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MASTERARBEIT

Titel der Masterarbeit

Role of the Th- 17 cytokines in the host response to *Salmonella enterica* Serovar Typhimurium

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Master of Science (MSc)

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Abstract

Salmonella typhimurium is a human foodborne pathogen, which upon ingestion causes inflammatory diarrhea but can also lead to bacteremia and death in the most severe cases. In immunocompetent patients the immune system confines the bacteria to the intestine and after a few days the infection is cleared. In children, elderly or patients with primary or secondary immune deficiencies *S. typhimurium* can spread to the blood stream and lead to bacteremia. However, attenuated variants of this bacterium still trigger a strong innate and adaptive immune response, which is why recently research has been done on *S. typhimurium* and its use as an immunization agent.

Key players of the innate immune responses to *S. typhimurium* are antigen-presenting cells including monocytes/macrophages and dendritic cells. Recently, it has been proposed that dendritic cells secrete a novel cytokine called interleukin (IL-)23, in addition to other pro- inflammatory cytokines such as IL-1β. While there are many studies of *S. typhimurium* infection in a mouse model, little is known about the induction and role of these cytokines during *S. typhimurium* infection in humans. To investigate this, we isolated monocytes and differentiated dendritic cells (antigen presenting cells, APCs) from blood samples collected from healthy human subjects and infected both cell types with *S. typhimurium.*

Infection of APCs with *S. typhimurium* resulted in high level of secretion of IL- 23 in addition to IL-1β. When these cells were infected with mutant strains lacking different pathogen associated molecular patterns we found, that in contrast to the mouse model, IL- 1β secretion was not dependent on either one of the two *Salmonella* pathogenicity islands (SPI) type-three secretion systems (T3SS). However, significant lower levels of IL- 1β and IL- 23 were observed in APCs when infected with a mutant defective in lipid A acylation. This indicates that activation of TLR4 is necessary for secretion of these cytokines.

It is thought that two signals are needed for the mature IL-1β to be secreted. While a first signal (often provided by LPS- mediated activation of TLR4) is needed for the production of pro-IL-1β, a second signal is needed to activate Caspase- 1 to cleave the pro- IL1-β. In this study we showed that the TLR adaptor protein MyD88, which links multiple pathogen recognition receptors to transcription factors is essential for the production of IL-1β and IL-23 in human DCs upon *S. typhimurium* infection.

Taken together we show that during a *S. typhimurium* infection LPS plays a major role in stimulating hDC to secrete the cytokines IL-1β and IL- 23 through MyD88. Furthermore we show that the supernatant of hDCs infected with *S. typhimurium* activates memory T cells to secrete the Th17 cytokine IL- 17. Our results also indicate, that the molecular response of APCs upon *S. typhimurium* infection is different in human and mouse, and therefore further studies in primary human cells should be performed.

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1. Introduction

The Gram negative facultative anaerobe bacteria species *Salmonella* has more than 2000 serovars, with only a small fraction commonly associated with disease in humans. Diseases caused by *Salmonella* range from self-limiting gastroenteritis to the severe typhoid fever. Most *Salmonella* serovars are not host species specific and cause zoonotic infections, meaning that the bacteria can be transferred between human and non- human species. *Salmonella enterica* serovar Typhimurium (STM), for example, was named according to its typhoid fever inducing potential in mice, but belongs to the non-typhoidal *Salmonella* (NTS) because it is one of the most frequent causes for food borne gastroenteritis in humans (Ibarra et al. 2009). Frequent sources of *Salmonella typhimurium* infection are eating undercooked poultry, chicken eggs as well as contact with reptiles such as pet tortoises and snakes. Another frequent source, which is a problem in Africa is drinking polluted surface water or standing water.

For immunocompetent humans a non- typhoidal *Salmonella* (NTS) infection is generally confined to the intestine, yet the same infection can lead to bacteremia in immunocompromised humans. The compromised immune system cannot confine the STM infection to the intestine and the bacteria spread to the bloodstream even when only small amounts are ingested. This is especially true for children, elderly and those with primary or secondary immune deficiencies including Acquired Immune Deficiency Syndrome (AIDS). In sub- Saharan Africa infection with NTS is currently a leading cause of hospital admissions of adults and among the most common bacteria isolated from the blood of hospitalized adults whom the vast majority are HIV positive. Moreover, *Salmonella* infection in AIDS patients is associated with high acute mortality rates (47%)(Raffatellu et al. 2008, Laupland et al. 2010).

To provide new treatment options for patients with bacteremia, it is important to understand which mechanisms of the immune response are essential to prevent *Salmonella* from spreading to the bloodstream.

A bugs live- virulence factors for active invasion

The mucosal surface constitutes a barrier against the systemic spread of pathogens like *Salmonella*. To overcome this barrier and successfully colonize the intestine, *Salmonella typhimurium* has a repertoire of virulence factors, which enable penetration through thick mucous layers, surface adherence and induction of host inflammatory response (Liu et al. 2009). *Salmonella typhimurium* is facultative intracellular and during infection can be found in a variety of phagocytic and non-phagocytic cells in vivo (Ibarra et al. 2009). This means that besides passive uptake, STM also actively invades all kinds of cell types, including epithelial cells, goblet cells, macrophages, dendritic cells.

To manipulate the host cells *Salmonella typhimurium* encodes two Type III Secretion Systems (T3SS). The biological function of the T3SSs is the translocation of proteins from the bacterial cytoplasm into the host cell in a molecular syringe-like manner. In contrast to type II secretion systems, the translocation of effector proteins is independent of an N- terminal sequence. The core structure consists of more than 20 different protein subunits and shows homology to the flagella assembly system(Kuhle et al. 2004).

The T3SS encoded by genes on the *Salmonella* Pathogenicity Island 1 (SPI-1) is used extracellularly to inject effector proteins (including SipA, SipB, SipC, SopB, SopE, SopE2, SopD) directly into the cytosol of host cells. Some of these proteins interact with the cytoskeleton filament actin to stimulate growth and rearrangements near the injection site. This results in membrane ruffling and the engulfment and uptake of the bacteria in a *Salmonella* Containing Vacuole (SCV). Right after internalization *Salmonella*'s effector proteins turn the cytoskeleton back to its resting state. (McGhie et al. 2009). However, Haenisch et al. have recently shown that *Salmonella* invasion is dependent on the effector proteins injected through the SPI-1 T3SS and actin remodeling, yet separable from membrane ruffling.

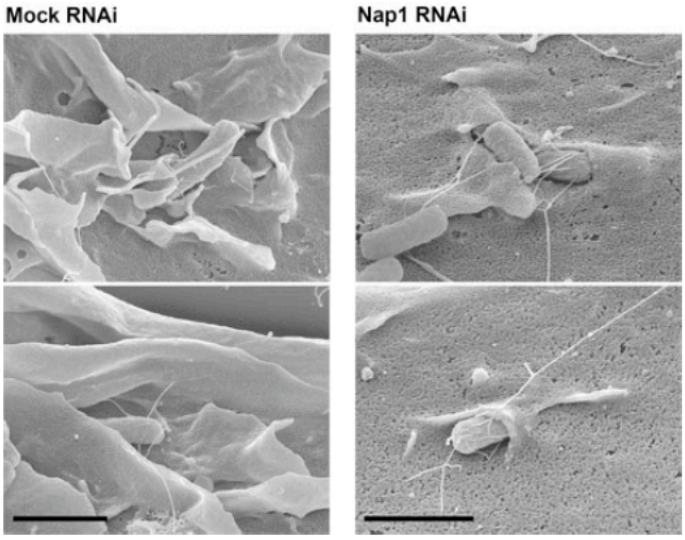


Fig. 4. WAVE-complex depletion abrog
Salmonella-induced membrane ruffling.
Mock and Nap1 RNAi VA-13 fibroblast
were infected with wild-type *Salmonella*
typhimurium, fixed and prepared for
inspection by scanning electron microsc
Note the dramatic induction of ruffles
emanating from the dorsal surface of m
RNAi-treated fibroblasts challenged with
Salmonella (rod-shaped objects). Rufflir
not induced in Nap1 RNAi cells. Instead
bacteria appear to be enveloped by the
cell surface in a 'zipper-like' fashion. Ba
equal 2 μm.

Figure 1-1, Figure and Legend taken from Haenisch et al. 2010

Life in the host cell

While the SPI-1 T3SS is not required for systemic infection, strains deficient in the type III secretion system encoded by the *Salmonella* pathogenicity island 2 are highly attenuated in murine salmonellosis (Shea et al. 1996). Furthermore SPI-2 T3SS mutant strains showed reduced survival and proliferation inside host cells. (Ochman et al. 1996, Cirillo et al. 1998) While the expression of SPI-2 T3SS specific genes is turned on after the bacteria entered the cell, the SPI-1 T3SS is repressed. Uchiya et al. have shown that the SPI-2 encoded protein SpiC is secreted by intracellular STM through the SPI-2 T3SS into the host cell cytosol of murine macrophages (Uchiya et al. 1999). Moreover, this group was able to show that SpiC interferes with endosome- lysosome fusion in a dose dependent matter. This prevents the *Salmonella* containing vacuole from fusing with lysosomes, thereby avoiding uptake of lysosomal hydrolases in the SCV that would kill the intracellular bacteria. SPI-2 also encodes for a protein called SifA, a cytoskeleton- modulating gene which is suggested to be important for systemic infection. Work by Stein et al. (1996) and by Brumell et al. (2001) suggest that the formation of *Salmonella*-induced filaments (SIF) by SifA does not affect survival and replication in mouse epithelial cells (Stein et al. 1996) but attenuates virulence and proliferation in mouse macrophages (Brumell et al. 2001). Besides modulating the host cell cytoskeleton SifA was also shown to maintain the integrity of the SCV and inhibit the release of STM into the cytoplasm (Beuzo et al. 2000).

Additionally, *Salmonella* constitutively expresses SspH1 and SepH2, which are translocated by both the SPI-1 T3SS and the SPI-2 T3SS and lead to dose dependent down regulation of nuclear factor kappa B (NFκB) (Miao et al. 1999). This pathogen also activates the Protein Kinase A pathway, which results in cytokine IL- 10 expression by infected mouse macrophages, resulting in an anti- inflammatory effect. (Uchiya et al 2004).

Salmonella typhimurium utilizes another important type III secretion system for the assembly of flagella. One function of flagella is the propeller- like rotation that the bacterium uses for motility. In *Salmonella* infected mouse macrophages flagellin, the extracellular monomer of flagella is translocated in the host cell cytosol by the SPI-1 T3SS, which results in caspase- 1 activation and cell death by pyroptosis. Pyroptosis is beneficial for the host, since this cell death results in secretion of pro-inflammatory cytokines. However, a recent study by Sano et al (Sano et al. 2007) has proposed that *Salmonella* induces another form of cell death called oncosis, by which the bacterium exits Macrophages in a flagellum- dependent. This indicates that flagellin is used by both, APCs for a pro- inflammatory immune response and by *Salmonella* to exit the host cells after successful invasion.

Host response to mucosal pathogens

When pathogens invade the mucosa, professional antigen presenting cells (APC) have the first important interaction with the pathogen and play an important part in the immune response. Macrophages and Dendritic Cells (DC) guard the sites of infection and orchestrate the host response. Upon contact with a pathogen these cells upregulate and secrete pro- inflammatory cytokines, like IL- 1β and IL- 23, which stimulate T cells to secrete cytokines, thereby amplifying the inflammatory response. The T cell cytokines IL- 22 and IL- 17 activate epithelial cells to secrete antimicrobial peptides into the gut lumen and neutrophil chemoattractants in to the tissue. Antimicrobial peptides and neutrophils contribute to clearance of the bacterial infection. However, several studies have shown that certain host immune responses are exploited by pathogens to successfully colonize the gut and achieve transmission to the next host (Stecher et al. 2007). It is not fully understood which mucosal responses are beneficial for the host and which are used by pathogens to colonize the gut.

Detecting *Salmonella*

Antigen presenting cells developed a range of mechanisms to sense intracellular and extracellular "danger" signals from different patterns of the pathogenic microorganism. Pathogen- associated molecular patterns (PAMPS) are highly conserved and essential components for bacteria survival. Pattern recognition receptors (PRRs) on the host cell target these structures. There are several classes of PRRs such as Toll Like Receptors (TLRs), Retinoic acid- inducible gene (RIG)-I-Like Receptors (RLRs) and Nucleotide- binding oligomerization domain (NOD)-Like receptors (NLRs). Activation of these receptors trigger downstream signaling cascades resulting in secretion of pro- inflammatory cytokine and type 1 interferon production (Kumar et al. 2009).

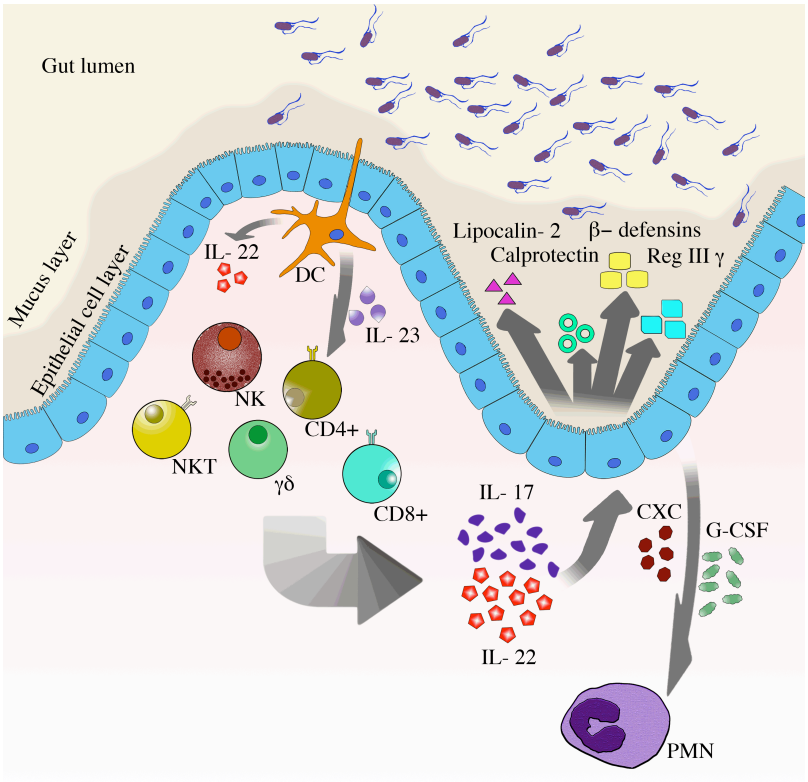


Figure 1-3, T cell cytokines and the gut mucosal barrier. Dendritic cells activated by pathogens secrete several cytokines, which further stimulate subsets of T cells to secrete IL-17 and IL-22. T cells promote amplification of the host response by stimulating the intestinal epithelium to secrete CXC chemokines (neutrophil chemoattractants) and antimicrobial peptides (Figure and Legend adapted from Blaschitz and Raffatellu, 2010, J. Clin Immunol).

All 12 known mammal TLRs are transmembrane glycoproteins with an intracellular Toll/IL-1 receptor (TIR) domain. As reviewed by Jin et al. (2008) these receptors form hetero or homodimeres resulting in an essential horseshoe like structure which initiates downstream signaling. Besides bacterial patterns also PAMPs of fungus, viruses and parasitic protozoa are recognized through different cell surface and in intracellular vacuole TLRs. Highly conserved and very important structures for Gram negative bacteria are lipopolysaccharides (LPS), which is the major component of the outer membrane. Since LPS is an essential structural component of the bacterial cell wall, it is a good target for PRRs.

The heterodimer TLR4-MD2 binds CD14 boud to LPS, which leads to the homodimerization of the two TLR4- TIR domains. This results in the recruitment of the adaptor protein pair TRAM- TRIF or MAL- MyD88 with TRIF and MyD88 relaying the signal. MyD88 stimulation results in activation of nuclear factor- κB (NF-κB) and the production of pro- inflammatory cytokines. TRAM stimulates sustained NF-κB and activates interferon regulatory factor 3 (IRF-3) resulting in interferon- β and CC- chemokine ligand 5 production. While MyD88 signaling results in large amounts of pro- inflammatory cytokines, TRIF signals modulate the immune response (Bryant et al. 2010). Another important PRR for detecting bacterial pathogens is TLR5, which recognizes only one protein PAMP, bacterial flagellin. The extracellular flagellin binds to TLR5, which recruits MyD88 and also stimulates NF-κB, resulting in secretion of large amounts of pro- inflammatory cytokines. Additionally the PI3 kinase is activated in what appears to be a negative feedback loop. (Miao et al. 2007). Signaling through Toll-like receptors leads to production of the pro- inflammatory cytokines including interleukin (IL)- 12, IL- 23, IL- 6, tumor necrosis factor (TNF)- α and the inactive pro forms of IL- 1β and IL- 18. While IL- 12 is built up by the subunits p35 and p40, IL- 23 consists of the heterodimer p40 and p19.

As mentioned earlier, an additional mechanism of bacterial recognition by APCs includes detection of a cytosolic danger signal. For instance, STM translocates flagellin through the SPI-1 T3SS into the cytosol of APC, where Ipaf/Nlrc4 recognizes it. Ipaf/Nlrc4 belongs to the NOD Like Receptor family, which includes 22 cytoplasmic receptor proteins in human and many more in mice. The role of most of the NLRs is unknown. All NOD- like receptors share a domain, called NACHT

domain, and have additional domains for interaction with host proteins. An example for a domain linking NLRs with a host protein is the Caspase activation and recruitment domain (CARD), which mediate the formation of larger protein complexes via direct interactions between individual CARDs. Binding of flagellin to Ipaf/Nlrc4 leads to oligomerization through homodimerizations of the CARDs on the receptor and caspase- 1. These complex triggers a "danger" signal in the activation of caspase-1 (Miao et al. 2007). Perhaps the most studied NOD like receptor is NLRP3 also known as cryopyrin. With an unknown mechanism NLRP3 is able to respond to multiple stimuli resulting in the activation of caspase-1. During *S. typhimurium* infection NLRP3 and Ipaf/Nlrc4 recruit ASC and Caspase- 1 and form an inflammasome, which cuts and activates pro-IL-1β as shown by Broz et al.

Some NLRs signal through the adaptor protein ASC, a bridge protein that seems to stimulate the interaction between the NLRs and the Caspase-1 CARD domain. Mariathasan et al have shown that Caspase-1 activation is significant decreased in ASC knock out murine macrophages, but not eliminated (Mariathasan et al. 2004).

Inflammatory host cell death

Caspase-1 was first found because of its protease activity that processes the precursors pro- IL-1β and pro- IL- 18 into mature inflammatory cytokines. It was later found that caspase-1 activation results in cell death, now termed pyroptosis. Pyroptosis is a caspases -1 dependent rapid cell death, characterized by plasma-membrane rupture and release of pro- inflammatory intracellular contents. (Bergsbaken et al. 2009) Activation of some NLRs via a broad range of stimuli results in caspase-1 activation and the formation of inflammasomes. An inflammasome is a multi- protein complex, which promotes the secretion of pro-inflammatory cytokines and the infection- induced cell death, pyroptosis. The four known inflammasomes differ in the NLRs interacting with the complex and initating the inflammasome formation. The adaptor protein ASC has been implicated in activation of the four inflammasomes, with the role of bridging the interaction between NLRs and Caspase- 1.

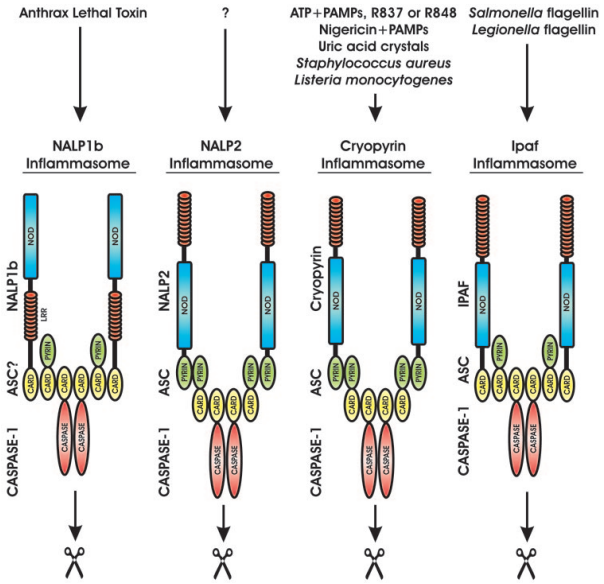


Figure 1-4, Ligands and composition of the four known Caspase-1 inflammasomes. The NLR proteins NALP1b, NALP2, Nlrp3 (cryopyrin), and Ipaf/Nlrc4 assemble a Caspase-1-activating inflammasome complex in response to specific microbial or endogenous danger signals. The murine Nalp1b inflammasome recognizes the cytosolic presence of anthrax lethal toxin (LT), whereas the stimulus that triggers the NALP2 inflammasome remains to be identified. The cryopyrin/NLRP3 inflammasome recognizes multiple PAMPs in combination with ATP or nigericin, as well as endogenous danger signals such as uric acid crystals, which may be released by dying cells. Finally, the Ipaf/Nlrc4 inflammasome senses the cytosolic presence of *Salmonella* and *Legionella* flagellin. The adaptor protein ASC is essential for these four inflammasome complexes, although its role in the Nalp1b inflammasome remains to be formally established (Figure and Legend adapted from Lamkanfi et al., 2007, J. of Leukocyte Biology)

Using ASC- GFP fusion protein expressing macrophages, Fernandes-Alnemri et al. have recently shown that upon stimulation with pro- inflammatory agents such as LPS the formation of a large supramolecular assembly of ASC, termed pyroptosome is induced. The formation of this complex is driven by sub physiological concentration of potassium. One pyroptosome per cell is formed which recruits and activates Caspase- 1 resulting in pyroptosis (Fernandes-Alnemri et al. 2007).

Pyroptotic cells are characterized by DNA damage, nuclear condensation, actin cytoskeleton destruction, pore formation which leads to ionic gradient shifts, water influx, cell swelling and finally membrane rupture releasing pro- inflammatory cytokines (Bortoluci et al. 2010).

The pro- inflammatory cytokines secreted by APCs activate receptors on T cells, including the subsets present in the gut mucosa. Recent studies ascribe an important role to a new subset of T helper cells, termed Th17, in orchestrating the mucosal

responses to pathogens, including *S. typhimurium*. Th17 cells are characterized by the production of the cytokines IL- 17A, IL- 17F, IL- 22 and in humans IL- 26. (Wilson et al. 2007) In the past it was hard to define Th17 cells, because other subsets of T cells also secrete these cytokines. However, several studies have shown that the early contribution of Th- 17 cells to the innate immune defense plays a major role in bacterial pathogen clearance. Essential for Th- 17 cell activation is the pro-inflammatory cytokine IL- 23. (Godinez et al. 2009)

When mice defective in Th 17 responses (IL- 17 or IL- 22 knock outs or with depleted IL- 17/IL- 22 receptors) were infected with pathogens like *S. typhimurium* (Intestine), *Klebsiella pneumoniae* (Lung), *Citrobacter rodentium* (Colon) and

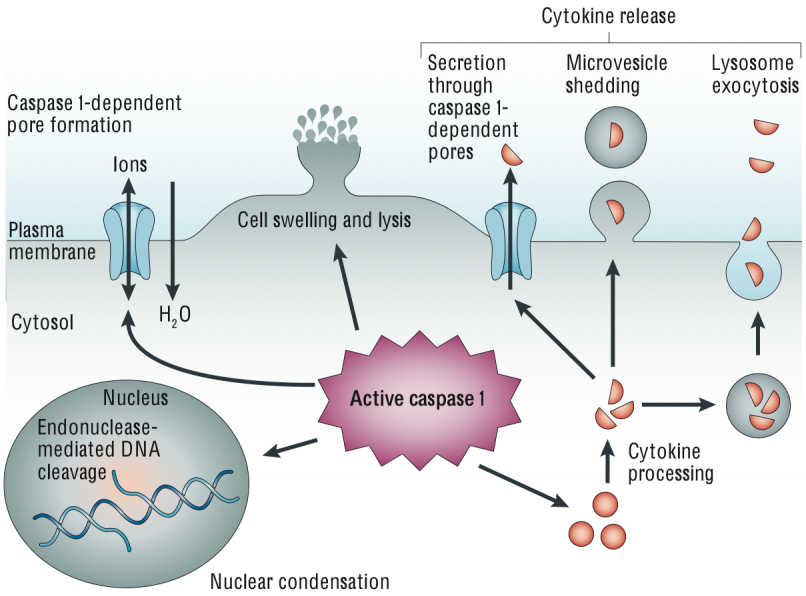


Figure 1-5, Host cell pyroptosis, overview of the different functions of active caspase-1 (Figure and Legend adapted from Bergsbaken et al. 2009)

Candida albicans (Oral) they showed a significant increase in bacterial dissemination. (Liu et al. 2009)

Several responses orchestrated by IL- 17 and IL- 22 might contribute to helping the host confine pathogens to the gut. Different studies have shown upregulation of chemokines (CXCL-8, CCL20) and antimicrobial peptides (iNos, lipocalin-2) in intestinal epithelial cells by in vitro stimulation with Th17 cytokines (Raffatellu et al. 2009, Dambacher et al. 2010, Brand et al. 2006). Furthermore, IL- 17 and IL- 22 contribute to the formation of tight junctions, to the increase in trans- epithelial resistance in polarized cells, to granulopoiesis and neutrophil accumulation to clear the pathogen infection (Blaschitz et al. 2010).

The signal starts with a few DCs and Macrophages secreting pro- inflammatory cytokines like IL-1β and IL-23 after recognizing pathogen associated molecular patterns from the first invading *Salmonella*. These cytokines are capable of stimulating several Th17 cells to secrete cytokines including IL- 17 and IL- 22. These cytokines stimulate epithelial cells to secrete antimicrobial peptides and neutrophil chemoattractants to fight the *Salmonella* infection in high numbers. We hypothesize that this signaling cascade from pathogen detection to antimicrobial peptide and neutrophil accumulation involves cells from the innate and the adaptive immune system and proceeds immediate and is triggered by complex relay of signas.

Specific Aims

Most of the studies investigating the host response to *Salmonella* and other pathogens have been conducted in the mouse. Using a mouse model has many advantages, compared to human studies, including the ability to use knock out mice, established methods, use of a controlled environment and easy access to biological replicates. However, there are also disadvantages. For instance, when investigating *Salmonella* pathogenesis, one obvious disadvantage is the different disease caused in mice and humans by *S. typhimurium* infection. While *S. typhimurium* infection in humans is localized to the gut and results in diarrhea, the same infection spreads from the gut and results in bacteremia/typhoid- like disease in mice. To achieve a similar course of disease as in humans, mice are often pre- treated with the antibiotic Streptomycin. This treatment leads to similar inflammation of the gastrointestinal tract in mice. However, compared to immune competent humans, in the mouse model the bacterium is not confined to the gut but spreads to different organs resulting in a systemic infection and death after a few days. Because the same pathogen*, S. typhimurium*, causes diarrhea in humans and typhoid fever in mice, it is likely that differences in the host response may be responsible for these different disease manifestations.

As described earlier, a first step in the activation of the host response by *S. typhimurium* results from the interaction with APCs, which then lead to activation of TH17 cells. These early events in host- *Salmonella* interaction have been extensively investigated in mice, however very little is known about these mechanisms of activation in humans. In this project, I aimed to determine the mechanisms of

interaction of *S. typhimurium* with human antigen presenting cells and T cells, by pursuing the following specific aims:

Specific Aim 1: Determine the mechanism by which *S. typhimurium* induces cytokine expression in human APC

In this aim, we wanted to determine the contribution of pathogen associated molecular patterns, TLR and NLR signaling to the secretion of pro- inflammatory cytokines by human antigen presenting cells in comparison to mouse antigen presenting cells during infection with *Salmonella typhimurium*.

Specific Aim 2: Determine the mechanism by which T cells secrete IL- 17 and IL- 22 during *S. typhimurium* infection

In this aim, we wanted to investigate the mechanism leading to Th17 cytokine production by human Th17 cells when T cell were stimulated with supernatants from APCs infected with *S. typhimurium*.

2. Material and Methods

2.1. Cell Biology

2.1.1. Media

The two basic media used for the cell culture experiments are Roswell Park Memorial Institute medium 1640 and Dulbecco's Modified Eagle Medium. The containing phosphate- buffer system in both media is intended to be used in a 5% carbon dioxide atmosphere.

After the addition of further ingredients the final medium was always filter sterilized. (Filter Units MF75™ Series, Nalgene® Labware, United States, cat#166).

2.1.1.1. Full- Medium

RPMI Medium 1640 containing L- Glutamine (GIBCO® through Invitrogen™, United States, cat# 11875) was supplemented by 10% (v/v) heat inactivated Fetal Bovine Serum (GIBCO® through Invitrogen™, United States, cat# 10082), 1% (v/v) Penicillin/Streptomycin (5000 units/ml Penicillin, 5000µg/ml Streptomycin, GIBCO® through Invitrogen™, United States, cat# 15070), and 1x L- Glutamin (200mM, 29.2 mg/ml in 0.85% NaCl, GIBCO® through Invitrogen™, United States, cat# 25030).

2.1.1.2. Infection- Medium

For infection experiments the Full- Medium could not be used since it contains the antibiotics Penicillin/Streptomycin which kills the bacteria used for the infection. Therefore the RPMI Medium 1640 was supplemented with 10% heat inactivated Fetal Bovine Serum and 1x L- Glutamin, without the addition of Penicillin/ Streptomycin.

2.1.1.3. hDC- Medium

To differentiate human progenitor cells to human dendritic cells the Full- Medium was supplemented with 50ng/ml human recombinant GM- CSF (PeproTech©, United States, cat# 300-03) and 10ng/ml human recombinant IL- 4 (PeproTech©, United States, cat# 200-04R).

2.1.1.4. mDC- Medium

Bone marrow derived mouse cells need Full- Medium supplemented with recombinant GM- CSF and IL- 4 to differentiate to mouse bone marrow derived dendritic cells. Therefore recombinant murine GM- CSF (PeproTech, United States, cat# 315-03) to a final concentration of 10ng/ml and recombinant mouse IL- 4 (eBioscience, United States, cat#14-8041) to a final concentration of 1ng/ml was added to the Full- Medium. Additionally to further maintain the physiological pH, HEPES (Invitrogen™, United States, cat# 15630) was added to a concentration of 25mM.

2.1.1.5. mMϕ- Medium

To differentiate bone marrow derived mouse progenitor cells in mouse macrophages the Full- Medium was supplemented with 30% (v/v) L929-conditioned medium. Cells of the immortalized mouse fibroblast cell line L929 were cultured until full density in Corning TC surface flasks. Supernatant was taken off, filtered and frozen at -20°C until use. Besides other factors the mouse fibrosarcoma cell line L929 secretes the mouse macrophage colony stimulating factor (M-CSF) which is necessary for the differentiation of bone marrow derived progenitor cells to macrophages.

2.1.2. Isolation and preparation of mouse primary cells

All the necessary trainings for animal work and all experiments were approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. The animals had free access to food and water.

2.1.2.1. Isolation of mouse progenitor cells and differentiation to macrophages and dendritic cells

4-12 week old C57BL/6 mice were used for the isolation of bone marrow-derived progenitor cells. Mice were sacrificed by CO_2 suffocation and subsequent cervical dislocation. Both legs of one mouse were skinned, cleaned from tissue, separated from the body above the hip and the feet were cut off. The bones were kept in 15ml RPMI completed with antibiotics on ice and were used immediately or stored on ice for up to 24h. In the lid of a Petri dish the legs were carefully cleaned from any remaining tissue using tweezers and scalpels. It is important to make sure that all the

tissue is removed from the bones since associated cells can contaminate the marrow preparation and potentially overgrow the macrophages. After separating femurs and tibias the knob ends were cut off. Using 5ml of ice cold RPMI, the femur and tibia were flushed from both ends of each bone using a 26G needle. The effluent, which contains the bone marrow stem cells, was collected in the bottom part of the petri dish. A single cell suspension was created by passing the cells through a 18G needle using a syringe. The bone marrow derived progenitor cells of each mouse were pooled and spun down for 5 minutes at 1500rpm at 4˚C. The supernatant was discarded and cells were used to generate either mouse bone marrow derived macrophages or mouse bone marrow derived dendritic cells.

2.1.2.2. Generating mouse Mϕ from bone marrow derived progenitor cells

After the cells were isolated from the bone marrow and spun down the pellet was resuspended in 10ml of mMϕ- Medium. 2ml of the cell suspension was distributed to each of five petri dishes already containing 18ml of the differentiation medium. Cells were incubated at 37˚C and 5% CO_2. On day three after the start of the differentiation 10ml of the mMϕ- Medium was added to each petri dish. Cells were fully differentiated on day seven. To harvest the cells the supernatant was discarded and 5ml of pre- warmed infection- Medium was added to each dish. Using a cell scraper, the cells were carefully detached from each of the five dishes and pooled in a 50ml tube. Cells were spun down for 5min at 1500rpm at room temperature, resuspendend in 10ml infection- Medium and counted using a hemocytometer.

2.1.2.3. Generating mouse dendritic cells from bone marrow derived progenitor cells

After the cells were isolated from the bone marrow and spun down the pellet was resuspended in 10ml of mDC- Medium. 2ml of the cell suspension was distributed to each of five petri dishes already containing 18ml of differentiation medium. Cells were incubated at 37˚C and 5% CO_2. On day three after the start of the differentiation 10ml of mDC- Medium was added to each petri dish. Cells were fully differentiated on day seven. A mouse CD11c positive selection kit (EasySep mouse CD11c positive selection kit, StemCell Technologies, United States, cat# 18758) was used to select for dendritic cells. 25ml of the supernatant was removed and

pooled in 50ml tubes. The remaining 5ml were used to detach the cells from the petri dishes with a cell scraper. Pooled cells from one mouse were spun down at 1500rpm at 4˚C for 5 minutes. The supernatant was discarded, cells were resuspended in 10ml tissue grade D-PBS (Dulbecco's Phosphate- Buffered Saline, Invitrogen™, United States, cat# 14190) including 2% (v/v) FBS and 1mM EDTA (Ethylenediamine Tetraacetic Acid, Tetrasodium Salt Dihydrate, FisherScientific, United States, cat# BP 121-500) and counted using a hemocytometer. $5x10^5$ cells were used for FACS to analyze the ratio of dendritic cells to other cells prior to positive selection. The rest of the cells was spun down (1500rpm, RT, 5min) and resuspended to a concentration of $1x10^8$ cells/ml. Then selection was performed according to the StemCell Technologies manual EasySep© protocol using the purple EasySep© magnet (StemCell Technologies, United States, cat# 18758). For the selection the recommended 5ml polystyrene stubes (Falcon™ by BDBioscience, United States, cat# 352054) were used. The optional FcR blocker was added. Cells were seperated by incubation in the magnet four times instead of the suggested two times. Cells were resuspended in three ml PBS including 2% (v/v) FBS and 1mM EDTA. Cells were counted, spun down at 1500rpm at RT for 5min and resuspended to a concentration of $1x10^6$ cells/ml in infection- Medium. $5x10^5$ cells were set aside and used for FACS to assess the purity of mouse dendritic cells after positive selection. All the remaining cells were used for experiments.

2.1.3. Isolation and preparation of human primary cells

Procedures involving human blood samples were performed in consideration of the University of California, Irvine guidelines.

2.1.3.1. Isolation and preperation of human monocytes

To isolate lymphocytes from human blood samples, 15ml Lymphocyte Separation Medium (Mediatech, Inc.®, Cat. Nr. 25-072-CI) was added to 50ml tubes (Sarstedt®, Cat. Nr. 93.1645). 30ml of human blood from healthy volunteers was carefully added on top without mixing the two resulting phases. The blood cells were separated by centrifugation for 15min at 2000rpm at room temperature. This results in four phases: a plasma phase that also contains other components on top, a layer of mononuclear cells, followed by the Lymphocyte Seperation Medium, and a layer of erythrocytes and granulocytes which are present in form of a pellet. The

mononuclear cell phase containing the progenitor cells was transferred to a new 50ml tube. To pellet the cells, the solution was spun down for 8min at 2000rpm at room temperature. The supernatant was discarded, the pellet was resuspended in 30ml PBS and spun down again for 8min at 1000rpm at room temperature. The washing step was repeated one more time. Cells were allowed to adhere to culture plates for 2h at 37 °C and 5 % CO_2. The supernatant including non- adherent cells was carefully removed and discarded. Full- Medium was added and the resulting monocytes were detached by repeated pipetting, counted using a hemocytometer and used immediately.

2.1.3.2. Differentiation of human Monocytes to human Dendritic Cells

To differentiate cells to human dendritic cells, isolated monocytes were resuspended in hDC- medium and cultured in TC- treated 6- well plates under humidified atmosphere of 5% CO_2 at 37°C. Half of the medium was replaced every 2 days with fresh medium and monocyte derived DCs were collected after 6 days by pipetting up and down. The cells were pelleted (5min, 1000rpm, RT), resuspended in 5ml of infection- Medium and counted using a hemocytometer.

2.1.4. Isolation of human T cells

Human T cells were isolated from human blood samples using immunomagnetic isolation kits according to the product instructions from StemCell Technologies. (EasySep® Human Memory CD4+ T Cell Enrichment Kit, cat# 19157, EasySep® Human Naïve CD4+ T Cell Enrichment Kit, cat#19155, EasySep® Human CD4 Positive Selection Kit, cat# 18052, all StemCell Technologies, United States)

2.1.5. Infection of macrophages and dendritic cells with *Salmonella typhimurium*

The bacteria culture was grown over night and prepared for infection. Cells were seeded in a concentration of $1x10^6$ cells/ml. Macrophages were seeded in tissue culture treated flat bottom 24 well plates (Corning Inc., United States, cat# 3524) at a total of $2.5x10^5$ to $1x10^6$ cells/ well. For dendritic cells tissue culture treated flat bottom 48 well plates (Corning Inc., United States, cat# 3548) were used for infection. Tissue culture treatment cross links carboxyl and amine groups and gives the plastic a negative charge (F. Grinnell 1978 Int. Rev.Cytol 43. p.65) to help cells

attach to the wells. Immediately after seeding, cells were infected with 50µl of bacteria suspension, if not stated otherwise. All experiments were performed with uninfected negative controls and positive controls. The cells were incubated for one hour at 37˚C at 5% CO_2 before the supernatant was carefully removed and discarded without touching the cells. A volume to reach a concentration of $1x10^6$ cells/ml of new Infection- Medium containing 100ng/ml Gentamicin was added to the cells and incubated for an hour. The gentamicin concentration was lowered to 25ng/ml 2 hours post infection by changing the medium. The antibiotic gentamicin was added to the wells to kill extracellular bacteria and protect the cells from being outgrown by the bacteria and killed. Lowering the concentration to 25ng/ml is essential since prolonged exposure to high concentration of the antibiotic might lead to cell death. Cells were incubated for 24 hours at 37˚C and 5% CO_2 before the supernatant was carefully removed and stored at -20˚C If cells were further used, PBS or infection-Medium was added to the wells and cells were detached by pipetting up and down.

2.1.6. Co- culture of human dendritic cells and human T cells

One day after the infection, the supernatant was removed from human dendritic cells and the adherent cells were resuspended in fresh infection- Medium to a concentration of $1x10^6$cells/ml by repeated pipetting. 100µl of this solution were used to generate a $1x10^5$ cells/ml dilution with infection- Medium. $1x10^4$ dendritic cells per well were seeded in a tissue culture coated 96 well plate (Corning® 96 Well Clear Round Bottom; cat# 3799; Corning Inc.). 100µl of a $1x10^6$cells/ml total (memory and naïve) T- cell suspension was added to the wells. Every condition was prepared in at least four replicates. After 2 and 5 days co- culturing at 37 °C and 5 % CO_2 the supernatants of the replicates were pooled and frozen for analytic assays. The cells of all replicates were detached by adding 20µl/well PBS and pipetting up and down, were pooled and subjected to FACS analysis.

2.1.7. (Re-) Stimulation of T cells with supernatant of DCs

$1x10^5$ T cells in a volume of 100µl per well were seeded in a 48 well plate. 100µl/well supernatant of uninfected (negative control) and infected human dendritic cells was added to stimulate human T cells. T cells were pre- activated by CD3 and CD28 receptor stimulation. Therefore 10µl CD3/CD28 beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen, United States, cat#111.32D) for each condition

were transferred to a 5ml polystyrene tube (Falcon™ by BDBioscience, United States, cat# 352054). The beads were diluted with 1 ml infection- Medium and were vortexed for 5 seconds. The tube was placed in a magnet for one minute, the supernatant was discarded, and the beads were resuspended in infection- Medium with the initial volume the beads were taken from the vial. 10µl of beads were added to each well. The total volume per well was brought up to 400µl with infection- Medium.

2.2. Microbiology

2.2.1. Strains and Media

In all experiments the *Salmonella enterica* serotype Typhimurium (*S. typhimurium*) strain IR715 was used. This strain is a spontaneous nalidixic acid-resistant derivative of the strain ATCC 14028 (American Type Culture Collection). Cells were also infected with six different isogenic deletion mutants.

Table 1

Designation	Strain type	Genotype	Medium
STM	IR715	ATCC14028 Nal^R	LB
invA	IR715	*invA::tetRA*	LB
spiB	IR715	*spiB::KSAC*	LB
fliC	IR715	*fliC::Tn10 fljB::MudJ - TetR, KmR*	LB
msbB	IR715	*msbB:KSAC*	LB-0
invAspiB	IR715	SPN487 Δ*invA*(-9 to +2057) Δ*spiB*(+25 to +1209)	LB
invAspiBflhDC	IR715	SPN496 Δ*invA*(-9 to +2057) Δ*spiB*(+25 to +1209) P(*flhDC*5451)::Tn10dTc(del-25)	LB

Luria Bertani (LB) broth and LB agar (Difco™ through BD- Diagnostics, United States, cat#244620, cat#244520) is a nutritionally rich medium widely used for the growth of bacteria in molecular biology and microbiology. The main ingredients of the broth are tryptone, as a source for peptides and peptones, yeast extract as source for vitamins and trace elements, and sodium chloride. The composition of the LB-agar only differs in the additional of agar.

The *msbB* mutant which is deficient in lipid-A acylation has defects in the cell wall and it was shown to have additional mutations unless grown in a medium with a

lower osmolarity. Therefore, we grew the *msbB* mutant in LB-0, consisting of 10g/l peptone, 5g/l yeast extract, 2mM $MgSO_4$ and 2mM $CaCl_2$.
After the preparation, each medium was always autoclaved.

2.2.2. Preparation of the inoculum

For the infection of human and mouse cells, 5ml of broth medium was inoculated with *S. typhimurium* directly taken from a -80˚C frozen stock (1:1 mix of over night culture and 60% Glycerol in H_2O) between 17 and 20h before the infection. The culture was incubated statically at 37˚C to minimize the cytotoxicity. The OD_{600} of a 2 fold dilution of the bacteria culture was measured using a spectrophotometer. An OD_{600} of 1 equals $1x10^9$ bacteria per ml. $1x10^9$ bacteria were spun down for 2 minutes at 10,000 rpm at room temperature. The bacteria pellet was resuspended in 1ml of infection- Medium. Cell infections were performed at a density of 0.1, 1 or 10 bacteria per cell, called "multiplicity of infection" (MOI) of 0.1, 1 or 10 respectively. The volume in which bacteria were added to each well for the infection never exceeded 50µl/well. 10-fold serial dilutions in infection medium were performed to obtain the right concentrations for the infection. As a control for the dilution and the correct MOI, the bacteria suspension was further diluted to a concentration of $1x10^3$ bacteria/ml. 100µl of this dilution were plated on LB- agar plates, incubated for 18-24 hours, and the bacteria colonies were counted.

2.2.3. UV killing

Coohill and Sagripanti (2009) stated that *Salmonella* is successfully killed with an irradiation of $100J/m^2$ from ultraviolet light. Bacteria cultures were grown for 17 to 20h and prepared for infection. 500µl of the final concentration was transferred to the bottom of a Petri dish. The bacteria suspension was exposed to irradiation of 0.25 J by an UV Stratalinker 18000 (Stratagene). 50µl/ well were used to infect cells and 100µl of the final concentration were plated and incubated for 18-24h as a control for the killing.

2.2.4. Invasion Assay

To quantify the intracellular bacteria, 24h after the infection assay cells were resuspended in PBS. 200µl of a $1x10^5$ cells/ml solution were centrifuged for 5min at 800 x rcf at room temperature. The supernatant was discarded and the cells were

lysed by addition of 500µl H_2O. After an incubation of 10min, 100µl of the cell lysate was plated on LB- agar plates and were inubated over night at 37˚C. The colonies were counted the next day.

2.3. Immunochemistry

2.3.1. Flow cytometry analysis

Fluorescence activated cell sorting (FACS) was used as an analytic assay for experiments with human dendritic cells, human T cells and mouse dendritic cells. The cell suspension was spun down at 800 x rcf for 5 minutes at room temperature. To preserve the cells' shape and structure, cells were resuspended in 500µl fixation buffer (4% Paraformaldehyde in PBS) and incubated for 20 minutes at 37˚C. Cells were spun down at 800 x rcf for 5 minutes at room temperature and were resuspended in 50-100µl PBS. To stain the cells, 5µl of fluorescent antibody solution was added, and the suspension was incubated at room temperature in the dark for 30 minutes. Cells were then washed by adding 1 ml PBS, spun down at 800 x rcf for 5 minutes and resuspended in 200µl PBS. Cells were either kept for 24h in the dark at 4˚C or were immediately analyzed using a flow cytometer (BD FACScalibur™, BD Bioscience, United States). The data were analyzed using FlowJo software (Tree Star Inc., United States).

For T- cell proliferation experiments the cells were incubated with carboxyfluorescein diacetate succinimidyl ester immediately after isolation. CFDA-SE enters cells by diffusion and is cleaved by intracellular esterase enzymes to form carboxyfluorescein succinimidyl ester. CFSE emits a detectable fluorescence in FL3 when excited with 488nm and is retained in the cell by binding covalently to intracellular lysine residues and other amine sources. If a stained cell divides, the dye is divided equally between the two daughter cells. Fluorescence is detectable in cells following up to 8 successive cell divisions.

2.3.2. List of antibodies

Name	Target	Conjugate	Company	Use
anti- human IL- 17A (monoclonal)	human IL-17A	PE	eBioscience™ (cat# 12-7179)	IC Flow
anti- human IL-22 (monoclonal)	human IL-22	Alexa Fluor® 647	eBioscience™ (cat# 51-7229)	IC Flow
anti- human IFN- γ (monocolonal)	human IFN- γ	FITC	eBioscience™ (cat# 11-7319)	IC Flow
PAb hTLR 4 (Polyclonal)	human TLR5	No conjugate	InvivoGen (cat#pab-hstlr4)	neutralization
PAb hTLR5 (Polyclonal)	human TLR5	No conjugate	InvivoGen (cat#pab-hstlr5)	neutralization
anti- mouse CD11c	mouse CD11c receptor, marker for DC	PE-TAC-Magnetic Particle	StemCell (cat# 18758)	Flow, Positive selection
anti- mouse CD11c (monoclonal)	mouse CD11c receptor, marker for DC	PE	eBioscience™ (cat# 12-0114)	Flow

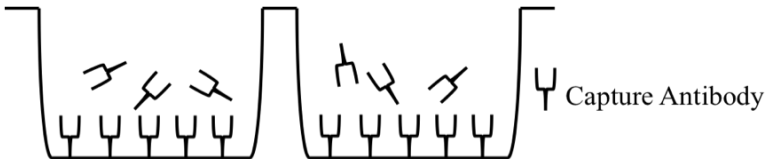
2.3.3. Enzyme linked immunosorbent assay

Sandwich enzyme linked immunosorbent assays were performed to analyze the mature secreted protein amounts in experiments. Samples were thawed an hour

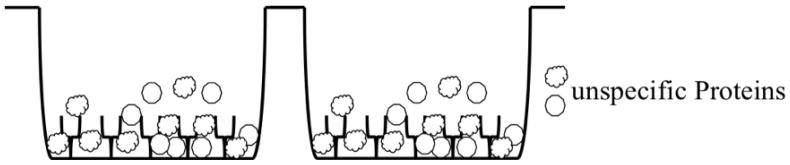
before the incubation step and immediately frozen again or aliquoted and stored at 4˚C over night according to sample stability.

2.3.3.1. Assay protocol

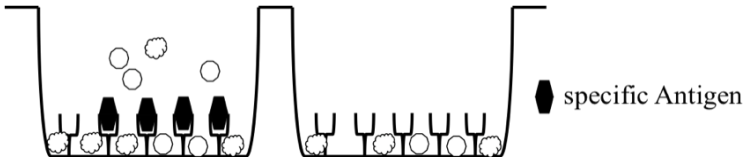
To attach the capture antibodies to the solid phase represented by wells of a 96 well plate (Corning® 96 Well clear flat bottom polystyrene high bind microplate, Corning, United States, cat#9018) the antibodies were diluted according to the certificates of analysis and 100µl were added to each well. Plates were sealed with Parafilm and incubated at 4˚C over night.



On the next day wells were washed five times by adding 300µl/well wash buffer (1x PBS at pH7.4, 0.05%(v/v) Tween- 20), incubation for 60 seconds and discarding of the liquid. To avoid unspecific binding of sample proteins to the well, resulting in a wrong positive result, wells were incubated for one hour at room temperature with 200µl blocking solution, containing BSA (1x Assay Diluent or 1%BSA in PBS).

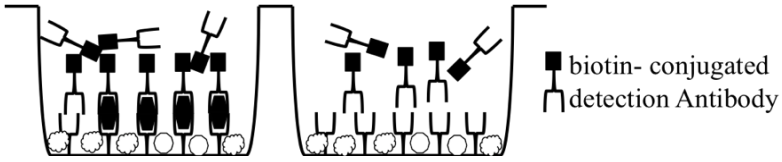


Wells were washed for a total of five times. The standard protein was diluted with assay diluent (from kit or 0.05% (v/v) Tween, 0.1% (w/v) BSA in PBS) according to the certificate of analysis for the top standard. The top standard was further diluted by seven 2- fold dilutions. All seven standard dilutions and a blank negative control (assay diluent only) were analyzed in every assay. Wells were loaded with 100µl of standard dilutions and diluted samples (in assay diluent) in duplicates.

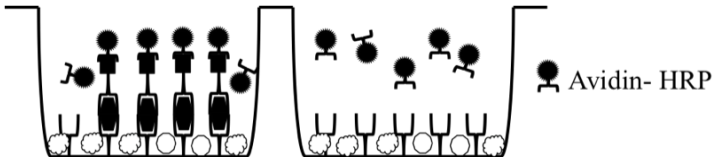


The highly specific antigen competes with the unspecific blocking protein and binds to the antibodies bound to the solid phase. Wells were washed five times, thereby eliminating unspecific binding proteins. The biotin- conjugated detection antibody

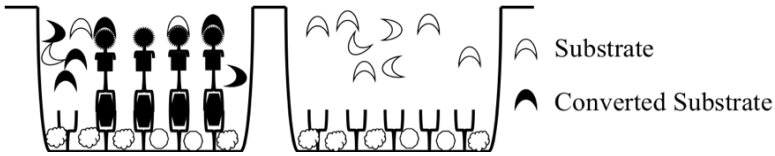
was diluted according to the certificate of analysis. 100µl were added to each well and the plate was incubated for one hour at room temperature.



Wells were washed five times to remove excess of detection antibody. The biotin binding (strep-) avidin (depending on the kit) linked to the enzyme horseradish peroxidase was diluted according to the certificate of analysis and 100µl were added to each well. The plate was incubated for 30 minutes at room temperature.



The wells were washed seven times with 300µl wash buffer and incubation for two minutes, removing the unbound avidin- HRP complex to avoid false positive reactions with the substrate. This will decrease the background signal. 100µl of the substrate solution Tetramethylbenzidine (TMB) was added to the wells.



After 15 minutes incubation the reaction was stopped by adding 50µl 2N HCl. The optical density was measured no later than 30 min after the addition of HCl with a spectrophotometer at a wavelength of 450nm. As a correction factor also the OD_{595} was measured and subtracted from the OD_{450}. The amount of cytokine was calculated using the standard curve generated in Microsoft Excel.

2.3.3.2. List of ELISA kits

Cytokine	Company	cat#
hIL-1β	eBioscience	88-7910
hIL-23	eBioscience	88-7237
hIL-17	eBioscience	88-7976
hIL-22	PeproTech	900-K246
hTNF- α	eBioscience	88-7346
mIL-1β	eBioscience	88-7913

mIL-1β	BioLegend	432605

2.3.3.3. Sample stability

Samples were handled in consideration of their freeze- thaw cycle and storage stabilities. (Information obtained from ELISA manuals by BenderMedSystems)

Cytokine	Freeze- thaw at -20˚C	Stored 24h at -20˚C	Stored 24h at 2˚-8˚C	Stored 24h at RT	Stored 24h at 37˚C
Mouse IL- 1β	5x	OK	Significicant loss	Significicant loss	Significicant loss
Mouse IL- 6	5x	OK	OK	OK	Significicant loss
Mouse TNF- α	5x	OK	OK	Significicant loss	Significicant loss
Human IL-22	3x	OK	OK	OK	OK
Human IFN- γ	1x	OK	OK	OK	Significicant loss
Human IL- 17	5x	OK	OK	OK	OK
Human IL- 1β	5x	OK	Significicant loss	Significicant loss	Significicant loss
Human IL- 23	3x	OK	OK	OK	OK

2.3.3.4. Sample dilution

To meet the standard- and limits of detection of the different ELISAs, samples were diluted prior to loading plates.

Cytokine	Dilution factor
Mouse IL- 1β	2-4
Mouse IL- 6	50
Mouse TNF- α	10 to 20
Human IL-22	undiluted
Human IFN- γ	undiluted
Human IL- 17	5
Human IL- 1β	10
Human IL- 23	2 to 4

2.3.4. Caspase 1 activity assay

The caspase 1 activity after *Salmonella typhimurium* infection was measured using a Carboxyfluorescein FLICA Caspase Assay Kit (Caspase 1 FLICA, Immunochemistry Technologies, LLC, United States, cat# 97). A cell permeable, and non- cytotoxic inhibitor is coupled to a carboxyfluorescein- labeled fluoromethyl ketone peptide, which emits green fluorescence when excited at 490nm. The amino acid sequence YVAD of the inhibitor protein covalently binds to the active site of the caspase 1 enzyme. Moreover, cells were stained with propidium iodide (PI) to distinguish between live cells and dead cells. PI stains DNA of necrotic, dead, and membrane- compromised cells.

Primary human dendritic cells were grown and infected for 1 to 12 hours before the supernatant was frozen away and the cells were resuspended in infection- Medium containing 25ng/ml Gentamicin at a concentration of $1x10^6$ cells/ml. Induced and uninduced cells were split to a volume of 300µl in a 5ml polystyrene round bottom tube (BD Falcon™, BD Bioscience, United States, cat# 352054) for each condition. 10µl 30x FLICA solution was added directly to the cell suspension. The suspension was mixed and incubated for one hour at 37˚C under 5% CO_2 protected from light. Every 20 minutes the cells were mixed by swirling the tube. Cells were washed twice by adding 2ml wash buffer, spinning down at 400g for 5 minutes at room temperature, gently vortexing the pellet and discarding of the supernatant. The cell pellet was resuspended in 200µl wash buffer. Cells that were analyzed by PI screening were treated with 2µl PI. Samples were kept on ice and analyzed within four hours.

2.3.5. Toll- like receptor inhibiton

To analyze the contribution of certain surface receptors to cytokine production the receptors were blocked. Antibodies were resuspended in tissue culture grade PBS according to the manufactures instructions, aliquoted and frozen at -20˚C until use. Cells were seeded in a concentration of $1x10^6$ cells/ml. Both blocking and isotype control antibodies were added in a concentration of 10µg/ml and cells were incubated at 37˚C and 5% CO_2 for 1 hour. Cells were infected with *Salmonella typhimurium* at a multiplicity of infection of 1. The cells were incubated for one hour at 37˚C and 5% CO_2 before the medium was replaced with infection Medium

containing 100ng/ml Gentamicin. Two hours post infection the gentamicin concentration was lowered to 25ng/ml by changing the medium. Cells were incubated for 24 hours at 37˚C and 5% CO_2 before the supernatant was carefully removed and used for analytic assays.

2.3.6. MyD88 inhibition

Inhibition of the TLR adaptor protein MyD88 was achieved by blocking homodimerization using an inhibitory peptide (MyD88 Homodimerization Inhibitory Peptide Set, IMGENEX, United States, cat# IMG-2005-1) containing a sequence (RDVLPGT) from the MyD88 TIR homodimerization domain. The peptide is linked to a protein transduction (PTD) sequence derived from antennapedia that renders the peptide cell permeable. A control peptide, which consists of this PTD sequence only, was used as a negative control with every experiment.

Human dendritic cells were prepared for infection and seeded in a 48 well plate at a concentration of $1x10^6$ cells/ml ranging from $2.5x10^5$ cells/well to $5x10^5$ cells/well. Either the MyD88 inhibitor or the control peptide were added to a final concentration of 100µM or 200µM. Cells were incubated for 24h at 37˚C under 5% CO_2. Cells were infected with *Salmonella typhimurium* (MOI=1). One and two hours after infection cells were subjected to gentamicin treatment as described earlier. 24 hours after infection the supernatant was carefully removed and used for analytic assays.

2.3.7. Caspase- 1 inhibition through ASC inactivation

In order to inhibit Caspase- 1 activation the adaptor protein ASC that bridges the receptor and the enzyme was blocked. As described, subphysiological concentration of intracellular potassium results in assembly of the pyroptosome. The extracellular concentration of potassium was increased, to keep intracellular potassium at a physiological level and thereby block activation of ASC.

Human dendritic cells were prepared for infection in infection- Medium containing 40mM KCl or 80mM KCl. At a concentration of $1x10^6$ cells/ml cell were seeded ranging from $2x10^5$ cells/well to $5x10^5$ cells/well in a 48 well plate. Cells were immediately infected at a multiplicity of infection of 1 with *Salmonella typhimurium*. One and two hours after infection cells were subjected to gentamicin

treatment. 24 hours after infection the supernatant was carefully removed and used for analytic assays.

3. Results

3.1. Determine the mechanism by which *S. typhimurium* induces cytokine expression in human APC

To investigate which bacterial patterns trigger the expression and secretion of pro-inflammatory cytokines by human antigen presenting cells, *Salmonella typhimurium* wild type and the different mutant strains (as in Table 1) were used to infect macrophages and dendritic cells. Previous mouse studies indicate an involvement of *Salmonella* Pathogenicity Island 1 T3SS, flagellum and lipopolysaccharide to trigger pro- inflammatory cytokine production. LPS and Flagellin stimulate TLR4 and TLR5 respectively to express cytokines including TNF, IL-6, pro- IL-1β, pro- IL-18 and the subunits p35, p40, p19 which form IL-12 and IL-23 (Bryant et al. 2010, Happel et al. 2003). Furthermore by the translocation of flagellin from the endosome to the host cytosol SPI-1 T3SS triggers NLR signaling resulting in activation of caspase-1, which in turn stimulates activation and secretion of IL-1β and IL-18 and pyroptosis (Miao et al. 2007).

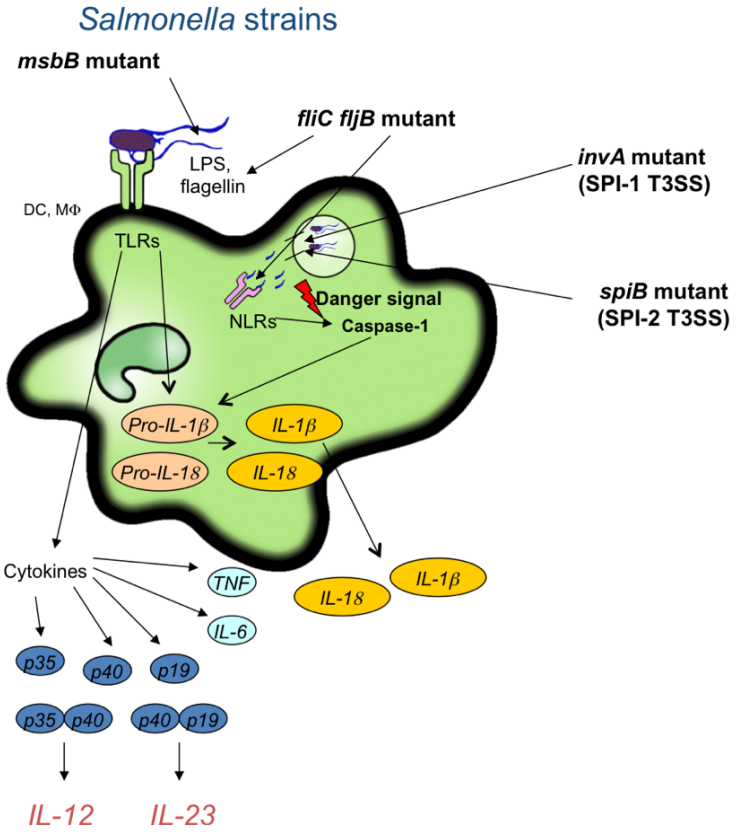


Figure 3-1, this figure shows the used *Salmonella typhimurium* mutant strains and how the defects are thought to have an effect on the signal cascade, leading to a decreased secretion of the pro- inflammatory cytokines;

Figure 3-1 shows a model based on the literature on the patterns from *S. typhimurium* that are detected by the receptors and trigger the expression and secretion of cytokines (Miao et al. 2007, Bryant et al. 2010, Happel et al. 2003). This model is largely based on studies in the mouse. Recent studies suggested that *S. typhimurium* mutants defective in the SPI- 1 T3SS would triggers less IL- 1β and IL-18 secretion in APCs due to reduced NLR signaling and therefore reduced caspase-1 activation. Defects in the SPI-2 T3SS are thought to reduce survival of intracellular STM and may contribute to NLR activation. A *fliC fljB* mutant cannot produce flagellin and hence is unable to assemble flagella. Thus, a *fliC fljB* mutant does not activate both TLR5 and IPAF signaling (Schmitt et al. 2001). A *msbB* mutant has a defect in lipid A acylation, therefore it does not activate TLR4 signaling.

These mutant strains were used to test whether some of the unknown pathogen-associated patterns and virulence factors may trigger IL- 1β and IL- 23 secretion in human APCs.

3.1.1. Induction of pro- inflammatory cytokines in mouse APC

Mouse APCs were isolated and infected as described in the Materials and Methods section. 24h after infection, the pro- inflammatory cytokines were quantified by ELISA.

3.1.1.1. Confirming induction of IL-1β secretion by mouse macrophages

To test whether we could reproduce published results, the IL-1β secretion by bone marrow derived macrophages upon infection with *S. typhimurium* and the mutant strains as in Table 1 was analyzed. Cells were infected with a multiplicity of infection (MOI) of 1 and 10 for 24 hours. Secretion of IL-1β was determined in the supernatant using an enzyme linked immunosorbent assay.

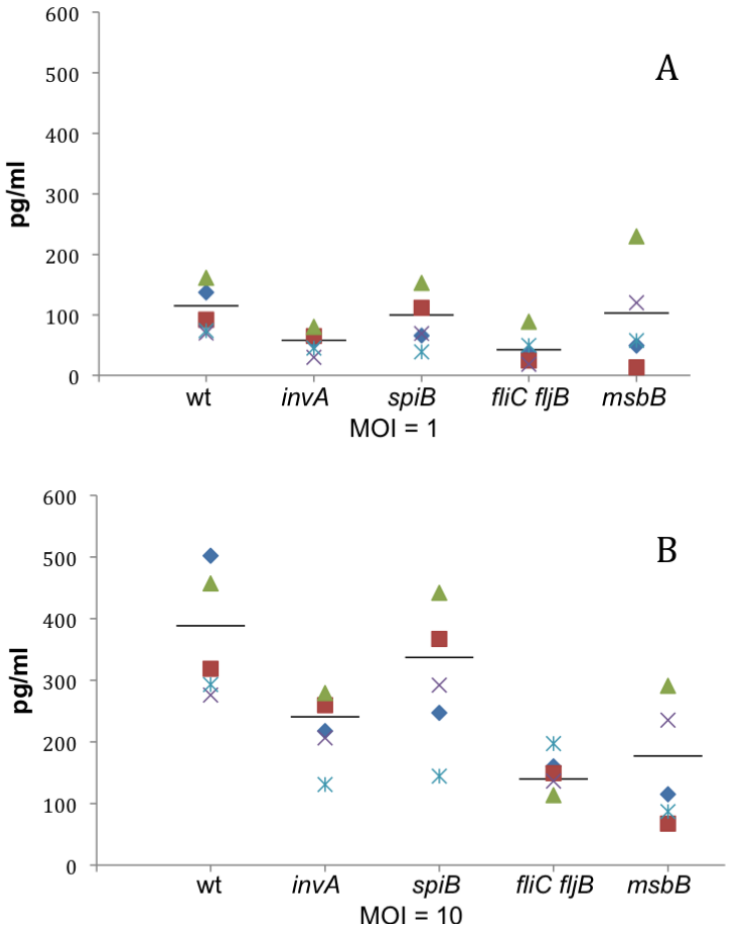


Figure 3-2 Mature mouse IL- 1β secreted by bone marrow derived macrophages ($1x10^6$ cells/ml) upon infection with *Salmonella typhimurium* and mutant strains, as determined by ELISA. Results of 5 biological replicates are shown; *A*, cells were infected with an MOI of 1. *B*, cells were infected with an MOI of 10; an MOI-dependent increase of IL-1β secretion was observed;

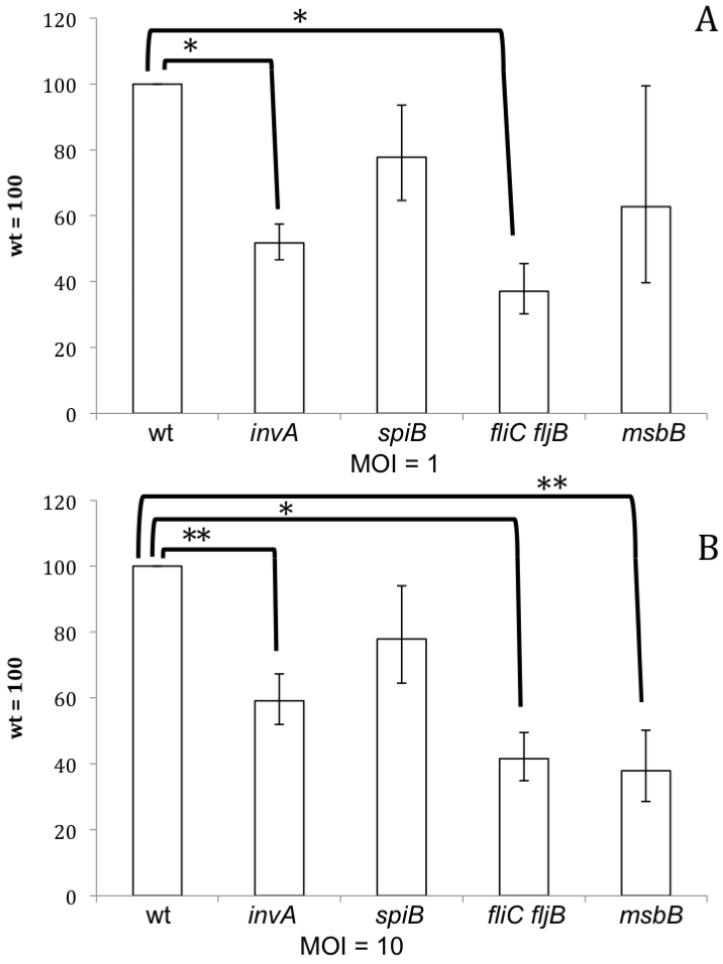


Figure 3-3 Paired analysis of mature mouse IL- 1β secreted by bone marrow derived macrophages ($1x10^6$ cells/ml) upon infection with *Salmonella typhimurium* and mutant strains quantified by ELISA. The average of 5 biological replicates is shown. The levels of IL- 1β secretion upon wild type STM infection are set to 100; * $P < 0.01$, ** $P < 0.05$; Error bars represent Standard Error; *A*, at an MOI of 1, significant lower amounts of IL- 1β secretion was detected when BMDM were infected with mutant strains defective in either the SPI-1 T3SS (*invA*) or flagellin production (*fliC fljB*) compared to infection with wild type STM; *B*, when BMDM were infected with an MOI of 10, there was less variation in IL- 1β secretion. Significant less secretion of IL-1β was observed when cells were infected with a SPI-1 T3SS mutant (*invA*), a flagellin mutant (*fliC fljB*) or a lipid-A acylation mutant (*msbB*).

As previous data from different groups suggested, we also found that mouse macrophages secrete significant less IL-1β when infected with a SPI- 1 T3SS defective mutant compared to infection with wild type STM. This likely occurs because a defect in the SPI-1 T3SS eliminates intracellular flagellin and reduces secretion of effector proteins in the host cytosol of macrophages, thereby reducing NLR signaling. This results in less caspase 1 activity and therefore less IL- 1β secretion. Furthermore, when we infected BMDM with a mutant strain not capable

of producing flagellin, the IL- 1β secretion was reduced to about 40% compared to wild type STM infection, consistent with the described role of IPAF signaling in IL-1β production. A similar decrease was observed when macrophages were infected with a lipid-A acylation defective mutant, suggesting involvement of TLR4 signaling as a "first signal".

Taken together, our results with bone marrow-derived macrophages are in agreement with the data from other groups. Therefore, our experimental setting and our mutant strains may provide useful tools to investigate the activation of the innate immune response in human cells.

3.1.1.2. Induction of IL-1β secretion by mouse dendritic cells

To investigate whether IL- 1β secretion by mouse dendritic cells is dependent on the same signaling pathway as in mouse macrophages bone marrow derived cells were differentiated to dendritic cells and infected with wild type STM and mutant strains as above. Prior to the infection, the purity of differentiated mouse dendritic cells was assessed by FACS before and after magnetic positive selection.

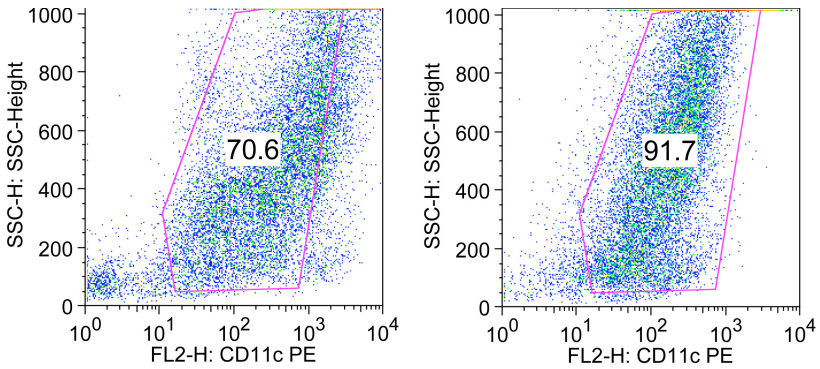


Figure 3-4 Example of FACS results before (left) and after (right) positive selection of bone marrow derived cells which have been differentiated to DCs over 7 days. Cells were stained with anti-CD11c antibodies.

Through magnetic positive selection the purity of dendritic cells was improved by an average of 13.45% (n=6).

The infection of mouse dendritic cells was performed with an MOI of 1 and 10. However, reliable amounts of IL-1β in the supernatant 24 hours after infection were only detected upon an infection with an MOI of 10.

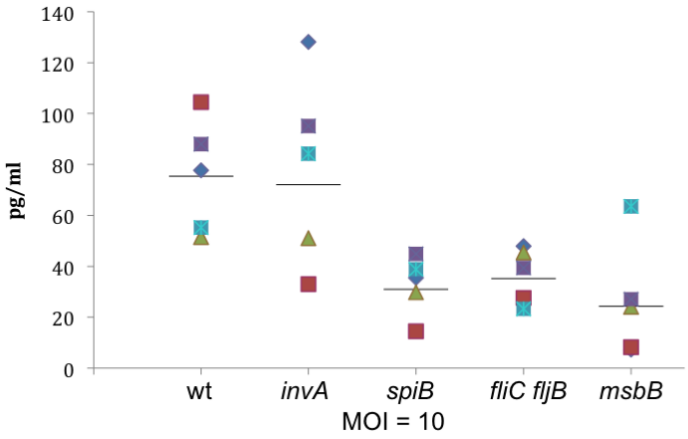


Figure 3-5 Mouse IL- 1β was detected by ELISA in the supernatants of bone marrow derived mouse dendritic cells (1x10^6 cells/ml) infected with wild type *S. typhimurium* and mutant strains with an MOI of 10. Results of 5 biological replicates are shown.

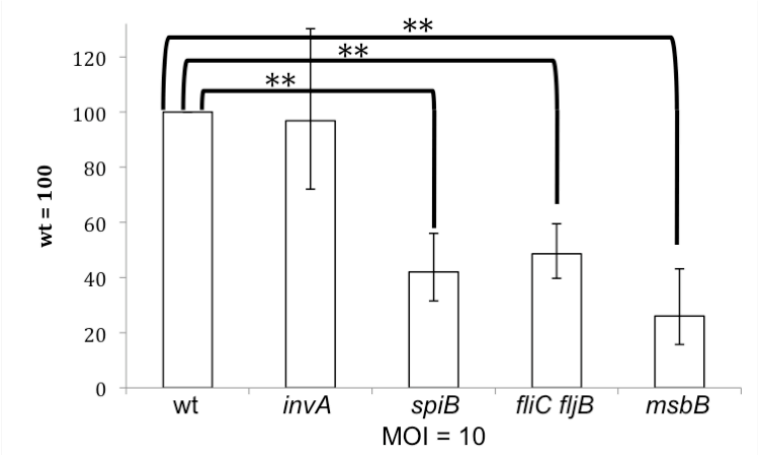


Figure 3-6 Paired analysis of mature IL- 1β secreted by mouse bone marrow derived DCs (1x10^6 cells/ml) upon infection with *S. typhimurium* and mutant strains with an MOI of 10 quantified by ELISA on supernatants. The average of 5 biological replicates is shown. The levels of IL- 1β secretion upon wild type STM infection are set to 100. ** $P < 0.05$; Error bars represent the Standard Error. Mouse DCs secreted a significant less amount of mature IL- 1β when infected with a *S. typhimurium* mutant strain defective in SPI-2 T3SS (*spiB*), flagellin (*fliC fljB*) or lipid- A acylation (*msbB*).

Significant lower levels of IL-1β were detected when DCs were infected with mutant strains defective in either the SPI- 2 T3SS (*spiB*), flagella (*fliC fljB*) or lipid- A acylation (*msbB*). SPI- 2 T3SS is activated in *Salmonella* containing vacuoles and translocates effector proteins in the host cytosol, where they may trigger NLR signaling, although it has not been shown yet. Flagellin in the lumen is recognized by TLR5, and in the cytosol by Ipaf. Infection with a knock out mutant strain that does not trigger either signaling pathway resulted in a significant lower amount of

mature IL- 1β compared to wild type. Because IL-1β secretion in mouse BMDM is triggered by Ipaf and not TLR5, we expect that Ipaf signaling contributes to IL-1β secretion in dendritic cells. It is yet to be determined whether Ipaf is activated through cytosolic flagellin delivered through SPI-2 T3SS, since the *spiB* mutant elicited the same levels of IL-1β as the *fliC fljB* mutant. Furthermore, TLR4 signaling seems to play an important role in IL- 1β production also in mouse dendritic cells, since infection with an STM mutant defective in lipid- A acylation resulted in significant less IL-1β.

Compared to mouse bone marrow derived macrophages, dendritic cells secreted lower amounts of the pro- inflammatory cytokine IL- 1β as shown in Figure 3-2 compared to Figure 3-5, indicating that macrophages are the major source of IL-1β levels during the inflammatory response. The secretion of this pro- inflammatory cytokine depends in both kinds of antigen presenting cells partly on TLR4 signaling, as shown by infection experiments with a *S. typhimurium* mutant strain defective in lipid- A acylation. Our results also indicate that IL- 1β secretion is dependent on the SPI- 1 T3SS in mouse macrophages but not dendritic cells. In contrast, DCs secrete less IL-1β when infected with a SPI-2 T3SS mutant strain. Further investigations are needed to test whether flagellin is translocated by the SPI- 2 T3SS in dendritic cells.

3.1.2. Induction of pro- inflammatory cytokines in human APC

Most studies investigating the mechanisms of secretion of pro- inflammatory cytokine production during *S. typhimurium* infection have been done in mouse cells. To investigate if the production of pro-inflammatory cytokine in mouse and human antigen presenting cells is triggered by similar mechanisms, the experiments performed in mouse APCs were also done in human APCs.

3.1.2.1. Induction of IL- 1β in human monocytes

Since mouse macrophages seem to be a major source of IL-1β, human monocytes were infected to see if the cytokine levels are comparable. To determine the PAMPs contributing to the production of this pro-inflammatory cytokine, human monocytes were infected with the *S. typhimurium* wild type and the same mutant strains used in the mouse experiments.

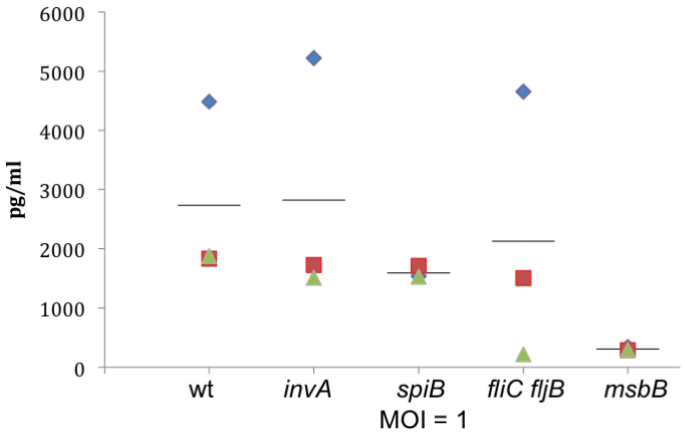


Figure 3-7 Human IL- 1β secretion in the supernatant of infected human monocytes. Human monocytes were infected with *S. typhimurium* wild type and the mutant strains at an MOI of 1. Results of 3 biological replicates are shown. Even at an MOI of 1, human monocytes secrete very high levels of mature IL- 1β.

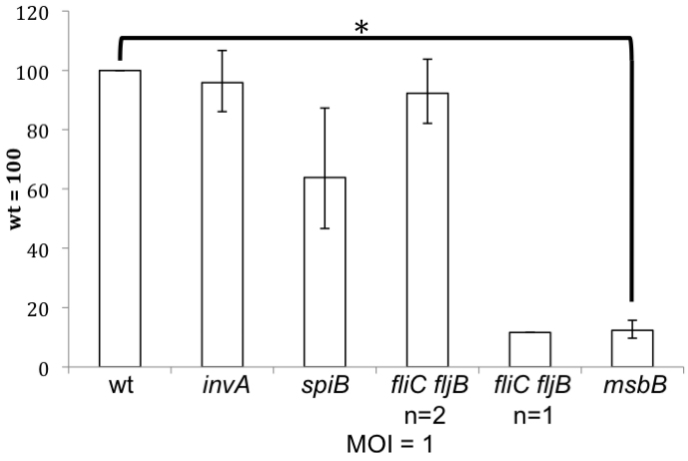


Figure 3-8 Paired analysis of mature IL- 1β secreted by human monocytes ($1x10^6$ cells/ml) upon infection with *S. typhimurium* and mutant strains at an MOI of 1, as detected by ELISA on supernatants. The average of 3 biological replicates is shown. The levels of IL- 1β secretion upon wild type STM infection are set to 100. * $P < 0.01$; Error bars represent the Standard Error. The concentration of IL- 1β detected when cells were infected with mutant strains defective in either the SPI- 1 T3SS (*invA*) or the SPI-2 T3SS (*spiB*) was compareable to the levels elicited by infection with the wild- type strain. Monocytes of two individuals secreted a similar amount of IL- 1β when infected with a flagellin mutant (*fliC fljB*) compared to wild type infection, while one individual secreted only about 10% of IL-1β compared to wild type infection. All individuals secreted around 10% IL-1β compared with wild-type infection when monocytes were infected with a mutant strain defective in lipid- A acylation (*msbB mutant*)

Consistent with the results obtained in mouse macrophages, human monocytes secreted very high levels of IL- 1β, even when infected at a multiplicity of infection

of 1. The significantly lower amount of IL- 1β secreted by human monocytes upon infection with a strain defective in lipid- A acylation indicates a major contribution of TLR4 signaling to mature IL- 1β production. This seems to be true for both mouse macrophages and human monocytes. In contrast, only mouse macrophages showed a significant reduction of IL-1β secretion when infected with a strain defective in the SPI-1 T3SS defective (*invA* mutant). Infection of both mouse macrophages and human monocytes with the SPI- 2 T3SS defective strain resulted in the same levels of IL- 1β secretion when compared to wild- type infection. Infection with a mutant strain defective in flagellin production which does not trigger TLR5 or Ipaf signaling in mouse cells resulted in 90% less IL-1β secretion in one out of three individuals, while in the two others the levels were comparable to the wild type strain. Further experiments will investigate the mechanisms of this variation in the individual host response to *S. typhimurium* flagellin. Reasons contributing to this variable response might be a genetic disorder/ polymorphism in the TLR5 or Ipaf receptor or a unmentioned concurrent/prior infection not mentioned by the healthy human blood donor.

3.1.2.2. Induction of IL- 23 in human monocytes

Besides IL- 1β, IL- 23 is suggested to be an important pro- inflammatory cytokine involved in T cell activation and differentiation. It has been shown that IL- 23 is essential for IL- 17 production (Godinez et al. 2009). Even though IL- 23 was not detected in mouse macrophages and DC infected with *S. typhimurium* (results not shown), supernatants of human cells were analyzed by ELISA for secreted IL- 23.

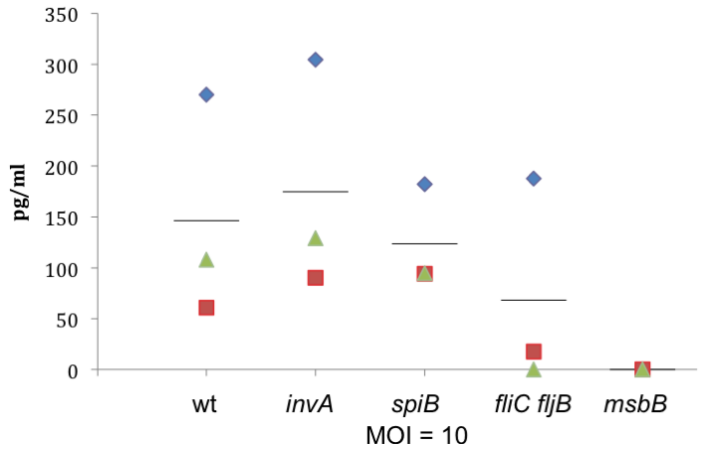


Figure 3-9 Human IL- 23 was detected by ELISA in the supernatants of human monocytes infected with wild type *S. typhimurium* and the mutant strains at an MOI of 10. Low levels of the cytokine were observed. Results of 3 biological replicates are shown.

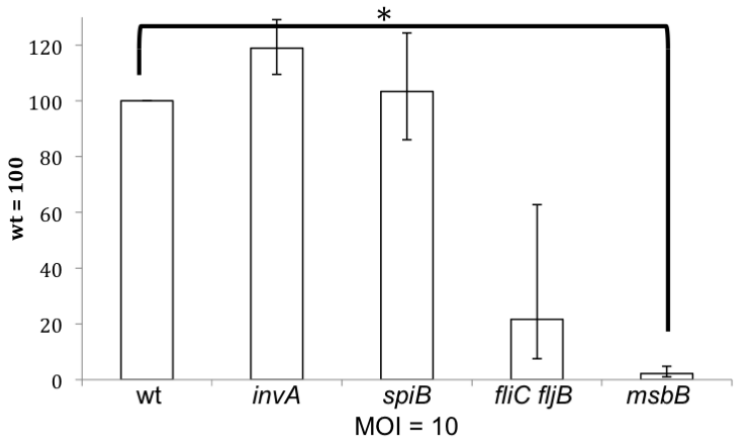


Figure 3-10 Paired analysis of IL- 23 secreted by human monocytes ($1x10^6$ celles/ml) upon infection with *S. typhimurium* and the mutant strains at an MOI of 10. The average of 3 biological replicates is shown. The levels of IL- 23 secretion upon wild type STM infection are set to 100. * $P < 0.01$; Error bars represent the Standard Error. Significant less IL- 23 was produced when human monocytes were infected with mutant strain defective in lipid-A acylation (*msbB*). The production of IL- 23 when monocytes were infected with a mutant strain defective in flagellin (*fliC fljB*) also seemed to be less but was not statistically significant.

Upon infection with wild type *S. typhimurium* human monocytes produce small amounts of IL- 23. The results from infections with mutant strains defective in different pathogen associated molecular patterns indicate a contribution of flagellin and LPS signaling to secretion of IL- 23, as shown by the lower levels of IL- 23 induced by infection with the *fliC fljB* mutant and the *msbB* mutant. This is likely dependent on reduced activation of TLR4 and TLR5.

3.1.2.3. Induction of IL- 1β in human DCs

We have shown that mouse macrophages are the major source of secreted IL-1β upon *Salmonella typhimurium* infection, while mouse dendritic cells secrete only small amounts. Furthermore, infection with mutant strains defective in SPI2- T3SS, flagellin and LPS triggered less IL-1β secretion by mouse DCs. To investigate if this applies also to human IL-1β secretion, human dendritic cells were infected with *S. typhimurium* wild-type and the mutant strains described above.

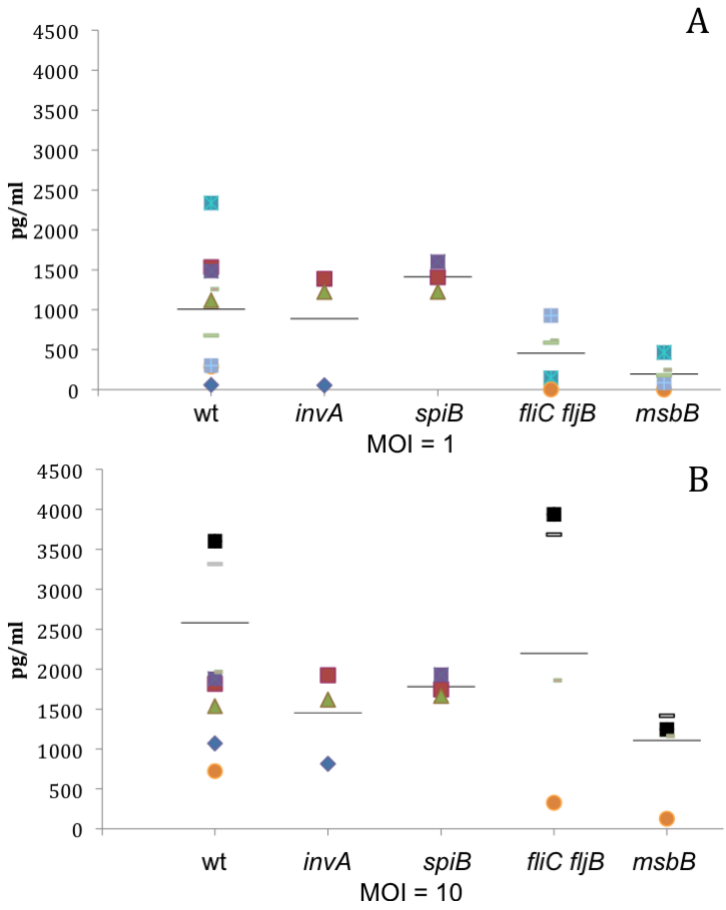


Figure 3-11 Human IL-1β was detected by ELISA in the supernatants of human dendritic cells infected with *S. typhimurium* and the mutant strains. *A*, cells were infected at an MOI of 1. *B*, cells were infected at an MOI of 10.

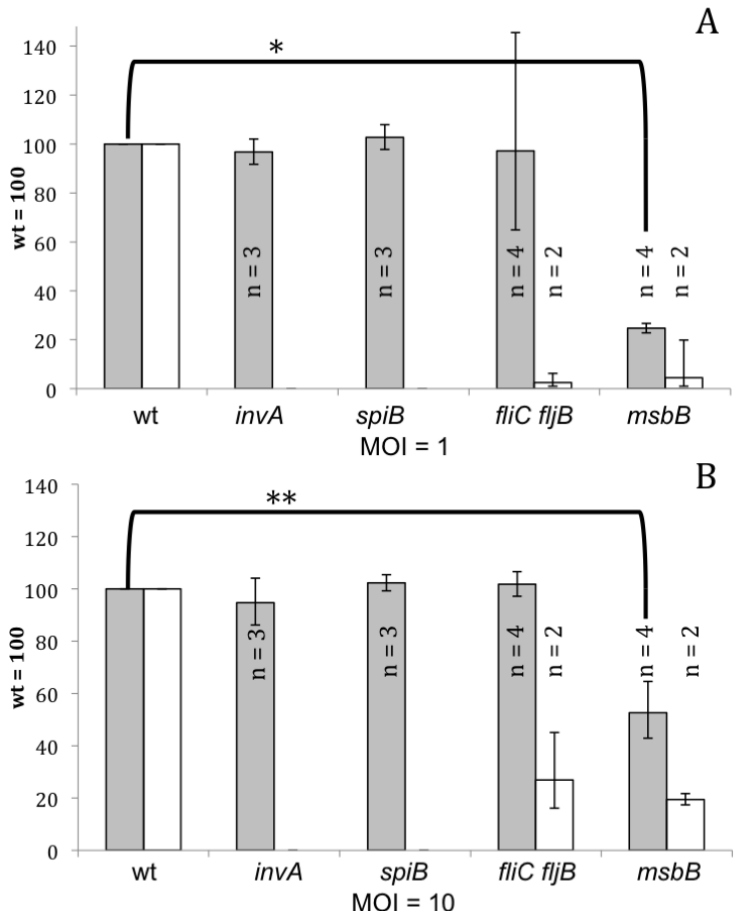


Figure 3-12 Paired analysis of mature IL- 1β secreted by human dendritic cells ($1x10^6$ cells/ml) upon infection with *S. typhimurium* and the mutant strains and quantified by ELISA on supernatants. The levels of IL- 1β secretion upon wild type STM infection are set to 100. * $P < 0.01$, ** $P < 0.05$; error bars represent Standard Error. *A*, infection at an MOI of 1; *B,* infection at an MOI of 10;
Compared to infection with wild type, infection with both mutant strains SPI1-T3SS (*invA*), and SPI2-T3SS (*spiB*) resulted in the same amount of IL-1β secretion. Out of 6 biological replicates 2 secreted less IL-1β when infected with a mutant defective in flagellin (*fliC fljB*). Infection with a mutant strain defective in lipid- A acylation (*msbB*) resulted in less IL-1β secretion in all 6 replicates. The trend of infection at an MOI of 1 and an MOI of 10 remains the same.

In contrast to what we observed in mouse DCs, human DCs seem to produce high levels of IL- 1β during *S. typhimurium* infection. While mouse DCs secrete lower levels of IL-1β compared to mouse macrophages, both human monocytes and DCs secreted similar levels of IL-1β.

Infection of human DCs with a mutant strain defective in lipid- A acylation (*msbB*) resulted in lower levels of IL - 1β secretion compared to *S. typhimurium* wild type infection. Because LPS triggers TLR4 signaling, these results suggest that signaling

through this receptor is a major mechanism for the production of the pro-inflammatory cytokine IL-1β also in human DCs. Infection of hDCs with a flagellin defective strain is comparable to the same infection in monocytes. Two out of six biological replicates secreted significantly lower amounts of IL-1β. For the two individuals, signaling triggered by flagellin (TLR5?Ipaf?) seems to be important for inducing high levels of IL-1β secretion. Why two out of six individuals respond differently to infection with a flagellin defective strain but similarly to infection with a lipid- A acylation defective strain and wild type strain has to be further investigated.

3.1.2.4. Induction of IL- 23 in human DCs

Infection of human monocytes with *Salmonella typhimurium* results in secretion of low amounts of IL- 23. To investigate if human DCs are a major source for this Th-17 promoting cytokine, these cells were infected with *Salmonella typhimurium* and their supernantant was collected to determine IL- 23 secretion. To determine if IL-23 secretion is triggered mainly by TLR signaling, human dendritic cells were infected with mutant strains defective in some TLR antagonists as described.

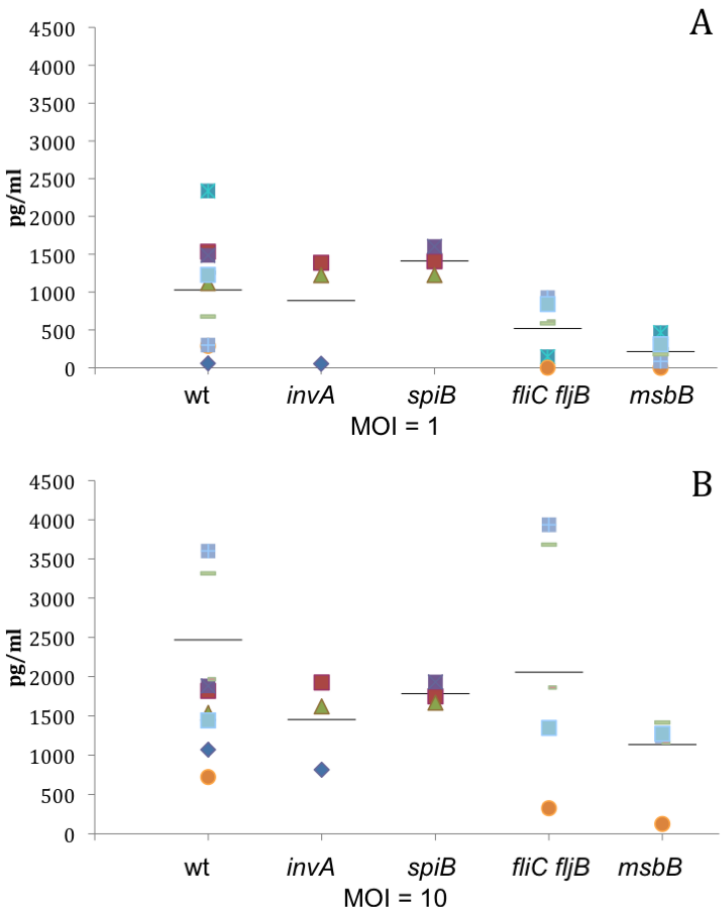


Figure 3-13 ELISA analysis of secreted IL- 23 in the supernatants of human DCs upon infection with *S. typhimurium* and the mutant strains. *A*, hDCs were infected with *S. typhimurium* at an MOI of 1 which lead to IL- 23 levels of up to 2400pg/ml. *B*, hDCs infected with wild type at an MOI of 10 secreted between 700 and 3700pg/ml of IL-23.

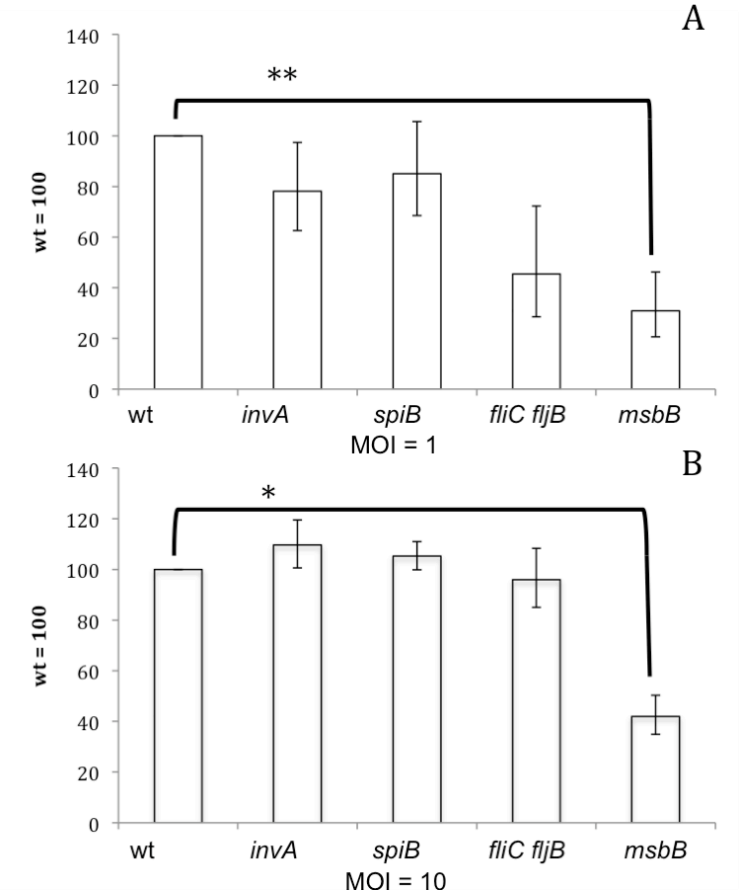


Figure 3-14 Paired analysis of IL- 23 in supernatants of hDCs upon wild type STM infection. The levels of IL-23 secretion upon infection with wild type are set to 100. The average of at least 3 biological replicates is shown. * $P < 0.01$, ** $P < 0.05$; error bars represent the Standard Error; *A*, infection at an MOI of 1 showed a slight but not significant decrease in IL- 23 secretion when infected with a mutant strain defective in flagellin (*fliC fljB*). Significantly less IL- 23 was produced when infected with a mutant strain defective in the acylation of lipid- A (*msbB*). *B*, hDC infected at an MOI of 10; compared to wild type infection the same amount of IL-23 was detected when hDCs were infected with mutant strains defective in SPI-1 T3SS (*invA*), SPI-2 T3SS (*spiB*) or flagellin (*fliC fljB*). hDCs secreted significantly lower amounts of IL- 23 when infected with a mutant strain defective in lipid- A acylation (*msbB*) compared to infection with wild type.

Our results show that human dendritic cells secrete high amounts of IL- 23 upon infection with *Salmonella typhimurium* suggesting that DCs but not monocytes are the major source for the Th17-promoting cytokine IL- 23. Infection of hDCs with a lipid- A acylation defective strain (*msbB* mutant) resulted in lower levels of IL- 23 compared to wild type infection. This indicates that TLR4 signaling has an important role in stimulating the production of this cytokine. However, at an MOI of 1, human DCs secreted slightly less IL- 23 when the assembly of flagella by *Salmonella*

typhimurium is impaired (*fliC fljB mutant*), suggesting a minor influence of TLR5 signaling in IL- 23 production. Furthermore, our results indicate that IL- 23 production is independent from both the SPI-1 and the SPI-2 T3SS.

3.1.3. Influence of TLR blocking on pro- inflammatory cytokine secretion

The approach of using mutant strains that do not activate certain signaling pathways leading to pro- inflammatory cytokine production resulted in a model that suggests a role for TLR4 and possibly TLR5 signaling in IL- 1β and IL- 23 production by human cells. To test this model with a different approach, we used antibodies to block these toll like receptors before human dendritic cells were infected with *Salmonella typhimurium* at an MOI of 1.

3.1.3.1. IL- 1β secretion by hDCs infected with *S. typhimurium* after treatment with antibodies against TLR4 or TLR5

To investigate if less IL- 1β is secreted when either TLR4 or TLR5 signaling is inhibited by antibodies, human dendritic cells were incubated with antibodies against these receptors one hour prior to infection with *S. typhimurium*.

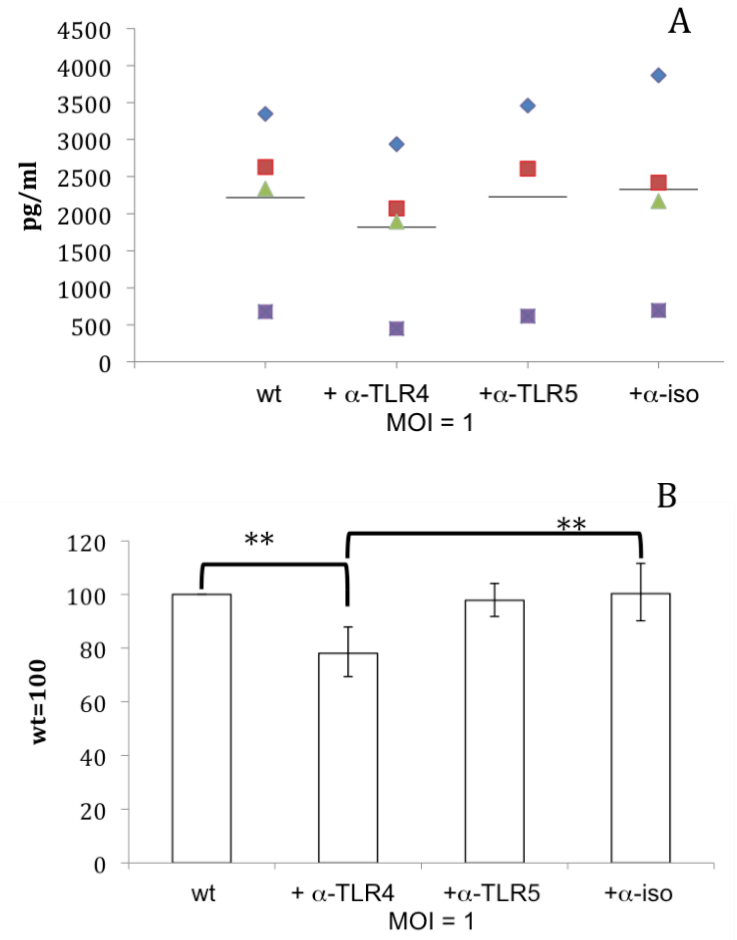


Figure 3-15 IL-1β secretion by hDCs treated with antibodies against TLR4, TLR5 or an isotype antibody for one hour before infection with wild type *S. typhimurium* at an MOI of 1. ** $P < 0.05$; error bars represent Standard deviation; *A,* pg/ml levels of IL- 1β range from 500 to 4000pg/ml. *B,* The paired analysis shows that IL- 1β secretion is significantly lower (about 20%) when TLR4 is blocked compared to wild type infection and compared to infection after incubation with an isotype antibody. The average of 3 biological replicates is shown.

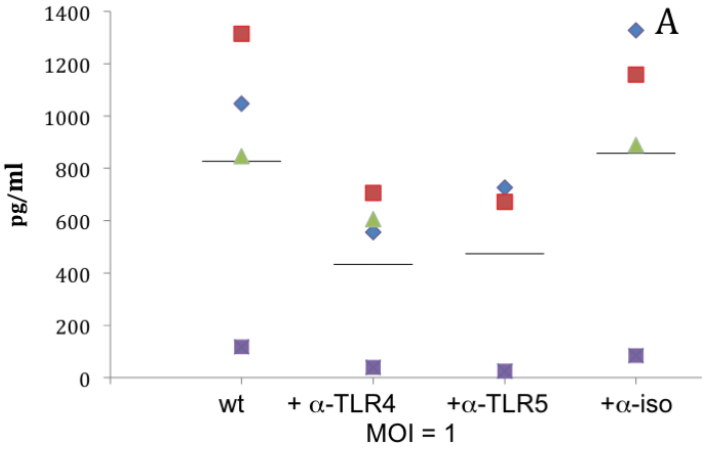
Infection of human dendritic cells with a *S. typhimurium* mutant strain defective in lipid- A acylation (*msbB* mutant) resulted in less IL- 1β secretion, as shown in Figure 3-12. The importance of LPS signaling through TLR4 in inducing IL- 1β production was confirmed when hDCs were infected after TLR4 was blocked with an anti- TLR4 antibody. Antibody treatment for one hour was sufficient to reduce IL- 1β secretion upon *S. typhimurium* infection. However, secretion of this pro-inflammatory cytokine was only reduced by 20%, indicating that besides TLR4 signaling IL-1β production may be triggered by other mechanisms. In contrast, blocking TLR5 signaling, which is triggered by flagellin did not result in reduced IL-

1β secretion. Nevertheless, a role of TLR5 in inducing IL-1β secretion cannot be excluded since infection with a mutant lacking flagellin (*fliC fljB* mutant) showed a significant decrease in IL-1β secretion by human DCs in two out of six biological replicates (Figure 3-12).

Taken together, our results indicate that LPS and TLR4 signaling contributes to the production of IL-1β in human dendritic cells. In contrast flagellin and the SPI-1 or the SPI-2 T3SS did not consistently contribute to IL-1β secretion. Moreover, our results suggest that the secretion of this very important pro- inflammatory cytokine is likely triggered by multiple redundant mechanisms.

3.1.3.2. IL- 23 secretion by hDCs infected with S. typhimurium after treatment with antibodies against TLR4 or TLR5

Our results in Figure 3-14 suggested that TLR4 and TLR5 activation may be important for IL- 23 secretion. To test this hypothesis further, TLR4 and TLR5 were blocked with antibodies one hour before infection with wild-type *S. typhimurium*. After infection and gentamicin treatement the Th 17 promoting cytokine IL- 23 was analyzed in the supernatant collected 24 hours later.



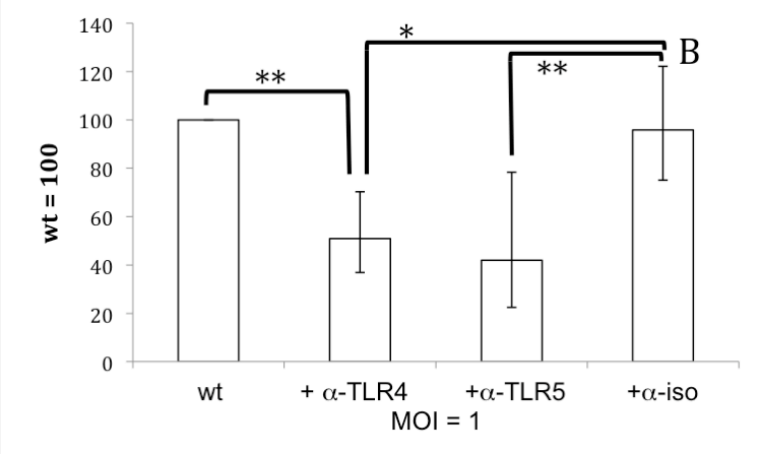


Figure 3-16 hDCs were treated with antibodies against TLR4, TLR5 or an isotype antibody for one hour before infection with wild type *S. typhimurium* at an MOI of 1. IL-23 levels in the supernatants were analysed by ELISA. *P<0.01, ** P < 0.05; error bars represent Standard deviation; *A,* pg/ml levels of IL- 1β range from 100 to 1300pg/ml when hDCs were infected with wild type *S. typhimurium*. *B,* IL-23 secretion by hDCs was significantly lower when cells were treated with antibodies against TLR4 or TLR5 before infection with *S. typhimurium* as shown in the paired analysis.

Our results shown in Figure 3-16 indicate that signaling through both TLR4 and TLR5 is important for induction of IL- 23 secretion. Blocking TLR5 prior to infection with *S. typhimurium* wild type reduced IL- 23 secretion in the supernatant to approximately 42% compared to infection without antibody treatment. After blocking TLR4, human dendritic cells infected with *Salmonella typhimurium* produce about half the amount of IL- 23 when compared to infection without antibody treatment. These results are consistent with the reduction of IL- 23 secretion that we observed when human dendritic cells were infected with an *msbB* mutant (which does not induce TLR4 signaling) and a *fliC fljB* mutant (which does not induce TLR5 signaling) as shown in Figure 3-14.

Taken together, our results suggest that IL- 23 secretion is severly reduced when TLR4 and TLR5 signaling is prevented. Thus, TLR signaling contributes to the secretion of the Th17 stimulating cytokine IL- 23 during *S. typhimurium* infection.

3.1.4. Determine the role of MyD88 signaling on cytokine secretion by hDCs

The adaptor protein MyD88 is involved in signaling pathways activated by most TLRs. Therefore, inhibition of this adaptor protein should reduce the expression of

most pro- inflammatory cytokines. Our previous data in Figure 3-12 and Figure 3-14 suggested that expression of IL-1β and IL-23 in human dendritic cells infected with *S. typhimurium* is at least partly dependent on TLR4 and TLR5. To investigate the contribution of MyD88 to the cytokine expression upon infection with *Salmonella typhimurium*, this adaptor protein was blocked using an inhibitory peptide for 24h before infection.

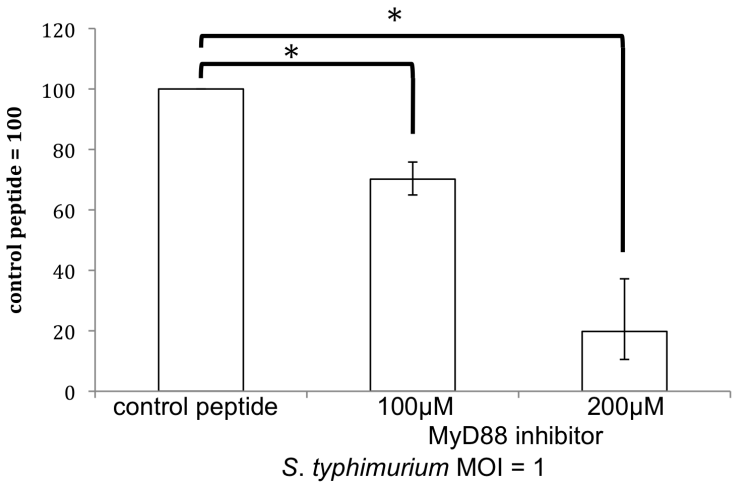


Figure 3-17 hDCs were incubated with a MyD88 inhibitory protein and a negative control peptide at a concentration of 100μM or 200μM for 24h before they were infected with *S. typhimurium* at an MOI of 1. Supernatants were analysed for secreted IL- 1β using ELISA. The average of at least 3 biological replicates is shown. Error bars represent Standard deviation; * $P < 0.01$; treatement with 100μM MyD88 inhibitor before infection resulted in less than 70% secreted IL-1β compared to the control peptide. Increasing the concentration of the inhibitor and the control peptide to 200μM before infection resulted in less than 25% secreted IL-1β.

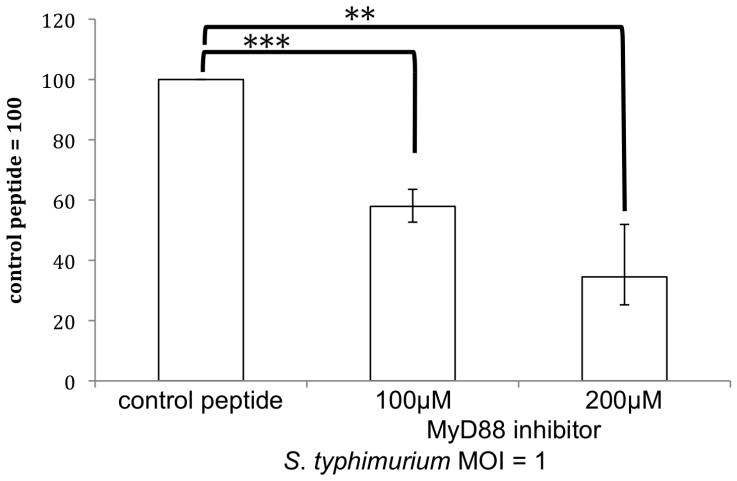


Figure 3-18 hDCs were incubated with a MyD88 inhibitory protein and a negative control peptide at a concentration of 100μM or 200μM for 24h before they were infected with *S. typhimurium* at an MOI of 1. Supernatants were analysed for secreted IL- 23 using ELISA. The average of at least 3

biological replicates is shown. Error bars represent Standard deviation; *** P < 0.07, ** P < 0.05; treatement with 100µM MyD88 inhibitor before infection resulted in 67% secreted IL-23 compared to the control peptide. Increasing the concentration of the inhibitor and the control peptide to 200µM before infection resulted in less than 35% secreted IL-23.

Blocking MyD88 with an inhibitory peptide at a concentration of 100µM 24h before infection with *S. typhimurium* at an MOI of 1 resulted in a decrease of more than 30% of IL- 1β and IL- 23 when compared to treatment with a control peptide. Moreover, when the concentration of the inhibitory and the control peptide are increased to 200µM, a further decrease of the secretion of both cytokines was detected. When the activation of MyD88 was blocked by the inhibitory peptide at a concentration of 200µM human dendritic cells infected with *S. typhimurium* secreted about 75% less IL- 1β and 65% less IL- 23 when compared to dendnrtic cells pre-treated with a control peptide. These results indicate that both IL- 1β and IL- 23 secretion depends on signaling through the adaptor protein MyD88.

3.1.5. Determine Caspase- 1 activity in hDCs upon *S. typhimurium* infection

Based on studies in the mouse, it is suggested that pro- IL- 1β needs to be cleaved in the mature form by Caspase- 1. Active Caspase- 1 also triggers the pro-inflammatory cell death called pyroptosis, with release of the mature form of IL- 1β into the lumen. To investigate if Caspase- 1 is activated in human dendritic cells upon infection with *Salmonella typhimurium,* active Caspase- 1 was stained with a fluorescence peptide and analyzed by FACS.

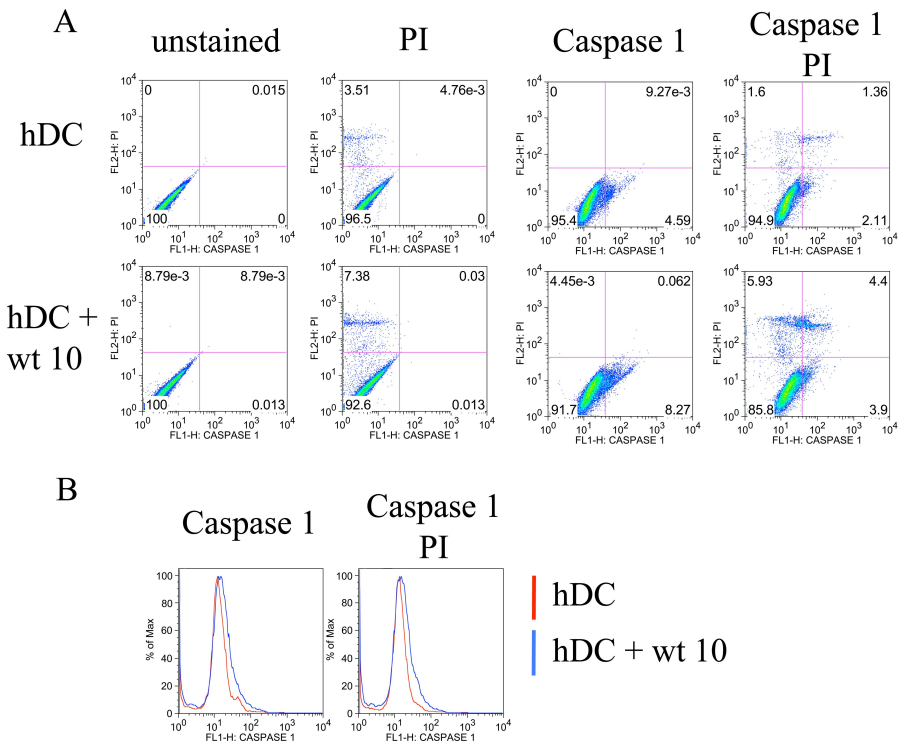


Figure 3-19 Caspase-1 activity assay. Caspase- 1 was induced by infecting human dendritic cells with wild type (wt) *Salmonella typhimurium* at an MOI of 10. 4h post infection the cells were divided in 4 tubes. One tube was left unstained, one was treated with PI (cell death), one was treated with FLICA (Caspase-1) and one was stained with both. After staining cells were analysed by FACS immediately. *A*, infected cells stained with PI showed a 2-fold increase in dead cells; active Caspase-1 showed a 2- fold increase when infected hDCs were stained with FLICA only; when hDCs were stained with both PI and Caspase- 1 there was an increase in both dead cells and cells with activated Caspase- 1. *B*, histogram showing an increase in Caspase- 1 activation when hDCs were infected with *Salmonella typhimurium*.

When human dendritic cells were infected with *S. typhimurium* and stained with propidium iodide (PI), a 2-fold increase in cell death was observed.When the same cell cultures were stained with FLICA, which covalently binds to the active form of caspase- 1, we also observed a 2-fold increase. Taken together, these results suggest that most human dendritic cells that die during *S. typhimurium* infection undergo caspase-1-mediated pyroptosis, thereby activating a pro- inflammatory response. This was also evident when cells were stained with both PI and FLICA to determine both, cell death and caspase-1 activation in the same population. The amount of dead cells positive for active Caspase- 1 increased from 1,36% to 4,4% upon infection with *S. typhimurium*. In addition the amount of live cells with active Caspase- 1 increases upon infection. These data indicate, that *S. typhimurium* infection of

human dendritic cells results in Caspase- 1 activation. This event likely triggers cleavage and release of mature IL-1β and also leads to pyroptosis.

3.1.6. Determine the contribution of ASC- dependent Caspase- 1 activation for IL-1β secretion during *S. typhimurium* infection of human DCs

In Figure 3-19 we have shown an increase in caspase-1 activity upon *S. typhimurium* infection of human dendritic cells. Furthermore, studies in mouse bone marrow derived macrophages indicate that caspase- 1 is required for the maturation and secretion of IL- 1β and IL- 18. In contrast, IL- 23 and IL- 12 expression is independent from caspase-1 activation.

To investigate the contribution of ASC-dependent caspase- 1 induction on secretion of mature IL- 1β and IL- 23, ASC activation was blocked by increasing the concentration of extracellular potassium before infection with *S. typhimurium*.

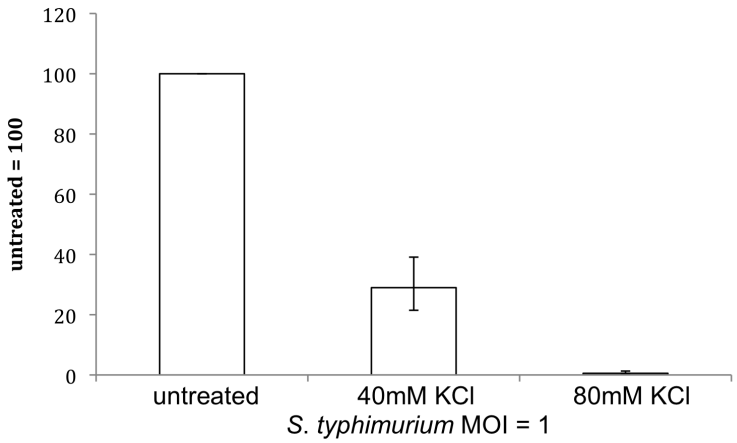


Figure 3-20 Paired analysis of mature IL-1β produced by hDCs cultured in 40mM or 80mM KCl containing medium upon infection with wild type *S. typhimurium* at an MOI of 1. The average of 2 biological replicates is shown. Error bars represent Standard Deviation. The IL-1β secretion of untreated infected hDCs was set to 100.

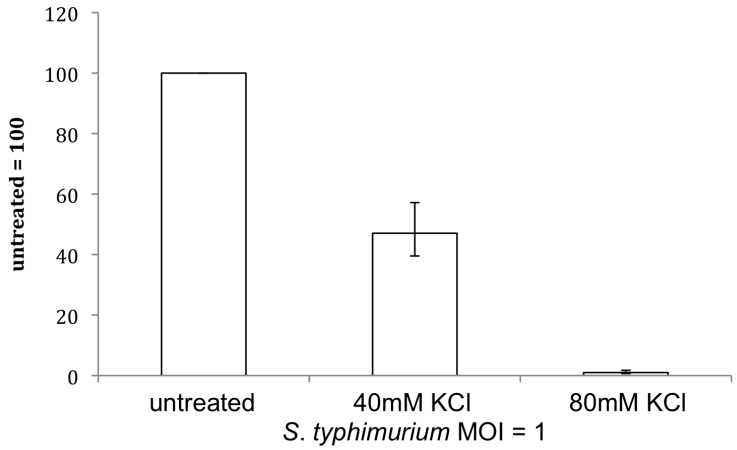


Figure 3-21 Paired analysis of IL-23 produced by hDCs cultured in 40mM or 80mM KCl containing medium upon infection with wild type *S. typhimurium* at an MOI of 1. The average of 2 biological replicates is shown. Error bars represent Standard Deviation. The IL-1β secretion of untreated infected hDCs was set to 100.

Recent studies have shown that inhibiting ASC results in lower levels of secreted IL-1β through inhibition of active Caspase- 1. We were also able to show a reduction in IL-1β secretion in cells treated with both 40mM and 80mM KCl, as shown in Figure 3-20. However, we also found that IL-23 secretion was decreased (Figure 3-21), even though IL- 23 secretion is not affected by ASC and caspase-1 activation. Most likely the reduction in both IL-1β and IL-23 secretion is a consequence of KCl cytotoxicity. Experiments with lower concentrations of KCl will be performed to address the role of ASC in pro- inflammatory signaling cascades during *S. typhimurium* infection of human DCs.

3.2. Determine the mechanism by which T cells secrete IL-17 during *S. typhimurium* infection

As we have shown thus far, human dendritic cells secrete pro- inflammatory cytokines upon *S. typhimurium* infection. These cytokines activate T cells, thereby amplifying the signal of the immune response. This relay of the signal is important for the activation of host defense mechanisms like the production of antimicrobial peptides and the recruitment of neutrophils, which ultimately lead to pathogen clearance. The T cell cytokines IL- 17 and IL- 22 are key factors in orchestrating the host response to pathogens.

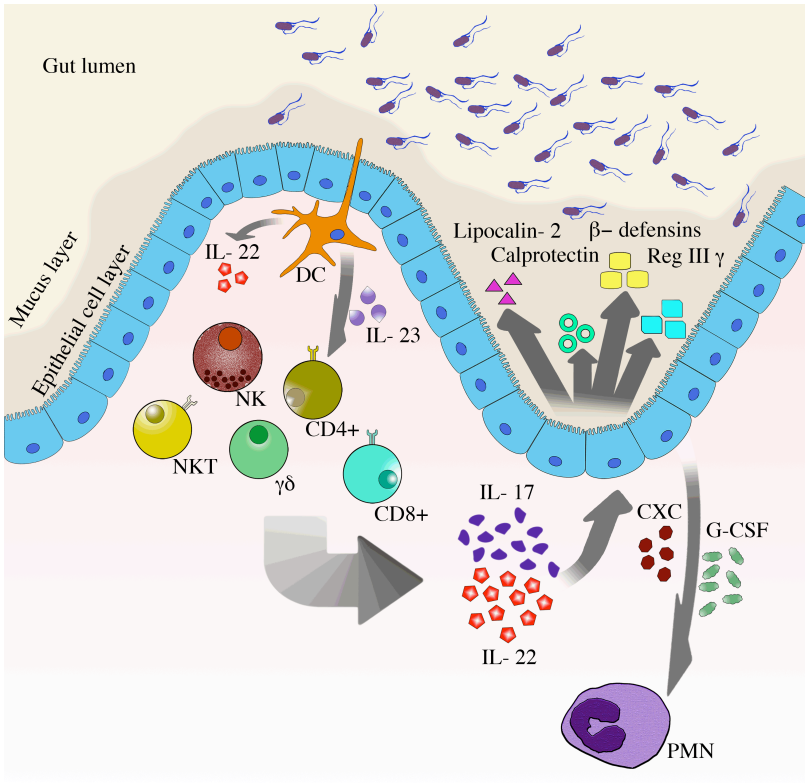


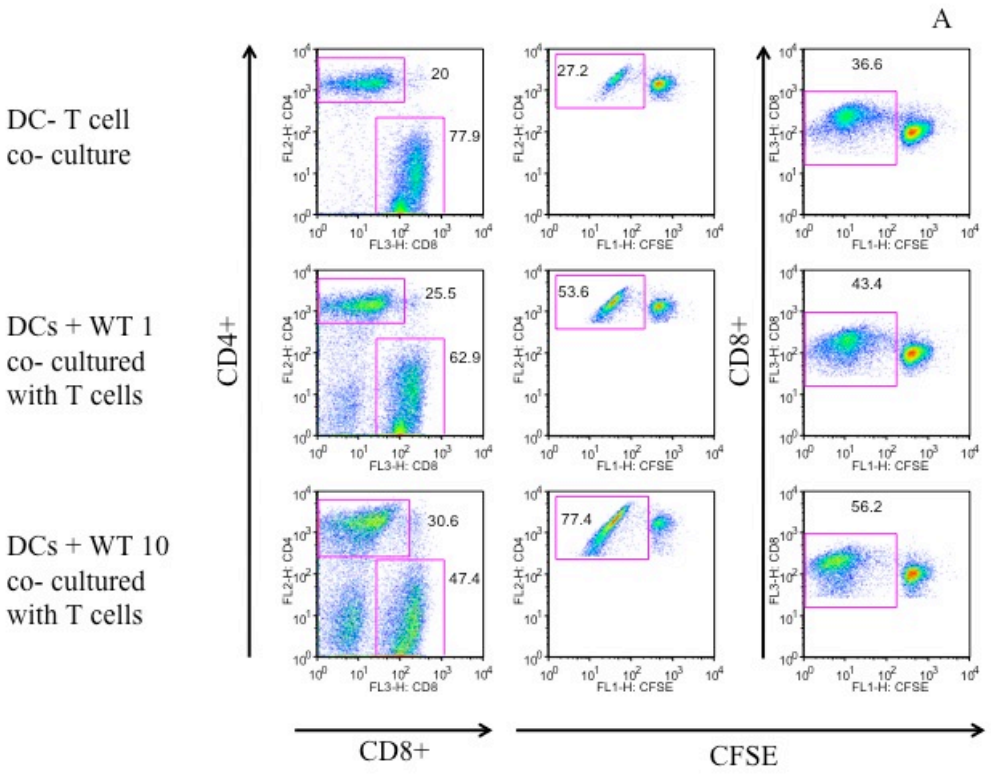
Figure 3-22, T cell cytokines and the gut mucosal barrier. Dendritic cells activated by pathogens secrete several cytokines, which further stimulate subsets of T cells to secrete IL-17 and IL-22. T cells promote amplification of the host response by stimulating the intestinal epithelium to secrete CXC chemokines (neutrophil chemoattractants) and antimicrobial peptides (Figure and Legend adapted from Blaschitz and Raffatellu, 2010, J. Clin Immunol).

We thus wanted to investigate if dendritic cells infected with *S. typhimurium* can trigger IL- 17 and IL- 22 expression by T cells. To investigate this, we co cultured *S. typhimurium* infected and uninfected hDCs with T cells and we analyzed the

expression of IL-17 and IL-22 and the proliferation of T cells. We also investigated if naïve and memory T cells secrete IL-17 upon stimulation with the supernatant of *S. typhimurium* infected and uninfected hDCs.

3.2.1. Determine T cell proliferation upon co- culture with dendritic cells infected with *S. typhimurium*

During infection antigen presenting cells interact with T cells. Besides stimulating mature T cells, APCs are also important to prime T cells to differentiate to either T helper cells or cytotoxic T cells. While both types of mature T cells are important for overcoming the infection, T helper cells are crucial for the response to bacterial pathogens. Here we tested whether co culture with human dendritic cells infected with *S. typhimurium* stimulates T cell proliferation in vitro. For the proliferation assay, T cells were stained with CFSE before co culture. To distinguish between helper and cytotoxic T cells, the surface proteins CD4 and CD8 were stained using fluorescently labeled antibodies.



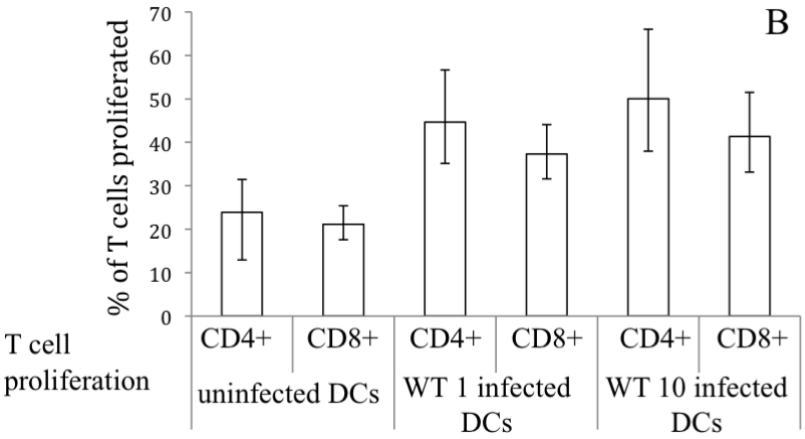


Figure 3-23 T cell proliferation. hDCs were infected with wild type *S. typhimurium* at an MOI of 1 or 10, treated with Gentamicin and cultured for 24h. Then, DCs were co- cultured with CFSE stained T cells. 5 days after co- culture T cell proliferation was determined by FACS. *A*, a plot of total T cells is shown in the first column. The gated cells on top represent the T helper cell population while the gate at the bottom of the charts include the cytotoxic T cell population. In the charts of the second and third column CFSE was plotted against CD4 and CD8 respectively showing the proliferated T cells. Gated were proliferated T cells, which contained less CFSE. A significant increase in proliferated cytotoxic and helper T cells were detected when DCs were infected with *S. typhimurium* before co-culture compared to non- infected. *B*, the average of 5 biological replicates is shown. Both, the CD4+ and CD8+ T cell population increases ($P < 0.07$ and $P < 0.05$ respectively) when T cells and infected DCs were co- cultured compared to co- culture with uninfected DCs. Error bars represent Standard Error.

DCs and T cells were obtained from two different donors and therefore also uninfected DCs stimulate T cells to proliferate because of an allograft reaction, when compared to T cells alone. However, when T cells were co- cultured with *Salmonella typhimurium* infected DCs, the proliferation of T cells significantly increases compared to uninfected DCs, with both CD4+ and CD8+ population increasingly significant. Furthermore, as shown in Figure 3-23, DCs infected with *S. typhimurium* slightly shift the proliferation of immature T cells to differentiate into T helper cells.

3.2.2. Determine induction of T cell cytokines upon co- culture with dendritic cells infected with *S. typhimurium*

T helper cells are important in host defense against bacterial pathogens, including *S. typhimurium*. A novel subset of Th cells, termed Th17, are major source of the cytokines IL- 17 and IL- 22, which play a major role in the innate immune defense against pathogens including *S. typhimurium*. Furthermore, the Th1 cytokine IFN-γ

was analyzed because of its importance in bacterial defense but also as a control since it is well known to be upregulated upon *S. typhimurium* infection. To investigate if dendritic cells can induce secretion of these cytokines by T cells, human DCs were infected with *S. typhimurium* and co- cultured with total T cells.

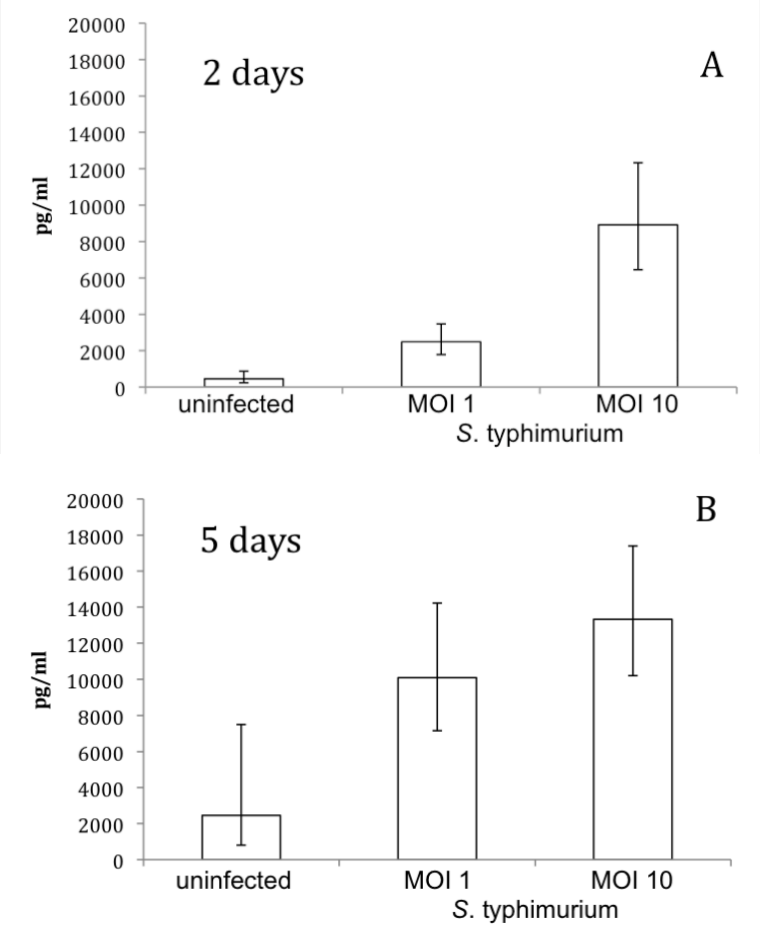


Figure 3-24 IFN-γ induction by *S. typhimurium* infected hDCs co- cultured with T cells detected by ELISA of supernatants. DCs were either uninfected or infected with *Salmonella typhimurium* MOI 1 or 10, subject to gentamicin treatment and 24h post infection co cultured with T cells. The average of 4 biological replicates is shown.Error bars represent Standard Error. *A*, two days after start of co-culture supernatants were analysed by ELISA. IFN- γ secretion increases from 2500pg/ml to 8900pg/ml with an increase of the MOI from 1 to 10. *B*, five days of co- culture increases the IFN-γ levels further to 13300 pg/ml at an MOI of 10. After five days the difference in IFN-γ secretion between hDCs infected at an MOI of 1 and 10 co- cultured with T cells is negligible.

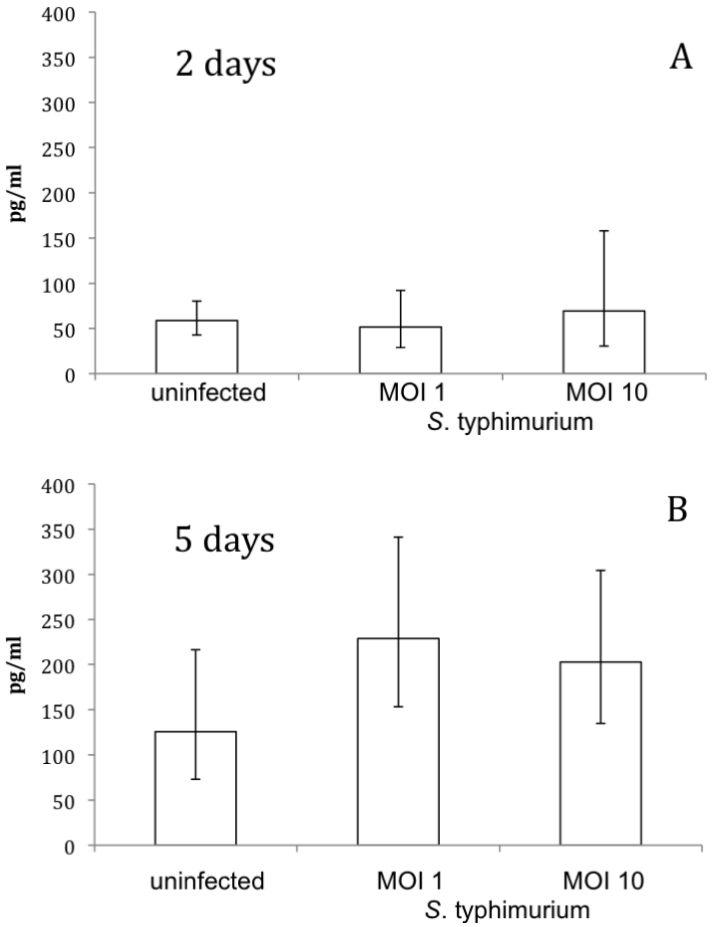
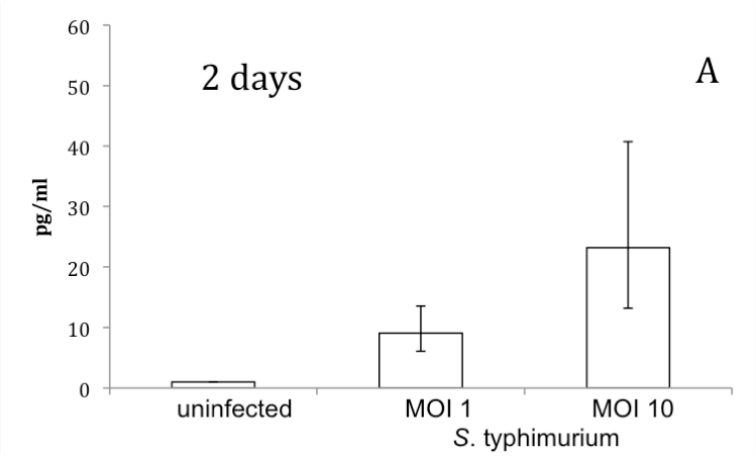


Figure 3-25, IL- 22 induction by *S. typhimurium* infected hDCs co- cultured with T cells detected by ELISA of supernatants. DCs were either uninfected or infected with *Salmonella typhimurium* MOI 1 or 10, subject to gentamicin treatment and 24h post infection co cultured with T cells. The average of 4 biological replicates is shown.Error bars represent Standard Error. *A*, two days after start of co-culture supernatants were analysed by ELISA. Compared to uninfected hDC co cultured with T cells the infected hDC T cell co culture does not secrete any IL- 22. *B*, after five days of co- culture more IL- 22 was detected in T cell co culture with infected hDCs but compared to the uninfected hDC T cell co culture there is no significant difference.



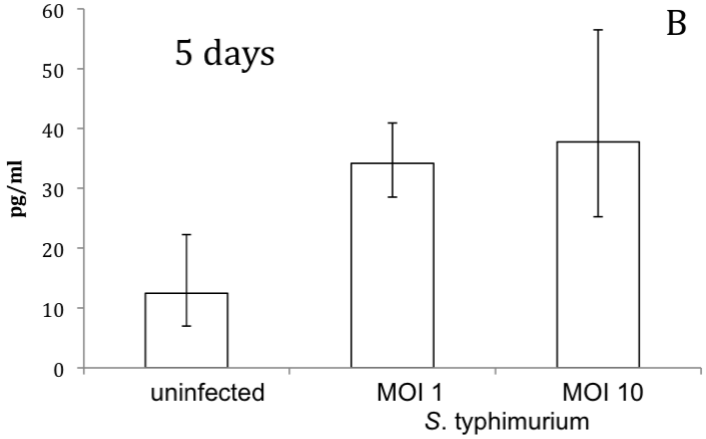


Figure 3-26 IL-17 induction by *S. typhimurium* infected hDCs co- cultured with T cells detected by ELISA of supernatants. DCs were either uninfected or infected with *Salmonella typhimurium* MOI 1 or 10, subjected to gentamicin treatment and 24h post infection co cultured with T cells. The average of 4 biological replicates is shown. Error bars represent Standard Error. *A*, already two days after start of co- culture IL-17 secretion is induced in T cells by hDCs infected with *S. typhimurium B*, after five days the difference in IL-17 secretion between hDCs infected at an MOI of 1 and 10 co- cultured with T cells is negligible. However, levels of IL- 17 are low.

When human dendritic cells were infected with *Salmonella typhimurium* prior to co-culture with a mix of total T cells, high amounts of IFN- γ were detected in the supernatants. The secretion of this cytokine started within the first two days of co-culture and was higher at an MOI of 10. In contrast to IFN- γ, IL- 22 was detected only after five days of co- culture. Additionally there was no difference between the multiplicity of infection and a high variation between the individuals. One potential pitfall is that IL- 22 was only discovered in recent years and the antibodies to quantify the cytokine may not have the same quality as the ones for IFN- γ. Co-culturing T cells with infected hDCs resulted in secretion of IL- 17 after only two days. The levels increased with the multiplicity of infection and with days incubation suggesting an expansion of Th17 cells.

3.2.3. Determine if supernatant of hDCs is sufficient to induce IL- 17 secretion by T cells

The results in Figure 3-26 show that infected human dendritic cells can stimulate primary T cells to secrete IL- 17. To further investigate the mechanism by which T cells can be stimulated to secrete this cytokine, the supernatant of infected human dendritic cells was used to stimulate naïve and memory T cells.

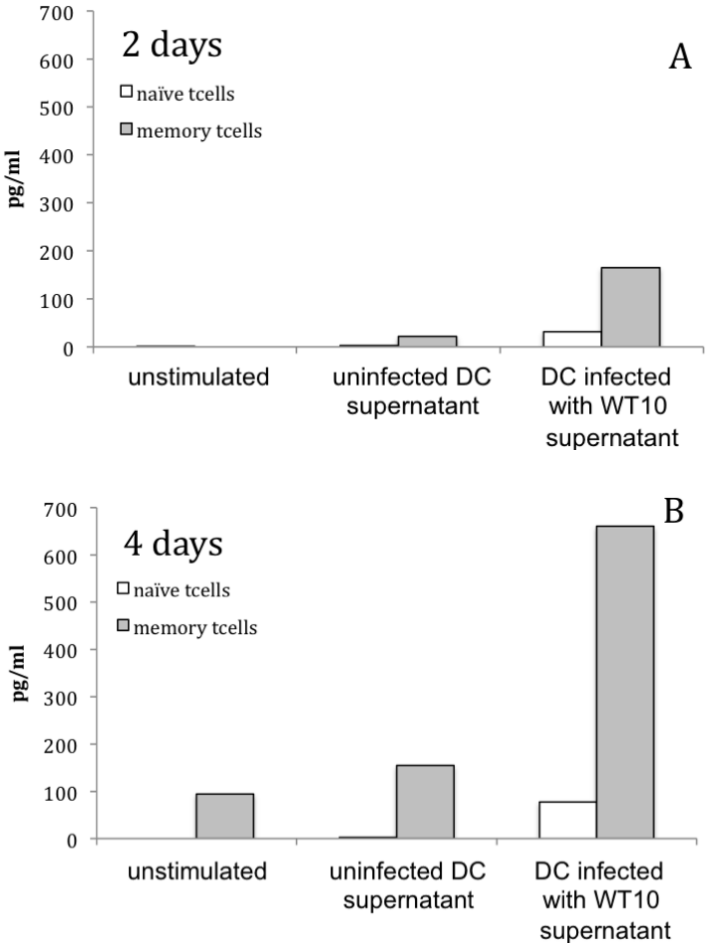


Figure 3-27 IL- 17 secretion upon stimulation with infected hDC supernatants detected by ELISA. Naive and memory T cells were activated by CD3/CD28 beads and stimulated either with supernatant from uninfected human DCs or DCs infected with wild type *S. typhimurium*. *A*, after two days memory T cells stimulated with the supernatant of infected DCs already produced IL- 17. *B,* after four days IL- 17 was detected in all memory T cell cultures. However, compared to unstimulated or stimulated with uninfected DC supernatant, the memory T cell culture stimulated with infected DC supernatant secreted a very high amount of IL- 17. The naive T cells started to secrete small amounts of IL- 17 after four days.

We found that secretion of IL- 17 by activated memory T cells can be induced by only the supernatant of human dendritic cells infected with *Salmonella typhimurium* (i.e. without DC-T cell contact). Secretion of IL- 17 increased about four fold from day 2 to day 4 after stimulation. Naïve T cells secreted lower amounts of IL- 17 upon stimulation. Taken together, our results indicate that memory T cells may respond to stimulation by DCs in an antigen independent fashion.

4. Discussion

Salmonella typhimurium infection triggers an acute inflammation that is characterized by a signal cascade resulting in the production of antimicrobial peptides and neutrophil recruitment, which ultimately leads to clearance of the infection. Upon contact between the pathogen and antigen presenting cells, pro-inflammatory cytokines are produced, which in turn stimulate resident T cells (Godinez et al. 2009). These active T cells relay and thereby amplify the signal by secreting cytokines that stimulate epithelial cells. Lung and gut epithelial cells respond to this composition of cytokines by releasing antimicrobial peptides and CXC chemokines. The antimicrobial peptides are released in the gut lumen to suppress bacterial growth. Neutrophils recruited to the site of infection by the secreted CXC chemokines are thought to kill the bacteria and prevent the infection from spreading to the blood stream. Together, these effects lead to clearance of the infection.

While most studies on *S. typhimurium* pathogenesis are performed in the mouse or by using mouse cells, it is not yet understood how the immune response to *S. typhimurium* is triggered in human cells. This is particularly important because mouse infection with *S. typhimurium* does not fully recapitulate the disease in humans, as explained in details in the introduction. In this study we were able to show that mouse and human immune cells partly respond to different pathogen associated patterns. Furthermore, we began investigating the mechanisms by which *S. typhimurium* induces secretion of the pro- inflammatory cytokines IL- 1β and IL-23. We also show that DCs infected with *S. typhimurium* trigger IL- 17 and IL- 22 secretion by memory T cells isolated from human blood samples.

The interaction of *Salmonella typhimurium* and murine macrophages is the best-established model of interaction between this bacterium and a host cell. The group of Wright et al observed first that C3H/HeJ mice are hyper susceptible to infection with *Salmonella typhimurium*. Later it was established that these mice have a genetic defect in the Toll-like receptor 4 and are therefore unable to trigger an immune respond to the lipid A region of the lipopolysaccharide, the main component of the gram negative bacteria cell wall (Wright et al. 2009). More recent studies discovered that LBP binds to LPS and CD14. This complex stimulates the homodimerization of TLR4, which triggers the induction of pro- inflammatory cytokine production (pro-

IL- 1β, pro- IL- 18, TNF, IL- 6, IL- 23, IL- 12). When we infected mouse antigen presenting cells, we were also able to show that lower amounts of pro- inflammatory cytokines are secreted when TLR4 signaling was abrogated. This was achieved by infection of murine macrophages with a *S. typhimurium* lipid- A acylation mutant strain, which resulted in 50% reduction of IL-1β secretion. This also shows that LPS is likely not the only pattern that triggers the secretion of this important cytokine.

Since higher organisms are exposed to many different foreign substances and microorganisms, the immune response has to be highly regulated. It has to tolerate the resident microbiota but respond to pathogenic bacteria to clear the infection. Recent research has shown that host cells detect bacterial flagellin, the monomer of the motility protein complex flagella, to determine the danger signal and modulate the immune response. Thus far, two receptors that recognize flagellin have been identified in host cells. One is the cell surface receptor TLR5, which triggers MyD88 signaling that leads to NFκB regulated expression of pro- inflammatory cytokines. Studies in mice have shown that host cells sense intracellular flagellin with the NLR Ipaf, which activates caspase- 1. Both TLR signaling and NLR signaling pathways need to be activated to secrete the mature form of the pro- inflammatory cytokines IL- 1β and IL- 18 that stimulate an immediate immune response. This response helps the host discriminate between pathogens and commensals. During *S. typhimurium* infection, flagellin is translocated by T3SS, encoded by the *Salmonella* Pathogenicity Island 1 into the host cytosol. (Miao et al. 2007)

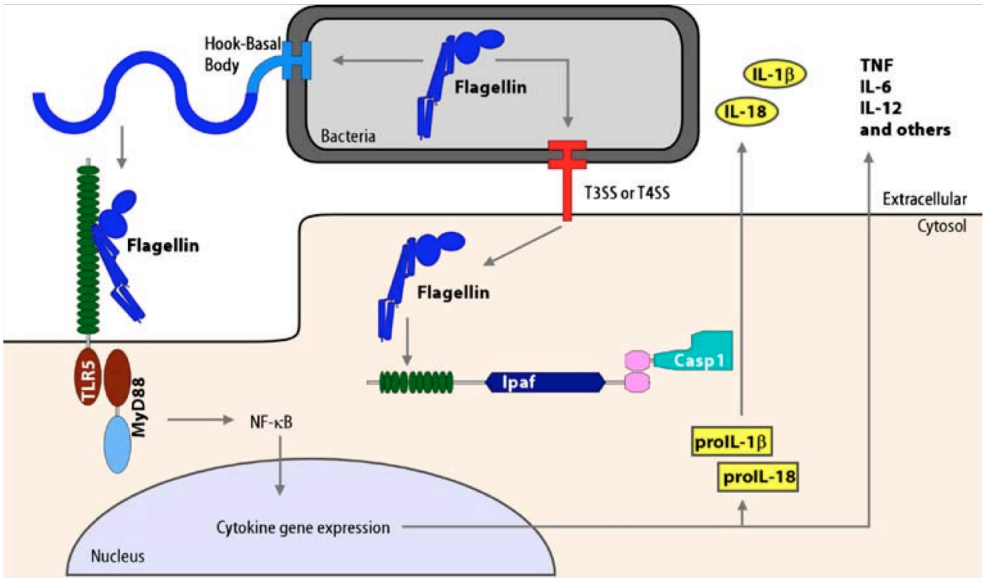


Figure 4-1, TLR5 and IPAF respond to extracellular and cytosolic flagellin; TLR5 signaling leads

activation of transcription factors through MyD88 resulting in pro- inflammatory cytokine expression; IL-1β and IL- 18 require dual stimuli, expression by TLR5 and other TLRs and activation through Caspase- 1 triggered by IPAF or other NLRs; (Figure and Legend adapted from Miao et al. 2007)

To test if the model presented in **Figure 4-1** holds true with human APCs, we infected human APCs with a mutant strain lacking the two *S. typhimurium* flagellins *fliC* and *fljB*, as well as mutant strains defective in either the SPI-1 or the SPI-2 T3SS. Before we tested the model in human APCs we tried to reproduce it with our experimental set up in mouse APCs. In agreement with the published results, we observed a significant decrease of IL- 1β secretion by mouse macrophages infected with either a mutant lacking either flagellin or the SPI-1 T3SS, responsible for the translocation of flagellin, compared to infection with a wild type strain of *Salmonella typhimurium*. In agreement with other groups, we also show that mouse macrophages are the major source of IL- 1β. Zenk et al have recently shown that the response to *Salmonella typhimurium* differs in macrophages and dendritic cells, even if these APCs are closely related (Zenk et al. 2009). To test if there are differences in the production of pro- inflammatory cytokines between these two cell types, we repeated the mutant infection experiments with mouse dendritic cells. The results of these infection experiments show the same major contribution of LPS and therefore TLR4 signaling to IL- 1β production as in macrophages. Flagellin, which triggers TLR5 and Ipaf signaling, is also a main contributor to IL- 1β production in both cell types, as shown in this study by infection with a mutant strain defective in flagella assembly. However, in contrast to macrophages our results suggest that IL- 1β secretion in mouse dendritic cells depends on the SPI- 2 T3SS rather than on the SPI-1 T3SS. As described, in macrophages intracellular *S. typhimurium* injects flagellin through the SPI-1 T3SS into the cytosol, which triggers caspase- 1 activation that leads to pro- IL- 1β cleavage and secretion. Based on this model, our results suggest that *S. typhimurium* may translocate flagellin in mouse dendritic cells through the SPI- 2 T3SS.

While there have been extensive studies on *Salmonella typhimurium* infection in the mouse model, experimental data on infection of human cells is still limited. Even though the response to *S. typhimurium* infection in mice and human is similar in some ways, it was not known whether the molecular mechanisms are the same. Our

results indicate that the mechanisms of induction of pro-inflammatory cytokines in human and mouse antigen presenting cells infected with *S. typhimurium* may be different. For instance, intracellular flagellin does not appear to trigger maturation and secretion of IL-1β in human cells, since neither mutant strain lacking flagellin or the SPI-1 T3SS showed any reduction in IL-1β secretion. However, we observed differences between individuals. While in one out of three patients infection with the *fliC fljB* mutant almost eliminated IL-1β production, the IL-1β secretion in another patient was reduced to about 40% when infected with the SPI-2 T3SS mutant.

To investigate the role of human dendritic cells in the host response to *S. typhimurium*, we infected human dendritic cells with *S. typhimurium* and the same mutant strains. In contrast to the mouse model, human DCs secreted high levels of IL-1β, almost to the levels of human monocytes. Furthermore, human DCs responded to the same PAMPs as human monocytes. Two out of six patients showed a significant decrease of IL-1β when infected with the non-flagellated mutant, while all patients secreted less IL-1β when infected with a *S. typhimurium* mutant defective in lipid- A acylation. Mutation in either the SPI-1 or the SPI-2 T3SSs did not affect IL-1β secretion by human dendritic cells.

For both, human Monocytes and human DCs our results support the model drawn and reviewed by Di Virgilio (Di Virgilio et al. 2007) in which the expression of pro-IL-1β is triggered by multiple PAMPs, and probably also damage associated patterns (DAMPs) that are unknown for human cells.

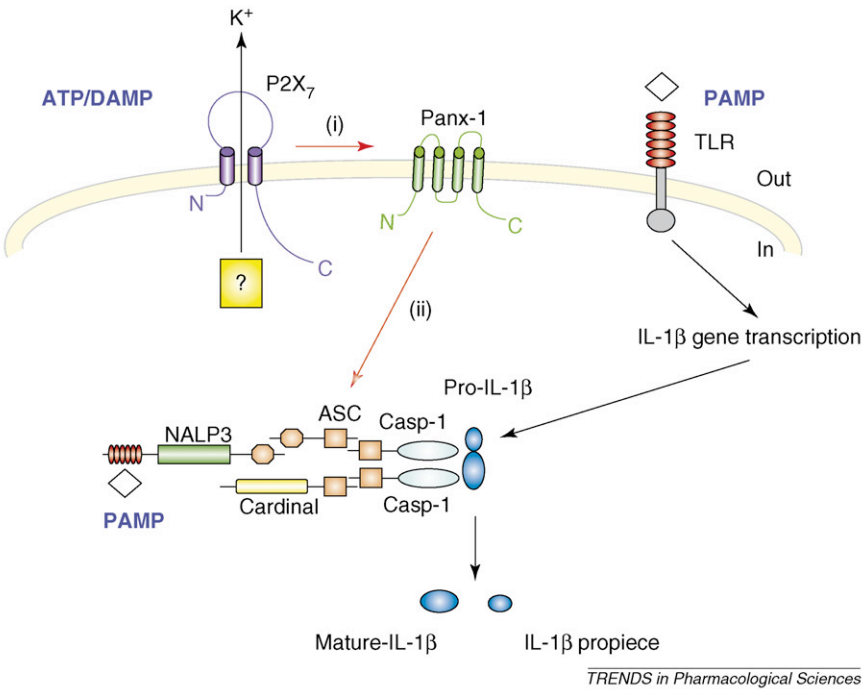


Figure 4-2, Hypothetical sequence of events leading to P2X7 receptor and panx-1-mediated inflammasome activation. PAMPs bind to TLRs and drive IL-1β gene expression and accumulation of the pro-cytokine. Extracellular ATP binds to the P2X7 receptor and triggers K+ efflux and panx-1 activation. The functional significance of K+ efflux is unknown, although it might facilitate or even precipitate inflammasome activation. Likewise, the mechanism of panx-1 activation by the P2X7 receptor is unknown. Panx-1 in turn activates the inflammasome. The activated inflammasome then cleaves pro-IL-1β Thus, stimulation of the inflammasome by extracellular ATP can be split into two steps: (i) recruitment and activation of panx-1 by the P2X7 receptor, and (ii) activation of the inflammasome by panx-1. (Figure and Legend adapted from De Virgilio et al 2007)

Even though multiple different pathogen associated molecular patterns can stimulate antigen presenting cells to produce pro- IL-1β, our experimental set up allowed us to show the contribution of two PAMPs, LPS and flagellin We also tested this by blocking the receptors TLR4 and TLR5 with antibodies. We were able to show that lipid- A is a major inducer of IL- 1β, while flagellin was needed for full induction of IL-1β in one out of three individuals.

Furthermore we were able to show that MyD88 is the major adaptor protein linking the signal from the receptors to the transcription factor NFκB thereby promoting pro- IL-1β expression. This was accomplished by blocking MyD88 during infection with the *S. typhimurium*.

By inhibiting potassium efflux using high extracellular potassium, ASC is inhibited, thereby caspase- 1 is inactive and both pyroptosis and activation of pro- IL-1β are prevented Even though we observed a decrease in IL-1β secretion, also IL- 23 secretion was decreased, which is not dependent on ASC/ Caspase- 1 The lower

amount of cytokine observed in the supernatant most likely resulted from toxicity of the increased KCl concentration. Therefore, these studies need to be further optimized to determine whether ASC needs to be activated for full IL-1β secretion. Even though IL-1β is known for a long time the molecular mechanism of its induction is not completely understood. Further studies will define the mechanisms of induction of IL-1β in human antigen presenting cells.

Another cytokine important for the stimulation of T cells is IL- 23. IL- 23, which has only been discovered in recent years, belongs to the IL- 12 family, and it is formed by the interaction of two subunits, p19 and p40. The p40 subunit is also shared with IL- 12, but while IL- 12 stimulates Th1 cells, IL- 23 appears to be important for Th 17 survival and the induction of IL- 17. Expression of the two subunits of IL- 23 is induced upon stimulation of TLR receptors.

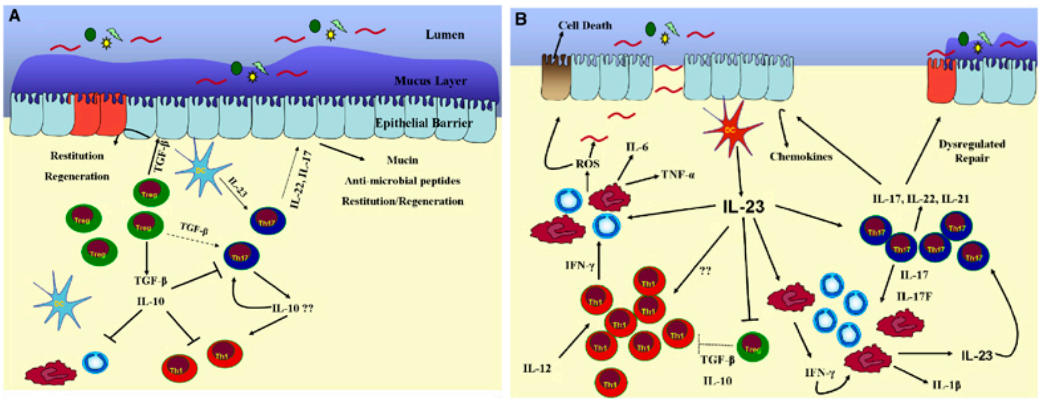


Figure 4-3, The IL-23 axis and control of the balance between immune homeostasis and pathology in the intestine. (A) Under steady state conditions, the intestine is populated by an array of immune cells, including a significant population of Tregs that prevent aberrant immune responses through production of molecules such as TGF-β and IL-10, despite the presence of Th1 and Th17 cells. TGF-β also plays a key role in the restitution and regeneration of the epithelium. In addition, it supports the differentiation of small numbers of Th17 cells, which produce cytokines like IL-17 and IL-22 in response to the low levels of IL-23. These direct mucin and anti-microbial peptide production, promoting host defense and preserving the epithelial barrier. Maintenance of an intact epithelial barrier prevents translocation of the microbial flora, thus avoiding excessive immune activation. (B) Breakdown of intestinal immune homoestasis leads to upregulation of IL-23 expression. This inhibits the accumulation of Tregs, allowing the expansion of Th1 and Th17 cells as well as leading to innate immune activation. IL-23 also promotes a pathogenic phenotype from Th17 cells, inhibiting IL-10 production and promoting expression of pro-inflammatory mediators. Significant damage to the epithelium due to this immune response allows the translocation of flora from the lumen into the underlying *lamina propria*, further exacerbating the inflammation and potentially activating the systemic immune response. Over-production of protective factors from Th17 may lead to a dysregulated repair response that may compound host damage (ROS, reactive oxygen species). (Figure and Legend adapted from De Virgilio et al. 2007)

We were able to detect IL- 23 in the supernatant of human monocytes and dendritic cells only upon infection with *S. typhimurium*. Whereas both human monocytes and DCs secrete IL- 1β, IL- 23 is mainly produced by DCs. With the use of mutant strains defective in different pathogen associated molecular patterns we were able to show that SPI-1 and SPI-2 T3SS are not necessary for the induction of IL- 23 production. However, the infection with a mutant strain defective in lipid A acylation resulted in significant less IL- 23 in the supernatant compared to infection with wild type *S. typhimurium*. Blocking TLR4 and TLR5 with antibodies before infection resulted in a decrease of more than 50% of IL- 23 secretion in both experiments. This indicates that signaling through both TLR4 and TLR5 induces IL-23 expression during *S. typhimurium* infection of human DCs.
The adaptor protein MyD88 has a major role in the signaling cascade leading to IL-23 expression. As reviewed by Ahem et al. (Ahem et al. 2008) high levels of IL- 23 in the intestine correlate with high levels of Th17 cytokines. While in the past IL- 23 was thought to be important for the differentiation of T cells to Th17 cells, it now has been shown that it is an important cytokine for mediating protective and pathologic functions but not for Th17 differentiation.

Infection of human DCs with *S. typhimurium* before co- culture with T cells resulted in induction of IFN- γ and IL- 17 after just two days, with a further increase after five days. We also observed a slight increase in IL- 22 secretion after five days of co- culture. Furthermore, we were able to show that the supernatant of human DCs infected with *S. typhimurium* for 24h is sufficient to induce IL- 17 secretion by memory T cells collected from human blood samples after just two days. These results suggest that cytokines secreted by infected DCs may stimulate memory T cells rather quickly to secrete IL- 17 and orchestrate the host response against bacterial infections. Even though T helper cells are usually categorized as components of the adaptive immune response, the fast and unspecific response of the memory T cells that we observed indicates that these cells might also play a role in the innate immune response to infections.

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Zusammenfassung

Salmonella typhimurium ist ein gram- negatives Bakterium und eine der häufigsten Ursachen für eine Lebensmittelvergiftung. Der Krankheitserreger kann häufig in zu kurz gekochten Eiern, Hühnerfleisch bzw. in Dritte- Welt- Ländern auch in stehendem Gewässer vorgefunden werden. Der Verzehr kontaminierter Lebensmittel kann zu akutem Durchfall (Diarrhö), Sepsis und in schweren Fällen bis zum Tode führen. Im Gegensatz zu immunkompetenten Personen kann die Infektion bei Kindern, älteren Personen und Patienten mit primärer oder sekundärer Immunschwäche dazu führen, dass sich die Bakterien rasch ausbreiten und in den Blutkreislauf gelangen. Eine Sepsis (Blutvergiftung) ist die Folge, welche in Dritte- Welt- Ländern häufig nicht rechtzeitig behandelt werden kann und bei den Patienten zum Tode führt.

Als Schlüsselspieler für die Immunabwehr während einer *Salmonella typhimurium* gelten Antigen- Präsentierende- Zellen (APC) wie Monozyten/ Makrophagen und Dendritische Zellen (DC). Dendritische Zellen produzieren während einer Bakterieninfektion Zytokine welche eine Entzündung stimulieren. Eine wichtige Rolle nehmen dabei die Zytokine Interleukin (IL)- 1β und das kürzlich entdeckte IL- 23 ein. Charakteristisch für eine solche Entzündung ist die Aktivierung von T Zellen durch APC und den folgenden Influx von Bakterien bekämpfenden Zellen wie Neutrophile Granulozyten.

Zur Untersuchung des molekularen Ablaufs wird in den meisten Fällen die Maus als Modellorganismus zur Hilfe gezogen. Es gibt jedoch kaum Informationen wie und wodurch diese neuen Entzündung stimulierenden Zytokine im Menschen induziert werden und welche Rolle sie spielen während eine *Salmonella typhimurium* Infektion.

Um dies zu untersuchen isolierten wir humane Monozyten aus Blutspenden gesunder Personen und differenzierten diese zu Makrophagen und Dendritischen Zellen. Wir infizierten die gewonnenen Zellen mit *Salmonella typhimurium* und Mutanten.

Die Infektion von APCs mit *S. typhimurium* resultierte hohen Levels an IL- 1β und IL- 23. Durch Infektionsexperimente mit Mutanten welche bestimmte pathogen assoziierte molekulare Muster nicht mehr produzieren, konnten wir zeigen dass im Gegensatz zum Maus Modell die Produktion von IL-1β nicht von einem der beiden *Salmonella* pathogenicity island (SPI) Type 3 Secretion System abhängt. Hingegen wurden deutlich geringere Mengen an IL-1β und IL- 23 bestimmt wenn diese Zellen mit einer Mutante

infiziert wurden, welche einen Defekt in der Lipid A Acetylierung aufweist. Dies lässt vermuten dass die Aktivierung des Oberflächenrezeptors TLR4 eine große Rolle bei der Induktion von Entzündung stimulierenden Zytokinen spielt. Viele Studien weisen darauf hin dass die Produktion von IL-1β zwei Signale benötigt. Während das erste Signal (oft induziert durch LPS und dessen Aktivierung von TLR4) die Expression von pro- IL-1β stimuliert, wird ein zweites Signal benötigt um mittels Aktivierung der Caspase- 1 pro-IL-1β zu spalten und dabei zu aktivieren.

Weiters zeigen wir in dieser Studie, dass das TLR Adapter Protein MyD88, welches oft die Brücke zwischen Pathogen erkennenden Rezeptoren und Transkriptionsfaktoren bildet, essenziell für die Produktion von IL-1β und IL- 23 in humanen DCs während einer *S. typhimurium* Infektion ist.

In dieser Studie konnten wir zeigen dass LPS eine große Rolle bei der Stimulation von humanen Dendritischen Zellen während einer *S. typhimurium* Infektion einnimmt. Dadurch kommt es zur Produktion der Entzündungs- stimulierenden Zytokine IL- 1β und IL- 23 mittels des Adapter Proteins MyD88. Weiters konnten wir zeigen, dass die Stimulation von Memory T Zellen mit dem Überstand von *S. typhimurium* infizieren DCs ausreicht, so dass diese das Zytokine IL- 17 produzieren. Außerdem, zeigten wir in dieser Studie dass die molekulare Antwort von APCs auf eine *S. typhimurium* Infektion im Menschen sich von der in der Maus unterscheidet.

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