA. In this way EspG-EspG2 induces actin cytoskeletal rearrangements[53] and A/E leasions.[52]

CNF1

CNF1 is a 115 kDa single chain AB,[55] Rho-activting[56] toxin that deamidate the catalytically active glutamine residue of GTPases of the Rho family, like RhoA, Rac1, and cdc42. The loss of the functional amide group prevents hydrolysis from GTP to GDP. UPEC expressing CNF1 appears to downregulate the phagocytosis of neutrophils. Possibly neutrophils are in the presence of CNF1 not able to remodel their membranes and react on opsonized bacteria.[55]

Helicobacter pylori

Helicobacter pylori causes peptic ulcer disease, mucosal associated lymphoid tissue (MALT) lymphoma's and gastric carcinoma's. Over 90% of the bacteria attach to the mucus layer of the stomach during the infection.[58] *H. pylori* produces urease, an enzyme which hydrolysis urea and forms a protection cloud of ammonia around the bacteria. This provide survival in acidic environments.[58,59] Two *H. pylori* strains are known. *H. pylori* type I contains a cag PAI and is highly virulent. *H. pylori* type II lacks this Cag PAI and is less virulent.

Once *H. pylori* adhere to host cells, T4SS is activated.[44] T4SS contains specialized adhesins, like CagL, which mediates integrin α5β1 adherens.[40] The activated T4SS injects the effector protein CagA into the host cell cytoplasm of professional phagocytes.[44,58,60] Only *H. pylori* type I is able to inhibit phagocytosis.[61]

CagA

CagA is recognized as a self protein[58] and therefore phosphorylated[58,60] and proteolytically cleaved when in is injected in the cytoplasm. The stable 35-45 kDa C-terminal tyrosine-phosphorylated fragment is the active part of CagA.[60] After activation, CagA affects many cellular pathways, induces

morphologically changes in the host, like actin reorganization[58] and pedestal formation,[59] variations in the host cell cycle and has some autocrine effects.[58] Further CagA results in the dysfunction of tight junction, in the cross linking of actin filament and imparement of the cell motility and elongation.[62] However, although CagA targets components of the phagosomal machinery, it seems neither to be involved in the inhibition of phagocytosis, nor in the prolonged survival inside the phagosomes.[60] The precise way *H. pylori* inhibit the phagocytosis is not yet fully understand.

Pseudomonas aeruginosa

Pseudomonas aeruginosa is an opportunistic plant and human pathogen, which may cause acute infections.[23] Once activated, the T3SS injects four effector proteins in host cytoplasm:[40,63] ExoS, ExoU, ExoY, and ExoT. Bacterial isolates from acute infection are scanned for the presence of these effector proteins. 58-72% of all the tested isolates contained an ExoS gene, 28-42% ExoU, 89% ExoY and 92-100% ExoT. In all the isolate either the ExoS or the ExoU gene present, however, they were hardly ever found together in one isolate.[23]

ExoS

ExoS is a 48 kDa bifunctional effector protein, with a GTPase-activating (GAP) domain on the N-terminus and a ADP ribosyl transferase (ADPRT) domain on the C-terminus.[23,64] Futher, ExoS contains a T3SS secretion signal, a binding site for the chaperon SpcS, and a membrane localization domain. Due to the initial membrane localization, ExoS traffics to the ER and the Golgi where it modifies its substrates.[23]

The GAP domain targets RhoA, Rac, and cell division cycle 42 (Cdc42).[14,23,39] The GAP domain of ExoS, contains an arginine finger domain, which is highly similar to the eukaryote arginine finger domain. The arginine finger hydrolysis GTP into GDP.[7,23,39,63] The small GTPase proteins are inactive in the GDP form. Now, they are no longer able to organize the host cell cytoskeleton, which leads to a disruption of the actin cytoskeleton.[23,63] This inhibits the phagocytosis by macrophages.[14,23,39,63,64]

The ADPRT domain requires the eukaryotic cofactor 14-3-3 for activation. ADPRT targets the small GTPase which affects cellular proliferation, survival and cytoskeletal structure. Targeting of exrin, radixin and moesin (ERM) family of proteins, effects many actin processes, like phagocytosis, adhesion and cell shape maintenance.[23] However, only the GAP domain is shown to have an antiphagocytic effect, not the ADPRT domain.[64] Probably, the disruption of the cytoskeleton by the ADPRT domain may result in reduced cell-cell adherence. This could facilitate bacterial penetration through the epithelial barriers. Which should indicate that the GAP domain and the ADPRT domain both are required for an effective infection.[23]

ExoT

ExoT is a 49 kDa protein[23] and share 76% homology with ExoS.[23,65] It also contains a GAP domain, an ADPRT domain, and a chaperone binding site for the SpcS chaperone.[23] The GAP domain targets

RhoA, Rac, and Cdc42,[14,23,39,65] and the ADPRT domain CRKI, CRKII, and phosphoglycerate kinase, but needs also coactivation of the eukaryotic cofactor 14-3-3, like ExoS.[23] The GAP domain of ExoT contains an arginine finger domain, just like ExoS.[39,65] ExoT disrupt the actin cytoskeleton similar to EcoS and inhibits phagocytosis as well.[14,23,39,65]

ExoU

ExoU is a 74 kDa protein and thereby the largest effector protein of *P. aeruginosa*. ExoU bind to the SpcU chaperone. After injection into the host cytoplasm, ExoU targets phospholipids, lysophospholipids and neutral lipids. The injection of ExoU leads to rapid cell lysis and thus necrosis.[23]

ExoY

ExoY, a 42 kDa protein with an unknown chaperone, contains an adenyly cyclase domain.[23,66] It binds to ATP and transforms it into cAMP. The elevated levels of cAMP result in the disruption of the actin cytoskeleton, increased endothelial permeability and the inhibition of phagocytosis.[23] Although ExoY results in the inhibition of phagocytosis. However, when the adenylyl cyclase domain in mutated, it still inhibits phagocytosis but less efficient. This indicates, that ExoY, just like ExoS and ExoT, contains more than one active domain.[66]

Photorhabdus luminescens

Photorhabdus luminescens is an entomopathogenic bacterium. It is highly pathogenic for the *Spodoptera littoralis*, a cotton leafworm. Several *Spodoptera* types are known to have haemocytes with phagocytic functions. *P. luminescens* uses a T3SS to deliver its effector protein into the target cells,[67] after adhesion.[40] The T3SS of *P. luminescens* belongs to the Ysc-family of T3SS and is therefore likely to be an extracellular pathogen.[67] The effector protein LopT injected into the insect haemocoel and inhibits the phagocytosis.[24,67] However, most probably, also other factors than LopT alone are involved in the antiphagocytic strategies of *P. luminescens*.[67]

LopT

LopT is a strong antiphagocytotic factor and is highly homologous to YopT of *Yersinia*. LopT shows the same modification of RhoA as YopT. LopT is shown to be expressed and injected *in vivo* in insect organs.[67]

Bordetella

Bordetella have three pathogenic subspecies, which are shown to have antiphagocytic factors. *B. pertussis* infects only humans and causes pertussis. *B. parapertussis* infect both human and sheep. It also causes pertussis, although in with a milder disease outcome. *B. bronchiseptica* causes persistent and mostly asymptomatic respiratory infections in mammals and humans, mostly when they are immunocompromized. All three species secrete adenylate cyclase toxin (ACT), dermonecrotic toxin (DNT) and tracheal cytotoxin (TCT). Only *B. parapertussis* and *B. bronchiseptica* express T3SS effector proteins.[68] *B. bronchiseptica* is known to secrete 3 different proteins present in the T3SS

machinery. BopN which has 19% similarity with YopN of *Yersinia,* BopD, which has 20% similarity with YopD of *Yersinia*, and BscN. BopN is most likely involved in the T3SS regulation, BopD is probably present in the translocase comples, and BscN is involved in targeting effector proteins into the host cell.[69]

The T3SS of *B. bronchiseptica* is thought to resist phagocytosis, reduce the proinflammatory response,[23,69] probably by the inhibition of NF-κB activation, and induce apoptosis of macrophages. Injected effector proteins are not yet found, however they probably show some similarity with YopJ of *Yersinia*.[69] The T3SS effector proteins are also thought to activate the ERK 1/2 MAPK pathway in DCs, resulting in the upregulation of CD86 and MHCII on the membrane surface.[70]

Besides the T3SS effector proteins, *Bordetella* species secretes toxins which are able to target host myeloid phagocytic cell that express the $\alpha_M\beta_2$ intergrin receptor, like macrophages, neutrophils and DCs.[71] These toxins penetrate the host membrane.[68]

DNT

DNT activates Rho GTPases by deamidation or transglytamination,[56] which forces Rho in a constitutively active state. This results in the assembly of actin stress fibers, focal adhesion and a motility shift of Rho.[72] These modifications may play a role in the antiphagocytosic strategy of *Bordetella*.

ACT

ACT penetrates phagocytes through $\alpha_M\beta_2$ intergrin receptor,[68,71] through which it delivers its adenylate cyclase (AC) domain into the host cytoplasm.[68] The AC domain binds to the intracellular calmodulin, which results in an uncontrolled conversion of ATP into cAMP.[53,68,71] This rapid increase of cAMP levels suppresses phagocytosis, chemotaxis, and superoxide production. ACT causes a selective inactivation of RhoA in macrophages with the increased cAMP signaling.[71] This leads to actin cytoskeleton rearrangement,[73] inhibition of microfilament assembly in neutrophils,[74] impairment of phagosome lysosome fusion,[73] and macrophage membrane ruffling. The ACT-induced increase of cAMP levels results in blockage of CR3 complement-mediated phagocytosis. These are all ATC dose-dependent effects.[71] Another effect of ACT is to inhibit the p38 MAPK pathway in DCs, thereby inhibiting the CD40 and IL-12 expression.[70]

Finegoldia magna

Finegoldia magna is a commensal gram positive anaerobic cocci (GPAC), present on the human skin and mucous membrane. It is formally called *Peptostreptococcus magnus*. *F. magna* encodes four albumine binding protein homologues, which are virulence factors involved in the antiphagocytic process; three in the genome and one in a plasmid. Further, *F. magna* encodes for many sortase genes which are involved in the bacterial adherence and the antiphagocytic strategies.[75]

Bacillus anthracis

Bacillus anthracis encodes for three toxins locates on its PAI; lethal factor (LF), edema factor (EF) and protective antigen (PA). First, adhesion is provide by PA, after which LF and EF can be transported into the host cytoplasm. The edema toxin (EdTx) is an adenyl cyclase which increases the cAMP levels in the host cell, just like the ACT toxin in *Bordetella.* This results in the impairment of phagocytosis by macrophages. Therefore it is likely that EdTx also results in antiphagocytosis. It is shown that EdTx downregulates genes in macrophages that are necessary in the actin cytoskeleton remodeling, e.g. protein kinase A (PKA).[76]

Chapter 5: Discussion

When bacteria has entered the body, the immune system will be activated. The phagocytes start to ingest the bacteria.[5] Bacteria developed several strategies to avoid or survive phagocytosis,[8] like immune evasion, antiphagocytosis, or induction of apoptosis. After phagocytosis, some bacteria are able to survive inside the phagosome.[4] Some of these bacterial strategies are mixed up in the literature, like immune evasion and antiphagocytosis. Bacteria can express multiple T3SS types, as described in chapter 2 and have therefore most likely multiple strategies to evade the immune system.[24] However, a clear distinction between the different strategies can be made.

Immune evasion is latterly the evasion of the immune system. Bacteria hide from the immune system by building a polysaccharide capsule around themselves, which only result in a weak immune activation.[5,8,29,30] To evade opsonization-depended phagocytosis, bacteria degrade opsonins present on the bacterial surface.[8,31-35] Antiphagocytosis on the other hand, is the active impairing of the phagocytosis machinery by injection of bacterial effector proteins.[4] Effector proteins which interfere with the phagocytic pathways are actively injected into the host cell with the T3SS injectosome, however, T4SS, T6SS, and T7SS are possible candidates as well.[15] The injected effector proteins causes disruption of the cytoskeleton, signal transduction pathways, and vesicle transport. Thereby, they actively impair the phagocytic machinery.[14] Intracellular survival mechanisms mostly involve protein secretion. Bacteria can inhibit the phagosomal maturation and evasion of the phagosome-lysosome fusion.[6] Other bacteria survive inside the phagolysosome by using the low pH to survive,[4] or secrete proteins that neutralizes the oxidative attack inside the lysosome.[8] The last strategy is the phagosomal escape by the secretion of pore forming enzymes.[4,36] Therefore the different phagocyte escape mechanism are clearly different from each other.

Several bacteria uses antiphagocytosis to remain extracellular, like *Yersinia spp.,*[5,42] *E. coli,*[40] *H. pylori,*[44] *P. aeruginosa,*[23] *P. luminescens,*[67] *Bordetella spp.*,[68,71] *B. anthracis,*[76] and *F. magna.*[75] The best studied bacteria in order of antiphagocytosis is Yersinia. Its adhesins initiate the T3SS activation, although they also function in the immune evasion strategies by the cleavage of C3b components.[43,45] The T3SS of Yersinia injects six effector proteins.[7] YopH, YopE, YopO, and YopT target different host proteins, but result all in destroying the cytoskeleton and thereby inhibit phagocytosis.[5,42] The other two, YopJ and YopM, do not inhibit phagocytosis.[7] *P. luminescens* injects the T3SS effector protein LopT. This effector protein is highly comparable to YopT of *Yersinia*. LopT shows the same RhoA modifications and also contain an antiphagocytotic function, just like YopT.[67] *F. magna* secretes albumine binding protein homologous for the antiphagocytotic effect, however the mechanism is not fully understand.[75]

The T3SS effector protein of *E. coli,* EspB, inhibit phagocytosis by the inhibition of myosin-actin interactions.[54] EspG/EspG2 result in microtubule disruption and antiphagocytosis.[51-53] CNF1 is a Rho-

activating toxin[56] and results also in the impairment of cytoskeletal remodeling[55]. A still unkown effector protein of *E. coli* inhibits the PI-3K pathway and inhibit phagocytosis at the start.[14,39,57]
P. aeruginosa injects four T3SS effector proteins, ExoS, ExoT, ExoU, and ExoY.[23] ExoS and ExoT both contains a GAP domain which inactivated small GTPase proteins, leading to disruption of the actin cytoskeleton and inhibition of the phagocytosis process.[23,63] ExoY transforms ATP into cAMP resulting in the actin cytoskeleton disruption as well. ExoY most probably contains also another domain with antiphagocytotic function, although not yet identified. ExoU induces cell lysis and necrosis and has no antiphagocytic function.[23]

Bordetella contains a T3SS, however, no injected effector proteins are yet identified.[69] The inhibition of phagocytosis is regulated with the secreted toxins, DNT and ACT, which penetrate through the host cell membrane.[68] DNT forces Rho in an constitutively active state, which may plays a role in the antiphagocytotic process.[72] A part of ACT binds calmodulin and convert ATP in cAMP. The highly increased cAMP levels result in inhibition of phagocytosis.[71] Also *B. anthracis* uses toxins in stead of T3SS effector proteins. These toxins result also in an cAMP increase-dependent phagocytosis inhibition.[76]

H. pylori is the only bacteria known so far uses a T4SS, instead of T3SS, to inject the antiphagocytotic effector proteins.[44] The effector protein, CagA, interferes with actin reorganization[58] and tight junction disruption.[62] However, CagA appears not to be the antiphagocytotic effector protein of *H. pylori*.[60] Other effector proteins with possible antiphagocytotic function are not yet identified.

Antiphagocytosis is the only mechanism by which the bacteria remain actively extracellular by impairing the phagocytic machinery of the host cell. It is mostly characterized by T3SS injection of effector proteins, however, there are some exceptions. *H. pylori* is thought to use a T4SS to inject effector proteins, although the mechanism is similar to T3SS. Other bacteria, like *Bordetella* and *B. anthraxis* secrete toxins which are able to penetrate the host cell membrane. Although the effector proteins are not injected, the bacteria are able to impair the phagosomal machinery remaining extracellular, which is similar to the other antiphagocytotic bacteria. All the antiphagocytotic effector proteins or toxins target different host cell proteins, but in the end they all impair the host cytoskeleton either in a direct or indirect way. Therefore is can be stated that antiphagocytosis is a mechanism of bacteria to remain actively extracellular even after recognition by the immune system, by injecting effector proteins into the host cell cytoplasm or secrete toxins which penetrate through the host cell membrane which target host cell proteins resulting either direct or indirect in the impairment of the cytoskeleton assembly, resulting in the inability of the host cell to manipulate their membrane to engulf the recognized bacteria.

References

1. Tissières P and Pugin J. The role of MD-2 in the opsonophagocytosis of gram-negative bacteria. *Current opinion in infectious diseases.* 2009;22:286-291.
2. Tan SY, and Dee MK. Medicine in stamps. Elie Metchnikoff (1845-1916): discoverer of phagocytosis. *Singapore medicine journal.* 2009;50(5):456-457.
3. Kindt TJ, Goldsby RA, and Osborne BA. Immunology, sixth edition. *W. H. Freeman and Company* New York. Chapter 2 and 3. 2007. ISBN-13: 978-0-7167-8590-3.
4. Flannagan RS, Cosío G and Grinstein S. Antimicrobial mechanisms of phagocytes and bacterial evasion strategies. *Nature revieuws microbiology.* 2009;7:355-366.
5. Sansonetti P. Phagocytosis of bacterial pathogens: implications in the host response. *Seminars in immunology.* 2001;13:381-390.
6. Greenberg S and Gristein S. Phagocytosis and innate immunity. *Current opinion in immunology.* 2002;14:136-145.
7. Shao F. Biochemical functions of *Yersinia* type III effectors. *Current opinion in microbiology*. 2008;11;21-29.
8. Urban CF, Lourido S, and Zychlinsky A. How do microbes evade neutrophil killing? *Cellular microbiology*. 2006;8(11).1687-1696.
9. Mantovani A. From phagocyte diversity and activation to probiotics: back to Metchnikoff. *European journal of immunology.* 2008;38:3269-3273.
10. Fahlgren A, Westermark L, Akopyan K, and Fällman M. Cell-type specific effects of *Yersinia pseudotuberculosis* virulence effectors. *Cellular Microbiology.* 2009;11(12):1750-1767.
11. Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL, and Gregory CD. Human CD14 mediates recognition and phagocytosis of apoptotic cells. *Nature*. 1998;392:505-509.
12. Suzuki E and Umezawa K. Inhibition of macrophage activation and phagocytosis by a novel NF-κB inhibitor, dehydroxymethylepoxyquinomicin. *Biomedicine and Pharmacotherapy.* 2006;60:578-586.
13. Wilson M, McNab R, and Henderson B. Bacterial disease mechanisms; an introduction to cellular microbiology. *University press, Cambridge, United Kingdom.* 2002;1-15:46-56:61-75. ISBN:0 521 79250 9 Hardback.
14. Knodler LA, Celli J, and Finlay BB. Pathogenic trickery: deception of host cell processes. *Nature revieuws molecular cell biology*. 2001;2:578-588.
15. Tseng TT, Tyler BM, and Setubal J. Protein secretion systems in bacterial-host associations, and their secription in the gene ontology. *BMC microbiology.* 2009;9(suppl I):S2.
16. Fronzes R, Christie PJ, and Waksman G. The structural biology of type IV secretion systems. *Nature reviews microbiology.* 2009;7:703-714.
17. Ghosh P. Process of protein transport by the type III secretion system. *Microbiology and molecular biology reviews.* 2004;68(4):771-795.
18. Yip CK, Kimbrough TG, Felise HB, Vuckovic M, Thomas NA, *et al.* Structural characterization of the molecular platform for type III secretion system assembly. *Nature.* 2005;435:702-707.
19. Marlovits TC, Kubori T, Lara-Tejero M, Thomas D, Unger VM, and Galán JE. Assembly of the inner rod determines needle length in the type III secretion injectisome. *Nature.* 2006;441:637-640.
20. Galán JE and Wolf-Watz H. Protein delivery into eukaryotic cells by type III secretion machines. *Nature reviews.* 2006;444:567573.
21. Thomas NA and Finlay BB. Establishing order for type III secretion substrates – a hierarchical process. *TRENDS in microbiology.* 2003;11(8):398-403.
22. Arnold R, Brandmaier S, Kleine F, Tischler P, Heinz E, *et al.* Sequence-based prediction of type III secreted proteins. *PLoS pathogens.* 2009;5(4):e1000376.
23. Hauser AR. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nature reviews.* 2009;7:654-665.
24. Troisfontaines P and Cornelis GR. Type III secretion: More systems than you think. *Physiology.* 2005;20:326-339.
25. Llosa M, Roy C, and Dehio C. Bacterial type IV secretion systems in human disease. *Molecular microbiology*. 2009;73(2):141-151.

26. Cascales E and Christie PJ. The versatile bacterial type IV secretion systems. *Nature reviews microbiology.* 2003;1:137-149.
27. Filloux A, Hachani A, and Bleves S. The bacterial type VI secretion machine: yet another player for protein transport across membranes. *Microbiology*. 2008;154:1570-1583.
28. Bingle LEH, Bailey CM, and Pallen MJ. Type VI secretion: a beginner's guide. *Current opinion in microbiology.* 2008;11:3-8.
29. Burns SM and Hull SI. Loss of resistance to ingestion and phagocytic killing by O^- and K^- mutants of a uropathogenic *Escherichia coli* O75:K5 strain. *Infection and immunity*. 1999;67(8):3757-3762.
30. Segura M, Gottschalk M, and Olivier M. Encapsulated *Streptococcus suis* inhibits activation of signaling pathways involved in phagocytosis. *Infection and immunology.* 2004;72(9):5322-5330.
31. Fan H, Wang Y, Tang F, and Lu C. Determination of the mimic epitope of the M-like protein adhesion in swine *Streptococcus epui* subsp*. Zooepidemicus*. *BMC microbiology.* 2008;8:170-179.
32. Rhen M, Eriksson S, and Pettersson S. Bacterial adaptation to host innate immunity responses. *Current opinion in microbiology.* 2000;3:60-64.
33. Brodsky IE and Medzhitov R. Targeting of immune signalling networks by bacterial pathogens. *Nature cell biology.* 2009;11(5):521-526.
34. Rooijakkers SHM, van Wamel WJB, Ruyken M, van Kessel KPM, and van Strijp JAG. Anti-opsonic properties of staphylokinase. *Microbes and infection.* 2005;7:476-484.
35. Rooijakkers SHM, Ruyken M, Roos A, Daha MR, Presanis JS, *et al.* Immune evasion by a staphylococcal complement inhibitor that acts on C3 convertases. *Nature immunology.* 2005;6(9):920-927.
36. Balraj P, Renesto P, and Raoult D. Advances in *Rickettsia* pathogenicity. *Rickettsiology and rickettsial diseases-fifth international conference – New York academy of sciences.* 2009;1166:94-105.
37. Bliska JB. *Yersinia* inhibits host signaling by acetylating MAPK kinases. *ACS chemical biology.* 2006;1(6):349-351.
38. Bergman MA, Loomis WP, Mecsas J, Sternbach MN, and Isberg RR. $CD8^+$ T-cells restrict *Yersinia pseudotuberculosis* infection: Bypass of anti-phagocytosis by targeting antigen-presenting cells. *PLoS pathogens.* 2009;5(9):e1000573.
39. Celli J and Finlay BB. Bacterial avoidance of phagocytosis. *TRENDS in microbiology.* 2002;10(5):232-237.
40. Kline KA, Fälker S, Dahlberg S, Normark S, and Henriques-Normark B. Bacterial adhesins in host-microbe interactions. *Cell Host & Microbe.* 2009;5:580-592.
41. Yuan M, Deleuil F, and Fällman M. Interaction between the *Yersinia* tyrosine phosphatase YopH and its macrophage substrate, Fyn-binding protein, Fyb. *Journal of molecular microbiology and biotechnology.* 2005;9:214-223.
42. Adkins I, Köberle M, Gröbner S, Bohn E, Autenrieth IB, and Borgmann S. *Yersinia* outer proteins E, H, P, and T differentially target the cytoskeleton and inhibit phagocytic capacity of dendritic cells. *Inernational journal of medical microbiology.* 2007;297:235-244.
43. Grosdent N, Maridonneau-Parini I, Sory MP, and Cornelis GR. Role of Yops and adhesins iin resistance of *Yersinia enterocolitica* to phagocytosis. *Infection and immunity.* 2002;70(8):4165-4176.
44. Rottner K, Stradal TEB, and Wehland J. Bacteria-host-cell interactions at the plasma membrane: stories on actin cytoskeleton subversion. *Developmental cell.* 2005;9:3-17.
45. China B, N'Guyen BT, de Bruyere M, and Cornelis GR. Role of YadA in resistance of *Yersinia enterocolitica* to phagocytosis by human polymorphonuclear leukocytes. *Infection and immunity.* 1994;62(4):1275-1281.
46. Deluil F, Mogemark L, Francis MS, Wolf-Watz H, and Fällman M. Interaction between the *Yersinia* protein tyrosine phosphatase YopH and eukaryotic Cas/Fyb is an important virulence mechanism. *Cellular microbiology.* 2003;5(1):53-64.
47. Wiley DJ, Nordfeldth R, Rosenzweig J, DaFonseca CJ, Gustin R, *et al.* The Ser/Thr kinase activity of the *Yersinia* protein kinase A (YpkA) is necessary for full virulence in the mouse, mollifying phagocytosis, and disrupting the eukaryotic cytoskeleton. *Microbial pathogenesis.* 2006;40:234-243.
48. Kozubowski L, Lee SC, and Heitman J. Signalling pathways in the pathogenesis of *Cryptococcus. Cellular microbiology.* 2009;11(3):370-380.
49. Kresse AU, Guzman CA, and Ebel F. Modulation of host cell signaling by enteropathogenic and Shiga toxin-producing *Escherichia coli. International journal of medical microbiology.* 2001;291:277-285.
50. Mattoo S, Alto NM, and Dixon JE. Subversion of myosin function by *E. coli. Developmental cell.* 2008;14:8-10.
51. Caron E, Crepin VF, Simpson N, Knutton S, Garmendia J, and Frankel G. Subversion of actin dynamics by EPEC and EHEC. *Current opinion in microbiology.* 2006;9:40-45.

52. Shaw RK, Smollett K, Cleary J, Garmendia J, Straatman-Iwanowska A, *et al.* Enteropathogenic *Escherichia coli* type III effectors EspG and EspG2 disrupt the microtubule network of intestinal epithelial cells. *Immunity and infection*. 2005;73(7):4385-4390.
53. Matsuzawa T, Kuwae A, Yoshida S, Sasakawa C, and Abe A. Enteropathogenic *Escherichia coli* activates the RhoA signaling pathway via the stimulation of GEF-H1. *The EMBO journal*. 2004;23(17):3570-3582.
54. Iizumi Y, Sagara H, Kabe Y, Azuma M, Kume K, *et al.* The enteropathogenic *E. coli* effector EspB facilitates microvillus effacing and antiphagocytosis by inhibiting myosin function. *Cell Host & Microbe.* 2007;2:383-392.
55. Davis JM, Rasmussen SB, and O'Brien AD. Cytotoxic necrotizing factor type 1 production by uropathogenic *Escherichia coli* modulates polymorphonuclear leukocyte function. *Infection and immunity.* 2005;73(9):5301-5310.
56. Lerm M, Schmidt G, and Aktories K. Bacterial protein toxins targeting Rho GTPases. *FEMS microbiology letters.* 2000;188:1-6.
57. Celli J, Olivier M, and Finlay BB. Enteropathogenic *Escherichia coli* mediates antiphagocytosis through the inhibition of PI 3-kinase-dependent pathways. *The EMBO journal.* 2001;20(6):1245-1258.
58. Censini S, Stein M, and Covacci *A.* Cellular responses induced after contact with *Helicobacter pylori. Current opinion in microbiology.* 2001;4:41-46.
59. Segal ED. Consequences of attachment of *Helicobacter pylori* to gastric cells. *Biomedicine and pharmacotherapy.* 1997;51:5-12.
60. Odenbreit O, Gerbert B, Püls J, Fischer W, and Haas R. Interaction of *Helicobacter pylori* with professional phagocytes: role of the Cad pathogenicity island and translocation, phosphorylation and processing of CagA. 2001;3(1):21-31.
61. Allen LAH, Schlesinger LS, and Kang B. Virulent strains of *Helicobacter pylori* demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages. *Journal of experimental medicine.* 2000;191(1):115-127.
62. Bourzac KM and Guillemin K. *Helicobacter pylori*-host cell interactions mediated by type IV secretion. *Cellular microbiology.* 2005;7(7):911-919.
63. Frithz-Lindsten E, Du Y, Rosqvist R, and Forsberg A. Intracellular targeting of exoenzyme S of *Pseudomonas aeruginosa* via type III-dependent translocation induces phagocytosis resistance, cytotoxity and disruption of actin microfilaments. *Molecular microbiology.* 1997;25(6):1125-1139.
64. Deng Q and Barbieri JT. Modulation of host cell endocytosis by the type III cytotoxin, *Pseudomonas* ExoS. *Traffic.* 2008;9:1948-1957.
65. Garrity-Ryan L, Kazmierczak B, Kowal R, Comolli J, Hauser A, and Engel JN. The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of *Pseudomonas aeruginosa* by epithelial cells and macrophages. *Infection and immunity.* 2000;68(12):7100-7113.
66. Cowell BA, Evans DJ, and Fleiszig SMJ. Actin cytoskeleton disruption by ExoY and its effects on *Pseudomonas aeruginosa* invasion. *FEMS microbiology letters.* 2005;250:71-76.
67. Brugirard-Ricaud K, Duchaud E, Givaudan A, Girard PA, Kunst F, *et al.* Site-specific antiphagocytic function of the *Photohabdus luminescens* type III secretion system during insect colonization. *Cellular microbiology*. 2005;7(3):363-371.
68. Vojtova J, Kamanova J, and Sebo P. *Bordetella* adenylate cyclase toxin: a swift saboteur of host defense. *Current opinion in microbiology.* 2006;9:69-75.
69. Huam Yuk M, Harvill ET, Cotter PA, and Miller JF. Modulation of host immune responses, induction of apoptosis and inhibition of NF-kB activation by the *Bordetella* type III secretion. *Molecular microbiology.* 2000;35(5):991-1004.
70. Skinner JA, Reissinger A, Shwen H, and Yuk MH. *Bordetella* type III secretion and adenylate cyclase toxin synergize to drive dendritic cells into a semimature state. *The journal of immunology*. 2004;173:1934-1940.
71. Kamanova J, Kofronova O, Masin J, Genth H, Vojtova J, *et al.* Adenylate cyclase toxin subverts phagocyte function by RhoA inhibition and unproductive ruffling. *The journal of immunology*. 2008;181:5587-5597.
72. Horiguchi Y, Inoue N, Masuda M, Kashimoto T, Katahira J, *et al. Bordetella bronchiseptica* dermonecrotizing toxin induces reorganization of actin stress fibers through deamidation of Gln-63 of the GTP-binding protein Rho. *PNAS*. 1997;94:11623-11626.
73. Kalamidas SA, Kuehnel MP, Peyron P, Rybin V, Rauch S, *et al.* cAMP synthesis ans degradation by phagosomes regulate actin assembly and fusion events: consequences for mycobacteria. *Journal of cell science.* 2006;119:3686-3694.
74. Downey GP, Elson EL, Schwab III B, Erzurum C, Young SK, and Worthen GS. Biophysical properties and microphilament assembly in neutrophils: modulation by cyclic AMP. *Journal of cell biology.* 1991;114:1179-1190.

75. Goto T, Yamashita A, Hirakawa H, Matsutani M, Todo K, *et al.* Complete genome sequence of *Finegoldia magna*, an anaerobic opportunistic pathogen. *DNA research*. 2008;15:39-47.
76. Yeager LA, Chopra AK, and Peterson JW. *Bacillus anthracis* edema toxin suppress human macrophage phagocytosis and cytoskeletal remodeling via the protein kinase A and exchange protein activated by cyclic AMP pathways. *Infection and immunity.* 2009;77(6):2530-2543.