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TYPE III SECRETION SYSTEM-MEDIATED IMMUNE MODULATION BY ENTEROPATHOGENIC BACTERIA

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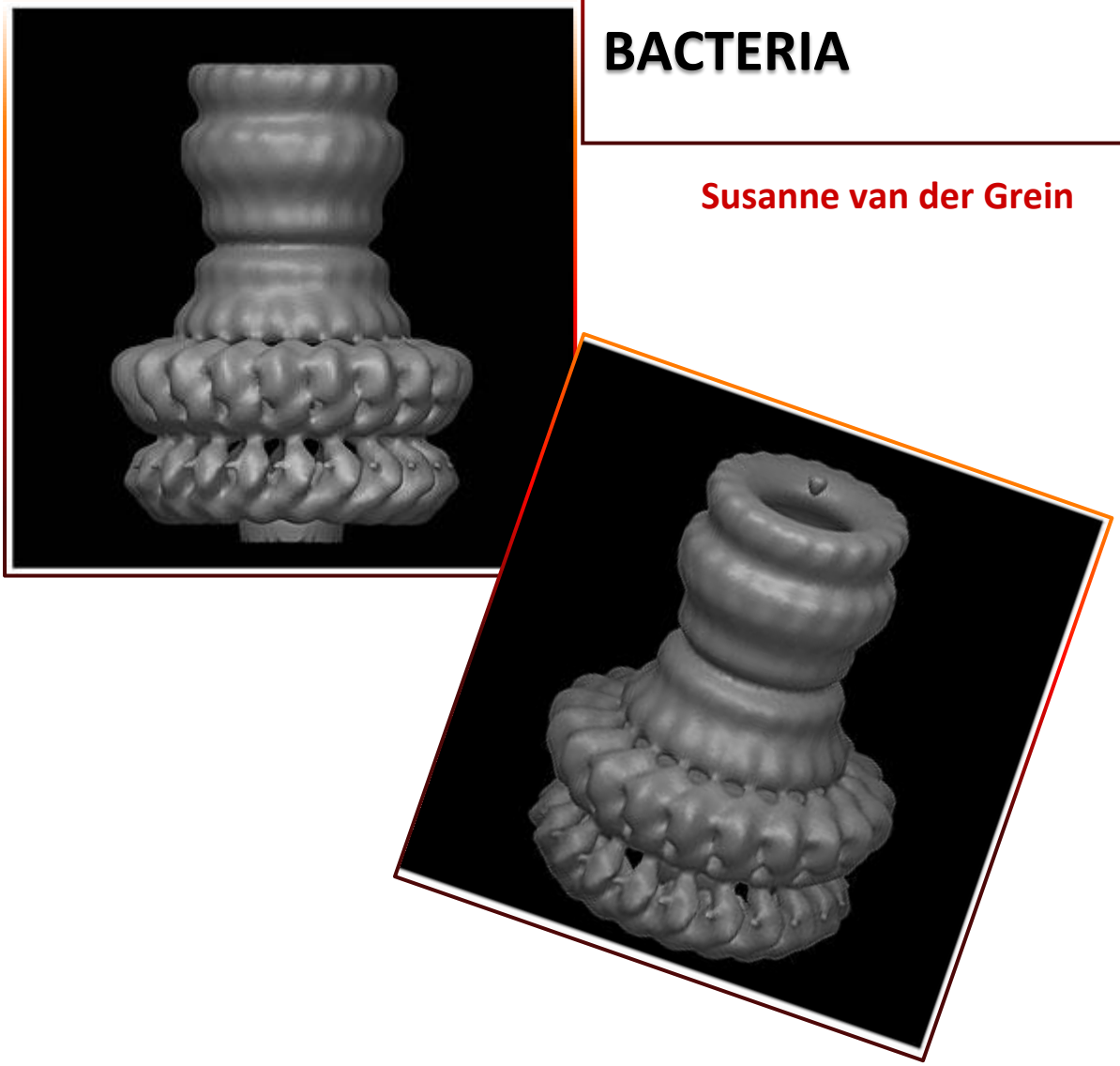

Master thesis

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Title: Type III secretion system-mediated immune modulation by enteropathogenic bacteria

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Figure on title page: Three dimensional modeling of *Salmonella typhimurium* type III secretion system needle complex.

From: Marlovits TC, Kubori T, Lara-Tejero M, Thomas D, Unger VM, Galán JE. Assembly of the inner rod determines needle length in the type III secretion injectisome. *Nature.* 2006, 441: 637-340.

LAYMAN'S SUMMARY

Four major bacteria species that cause intestinal disease are enteropathogenic and enterohaemorrhagic Escherichia coli (EPEC/EHEC), Yersinia, Salmonella and Shigella. These bacteria have a complex structural component called the type III secretion system (T3SS). The ability of these pathogens to cause disease depends on their T3SS. The T3SS resembles a needle that extends from the bacterial surface to the host cell. Through this hollow needle bacterial molecules are injected into the host cell that manipulate host cell functions to the bacterium's advantage. These molecules are called effectors. In the intestinal system many immune cells are present that protect the human body against pathogens invading the gut with our food. To be able to establish an infection, enteropathogenic bacteria must combat the intestinal immune system. To this end, T3SS-injected effectors interfere with several cell types with an immunological function, like intestinal epithelial cells and phagocytic cells. EPEC/EHEC, Yersinia, Salmonella and Shigella have each developed a set of effectors that manipulate the intestinal immune system in a manner specific to each bacterial species. EPEC/EHEC and Yersnina prevent inflammatory signaling in intestinal epithelial cells that would warn immune cells of an infection and prevent the uptake into phagocytic immune cells that would destroy the bacteria. Salmonella and Shigella initially also inhibit inflammatory signaling in epithelial cells, but later on stimulate inflammation to attract phagocytic immune cells. These cells are then exploited to spread further throughout the body while destruction of the bacteria by these cells is prevented. Each effector set is highly adapted to specific functions that supports survival and spreading of the bacterial species.

Type III secretion system-mediated immune modulation by enteropathogenic bacteria

EPEC/EHEC, Yersinia, Salmonella and Shigella are four major enteropathogenic bacteria species that cause gastrointestinal disease using a type III secretion system (T3SS). The T3SS complex forms a needle that injects bacterial effector proteins into host cells. T3SS effectors modulate cellular functions to the bacterium's own advantage. T3SS effectors interfere with the intestinal immune system to prevent eradication and establish enteric infection. Enteropathogenic bacteria encounter intestinal epithelial cells, phagocytic immune cells and cells of the adaptive immune system during the course of an infection. The enteropathogenic bacterial species have each developed a strategy to modulate the immune function of these cell types. While EPEC/EHEC and Yersinia actively prevent intestinal epithelial cell inflammation and phagocytic uptake, Salmonella and Shigella exploit inflammation and phagocytic cells to spread. These virulence strategies are carried out by a species-specific set of T3SS effectors. T3SS effectors have a variety of functions, but often share cellular targets, like the pro-inflammatory transcription factor NF-κB and regulators of cytoskeletal rearrangements. T3SS effector proteins are extensively adapted to a host cell specific virulence function. Together, the set of injected T3SS effectors manipulate the host's immune response to promote bacterial survival, for each species in a unique manner.

T3SS-EXPRESSING ENTEROPATHOGENIC BACTERIA

The human intestinal tract is often viewed as a potent immunogenic organ[1]. Daily, many harmless but also potentially dangerous microorganisms pass through the intestinal tract with our food. The epithelial cells that line the intestinal lumen (enterocytes) act as sentries to evoke either tolerance or a response to respectively commensal and enteropathogenic bacteria[1,2]. In the underlying mucosa, organized gut-associated lymphoid tissue (GALT) such as Peyer's patches are crowded by immune cells like macrophages, dendritic cells (DCs) and T and B lymphocytes[3]. The intestinal epithelium is very important in generating and regulating the mucosal immune response[2]. The epithelium covering GALT is termed follicle-associated epithelium (FAE) and contains microfold cells (M cells) that directly sample the contents of the intestinal lumen. M cells take up antigenic material from the gut lumen and shuttle it to antigen presenting cells populating the FAE dome region, initiating an appropriate response[1]. In addition, upon enteropathogenic bacterial invasion, enterocytes produce signals that alert immune cells of a possible infection. One major product of infected enterocytes is the pro-inflammatory cytokine interleukin 8 (IL-8)[4]. IL-8 is secreted basolaterally and acts as a principal chemoattractant summoning polymorphonuclear cells (PMNs, mainly neutrophils) to the site of infection[5]. PMNs and other phagocytic cells like macrophages, are considered very important in the clearance of enteric bacterial infection[6]. Full clearance also requires eradication of bacteria on the luminal side of the intestinal wall, where attachment of bacteria to enterocytes is the first step in host invasion. Transepithelial migration of PMNs is triggered by chemoattractant factors secreted on the apical side of enterocytes. The flip-side of PMN transepithelial migration is disruption of the epithelial barrier, contributing to diarrhea and clearing a path for bacteria to disseminate throughout the body[7]. Thus, PMN transepithelial migration is often an important aspect of both the pathogenicity and clearance of enteric bacterial infection.

Four major enteropathogenic bacteria species are EPEC/EHEC, Yersinia, Shigella and Salmonella. These food-borne pathogens can cause severe diarrhea and other intestinal problems as well as systemic disease in some cases[7]. A key virulence determinant of these and other Gram-negative bacteria is the type III secretion system (T3SS)[8]. The T3SS resembles a molecular syringe that spans both bacterial membranes and the host cell

membrane[9]. Through their T3SS, bacteria directly inject proteins termed effectors into the host cell cytoplasm to modulate cellular functions[8]. The T3SS is a multiprotein complex, composed of over twenty proteins. The T3SS complex can largely be divided into three parts: the basal body embedded in both bacterial membranes, an extracellular hollow needle-like filament, and the translocon which forms a pore in the eukaryotic target membrane (see Figure 1)[9]. The T3SS and its effectors are often encoded on pathogenicity islands in the bacterial genome[7]. Some of the T3SS-injected effectors modulate the host cell's immunological functions to promote bacterial survival. EPEC/EHEC, Yersinia, Shigella and Salmonella will come into contact with intestinal epithelial cells, phagocytes, and B and T cells of the adaptive immune system on their path of infection. In this review the modulating effect of their T3SS-injected effectors on the immune function of each of these cell types will be discussed. First the bacterial species will be introduced shortly. The mechanisms of host colonization of the enteropathogenic bacteria species EPEC/EHEC, Yersinia, Salmonella and Shigella are summarized in Figure 2.

Enteropathogenic Escherichia coli: EPEC and EHEC

The human pathogens Enteropathogenic and Enterohemorrhagic *Escherichia coli* (EPEC and EHEC respectively) are among the leading causes of diarrheal disease worldwide. In the intestinal tract EPEC and EHEC attach and colonize on the luminal side of the epithelial layer; the bacteria remain extracellular (see Figure 2a). EPEC and EHEC induce A/E (attaching and effacing) lesions, a striking histopathological feature characterized by intimate adherence to the apical enterocyte surface, formation of a cytoskeleton-based pedestal-like structure on which the bacteria lie and replicate, and destruction of microvilli. EPEC/EHEC carry a pathogenicity island termed the locus of enterocyte effacement (LEE), which is of critical importance to pathogenesis and encodes the T3SS. Following initial contact with enterocytes, the T3SS is formed and inserted into the host cell membrane and injects effectors that are LEE-encoded or lie scattered throughout the EPEC/EHEC genome (non-LEE encoded, or Nle effectors)[7,10].

Yersinia

Enteropathogenic Yersinia species are *Yersinia pseudotuberculosis* and *Yersinia enterocolitica.* Enteropathogenic Yersinia initially contact M cells and exploit them to acquire access to the mucosal lymphoid tissue, where Yersinia bacteria replicate and form extracellular microcolonies (see Figure 2b). *Y. pseudotuberculosis* and *Y. entercolitica* share a virulence plasmid (pYV) that encodes the T3SS and effectors called Yersinia outer proteins (Yops). Upon interaction with phagocytic cells, the T3SS is assembled and secretes Yops to help the bacteria persist extracellularly[7,11].

Salmonella

Salmonella enterica are a group of pathogens that can cause a variety of intestinal diseases. Typhoidal strains, like *S. enterica* serovars Typhi and Paratyphi can cause severe systemic illness called enteric fever. In contrast, non-typhoidal strains like *S. enterica* serovars Typhimurium and Enteridis, can cause a possibly self-limiting gastroenteritis. Salmonella expresses two distinct T3SS, encoded by pathogenicity islands 1 and 2 (SPI-1 and SPI-2). Upon initial contact with M cells, the SPI-1 T3SS is activated to translocate effectors across the host cell membrane, triggering Salmonella internalization (see Figure 2c). The elicited inflammatory response compromises the epithelial barrier, causing bacteria to cross. Subsequently, bacteria infect also epithelial cells and phagocytes. Inside cells, Salmonella resides in so-called Salmonella containing vacuoles (SCV) where the bacteria replicate. The SPI-2-encoded T3SS is expressed by internalized bacteria and translocates effectors across the vacuolar membrane to support intracellular survival and replication[7,12].

Shigella

All four Shigella species (*S. flexneri, S. dysenteriae, S. boydii* and *S. sonnei)* can cause disease called bacillary dysentery or shigellosis in humans. Shigella shows tropism for colonic and rectal epithelial cells, which they can only

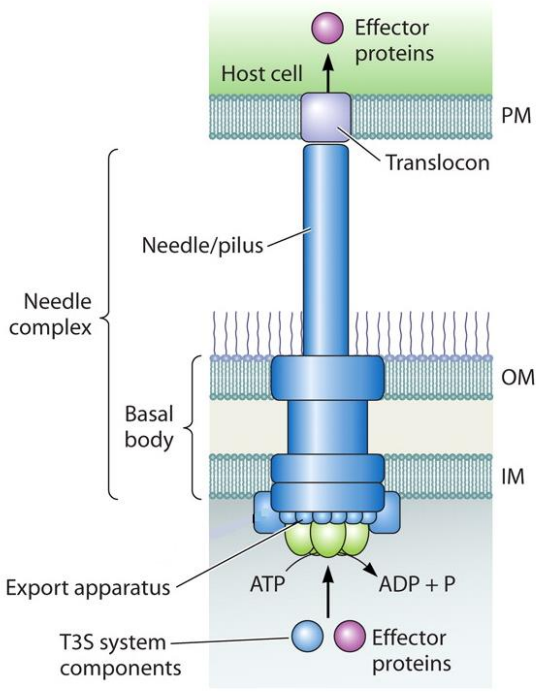

Figure 1: The T3SS complex forms a needle-like structure through which effectors are secreted.
The T3SS is a multiprotein complex composed of a basal body spanning both the outer (OM) and inner (IM) bacterial membrane, a needle/pilus that extends towards target cells, and a translocon at the tip of the needle that is inserted into the host cell membrane (PM). Bacterial effector proteins are transported through the hollow needle filament to host cells, at the cost of ATP.

infect basolaterally. The epithelial basolateral membrane is reached by either one of three ways. Initially, Shigella target M cells that shuttle or transcytose the bacteria from the intestinal lumen to the subepithelial FAE dome region (see Figure 2d). Secondly, Shigella can modify tight junctions to traverse the epithelial barrier, and finally Shigella can benefit from inflammation-induced PMN transepithelial migration disrupting the epithelial layer. Activated by host-cell adherence, Shigella uses its T3SS encoded on a large virulence plasmid to inject effectors that induce bacteria uptake. Inside epithelial cells, Shigella escapes the vacuole and replicates in the cytoplasm, from where it can spread directly to neighboring cells. A second set of T3SS effectors are delivered by intracellular Shigella and sustain cytoplasmic replication and survival[7,13].

IN CONTACT WITH INTESTINAL EPITHELIAL CELLS: MODULATING THE INFLAMMATORY RESPONSE

The first cells encountered by enteropathogenic bacteria are the epithelial cells lining the intestinal tract. Upon pathogen recognition, these enterocytes can initiate an inflammatory response. Key regulators of the intestinal epithelial cell's inflammatory response to enteropathogenic bacteria are the NF-κB family of transcription factors[14]. The term NF-κB is collectively used for homo- or heterodimers of Rel (RelA or p65, RelB, c-Rel) and NF-κB-proteins (p50 or NF-κB1, p52 or NF-κB2). The most abundant form in mammalian cells is a p65/p50 dimer[14]. In inactivate state, NF-κB is retained in the cytoplasm by its association with inhibitory protein IκB[15]. Various stimuli, such as TNFα, IL-1β and Toll-like receptor (TLR) signaling can lead to the activation of the TAK1 complex, which can phosphorylate and activate the IκB-kinase complex (IKK)[16]. Subsequently, IκB becomes phosphorylated by IKK, poly-ubiquitinated and degraded, exposing a nuclear localization signal on NF-κB which is then translocated to the nucleus[15]. Here it can exert its function as transcription factor and lead to the expression of several genes involved in inflammation, such as IL-8, IL-6, IL-1β and TNFα[16].

Also involved in regulation of inflammation are mitogen-activated protein kinases (MAPK) like c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinases (ERK1/2). MAPKs are a family of serine/threonine-specific protein kinases involved in the regulation of a multitude of cellular functions including cell proliferation and differentiation, cell survival and apoptosis[17]. MAPKs can become activated in a signaling cascade culminating in phosphorylation of MAPKs by a MAPK-kinase (MAP2K), preceded by the phosphorylation of a MAP2K by a MAPK-kinase-kinase (MAP3K)[17].

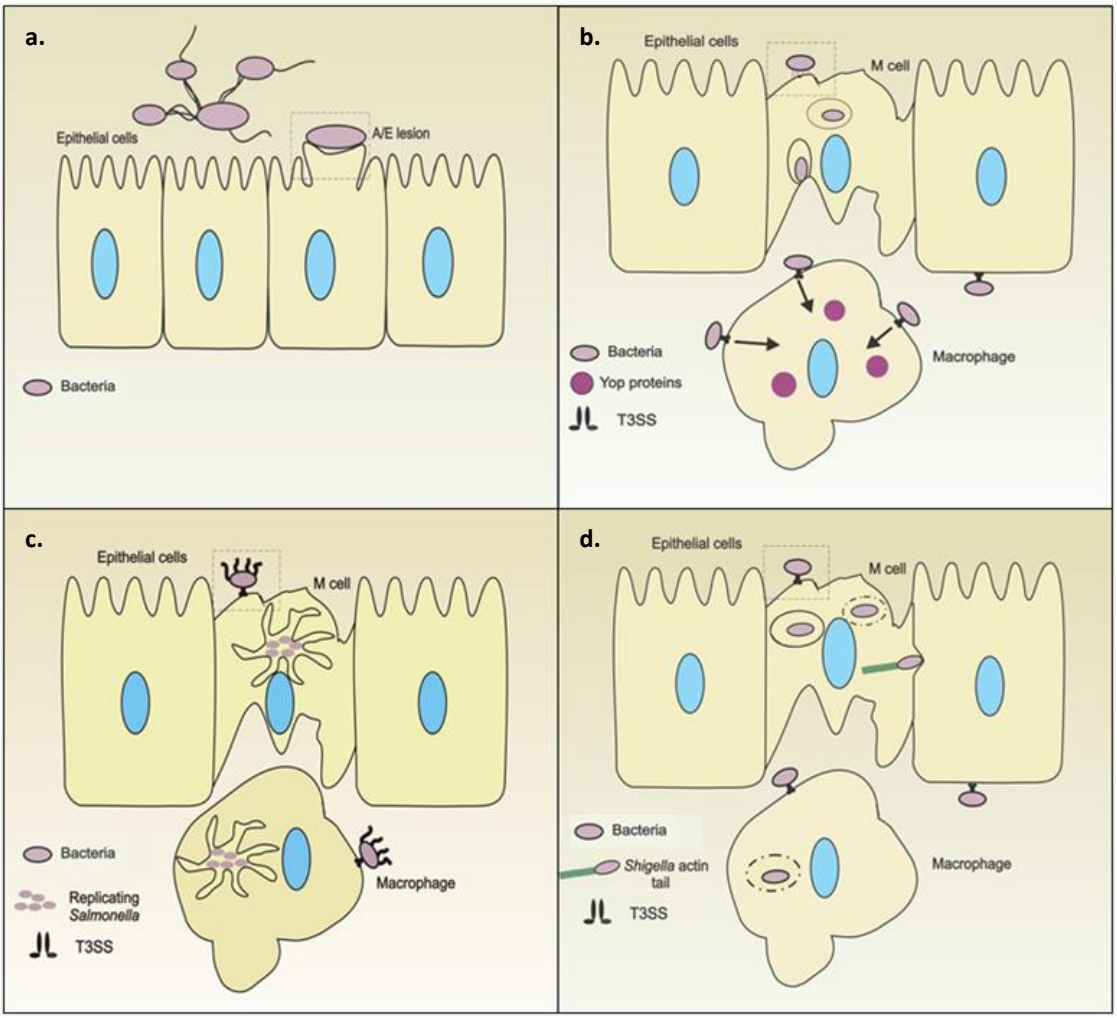

Figure 2: EPEC/EHEC, Yersinia, Salmonella and Shigella colonize by different mechanisms.
a. EPEC/EHEC attach to the intestinal epithelial layer and induce A/E lesions by a T3SS-depedent mechanism. *b.* Yersinia traverses the intestinal epithelium via M cells. In the FAE-dome region Yersinia replicates extracellularly. The T3SS effectors called Yop proteins target phagocytes. *c.* Salmonella invades the host via M cells and replicates intracellularly in epithelial cells and phagocytes in a vacuole. *d.* Shigella infects colonic/rectal epithelial cells basolaterally via M cell transcytosis and epithelial barrier or tight junction disruption. Shigella escapes the vacuole and replicates in the cytoplasm of epithelial cells. Shigella are propelled to neighboring cells by an actin-driven tail. Adapted from ref 7.

In response to TNFα, IL-1β or TLR signaling, activated TAK1 functions as a MAP3K ultimately bringing about the activation of JNK and p38[18]. JNK and p38 can activate transcription factor AP-1, which can transcribe several inflammation mediators, including IL-8[19]. ERK1/2 is involved in the induction of PMN transepithelial migration[20,21].

Enteropathogenic bacteria species are recognized by pattern recognition receptors (PRR) like TLRs and Nod1-like receptors (NLR) that recognize pathogen-associated molecular patterns (PAMPs)[3,22,23]. Recognition of enteropathogenic bacteria by TLR/NLR leads to initiation of an inflammatory response via NF-κB and MAPK activation[24]. However, EPEC/EHEC, Yersinia, Shigella and Salmonella species have been shown to downmodulate pro-inflammatory signaling in intestinal epithelial cells, especially in early stages of infection to prevent premature eradication by the host's immune system and ensure colonization by an initial low number of bacteria[25-28]. EPEC/EHEC, Yersinia, Shigella and Salmonella all inhibit IL-8 secretion from enterocytes, activity dependent on their T3SS[25-28]. Sharma *et al* (2006) show that the inflammatory response to EPEC infection is the net result of a balance between pro-

inflammatory extracellular factors that are shed or secreted by the bacteria and anti-inflammatory intracellular T3SS-injected effectors[27]. Infecting intestinal epithelial cells with increasing concentrations of bacterial supernatant induces proportionally increasing IL-8 production, whereas infection of cells with whole EPEC bacteria with increasing multiplicity of infection (MOI) is inversely correlated to IL-8 production[27]. Infection with a non-functional T3SS mutant increases IL-8 production up to bacterial supernatant levels[27]. At the start of infection inflammation ensues due to recognition of bacterial PAMPs. For example, LPS or flagella and the shed monomer protein flagellin have been shown to be major inducers of IL-8[29-31]. However, once efficient bacterial effector delivery to the host cell is established, the inflammatory response is actively being repressed. Inhibition of IL-8 secretion is caused by disruption of NF-κB and MAPK activation, for which enteropathogenic bacteria have developed various strategies.

EPEC/EHEC

Much research has been performed on inhibition of NF-κB by EPEC/EHEC. EPEC and EHEC strains repress NF-κB activation in epithelial cells from 2 hours post-infection onwards[32]. EPEC and EHEC encode several T3SS-injected effectors that exert NF-κB inhibitory function at several points along the NF-κB activation axis. In a study performed by Nadler *et al* (2010) interference with NF-κB activation could predominantly be attributed to effector NleE, aided by effector NleB[28]. NleE prevents both TNFα and IL-1β-induced NF-κB activation, while NleB suppresses only TNFα-induced NF-κB activation[33]. NleE is believed to act by preventing the activation of IKK whereas NleB is hypothesized to act upstream from TAK1 in the TNFα signaling cascade, since the IL-1β and TNFα pathway converge at the level of TAK1[28,33]. NleE and NleB do not account for the full immune-suppressive potential of EPEC and EHEC. In case of a *ΔnleEΔnleB* double deletion mutant there is residual repression of NF-κB in comparison to a T3SS-defective strain[28,34]. NleC, a zinc metalloprotease, was found to suppress NF-κB activation by cleaving both cytoplasmic and nuclear p65[34-37]. Expression of NleC might be regulated to occur later than NleE. At 2-4 hours of infection NleE-dependent NF-κB inhibition has a prominent role, whereas at 6 hours NleC-induced p65 degradation becomes more evident, suggesting either a build-up of injected NleC or an increase of the target molecule p65[36]. NleD, like NleC, is a zinc metalloprotease that cleaves JNK and p38, leading to their degradation[38]. NleD adds to the NF-κB-inhibition-mediated IL-8 repression by preventing AP-1 activation, since both NF-κB and AP-1 are required for maximal induction of IL-8 expression[29,38]. In cells infected with EPEC *ΔnleEΔnleC*, the IL-8 production levels reach the level of cells infected with a T3SS defective mutant[34,36]. Interestingly, cells infected with a *ΔnleEΔnleBΔnleCΔnleD* mutant, secrete more IL-8 than cells infected with a T3SS-deficient mutant[38]. This may be due to the fact that structural components of the T3SS are also considered PAMPs to which a pro-inflammatory reaction is instigated[39].

The EPEC and EHEC genome also contains two copies of the gene encoding effector NleH: *nleH1* and *nleH2*. The effect of NleH on NF-κB has for long been debated[40-42]. Gao *et al* (2009) show that NleH interacts indirectly with NF-κB via RPS3 (human ribosomal protein S3), a NF-κB complex subunit that directs specific NF-κB-dependent gene transcription[41,43]. NleH1, but not NleH2, inhibits TNFα-induced phosphorylation of RPS3 by IKK, necessary for its nuclear localization, thereby preventing its association with NF-κB in the nucleus and NF-κB-RPS3-dependent gene transcription[41,44]. NleH1 and NleH2 have been shown to be able to bind each other[45]. In cells co-transfected with both NleH1 and NleH2 in different molar ratios, increasing NleH2 leads to a diminished inhibition of NF-κB activity[45]. Thus, NleH2 may offer an extra level of regulation to NleH1-mediated NF-κB inhibition.

Taken together, several EHEC and EPEC effectors may cooperate to achieve full repression of IL-8 secretion. The actions of NleE, NleB, NleC, NleH and NleD are summarized in Figure 3.

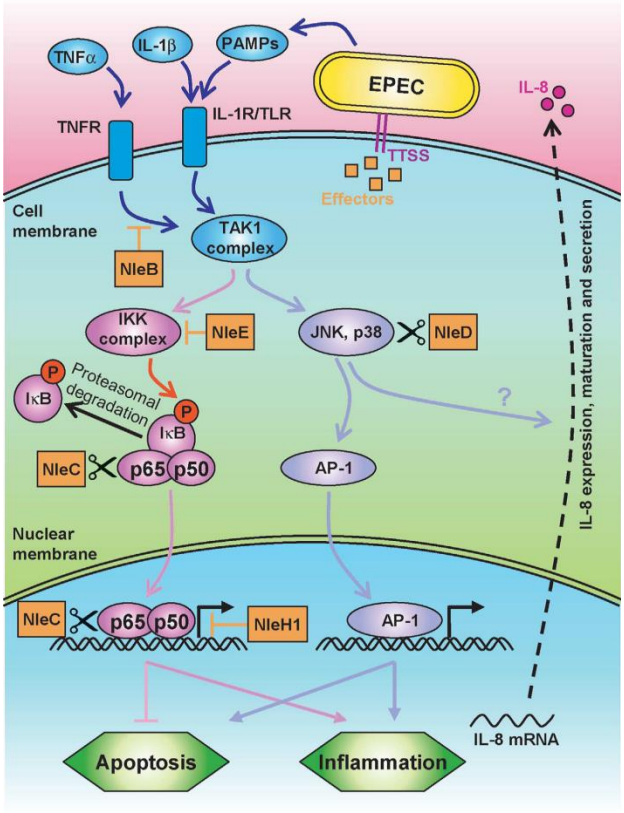

Figure 3: EPEC/EHEC Nle effectors target intestinal epithelial cell's inflammatory signaling.
EPEC/EHEC attaches to intestinal epithelial cells and inject effector proteins via their T3SS (=TTSS). Several Nle effectors target IL-8 production by suppressing NF-κB and AP-1 transcription factor activation which is triggered by cytokine or TLR signaling. NleB prevents NF-κB activation at a level upstream of the TAK1-complex, whereas NleE inhibits the IKK complex. NleC induces p65 degradation both in the cytoplasm and in the nucleus, while NleD cleaves JNK and p38 to prevent AP-1activation. NleH1 inhibits specific NF-κB-dependent transcription by binding a NF-κB complex subunit. From ref 38.

Yersinia, Salmonella and Shigella

Yersinia, Salmonella and Shigella also secrete effectors that interfere with NF-κB-mediated inflammatory signaling. Yersinia species encode the effector YopP/J (YopP in *Y. enterocolitica*, YopJ in *Y. pseudoturberculosis*) that exerts NF-κB and MAPK inhibitory function resulting in disrupted IL-8 and TNFα production by epithelial cells and macrophages respectively[46-49]. The mechanism of action of YopP/J remains under debate. YopP/J has similarity to a family of cysteine proteases that act as deubiquitinating and deSUMOylating enzymes[50], but has also been shown to have serine/threonine acetyltransferase activity[51-53]. YopP/J could deubiquitinate and/or acetylate and thereby inactivate several components of the NF-κB and MAPK pathways at the level of IKK, MAP2Ks, MAP3Ks or further upstream of these[51-54]. The Salmonella effector protein AvrA, a homologue of YopP/J inhibits NF-κB activation by a mechanism apparently distinct from YopP/J[55,56]. AvrA is a cysteine protease that deubiquitinates IκB, thus preventing its degradation and NF-κB activation[57]. In contrast, YopP/J blocks the NF-κB pathway upstream of IKK. Others have shown AvrA having only a moderate effect on NF-κB, and exerting acetyltransferase activity towards specific MAP2Ks that can activate JNK[58,59].

Intracellular replicating Shigella bacteria are mainly recognized by Nod1 receptors, which leads to NF-κB-mediated inflammation[60]. Shigella encode a family of highly homologous T3SS effectors called IpaH proteins that are secreted during the bacteria's intracellular phase[61]. A shigella *ΔipaH-null* mutant causes more severe inflammation in a murine lung infection model than WT bacteria[61], suggesting that IpaH proteins function to dampen the inflammatory response. The IpaH family includes the *Salmonella enterica* serovar Typhimurium effector SspH1 and comprises a novel class of E3 ubiquitin ligases, with no similarity to known eukaryotic HECT or RING type E3 ligases[62,63]. The IpaH proteins contain a variable N-terminal leucine-rich repeat (LRR) domain which determines binding partner specificity, a conserved C-terminal E3 ubiquitin ligase domain, and they

localize to the host cell's nucleus[63-65]. SspH1 downregulates NF-κB-dependent pro-inflammatory cytokine expression, possibly by acting as an E3 ubiquitin ligase for the mammalian kinase PKN1, which is part of the NF-κB pathway[26,62,66]. The Shigella IpaH effector IpaH9.8 is responsible for ubiquitinating a component of the IKK-complex, triggering its proteasomal degradation and subsequently perturbing NF-κB activation[67]. IpaH9.8 also localizes to the nucleus where it binds mammalian splicing factor $U2AF_{35}$, disrupting $U2AF_{35}$-dependent splicing of IL-8 mRNA[68].

Like IpaH9.8, the Shigella effector OspF localizes to both the nucleus and the cytoplasm[69]. In the nucleus OspF induces chromatin remodeling, rendering the NF-κB promotor site inaccessible which leads to suppression of IL-8 transcription[70]. This effect of OspF may be linked to its phosphothreonine lyase activity that irreversibly removes phosphate groups from threonine residues in the activation loop of MAPKs, thereby completely inactivating them[71]. Cytoplasmic activity of OspF may affect PMN transepithelial migration, possibly involving binding to ERK1/2 and altering its phosphorylation state[69,72]. SpvC, a non-typhoidal Salmonella strain T3SS-secreted effector, is homologous to OspF. SpvC has been shown to have phosphothreonine lyase activity towards MAPKs and to downregulate pro-inflammatory cytokine expression in infected cells[71,73,74].

Taken together, EPEC/EHEC, Yersinia, Salmonella and Shigella species encode many T3SS effectors that interfere with the intestinal epithelial cell's inflammatory response at early stages of infection, mainly by acting on the NF-κB and MAPKs signaling pathways. More T3SS-injected effectors that are not mentioned here play a role. The modulation of the epithelial cell's inflammatory response by T3SS effectors is complex. At least for Salmonella and Shigella, but maybe also Yersinia and even EPEC/EHEC, later on in infection pro-inflammatory signaling is stimulated in a T3SS-dependent manner[7,72.] The damage to the epithelial barrier brought forth by activated immune cells aids bacterial dissemination. Together, the recognition of bacterial PAMPs and pro-inflammatory signaling leads epithelial cells to call upon phagocytes to clear the infection. The effect of T3SS-injected effectors on phagocytic cells will be discussed next.

IN CONTACT WITH PHAGOCYTES: MODULATING THE INNATE CELLULAR RESPONSE

Phagocytosis is the process by which specialized innate immune cells can internalize and destroy extracellular antigenic material. Macrophages and neutrophils are examples of professional phagocytes. As mentioned earlier, EPEC and EHEC species colonize on the luminal side of the intestinal epithelial wall, whereas Yersinia replicates in the FAE dome region after traversing the epithelial layer via M cells. Both pathogens remain largely extracellular and therefore must actively prevent their uptake in phagocytic cells in which they could not survive. In contrast, Salmonella and Shigella species invade both epithelia and phagocytes, and combat phagocytic degradation by other means. Both strategies employ the T3SS and its effectors.

EPEC/EHEC

Indeed, EPEC/EHEC species have been shown to inhibit their own phagocytic uptake by macrophages[75-77]. EPEC/EHEC anti-phagocytic activity depends on T3SS-mediated effector delivery, since T3SS-deficient bacteria are efficiently internalized by macrophages whereas WT bacteria remain extracellular[75-77]. So far, T3SS effectors EspB, EspF, EspH and EspJ have been ascribed an anti-phagocytic role[75,77-80]. Together, these effectors cooperate to ensure the exclusion of EPEC/EHEC from the intracellular milieu of phagocytes. The exact mechanism of action of each of these effectors is unknown, however it is not unlikely that they all act as modulators of the cytoskeleton. The process of internalizing extracellular material requires extensive cytoskeletal rearrangements in the phagocytic cell and EPEC/EHEC are known to cause massive reorganization of the

cytoskeleton in epithelial cells, resulting in the distinctive A/E lesions[10]. The effector EspB blocks the function of myosins by occupying the actin binding domain on these proteins[80]. Interaction between the cytoskeleton filament actin and myosins is believed to provide the force needed to close the phagosome[80]. An EspB mutant bacterium that cannot bind myosin is unable to inhibit its own phagocytic uptake, demonstrating the importance of EspB to EPEC/EHEC anti-phagocytic activity[75,80]. EspF is also considered indispensable for suppressing bacterial phagocytosis, although its mechanism of action is poorly understood[77]. Surprisingly, the effector EspJ blocks the internalization of opsonized particles including material other than the bacteria itself, a process referred to as *trans*-inhibition of phagocytosis[79]. Blockage of the bacteria's own uptake is termed *cis*-inhibition. EPEC/EHEC *espJ* mutants were unable to inhibit the uptake of red blood cells opsonized with antibodies or complement, whereas an *espF* mutant suppressed opsono-phagocytosis *in trans* similar to WT bacteria[79]. Accordingly, EspJ did not play a role in *cis*-inhibition of phagocytosis of non-opsonized EPEC/EHEC bacteria. Thus, EspF and EspJ target distinct pathways of phagocytosis by very different mechanisms. A model is proposed where EspF-mediated *cis*-inhibition prevents phagocytic uptake of bacteria early on in infection to allow for colonization, whereas *trans*-inhibition occurs later to prevent internalization of A/E lesional material once the bacteria are firmly attached to epithelial cells[79]. Finally, EspH has emerged as an inhibitor of both phagocytosis and opsono-phagocytosis by macrophages. EspH markedly disrupts actin skeleton organization, by disrupting Rho GTPase signaling[78]. Proteins of the Rho GTPase family, like RhoA, Rac-1 and Cdc42, regulate cytoskeletal rearrangements necessary for phagocytosis[81].

Yersinia

During Yersinia infection, Yersinia attaches to phagocytes in the FAE dome region. The interaction between Yersinia proteins and host cell integrins triggers phagocytosis through the activation of Rho GTPase proteins and the initiation of phosphorylation events that activate the focal adhesion complex. However, four Yersinia T3SS effector Yops, have been ascribed a function in conferring resistance to phagocytic uptake by macrophages and neutrophils: YopH, YopE, YopT and YopO[82]. YopH is an abundant phosphotyrosine phosphatase that antagonizes several host cell signaling pathways[83]. YopH inhibits its own phagocytic uptake by targeting early phosphorylation events of the integrin signaling pathway. For example, YopH dephosphorylates components of the focal adhesion complex, causing focal adhesions to disassemble and disconnect from the actin cytoskeleton, which impairs the uptake of bacteria[83]. Besides preventing the internalization process itself, YopH activity also counteracts processes associated with phagocytosis, like the oxidative burst in macrophages and neutrophils[84,85], which is the release of reactive oxygen species (ROS) that can kill pathogens. Also, YopH blocks the degranulation of neutrophils by interfering with Ca^{2+} signaling[86]. The importance of YopH in blocking phagocytosis is illustrated by the fact that macrophages internalize 80% of *yopH* mutant bacteria, in comparison to 95% of virulence plasmid deficient (pYV^-) and 35% of virulence plasmid carrying (pYV^+) bacteria[87].

YopE, YopT and YopO are members of a bacterial effector family that act on Rho GTPases that control actin cytoskeleton organization. YopE exhibits GAP (GTPase activating protein) activity towards RhoA, Rac-1 and Cdc42 *in vitro*, stimulating the GTPase's inherent ability to hydrolyze its bound GTP to GDP, rendering the GTPase inactive and terminating the signaling event[88]. In a study performed by Grosdent *et al* (2002), it appeared that YopE may preferentially target Rac-1 over RhoA *in vivo*[82]. YopE was shown important for conferring anti-phagocytic activity towards non-opsonized bacteria, but not towards its opsonized counterparts. Under these experimental conditions, opsono-phagocytosis would be mediated by a RhoA-dependent pathway whereas phagocytosis of non-opsonized particles would depend on Rac-1[82]. YopT acts as a cysteine protease that cleaves off a lipid modification moiety from RhoA, causing the GTPase to dissociate from the membrane and become inactive[89]. At least

in vitro, Rac-1 and Cdc42 can also be targeted by YopT. The importance of YopT for Yersinia pathogenesis remains under discussion, as some strains lack YopT. YopO (or YpkA, Yersinia protein kinase A) also contributes to the anti-phagocytic activity of Yersinia, as a *ΔyopO* deletion mutant was more efficiently internalized by macrophages and neutrophils than the WT parental strain[82]. How exactly YopO inhibits Rho GTPase signaling is unsure, although interaction with RhoA, Rac-1 and actin, and serine/threonine kinase activity have been shown[11].

The anti-phagocytic actions of YopH, YopE, YopT and YopO allow Yersinia to proliferate as extracellular microcolonies in the macrophage-dense milieu of GALT like Peyer's patches. The uptake of Yersinia bacteria by phagocytic cells cannot fully be prevented though. However, Yersinia can trigger bacteria-containing macrophages to undergo apoptosis[90,91]. As described earlier, YopP/J interferes with MAPK and NF-κB signaling[46-49,92]. Besides suppressing the inflammatory response, this also results in the induction of apoptosis in macrophages, as NF-κB and MAPK regulate the expression of cell survival factors and apoptosis inhibiting genes[93]. Remarkably, the block in NF-κB signaling does not result in apoptosis in epithelial cells, unless they are simultaneously stimulated with TNFα[94]. This requirement is not met, as YopP/J-mediated inhibition of NF-κB and MAPK disrupts TNFα secretion, safeguarding epithelial cells from apoptotic death. In contrast, macrophages do not need the presence of TNFα to undergo apoptosis[94].

Shigella

In contrast to EPEC/EHEC and Yersinia species, Shigella and Salmonella bacteria are readily internalized by subepithelial resident macrophages after crossing the intestinal epithelial wall. To avoid phagocytic degradation, Shigella rapidly escape the phagosome and kill the macrophage, leaving the bacteria free to invade epithelial cells at their basolateral membrane[95,96]. In biopsies of Shigella-infected patients many dying phagocytic cells are observed[97]. Shigella escape the phagocytic vacuole via T3SS effector mediated lysis of the vacuolar membrane. Macrophage killing is also dependent on T3SS effectors. Following their escape from the phagosome into the cytoplasm, Shigella secrete T3SS effector IpaB. IpaB can bind and activate caspase-1, leading to cytotoxicity[98-100]. This type of caspase-1 dependent cell death is termed pyroptosis and is accompanied by the release of pro-inflammatory cytokines IL-1β and IL-18, that together with IL-8 subsequently recruit PMNs. PMNs cause damage to the epithelial barrier, enabling Shigella bacteria to spread further into the tissue. Eventually however, PMNs are responsible for eradication of the infection[13]. Shigella are unable to escape phagocytic vacuoles in neutrophils[101]. The host defense protein neutrophil elastase (NE) may account for this. NE is known to cleave virulence factors of Shigella, but also Salmonella and Yersinia species[13,101]. In neutrophils in which NE is inactivated, Shigella escapes phagosomes and bacterial survival increases.

Salmonella

The strategy of Salmonella to cope with the threat of phagocytic degradation is remarkable. For Salmonella, macrophages are an important cellular niche for proliferation. Moreover, in mice, mutant strains that cannot survive and proliferate in macrophages are attenuated in their ability to develop systemic infection[102]. After traversing the epithelial layer, Salmonella can invade FAE dome region-resident macrophages. Inside phagocytic cells, as in epithelial cells, Salmonella are enclosed in a specialized vacuolar compartment that is permissive for bacterial replication. This compartment is called the Salmonella containing vacuole (SCV) and shields the bacteria off from cellular agents that may recognize and destroy the bacteria. To survive and proliferate within macrophages, the bacteria require SPI-2 encoded T3SS effector secretion, that counteracts the phagocyte's microbicidal activity[103]. The SPI-2 encoded T3SS is expressed and assembled by intracellular Salmonella and secretes effectors across the vacuolar membrane. Withstanding the antimicrobial intracellular milieu of phagocytes includes actively preventing vacuolar acidification and avoiding fusion between the SCV and lysosomes that would

deliver hydrolytic enzymes to the bacteria-occupied compartments[104]. Also, the intraphagosomal production of ROS by the NADPH oxidase complex is inhibited. It was shown that WT Salmonella bacteria prevent the assembly of the NADPH oxidase complex at the phagosomal membrane, whereas T3SS-mutant Salmonella bacteria were not able to[104]. InWT-Salmonella-infected macrophages, the NADPH oxidase is excluded from the vicinity of SCVs, resulting in enhanced bacterial replication[105]. The SPI-2 encoded T3SS effectors may interfere with trafficking of NADPH oxidase complex components to SCVs. Also in neutrophils it was shown that the oxidative burst is decreased in WT Salmonella-infected cells compared to avirulent, SPI-2 mutant strains[104].

Thus, Salmonella can survive and replicate in macrophages. Therefore it may seem contradictory that Salmonella has also been shown to trigger programmed cell death in macrophages by at least two distinct mechanisms at different time points in infection[102,106,107]. SPI-1 encoded T3SS effectors mediate cell death within the first hours of infection. This type of 'early' cell death is triggered by activation of caspase-1 (pyroptosis). The effector protein SipB may be involved[107,108], although it is difficult to determine the exact effector function of SipB in inducing pyroptosis as SipB is also part of the translocation complex. As such, any effects ascribed to SipB-activity may reflect the function of effectors translocated in a SipB-dependent manner. SipB shows homology to the IpaB protein of Shigella species[109]. A distinct type of cell death with features op apoptosis occurs from 12 hours in infection onwards[110]. This delayed type of cell death is induced by SPI-2-encoded T3SS effector secretion across the vacuolar membrane. Effectors that are likely to play a role are SpvB and SseL[110,111]. SpvB causes actin depolymerization and is required for macrophage apoptosis by caspase-3 activation[103,112,113]. SseL contributes to SPI-2 T3SS dependent macrophage killing by targeting NF-κB pro-survival and anti-apoptotic signaling[111]. The early cell death is likely predominantly important during the intestinal phase of infection, since SPI-1 mutant bacteria are attenuated only when administered to mice orally[107]. The caspase-1 mediated early cell death likely elicits a pro-inflammatory reaction to draw phagocytic cells to the site of infection, leading to disruption of the epithelial barrier. The induction of apoptosis late in the infection cycle may facilitate systemic spread of the bacteria. Bacteria-containing apoptotic bodies may be internalized by bystander macrophages, transferring the bacteria to naive macrophage hosts.

Thus, EPEC/EHEC, Yersinia, Salmonella and Shigella have developed vastly different strategies to deal with the phagocytic cells they encounter. Despite the varying mechanisms of action, for each pathogen T3SS-injected effectors are of critical importance to dealing with phagocytes. When bacteria are internalized by specialized phagocytic cells that are professional antigen-presenting cells, this may implicate the start of an adaptive immune response. How T3SS effectors influence adaptive immunity will be discussed next.

IN CONTACT WITH DCs, T AND B CELLS: MODULATING THE ADAPTIVE IMMUNE RESPONSE

Not every pathogen will necessarily need to counteract the adaptive immune response. For many pathogens, their life cycle within the host is over before a suitable adaptive immune response can be mounted. However, pathogens that cause persistent infections may benefit from interfering with adaptive immunity. One may speculate that EPEC/EHEC are unlikely to come into contact with cells of the adaptive immune system like B and T lymphocytes, since the bacteria colonize on the luminal side of the intestinal epithelium. In contrast, Yersinia bacteria cross the intestinal epithelial wall and are likely to encounter lymphocytes in Peyer's patches, lymph nodes, spleen and liver where T and B cells reside and Yersinia replicates extracellularly. Yersinia can bind T and B lymphocytes and inject effectors using their T3SS[114], although it is uncertain how efficiently T and B cells are selected for

injection *in vivo*[115]. The injected T3SS effectors can impair lymphocyte activation as illustrated by the inability of T cells to produce cytokines and the inability of B cells to upregulate surface expression of costimulatory molecules following exposure to Yersinia bacteria[114]. The block in lymphocyte activation is the result of interference with early antigen receptor signaling events. The T3SS effector YopH was shown to be responsible for this effect[114]. As mentioned earlier, YopH is a tyrosine phosphatase that counteracts a plethora of cellular phosphorylation events. The presence of YopH in B and T cells results in hypo-phosphorylation of many components of the antigen receptor signaling complex, rendering it unable to further transduce the activating signal triggered by antigen binding[114]. A major target of YopH is for example the lymphocyte-specific protein tyrosine kinase Lck, a primary signal transducer in the T cell antigen receptor complex[116,117]. YopH activity effectively turns off T cell activation at one of the earliest steps by the dephosphorylation of Lck.

Counteracting antigen presentation

Preventing T cell activation by interfering with antigen presentation on DCs is a well-known immune evasion mechanism employed by many viruses. Research is only beginning to unravel the effects of T3SS-expressing bacterial pathogens on antigen presentation. EPEC/EHEC for example may not directly influence B and T cells, but have been shown to inject T3SS effectors into long DC processes that penetrate the epithelium and extend into the intestinal lumen[118]. These T3SS-injected DCs are less potent activators of T cells.

For Yersinia, the T3SS effector YopP/J interferes with antigen presentation by inhibiting DC maturation as shown by a decreased surface expression of class I and II MHC and costimulatory molecules[119]. In a murine model of infection, WT Yersinia do not trigger a significant T cell response whereas a strong response is induced by strains that lack YopP/J[119]. Thus, Yersinia infected DCs have a diminished immune stimulatory capacity, caused by YopP/J activity. Whether YopP/J also causes programmed cell death in DCs as it does in macrophages, remains under debate. Apoptosis of DCs might be harmful for Yersinia as it could lead to the development of a $CD8^+$ T cell response to cross-presented apoptotic vesicles on bystander DCs and subsequent immune activation. In contrast, apoptosis and phagocytosis of apoptotic structures may be beneficial to intracellularly replicating bacteria, like Salmonella and Shigella as it provides a novel route of infection. Yersinia however may induce a non-immunogenic type of programmed cell death in DCs. Like in macrophages, Shigella-infected DCs are rapidly killed in a T3SS dependent manner, thereby preventing antigen presentation[13,95].

Most research into adaptive immunity evasion by T3SS-expressing bacteria has been performed on Salmonella. *Salmonella enterica* infected individuals can remain in a chronic carrier state. Despite the prolonged contact with the bacteria, the host is often unable to develop an adaptive immune response that eradicates the infection and protects from subsequent reinfection[120,121]. *Salmonella enterica* suppresses T cell activation and proliferation through interference with the antigen presentation function of DCs[120,122]. Salmonella inhibits the presentation of bacteria-derived antigens on both class I and II MHC molecules to respectively $CD8^+$ and $CD4^+$ T cells[120,123]. Viability, maturation and antigen uptake efficiency are unaffected in Salmonella-infected DCs, however antigen processing and loading on MHC molecules are impaired[120]. The block in antigen processing and presentation is achieved by preventing the fusion of the SCV with lysosomes[122,124]. This way, the bacteria are kept away from the lysosomal degradative pathway that would process the bacteria into presentable peptides that could prime a T cell response. The SPI-2 encoded T3SS and its secreted effectors are responsible for interfering with antigen presentation to evade DC-mediated T cell activation[120,122,125]. Salmonella strains carrying SPI-2 mutations can be detected in lysosomes and are no longer able to avoid DC antigen presentation[122,125]. Mice vaccinated with SPI-2 expressing Salmonella are more susceptible to reinfection than mice vaccinated with SPI-2 deficient Salmonella and the latter group has enhanced $CD4^+$ and $CD8^+$ T cell activation[120]. Salmonella SPI-2 T3SS effector proteins SifA,

SspH2, SlrP, PipB2, SopD2, SseF and SseG were shown to be involved in the interference with antigen presentation by DCs[123]. *ΔsifA*, *ΔsspH2*, *ΔslrP*, *ΔpipB2* and *ΔsopD2* single mutants and a *ΔsseFΔsseG* double mutant strain induced a level of T cell proliferation comparable to a SPI-2 T3SS deletion mutant, illustrating the importance of each of these effectors for preventing DCs from activating T cells[123]. The exact molecular mechanism of action of these effectors remains to be elucidated. However, it is not unlikely that they hamper intracellular transport that should otherwise accommodate delivery of antigens to processing and loading compartments. In support of this, it was shown that effective presentation of Salmonella antigens by DCs can be restored by coating the bacteria with specific IgG antibodies[124,126]. By directing the bacteria towards a $Fc_{\gamma}R$-mediated internalization, Salmonella are rerouted towards the lysosomal degradation pathway and efficiently processed and loaded for presentation on class I and II MHC. This results in a subsequent robust T cell activation. By reverting Salmonella to a different uptake mechanism, the bacteria may also be transported to lysosomes in an unconventional manner that is not inhibited by T3SS effectors.

In addition, Salmonella can also induce the poly-ubiquitination of surface-expressed peptide-loaded class II MHC molecules, leading to their removal from the antigen presenting cell membrane[127]. This activity is mediated by SPI-2 T3SS secreted effectors and specifically affects class II MHC as other molecules that travel through the same compartments are left untouched[127].

Counteracting cell migration

Besides interference with antigen presentation, another virulence mechanism shared by several T3SS-expressing pathogens in counteracting the adaptive immune response is altering the motility of immune cells. Shigella can inject T3SS effectors into activated $CD4^+$ T lymphocytes. The activity of one of the effectors, IpgD, results in the inhibition of chemokine-induced T cell migration. IpgD prevents the formation of a polarized cell edge, an early step required for cellular migration[128]. The migratory capacity of T cells is essential to exerting its anti-pathogenic function. Thus, Shigella 'freezes' T cell immunity[128].

SrfH, also called SseL, is a Salmonella SPI-2 T3SS effector required for maintaining a long-term chronic systemic infection in mice[121]. SrfH/SseL can influence DC migratory capacity. Worley *et al* (2006) show that SrfH stimulates Salmonella-containing phagocytes to exit the intestinal epithelium and move into the bloodstream[129]. This way, SrfH accelerates the systemic spread of Salmonella infection. However, in a study performed by Lapaque *et al* (2009), SseL caused a blockade in DC migration to the spleen in mice infected with *S. typhimurium*[127]. Accordingly, mice infected with WT *S. typhimurium* had a lower splenic DC and $CD4^+$ T cell count than mice infected with SseL-deficient mice. SseL was shown to interact with host factor IQGAP1, an important regulator of the cytoskeleton and cell movement[121]. Thus, in this study SseL prevented Salmonella-containing DCs from entering peripheral sites where they could prime T cells. The apparent discrepancy might be explained by the use of different model systems in these two studies, reflecting the extensive level of adaptation of T3SS effectors towards strain and host cell type specific effects. Conversely, SrfH/SseL may have dual functions.

Taken together, T3SS effectors of enteropathogenic bacteria can modulate adaptive immunity, activity most clear in Salmonella species. T3SS effector interference with adaptive immunity is mainly aimed at antigen presentation by DCs. Despite the fact that Salmonella does not replicate in DCs, they may be an ideal target cell pool to establish systemic chronic infection. While T3SS effectors prevent antigen presentation to stop the onset of an adaptive immune response, Salmonella makes use of the DC's intrinsic migratory capacity to disseminate throughout the body from the initial site of infection. This example nicely shows the level of manipulation T3SS effectors can confer on a cell.

DISCUSSION

The intestinal lumen is continuous with the extracellular environment and therefore at a constant risk of pathogenic attack. For this reason the human intestinal tract is populated by many immune cells and endowed with detection and regulatory mechanisms to properly respond to invading pathogens and at the same time tolerate commensal flora. The four major enteropathogenic bacteria species discussed here; EPEC/EHEC, Yersinia, Salmonella and Shigella, depend on their T3SS to cause disease. Although this secretory protein complex is not their only virulence factor, T3SS mutant bacteria are dramatically attenuated in several *in vivo* models of infection[8]. To efficiently establish enteric infection, T3SS-expressing bacteria must combat the intestinal immune system. To this end, many T3SS effectors interfere with enterocyte pro-inflammatory signaling that warns immune cells of an infection, as well as interfere with innate phagocytic cells and adaptive immune cells. Thus, T3SS mutant bacteria are attenuated as they lose their ability to suppress the intestinal immune system[8]. EPEC/EHEC, Yersinia, Salmonella and Shigella have each developed their own strategy, making use of their T3SS, to circumvent intestinal immunity. EPEC/EHEC colonize extracellularly on the luminal side of the intestinal epithelium and prevent uptake of bacteria into phagocytic cells and impede pro-inflammatory signaling in enterocytes. Yersinia proliferate extracellularly as well, in subepithelial lymphoid tissue after crossing the intestinal epithelial barrier. Surrounded by immune cells, Yersinia actively prevent inflammation and phagocytosis. In contrast, Salmonella and Shigella readily invade gut epithelia and proliferate intracellularly. Both Shigella and Salmonella initially suppress enterocyte pro-inflammatory signaling, but later induce inflammation to cause breaching of the intestinal epithelial barrier to gain access to and attract naive target cells. While Shigella invades and quickly triggers phagocyte cell death to enhance inflammation, Salmonella uses phagocytic cells to proliferate. Salmonella hides out in a contained vacuolar compartment and counteracts intracellular microbicidal activity. In addition, enteropathogenic species that can cause persistent infection, interfere with adaptive immunity, mainly by suppressing DC antigen presentation.

Each of these intestinal immunity combat strategies is carried out by a specific set of bacterial effector proteins injected into host cells by the T3SS. A vast array of T3SS effectors have been described, and more will likely be identified in the future. During evolution, new T3SS effectors are formed by fusing protein-coding sequences to sequences governing T3SS-mediated secretion[130]. Indeed, the N-terminus of T3SS effectors generally contains the signal sequence for secretion, while the C-terminus can harbor a variety of functional sequences. These gene-fusion events are facilitated by the fact that effector protein sequences are mostly encoded on mobile genetic elements[130]. The genetic rearrangements have given rise to the broad spectrum of present-day existing T3SS effector proteins. The set of injected effectors differs greatly in number and functions between species. However, effector homologues between species do exist, like the earlier described Salmonella SipB and Shigella IpaB. In addition, many different effectors interfere with similar cellular targets. A common target of enteropathogenic bacteria is the cytoskeleton. T3SS effectors modulate cytoskeletal rearrangements to gain or prevent entry into cells or to accommodate spreading. Intracellularly replicating enteropathogenic bacteria also often interfere with vesicular trafficking to promote the delivery of materials that sustain proliferation or prevent the delivery of destructive agents to bacteria-occupied subcellular structures. Usually, T3SS effectors do not directly act on cytoskeletal elements, instead they alter regulatory factors like Rho GTPases. The interference with cellular regulatory factors is a common theme in T3SS effector activity. Hampering the intestinal epithelial cell's inflammatory response is mostly directed at the master regulator NF-κB. Often, T3SS effectors have enzymatic activity to add or remove molecular modifications like phospo or ubiquitin groups from their cellular targets, e.g. Shigella IpaH proteins that act as E3

ubiquitin ligases. As a consequence multiple cellular targets may be effected by a single effector. One clear example of a multifunctional effector is the abundant phosphotyrosine phosphatase YopH of Yersinia. Another striking feature of some effector protein sets is the apparent redundancy within a bacterial species. For EPEC/EHEC a set of Nle effectors inhibit NF-κB activation by interfering at several levels of the NF-κB signaling pathway. These effectors may act in concert to completely abolish NF-κB activation that would be detrimental to the infection. Shigella strains express a varying number of IpaH effectors. It has been proposed that IpaH proteins act synergistically[61]. As such, the number of IpaH copies carried by a strain, may reflect the severity of the strain-specific host response. Taken together, the T3SS delivers a refined package of effector proteins to take complete control of host cells, but often with individually subtle functions. These subtle effects allow T3SS effectors to modulate host cell functions in a way that they go by unnoticed, and without causing the cell's demise. It is not surprising that bacterial species have developed both general and unique effector functions, as establishing an infection is likely to require both common and species-specific anti-immunity activities. Salmonella uniquely expresses a second T3SS to support its intracellular lifestyle.

To maintain a specific virulence strategy, T3SS effectors must be delivered to host cells in a controlled manner. Also, once inside host cells T3SS effector function must be regulated. A T3SS effector may be required to function only early in infection whereas it should be silenced at a later time point, or be active in one cell type but not in others, illustrating the need for spatio-temporal regulations. Whereas Shigella and Salmonella species inhibit NF-κB mediated pro-inflammatory signaling at early time points in infection, inflammation is induced later on. Not much is known about the regulation of T3SS effector secretion. Real-time analysis have revealed a tight order of effector protein translocation for EPEC[131]. For Shigella, two known transcription factors are involved in effector secretion regulation. While effectors co-regulated by both VirB and MixE are most likely required during the full course of infection, effectors controlled by MixE alone are required during the bacterium's intracellular phase only[13]. Inside host cells, T3SS effectors may be subjected to cellular regulatory mechanisms, such as ubiquitination which controls protein turnover. Since the intracellular milieu differs between cell types, this might explain the apparent host cell type specific virulence function of T3SS effectors. Effectors that modulate the macrophage milieu to support Salmonella bacterial proliferation do not aid intracellular survival in DCs[132]. The ongoing evolutionary interplay between T3SS-expressing bacterial pathogens and host cells has resulted in an extensive level of host cell type and bacterial strain specific adaptation of T3SS effector function. Conversely, different cell types may be selected for effector injection with varying efficiency, or may even receive differing sets of effectors, although not much has been reported on this phenomenon.

As T3SS-mediated effector delivery offers possibilities of spatio-temporal control, this may explain the costly investment to maintain such a complex protein system. Indeed, whereas effector proteins are diverse, the T3SS translocation machinery is largely conserved and is often interchangeable between enteropathogenic bacteria species[8]. As such, the T3SS would be a valid drug target to treat enteropathogenic infections. In addition, drugs that act on the T3SS might have a decreased selective pressure for development of resistance, as the T3SS is not absolutely required for bacterial survival. Also, the fact that T3SS is very important to pathogenesis and specific to pathogenic bacteria, could prove T3SS-targeting drugs interesting candidates to further explore in future research.

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