Cross-talk between *Shigella* and cells of the adaptive immunity: The TTS effector IpgD inhibits T cell migration

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Christoph Konradt

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Mitglieder der Promotionskommission:

Vorsitzender: ....

1. Gutachter: Prof. Dr. Dr. h.c. mult. Jörg Hacker

2. Gutachter: Prof. Dr. Ulrich Dobrindt

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“meinem Vater”

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Summary

Zusammenfassung

Summary

Shigellosis, or bacillary dysentery, is a rectocolitis caused by the gram-negative, enteroinvasive bacteria of the genus *Shigella*. Shigellosis still remains a major public health burden with an estimated 80 million cases of bloody diarrhoea and 700.000 deaths per year, primarily in children under the age of 5. *Shigella* disrupts, invades, and causes inflammatory destruction of the colonic epithelium in humans through virulence effectors secreted by the type III secretion apparatus (TTSA). In contrast to the *Shigella*-induced manipulation of the host innate immune response, the impact of *Shigella* on the adaptive immunity has been poorly studied thus far. In order to understand why the naturally induced protective humoral response requires several infections to be primed and is of short duration, the work presented here investigates if *Shigella* is able to directly interact with T cells. Indeed, it has been shown that *Shigella* was able to invade and proliferate inside T cells. Furthermore, *Shigella* was able to inhibit T cell migration through a TTSA effector. Moreover, the *Shigella* effector IpgD, a phosphoinositide 4-phosphatase that specifically dephosphorylates phosphatidylinositol-(4,5)-bisphosphate (PIP2) into phosphatidylinositol-(5)-monophosphate (PI(5)P), was identified as the effector responsible for the observed inhibition. It could be demonstrated that IpgD was responsible for a reduction of intracellular PIP2 levels in T cells. Further experiments showed a reduced level of phosphorylated ezrin, radixin and moesin (ERM) proteins in infected, as well as with IpgD transfected, T cells. The ERM protein family plays an imported role in signal transduction and motility and their activity is closely related to the binding of PIP2. Therefore, the low level of PIP2 leads to a dephosphorylation of the ERM proteins which inhibits T cells response to chemokine stimulation. Indeed, IpgD transfected T cells show a reduced ability to re-localise the ERM proteins upon chemokine stimulation. Targeting T cell motility, via TTSA effectors, could explain the low level of specific T cell priming during *Shigella* infection. This is the first report of *Shigella* induced manipulation of T cell function and on the inhibition of T cell migration by a bacterial effector.

Zusammenfassung

Shigellose oder Bakterieruhr ist eine von Bakterien der Gattung *Shigella* ausgelöste Dysenterie Erkrankung des Dickdarms. Mit jährlich über 80 Millionen Fällen von blutigen Durchfällen und 700000 Todesfällen, hauptsächlich bei Kindern unter 5 Jahren, stellt *Shigella* immer noch ein ernsthaftes Gesundheitsproblem dar. *Shigella* destabilisiert das menschliche Dickdarmgewebe und dringt in dieses ein, wo es eine akute Entzündung auslöst, die das Gewebe weiterhin zerstört. Verursacht wird dies durch bakterielle Effektoren, die durch ein Type III Sekretionssytem (TTSA) sekretiert werden. Verglichen mit der Anzahl an Studien über die Manipulation der angeborenen Immunabwehr gibt es nur wenige Studien über die Interaktionen von *Shigella* mit dem adaptiven Immunsystem. Um zu verstehen, warum für die Entwicklung einer humoralen Immunantwort mehrere Infektionen erforderlich sind, wurde im Rahmen dieser Arbeit untersucht, ob *Shigella* in der Lage ist, direkt mit T-Zellen zu interagieren. Es konnte gezeigt werden, dass *Shigella* in T-Zellen eindringen und sich vermehren kann. Darüber hinaus zeigt sich, dass Shigella in der Lage ist, durch TTSA-Effektoren die T-Zell-Migration zu hemmen. Der *Shigella* Effektor IpgD konnte als der für die Hemmung verantwortliche Effektor identifiziert werden. Bei IpgD handelt es sich um eine 4-Phosphoinositid-Phosphatase, die Phosphatidylinositol-(4,5)-bisphosphat (PIP2) zu Phosphatidyl-inositol-(5)-monophosphat (PI(5)P) dephosphoryliert. Es wurde deutlich, dass der Effektor IpgD, neben der Menge an PIP2, auch die Menge an phosphorylierten Ezrin, Radixin und Moesin (ERM) Proteinen in T-Zellen reduziert. Die ERM-Protein-Familie spielt in der Signaltransduktion und bei der Motilität von T-Zellen eine wichtige Rolle und ihre Phosphorylierung ist eng an die Bindung von PIP2 gekoppelt. Daher führt eine geringe Menge an PIP2 zu einer Dephosphorylierung der ERM-Proteine, was eine Stimulierung der T-Zellen durch Chemokine hemmt. In der Tat zeigten IpgD-transfizierte T-Zellen eine verminderte Fähigkeit zur Relokalisierung der ERM-Proteine nach einer Chemokine-Stimulation. In dieser Arbeit konnte erstmals die Manipulation von T-Zell-Funktionen durch *Shigella* und die Hemmung der T-Zell-Migration, ausgelöst durch einen bakteriellenTTSA-Effektor, gezeigt werden.

Introduction

1. Introduction

Diarrhoeal diseases still remain a leading cause of preventable death, especially in developing countries. Moreover, after acute respiratory infections, diarrhoeal diseases are the second cause of death under the age of five years, responsible for the killing of 1.5 million children every year, representing 16 % of all deaths in this group. Rotaviruses, *Vibrio cholerae, Salmonella typhi,* Enterotoxigenic *Escherichia coli* (ETEC) and *Shigella* species are the main pathogens responsible for this burden. But although rotaviruses and ETEC are the most common causes of diarrhoea, *Shigella*, is the leading cause in mortality (WHO, 2008). Vaccination represents one of the most efficacious and cost-effective medical interventions and it is the only medical intervention proven to eliminate disease at a global level. However, vaccines are so far only available against rotaviruses, *V. cholerae* and *S. typhi*, but not for *Shigella* or ETEC. Epidemic outbreaks and an increasing appearance of antibiotic-resistant *Shigella* strains underline the need for the development of new vaccine candidates against *Shigella*. For this we need a better understanding of the host immune responses to a *Shigella* infection and a deeper knowledge of the manipulation of immune cells by this pathogen.

1.1. The Mucosal Immune System

The body's mucosal surfaces include the gastrointestinal, respiratory, and urogenital tracts as well as the exposed cornea/conjunctiva. The mucosal barrier provides both mechanical and immunological protection to the internal body environment from external agents. Furthermore, the mucus-covered surfaces also provide essential absorptive functions as the intake of food and air, as well as the paths required for reproduction and the liberation of waste. The human adult mucosa surface is about 200 times that of the skin, amounting to some 400 m^2 (Brandtzaeg et al., 1998). These surfaces are protected by the mucosa-associated lymphoid tissue (MALT). The mucosal immune system is exposed to the heaviest burden of environmental antigens. These antigens are derived from a variety of sources, including airborne allergens and food, as well as commensal and pathogenic microorganisms. Over 90 % of all human infections begin at mucosal sites. Consequently, the mucosa is

considered the "first line of defence" against many infections (McKenzie et al., 2004). The size and function defines the mucosa as the body's major immunological organ. A special emphasis must be put on the human intestinal mucosa as the major mucosal surface. The physical barrier plays a major role in the protection of the intestinal mucosa. Resident Goblet cells produce a dense network of glycoproteins that form a thick electrostatically charged glycocalyx known as mucus. Combined with the tight junctions between neighbouring cells, mucus acts as a major barrier against penetration of pathogens. Furthermore, mucosal secretions, particularly of the gut, contain many other factors that restrict infection. Specialized cells of the mucosa secrete factors like digestive enzymes, which act with bile, lactoferrin, and peroxidases to break down antigens and inhibit invading microbes. Granulocytic Paneth cells release lysozyme, type II phospholipase A2, and α defensins. Defensins are a set of small peptides that possess antimicrobial activity (Fellermann and Stange, 2001; Schutte and McCray, 2002). Mucosally-expressed defensins include the β- and α-defensins. Only the pathogens that can survive these harsh innate chemical defences have the ability to infect and cause disease.

1.1.1. Organisation of the Intestinal Mucosal Immune System

In the gut, protection is mediated by the gut-associated lymphoid tissue (GALT) and is composed of, depending on the localisation, numerous Peyer's patches (PP) (n=100–250), solitary lymphoid follicles (n=30,000) and mesenteric lymph nodes (MLN) (McKenzie et al., 2004). Unlike peripheral lymph nodes that sample systemic antigen through afferent lymphatics, PPs and lymphoid follicles sample luminal antigens through the gut mucosa. Antigen transport from the gut lumen to the PPs is mediated by specialized antigen-sampling microfold cells (M cells) present in the follicle-associated epithelium (FAE) (Neutra et al., 1996; Owen, 1999). The ability of M cells to transport antigens across the mucosal epithelium is widely exploited by pathogens to gain entry to the body (Sansonetti and Phalipon, 1999). The role of the innate mucosal immune system is to provide rapid "first-line" defence from challenging pathogens. The epithelial and subepithelial region of mucosal surfaces contains abundant immunocytes of many varieties. Indeed, in a healthy human

adult, the mucosal immune system contributes almost 80 % of all immunocytes. In the gut there are approx 10^{12} lymphoid cells per meter of small intestine (Czerkinsky et al., 1999). The majority of these immunocytes are B cells and mainly found in the gut lamina propria (LP). 80 – 90 % of the plasma cells, differentiated from B cells, found in the LP produce dimeric or some trimeric IgA, collectively called polymeric (p)IgA (Brandtzaeg, 2010). IgA forms an active barrier against mucosal infection through inhibiting adhesions and toxins, and enhancing pathogen clearance. However, most of the IgA are directed against epitopes on commensal bacteria.

1.1.2. Homeostasis versus infection

The intestine is colonised with more than 1000 species of microorganisms, most of them beneficial for the host (Cerutti and Rescigno, 2008). The immune system must therefore be able to distinguish between commensal bacteria and pathogens. This is accomplished by a combination of finely tuned, apparently antagonistic processes (Sansonetti, 2004). A physiological inflammation reflects a status of mucosal homeostasis that includes tolerance to commensal bacteria, resulting in part to their exclusion from mucosal surface by sIgA and other nonspecific defence mechanisms like mucus and defensins (Sansonetti and Di Santo, 2007). Commensal bacteria further modulate the fine-tuning of the T cell repertoire and regulate the differentiation of gut-resident T cells. Commensals mainly promote the differentiation of T lymphocytes into helper T_H2 and regulatory T cells, these T cells further promote local TGF-β secretion and IgA production. In a homeostatic situation the intestinal epithelial barrier is not disrupted and its cells are not activated thus there is no sensing of danger signals. In contrast, pathogens, unlike commensals, tip the immune balance towards inflammation. They can adhere and translocate through the epithelial barrier and also show an increased potential for invasion and destruction of tissues. The disruption of the epithelial barrier is associated with secretion of proinflammatory factors like the TREM family (Colonna, 2003) and HMGB1 (Lotze and Tracey, 2005). Furthermore, the sensing of pathogen-associated molecular motifs (PAMP) by a variety of immune cells results in the release of proinflammatory cytokines, which further leads to the recruitment of polymorphonuclear cells (PMN).

The release of proinflammatory cytokines and danger signals activates macrophages and dendritic cells (DC) resulting in rapid production of cytokines such as IL-6 and IL-12 that promotes T lymphocyte differentiation into T_H1 and T_H17 cells. An amplification loop reinforces the inflammatory response resulting in PMN-mediated pathogen but also tissue destruction (Sansonetti and Di Santo, 2007).

1.2. Link between innate and adaptive immunity

In the GALT, microorganisms act on intestinal epithelial cells (IEC) that, in turn, release signals that attract inflammatory and immune cells. IECs express various pathogen-recognition receptors (PRR), including Toll-like receptors (TRL), which are able to recognise PAMPs (Cario, 2002). Activation of microbe-induced pathways in IECs through these receptors mediates the recruitment of proinflammatory cells, like PMNs and macrophages, as well as immature DCs. Furthermore, the FAE constitutively expresses the chemokine CCL20 that is responsible for the recruitment of DC to the subepithelial region of the PPs (Tanaka et al., 1999). DC in the intestinal epithelium are able to open up junctional complexes and send dendrites into the lumen to sample lumenal microorganisms, in particular commensals (Rescigno et al., 2001). After pathogen recognition mucosal DCs migrate to the lymphoid follicles and the draining mesenteric lymph nodes to initiate an adaptive immune response. The induction of mucosal immunity in the gut is dependent on efficient activation of lymphocytes within the GALT. This activation is mediated mainly by DCs. The GALT compartment is also closely linked to the systemic lymphoid compartment. Typically, immune responses induced in the GALT result in concurrent induction of a systemic response. Contrarily, immune responses induced in systemic compartments rarely result in mucosal responses. For this reason, proximal responses typically require mucosal antigen delivery. Once activated, lymphocytes proliferate and differentiate under the further influence of local signals from the mucosal microenvironment. During this process, mucosal homing “programs” control the traffic of naive, activated, and memory lymphocytes from peripheral tissues to inductive tissues of the GALT, and finally to effector tissues of the LP and epithelia. Lymphocyte homing plays a crucial role in adaptive protection of the mucosa (McKenzie et al., 2004).

1.3. T lymphocytes

T lymphocytes or T cells are a subgroup of lymphocytes that play a crucial role in the development of adaptive immunity. They also play an important role in facilitating a more effective innate immune response to pathogens. T cell migration, activation, proliferation and differentiation are crucial steps in T cell function and involve a complex regulation of receptor expression levels, actin rearrangements, and signalling pathways activation. And it has been shown that pathogens, like *Yersinia, Salmonella* and *Bordetella,* are able to modulate some of this T cell functions (Gerke et al., 2005; Paccani et al., 2008; van der Velden et al., 2008). T cells originate from haematopoietic stem cells in the bone marrow. Haematopoietic progenitors that derived from haematopoietic stem cells migrate to the thymus, maturate into naive T cells, and accumulate in large numbers within lymphoid tissues where an adaptive immune response is initiated or they can circulate in either the blood or the lymph. Naive T cells are small, featureless cells with few cytoplasmatic organelles and the methylation pattern of the DNA. The chromatin structure resembles an inactive state of the naive T cell. These small lymphocytes have no functional activity until they encounter their specific antigen, recognised via the T cell receptor (TCR). After they have met their antigen naive T cells becomes activated, proliferate, differentiate and mature into fully functional lymphocytes and are termed effector T cells (Murphy et al., 2008).

1.3.1. T cell activation

Naive T cells circulate from the bloodstream into lymph nodes, spleen and MALT before re-entering the blood. During this time T cells encounter thousands of antigen-presenting cells (APC) and sample the peptides which are presented on the surface through the major histocompatibility complex (MHC) located on these cells. T cell activation begins with the interaction between an antigen-specific T cell and an APC presenting the specific antigen. This leads to substantial membrane and cytoskeletal rearrangements, resulting in the formation of the immunological synapse (IS), a micrometer-sized cluster of segregated proteins formed at the T cell–APC intercellular contact (Davis and Dustin, 2004). Important molecules in the IS are the

TCR and its counterpart the MHC class II as well as, the for an activation important, co-stimulatory molecules CD80/CD86 and CD28 and furthermore the adhesion molecules LFA-1 and ICAM-1 (Dustin, 2008). The ERM protein family also plays an important role by regulating, at least in part, the actin cytoskeletal rearrangement which is responsible for the central accumulation of the TCR–peptide–MHC complex in the IS (Dustin, 2008; Mempel et al., 2004). After the recognition of a specific antigen by the TCR complex the tyrosine-containing signal motifs, known as ITAM, get phosphorylated through kinases of the Src family. The phosphorylated ITAM then recruits ZAP-70, another tyrosine kinase. The activation of ZAP-70 results in the phosphorylation of scaffold proteins called LAT and SLP-76. The most important of the signalling protein, recruited and activated by these phosphorylated scaffolds, is phospholipase Cγ, which when activated, generates inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 has an important role in inducing changes in intracellular calcium concentrations, while DAG is involved in activating protein kinase Cθ and the small G protein Ras. These pathways ultimately result in the activation of three transcription factors, AP-1, NFAT and NFκB, which together induce transcription of the cytokine interleukin 2 (IL-2), which is essential for the proliferation and further differentiation of the activated T cell (Murphy et al., 2008).

1.3.2. T cell subtypes

Different types of T cells are required for the generation of an optimal defence against the diverse types of microorganisms that invade our body. T cells are defined on the basis of the expression of a T cell receptor (TCR). So far, two different kinds of TCR have been discovered. T cells that express an α/β-chain combination of the TCR, also called conventional T cells, carry the major burden of protection (Bonneville, 2005). The other are T cells with a TCR that is composed of a γ/δ-chain combination that recognizes phosphate-containing non-proteinaceous antigens without the need for a known presentation molecule (Chien and Konigshofer, 2007). γ/δ T cells that represent less then 5 % of the total T cell population are more considered to belong to the innate than to the adaptive arm of the immune system. The α/β T cells can be further distinguished into cytotoxic T cells and T helper Cells.

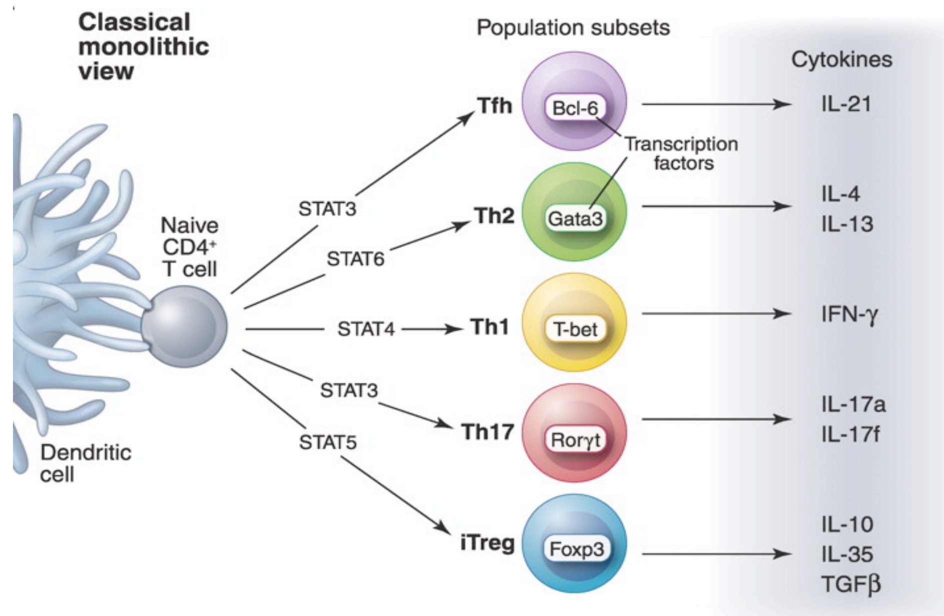


Figure 1.3.2.: Classical monolithic view on T helper cell subsets with lineages and master regulators (O'Shea and Paul, 2010).

Cytotoxic T cells, also called CD_8^+ T cells, are defined by the expression the surface receptor CD_8. Cytotoxic T cells recognize antigens that are presented by the MHC class I. They are responsible for the destruction of pathogen-infected cells. T helper or CD_4^+ T cells express the CD_4 receptor as a marker on their surface. CD_4^+ T cells have a more flexible repertoire of effector activities than CD_8^+ T cells, therefore they play a central role in all immune responses. After recognition of peptides, presented by MHC class II molecules, naive CD_4^+ T cells can differentiate into different T helper cell subsets with different immunological function. The main CD_4^+ T cell subsets that are currently distinguished are T_H1, T_H2, T_H17, T_{FH}, and T_{reg} (Figure. 1.3.2.). The T_H1 T helper cell subset is responsible for inducing a cell-mediated immunity, with interferon γ (IFN-γ) and interleukin 2 (IL-2) as the lead cytokines. A T_H1 immune response leads to an activation of macrophages, which supports the killing of intracellular pathogens, and also to an activation of cytotoxic T cells that then kill infected target cells. The T_H2 T helper cell subset plays a central role in the development of humoral immunity by promoting the maturation of B cells, antibody production and class switch of immunoglobulins. T_H2 T cells are also important for the defence against helminths. IL-4 and IL-5 are the lead cytokines for a T_H2 immune response (Mosmann and Coffman, 1989; Romagnani, 2005). A more

recently discovered T helper cell subset is the T_H17 subset (Oppmann et al., 2000). These T cells produce IL-17 as a cytokine marker and are apparently highly pathogenic because they have mostly been found in subjects suffering from autoimmune diseases (Zheng et al., 2007). However, it is likely that they also have a role in antimicrobial defence and initial evidence indicates that they might participate in immunity against extracellular bacteria by activating neutrophils (Happel et al., 2005). More recent findings indicate that T_H17 cells might also contribute to protection against intracellular bacteria by directing T_H1 cells to the site of bacterial replication (Khader et al., 2007). The fourth subset of T helper cells that express the CD_4^+ receptor are the regulatory T cells (T_{Reg}). T_{Reg} are defined by the expression of the markers CD25 and FoxP3. They control and counteract excessive immune responses therefore they are crucial for the maintenance of immunological tolerance. Their major role is to down-regulate T cell-mediated immunity towards the end of an immune response and to suppress auto-reactive T cells that have escaped the process of negative selection in the thymus (Belkaid and Rouse, 2005). Follicular helper T cells (T_{FH}) are another new discovered CD_4^+ T cell subset which is found in the B cell follicles of secondary lymphoid organs. Their particular function is to support the development of germinal centres and promote immunoglobulin class switch recombination and affinity maturation. T_{FH} T cells markers are CXCR5, ICOS, and Bcl6 (Fazilleau et al., 2009; Zhou et al., 2009). Memory T cells are induced during an immune response against a pathogen. Antigen-specific T cells get activated, proliferate, expand and differentiate into the different subsets of effector T cells. This expansion phase creates a large population of effector T cells, most of them will die during infection or undergo apoptosis after the infection. However, the expansion phase also creates cells that eventually will turn into memory T cells. These primed T cells are maintained long term after immunization. During a second infection with the same pathogen, the pool of memory T cells leads to an improved secondary immune response compared to the primary response, the host reacts by mounting a greater, faster, more efficient response. A secondary immune response is also more effective by showing a higher diversity or complexity of secondary effectors (Harty and Badovinac, 2008; Kaech and Wherry, 2007; Williams and

Rudensky, 2007). Both CD_4 and CD_8 T cells subtypes can turn into memory T cells. The memory T cells pool can be further distinguished in two populations, effector-memory T cells (T_{Em}) and central-memory T cells (T_{Cm}). Surface markers for T_{Em} are $CD62L^{lo}$, $CCR7^{lo}$ and for T_{Cm} $CD62L^{hi}$, $CCR7^{hi}$. Each T cell memory subset possesses distinct functional properties (Sallusto et al., 1999).

1.3.3. T cell migration

Immune cells are the most motile cells in the human body. The trafficking of T cells into and out of lymphoid organs, or into and out inflammatory sites, involves a high coordination and regulation level of adhesion molecules, chemoattractants, receptor expression levels and signalling pathway modulation (Kehrl, 2006). An impairment of T cell motility and migration capacity, by a pathogen would have a critical impact on immunosurveillance and the immune response against this pathogen. The impact of an imbalance in the T cell migration is seen in patients with the WHIM Syndrome (Wart, Hypogammaglobulinemia, Infection, and Myelokathexis syndrome). Patients with WHIM show an increased susceptibility to bacterial and viral infections. The WHIM Syndrome is caused by a function mutation in the CXC chemokine receptor 4 (CXCR4) resulting in a hyperactivity of the receptor (Kawai and Malech, 2009). Chemokines and lipid chemoattractants, identified because of their important roles in recruiting innate immune cells and effector T cells to the sites of inflammation, orchestrate T lymphocyte migration. Certain members of the chemokine superfamily (most notably CCL19 and CCL21, which both bind CCR7, CXCL13 and its receptor CXCR5, and CXCL12 and its receptor CXCR4 have key roles in promoting the organisation and function of secondary lymphoid tissues (SLO) (Cyster, 2005). Effector and memory T cells generated in the SLO migrate to the periphery for participation in the immune response and immune surveillance. The relocation of effector and memory T cells is non-random because of tissue-specific address codes, which are mediated by unique combinations of adhesion molecules and chemokine receptors that enable proper tissue homing (Kunkel and Butcher, 2002). There is also growing evidence that lipid chemoattractants, such as sphingosine 1-phosphate (S1P) and eicosanoids, which also engage the G-protein-coupled receptors (GPCR),

have a prominent role at different stages of the immune response in the navigation of distinct T cell subsets to their sites of infection and inflammation. Members of the eicosanoid family appear to contribute to the orchestrated trafficking of effector T cells to the sites of infection or inflammation. The leukotriene B4 (LTB4) receptor BLT1 is expressed on T_H1 and T_H2 T cells, CD_8 effector T cells, and in the spleen and lymph nodes. The prostaglandin D2 (PGD2) receptor (DP2) is expressed on T_H2 T cells and in the thymus (Ward, 2006). Migrating T lymphocytes first roll along the surface of the high endothelial venules (HEV), adhering as a result of interactions between selectin and integrin and their respective vascular ligands. After an activation of integrins through chemokines a firm integrin-mediated adhesion of lymphocytes to the endothelium of the microvasculature occurs, then the lymphocytes transmigrate through the vessel wall, and finally they migrate further into extravascular tissues (Figure. 1.3.3.) (von Andrian and Mempel, 2003). Chemokines induce multiple signalling pathways, including phosphatidylinositol 3-kinase (PI3K) and phospholipase C (PLC) cascades (Kinashi, 2007).

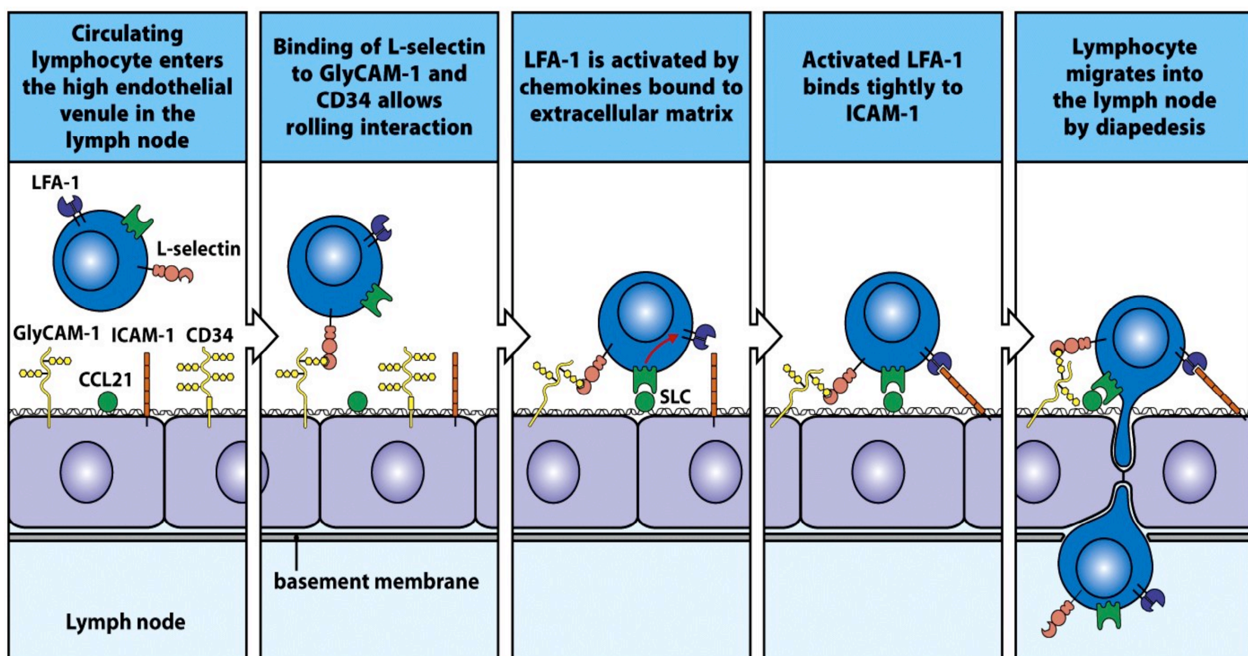


Figure 1.3.3.: Lymphocytes in the blood enter lymphoid tissue by crossing the walls of high endothelial venules (Murphy et al., 2008).

1.3.4. Phosphatidylinositol 3-kinase

The phosphatidylinositol 3-kinase (PI3K) family consists of three classes. Here we will focus only on the class I PI3K as they are the best understood class. The class I PI3K is divided into two subclasses, class IA and class IB. They are heterodimeric molecules composed of a 85 kDa regulatory and a 110 kDa catalytic subunits. Class IA enzymes include the α, β and δ p110 catalytic isoforms and the five isoforms of the p85 regulatory subunit, designated p85α, p55α, p50α, p85β, or p55γ. Class IA PI3K is activated by most receptors that trigger tyrosine kinase activity. The class IB PI3K consists of the p110γ catalytic subunit, which is activated by G-protein $\beta\gamma$ subunits and signals downstream of GPCRs. GPCRs, including chemokine and sphingosine-1-phosphate receptors, activate class IA PI3K (Vanhaesebroeck et al., 2005). Class I PI3K shows broad substrate specificity towards phosphatidylinositols (PIP), phosphatidylinositol 4-monophosphate (PIP1) and phosphatidylinositol 4,5-bisphosphate (PIP2). The resulting phosphatidylinositol 3,4,5-triphosphate (PIP3) lipids have important biological functions that rely on interactions with effector proteins. Class I PI3K resides mainly in the cytoplasm until recruited to active signalling complexes (Foster et al., 2003). PI3K plays an important role in T cell migration especially in controlling the trafficking among tissues. It is believed that a PI3K activation following antigen recognition reprograms the lymphocytes to reduce their capacity for lymph node re-entry to promote migration in inflamed tissue. Effector T cells rely on PI3K signalling to drive the homing to peripheral tissue (Fruman and Bismuth, 2009). Both class IA and IB PI3K subtypes have been implicated in migration of activated cells. It has been shown that especially p110γ plays an important role in trafficking of primed T cells (Thomas et al., 2008).

1.3.5. Phosphoinositides

Phosphoinositides (PI) are a family of phosphorylated derivatives of the membrane lipid phosphatidylinositol. They are glycerophospholipids that contain a hydrophobic diacylglycerol (DAG) backbone esterified to a polar inositol headgroup. Three of the five hydroxyl residues on the inositol ring can be phosphorylated individually or in combination to yield seven different phosphorylated phosphatidylinositols, or PIPs (Figure. 1.3.5.). Phosphorylation and dephosphorylation by lipid kinases and phosphatases can rapidly interconvert PIP species, contributing to the dynamic production of specific PIP lipids within different cellular compartments. Phosphoinositides make up a very small proportion of the lipids present within cellular membranes. PI makes up ≈ 4 % of cellular membrane phospholipids, and the other phosphorylated PIs together comprise ≈ 1 % (Skwarek and Boulianne, 2009). Classical PIP signalling results from the hydrolysis of PIP2 by phospholipase C isoforms, resulting in the production of diacylglycerol (DAG) and inositol-3,4,5-triphosphate (IP3), which act as second messengers. A second, very well studied phosphoinositide signalling pathway results from the activation of PI3Ks. Downstream of stimulation by growth factors, hormones, or other cellular signals, PI3Ks phosphorylate PIP2 and PIP1 to produce PIP3 and PI(3,4)P2. These lipids then activate the downstream protein kinase B (PKB)/Akt signalling pathway. PKB/Akt signalling is important for cell growth, survival, proliferation, and motility and also provides crosstalk between signalling pathways, including those activated by growth factors, insulin, Notch, BMP, and Shh. PIPs also play a role in the specification and maturation of various intracellular compartments. PIP binding is important for recruitment of proteins to specific membranes or domains of membranes, such as the localization of FYVE domain containing trafficking proteins to endosomes. However, it is important to emphasize that PIP binding can also play a direct role in regulating protein-protein interactions and catalytic activities. Furthermore, PI have also been implicated in regulation of membrane trafficking, cell polarity, motility, chemotaxis, and transcription (Skwarek and Boulianne, 2009).

Figure 1.3.5.: Metabolism of phosphatidylinositols: (A) Structure of phosphatidylinositol (PI). Positions 3, 4, and 5 on the inositol ring can be phosphorylated to produce seven different phosphoinositide species. (B) Metabolic pathways interconverting the different phosphoinositides (Skwarek and Boulianne, 2009).

1.3.6. The ERM proteins

Ezrin, radixin and moesin are highly homologous proteins and form together with merlin/schwannomin the ERM family of proteins (Bretscher et al.). Ezrin, radixin and moesin share a 75% similarity in their amino acid sequence compared with 45% of similarity with merlin. The ERM proteins belong to the erythrocyte protein 4.1 superfamily that is characterized by a conserved ≈ 300-residue globar N-terminal domain, the Four.1-Ezrin-Radixin-Moesin (FERM) domain. The FERM domain is followed by an α-helical domain and a charged C-terminal region, known as the C-terminal ERM-association domain (C-ERMAD), with the ability to bind the FERM domain or filamentous actin (F-actin). The FERM domain interacts with membrane components and most signalling molecules, it can bind directly or indirectly, via the adaptor proteins EBP50 and E3KARP, to the cytosolic tail of various transmembrane proteins. With the C-terminal part the ERM proteins bind to the actin cytoskeleton, therefore, the ERM proteins connecting the actin cytoskeleton with the plasma membrane (Charrin and Alcover, 2006). The ERM proteins exist under two

conformations, a “dormant” state, in which the protein presents a head to tail folded conformation, and an “active” state, in which the protein is unfolded and fully capable to interact with the membrane components and with the actin cytoskeleton (Fugure. 1.3.6.). The activation of the ERM proteins occurs through two steps, first by binding of PIP2 to the FERM domain leading to a conformational change and therefore rendering the conserved threonine residue more accessible to phosphorylation and second by the phosphorylation of the now accessible conserved threonine residue in the C-terminal domain (Fievet et al., 2004). LOK, ROCK and PLCθ are major kinases, responsible for ERM phosphorylation, identified so far in lymphocytes (Belkina et al., 2009). In T cells, the transmembrane proteins CD43, CD44, L-selectin, P-selectin, PSGL-1, intracellular adhesion molecules ICAM 1-3 and CD95, have been identified as ERM partners. The ERM proteins are involved in cell cortex organization at two important stages of T cell physiology, during polarisation and migration in response to chemokines and during the formation of the immunological synapse. ERM proteins play a crucial role in cell polarisation during T cell migration. T cells polarize in response to adhesins or chemokines, leading to the formation of two poles, a lamellopodia structure at the front edge and a posterior protrusion called the uropod. The ERM proteins are involved in the formation of the uropod. The ERM protein activation is modulated during T cell polarisation and cell migration. The threonine phosphorylation is rapidly downregulated upon chemokine stimulation. The ERM proteins are also involved in the formation of the immunological synapse (IS), an APC and a T cell form, during the formation of an IS, a tight interaction that assembles in seconds and persists for hours. Recognition of the peptide presented by the APC triggers the T cell receptor, and this induces several changes. Unstimulated T cells are covered in microvilli and, in order to form the synapse, the microvilli have to be locally disassembled at the site of interaction which is achieved through ERM proteins dephosphorylation. In addition, glycoproteins, such as CD43, have to be moved away, and the T cell receptor has to be recruited to the site of the synapse. Adhesion molecules have to then hold the two cells together. The ERM proteins have been implicated in all these processes (Allenspach et al., 2001). In unstimulated T cells, phosphorylated moesin is enriched

and associates with CD43 in the cell cortex, whereas ezrin is largely unphosphorylated and located in the cytoplasm. After stimulation, moesin is rapidly and locally dephosphorylated to collapse the microvilli in the contact area and release CD43. Ezrin binds to, and is needed for, the recruitment of ζ-chain associated protein kinase of 70kDa (ZAP-70), a downstream component of T cell signalling, to the synapse (Ilani et al., 2007).

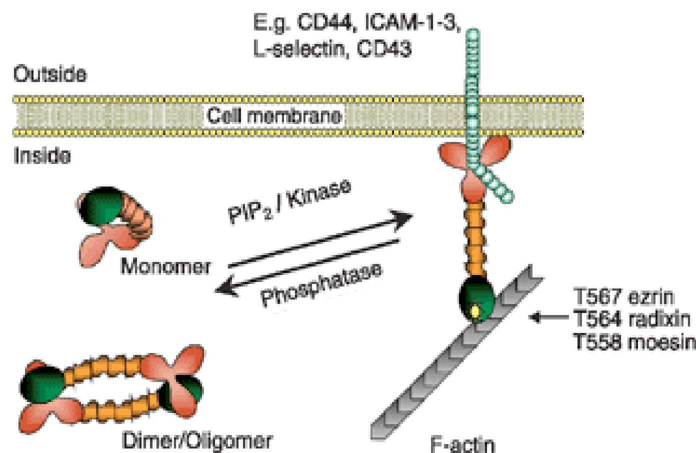


Figure 1.3.6.: ERM proteins exist under two conformations: a "dormant state", in which the protein is folded and an "active state", in which the protein is unfolded and fully capable to interact with membrane components and with the cytoskeleton (Charrin and Alcover, 2006).

1.4. *Shigella,* the causative agent of shigellosis

Shigellosis, or bacillary dysentery, is a rectocolitis caused by the enteroinvasive, non-motile, Gram-negative bacteria of the genus *Shigella*. The genus *Shigella* belongs to the class of γ Proteobacteria and the family of *Enterobacteriaceae*. *Shigella* was discovered by the Japanese microbiologist Kiyoshi Shiga in 1898. With a degree of homology of their chromosomal DNA close to 100 % the *Shigella* species are very closely related to the species *Escherichia coli* (Brenner et al., 1972). However, especially due to their particular metabolic profile and their capacity of causing dysentery, *Shigella* appears to be unique among the *Enterobacteriaceae*, so their species status is maintained. The genus *Shigella* is characterized by biochemical and serological properties and comprises four species: *S. dysenteriae* (Subgroup A), *S. flexneri* (Subgroup B), *S. boydii* (Subgroup C) and *S. sonnei* (Subgroup D). The four different species are further classified into different serotypes and subserotypes, depending on the structure of the somatic O-antigen of the lipopolysaccharide (LPS), the major bacterial surface antigen. To present, there are 15 serotypes and subserotypes reported for *S. dysenteriae,* 14 for *S. flexneri,* 20 for *S. boydii* and 1 for *S. sonnei* (Levine et al., 2007). However, only some of them are responsible for the endemic or epidemic forms of the disease.

1.5. Clinical features of shigellosis

Shigella infection occurs through the uptake of contaminated food or water in addition to a person-to-person transmission via the oral-faecal route. Studies on American volunteers have shown that the oral administration of only as few as a 100 bacteria was enough to cause shigellosis in 25 – 50 % of the cases (DuPont et al., 1969). The classical symptom associated with *Shigella* is dysentery, which develops after an incubation period of 1 to 4 days. However, the severity of dysentery varies widely from asymptomatic to severe infection. The typical clinical profile begins with fever, lower abdominal pain, watery diarrhoea and malaise. Later on, the diarrhoea progresses to dysentery, blood, mucus and pus appearing in the stools. The infection is always localized to the recto-sigmoid area (DuPont et al., 1969). The most common chronic complications, occurring mainly in the poorest regions, are

persistent diarrhoea and chronic malnutrition. Further reported complications are hypoglycaemia, seizures, rectal prolapse, toxic mega-colon, haemolytic-uremic syndrome, and leukemoid reaction that are often the cause of death, especially in the youngest individuals (Black et al., 1982).

1.6. Epidemiology of *Shigella*

The majority of cases of shigellosis occur in the developing world with only sporadic cases or outbreaks in developed countries. Shigellosis is associated with poor hygiene standards and inadequate water supply, situations that mainly appear in low-income countries. Despite a significant improvement in both water supply and sanitation in the last 20 years , the number of cases remains high. Indeed, shigellosis still remains a major public health burden with estimated at least 80 million cases of bloody diarrhoea and 700.000 deaths per year (WHO, 2005), preferentially in children under the age of 5, which account for 69 % of all cases and 61 % of all deaths. It is noteworthy that an increasing number of cases in industrialized countries have been recently reported. *S. flexneri* is the main cause of endemic shigellosis in developing countries whereas *S. sonnei* predominates in developed and transitional countries, causing sporadic diarrhoea and occasional outbreaks. Severe epidemic outbreaks are preferentially caused by *S. dysenteriae* type 1. Shigellosis caused by *S. boydii* is rather uncommon (Kotloff et al., 1999).

1.7. *Shigella* pathogenicity

1.7.1. The virulence plasmid

The enteroinvasive phenotype of *Shigella* is based on the presence of a 200-230-kb virulence plasmid (VP), harboured by all the virulent *Shigella*. The VP carries the genes that are required to express the invasive phenotype. Loss of the VP results in an avirulent strain (Sansonetti et al., 1982). A 30-kb region called the entry region or "*ipa/mxi-spa* locus" has been identified, which is essential for *Shigella* entry into epithelial cells (Parsot, 2005). Moreover, sequence analysis of the VP of *S. flexneri* 5 and 2a strains has shown a composition of a mosaic of ≈ 100 genes (Buchrieser et

al., 2000; Venkatesan et al., 2001). The regulation of genes encoded by the VP has been extensively studied. Transcription of genes of the entry region is regulated by temperature and two VP-encoded proteins: VirF, a member of the AraC family of transcription activators and VirB, a member of the ParB family of partition proteins. Binding of H-NS to the promoters of *virF* and *virB* prevents transcription of these genes under 37 °C. At 37 °C a conformational change in the DNA leads to an increased transcription of *virF* and an activation of *virB* through VirF (McKenna et al., 2003). VirB controls the transcription of around 15 genes on the VP, most of them are part of the type III secretion apparatus (TTSA). The TTSA, assembled at 37 °C, is only weakly active, secretion only becomes activated upon contact with a target cell. Activation of the TTSA leads to a further transcription of 12 genes encoded on the VP. The increased transcription of these genes, in condition of secretion, is controlled by MxiE, another transcription activator of the AraC family. MxiE not only controls the transcription of genes on the VP. It also controls the transcription of effectors located on the chromosome. Based on their expression profile, TTSA substrates are classified in three categories: (1) TTSA substrates expressed independently from the TTSA activity, (2) TTSA substrates expressed in condition of non-secretion and induced in condition of secretion, (3) TTSA substrates expressed only in the condition of secretion (Figure. 1.7.1.) (Parsot, 2005).

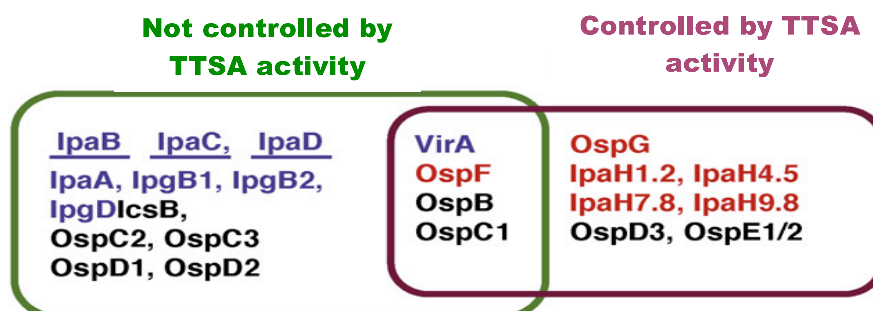


Figure 1.7.1.: Differential expression of effectors by the TTSA activity. Effectors produced independently of the TTSA activity are indicated in the green box, effectors whose production induced by the TTSA activity are indicated in the purple box and effectors the transcription of which was detected when the TTSA is not active and increased when the TTSA is active are shown in the overlap.. Effectors involved in entry are indicated in blue and effectors involved in dampening the host innate responses are indicated in red. IpaB, IpaC and IpaD (underlined) are the proposed translocators. Adapted from (Parsot, 2009).

1.7.2. The TTSA

The TTSA functions as a molecular syringe, which enables the bacteria to inject effector proteins directly into the host cell cytoplasm (Figure. 1.7.2.). The core of the TTSA, also called the needle complex, consists of a needle composed of the proteins MxiH and MxiI, and a basal body composed of the proteins MxiG, MxiJ, MxiD and presumably MxiM (Cordes et al., 2003). Spa47, an ATPase, provides the energy for the transit of substrates through the TTSA. MxiK and MxiN interact both with Spa47 and are required for the transit of needle components. The protein Spa32 controls the needle length and the switch of TTSA substrates specificity (Magdalena et al., 2002). Moreover, TTSA activity is regulated by external signals. To stabilize translocators and some of the effectors in the cytoplasm, and also to keep them in a secretion-competent state while they are stored, they are bound to specific chaperons. The VP encodes for four chaperons: IpgA, IpgE, Spa15, and IpgC (Page et al., 2001). Due to their sequence and their ability to interact and to form pores in lipid membranes, IpaB and IpaC are proposed to be translocators. IpaD is located at the tip of the needle and is probably required for insertion of IpaB and IpaC in the cell membrane (De Geyter et al., 2000).

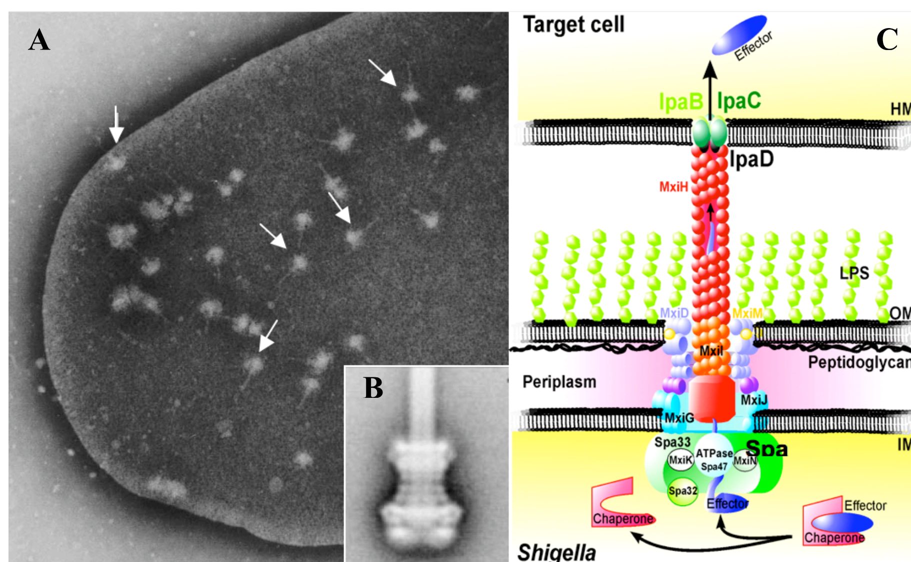


Figure 1.7.2.: The type three secretion apparatus.
A: TTSA on the bacterial surface (white arrows) (Blocker et al., 1999).
B: Averaged electron microscopical image of the TTSA complex (Blocker et al., 2001).
C: Architecture of the *Shigella* TTSA (Schroeder and Hilbi, 2008).

1.7.3. *Shigella* TTSA effectors

Effectors are defined as through the TTSA injected proteins which, once inside the cell cytoplasm, affect cellular functions. About 25 effectors have been identified so far that are encoded by the VP and 5-7 encoded by the chromosome (Parsot, 2005). The complete role of most of the effectors has just begun to be elucidated but for many effectors the exact function still remains unclear. As described in the next chapter, the effectors have a pivotal role in the infection process of *Shigella* by i.e. promoting entry into non-phagocytic cells or modulating the host inflammatory response. Their production and secretion is tightly and hierarchically regulated, suggesting a different importance of some effectors at different stages of the infectious process. Some *Shigella* strains also harbour genes for the production of toxins. The most known is the Shiga toxin encoded by the chromosome in *S. dysenteriae* 1. The Shiga toxin is a heterodimer showing a potent cytotoxicity inducing cell death by blocking eukaryotic protein biosynthesis (Sandvig et al., 1991). Two other enterotoxins produced by *Shigella* have been characterized: the chromosomally encoded *Shigella* enterotoxin 1 (SHET1) essentially expressed by *S. flexneri* 2a, and the VP-encoded *Shigella* enterotoxin 2 (SHET2). These two enterotoxins may account for the early diarrhoeal phase of shigellosis (Nataro et al., 1995; Noriega et al., 1995).

1.8. Innate Immunity to *Shigella* infection

1.8.1. Mechanisms leading to the induction of acute inflammation by *Shigella*

1.8.1.1. Cross-talk with intestinal epithelial cells (IEC)

Shigella cannot invade IEC through the apical pole but only through the basolateral side (Mounier et al., 1992; Perdomo et al., 1994). However, in polarised epithelial cells *Shigella* down-regulates the expression and therefore the production of antimicrobial cationic peptides, particularly of the human β-defensin hBD-3, via MxiE regulated effectors, suggesting also injection of effectors via the apical side of IEC as *Shigella* entry could not be observed (Sperandio et al., 2008). To enter IEC the

bacterium must first cross this epithelial barrier to establish an infection, which further causes the symptoms of shigellosis. Current evidence indicates that the route of entry is facilitated by the follicle-associated epithelium (FAE) that overlays the lymphoid follicles associated with the intestinal mucosa (Sansonetti and Phalipon, 1999). Once has *Shigella* reached the basolateral side of IEC it induces, via its effectors, a massive but local reorganization of the cell subcortical cytoskeleton resulting in the uptake of the bacterium. Effectors involved in the early steps of infection and mainly promoting bacterial entry at the basolateral side of polarized cells are IpaA, IpaB, IpaC, IpgB1, IpgB2 and VirA. IpaC plays a key role in inducing actin polymerization and bacterial invasion by recruiting the tyrosine kinase Src to the side of entry (Nhieu and Sansonetti, 1999). IpaA stimulates actin depolymerization by targeting vinculin and also stimulates a loss of stress fibres by the GTPase RhoA (Bourdet-Sicard et al., 1999). IpgB1 is assumed to play a major role in producing membrane ruffles by activating Rac1 through ELMO-Dock180, a Rac1 guanine nucleotide-exchange factor (Handa et al., 2007). IpgB2 is an IpgB1 homologue that binds to mDia1 and the Rho kinase ROCK. In this way IpgB2 induces the formation of stress fibres, probably interacting with the Rho binding domain of CRIK, ROCKI, ROCKII and mDia1 (Alto et al., 2006). But the specific involvement of IpgB2 in bacterial entry remains still unclear. The effector IpgD is a phosphoinositide 4-phosphatase that specifically dephosphorylates phosphatidylinositol-(4,5)-bisphosphate (PIP2) into phosphatidylinositol-(5)-monophosphate (PI(5)P). IpgD is responsible for dramatic morphological changes of the host cell, leading to a decrease in membrane tether force associated with membrane blebbing and actin filament remodelling. Although involved in the formation of the fully structured entry sites, it is not involved in invasion. Furthermore, IpgD, through the induction of the accumulation of PI(5)P, is involved in the activation of the host cell PI-3-kinase/Akt pathway. It is believed that this plays an important role in the host cell response for survival (Niebuhr et al., 2002; Pendaries et al., 2006). The translocator IpaB promotes host cell survival by inducing a cell-cycle arrest by targeting Mad2L2, an anaphase-promoting complex (Handa et al., 2007). Internalized bacteria rapidly lyse the phagocytic vacuole and gain access to the cytoplasm. Microtubules severely

hinder *Shigella* movement within the cell, but the effector VirA, delivered into the host cell cytoplasm, induces local microtubule degradation. It is also assumed that VirA contributes to the ruffle formation during *Shigella* entry (Yoshida et al., 2002). The effector IcsB allows *Shigella* to escape autophagic destruction, as autophagy is able to remove intracellular bacterial pathogens. IcsB binds IcsA, another virulence factor of *Shigella*, and hereby protects the recognition of IcsA by the autophagy protein Atg5 (Ogawa et al., 2005). IcsA, also called VirG, is an outer membrane protein that is responsible for the bacterial actin-based movement inside the host cell. IcsA is localized on one pole of the bacterium and binds and activates N-WASP (neural Wiscott-Aldrich syndrome protein). N-WASP recruits Cdc42 and the Arp2/3 complex. This causes actin nucleation and elongation, leading to bacterial movement inside the cell. Through the movement, *Shigella* is able to cause membrane protrusions that penetrate into neighbouring cells, allowing the bacteria to disseminate into adjacent cells (Pantaloni et al., 2001). Intracellular *Shigella* reprograms the gene expression of infected epithelial cells. Multiplying bacteria inside the cytoplasm release peptidoglycan (PGN), which is recognized by the intracellular pattern recognition receptor NOD1. PGN activates the NOD1-RICK signalling pathway, which activates the NF-κB and MAPK pathways leading to the production and secretion of IL-8 (Girardin et al., 2003; Philpott et al., 2000). IL-8 is a potent chemoattractant for Polymorphonuclear cells (PMN) cells. The *Shigella* virulence factor SepA, which is not secreted through the TTSA, enhances inflammation of infected tissues. SepA hydrolyses several peptides that have been described as specific substrates for cathepsin G, a serine protease produced by PMNs that is proposed to play a role in inflammation. (Benjelloun-Touimi et al., 1995; Benjelloun-Touimi et al., 1998).

1.8.1.2. Pyroptosis of resident macrophages

After crossing the epithelial barrier through M cells, *Shigella* has to face another line of defence: the resident macrophages in the intraepithelial pocket of M-cells. These cells are the first immune cells that *Shigella* contacts. After passing the M-cells, *Shigella* is rapidly phagocytosed by these macrophages. Once inside, the bacteria

escapes rapidly from the phagosome into the cytosol. Induced by the effector IpaB (Zychlinsky et al., 1994b), infected macrophages undergo a caspase-1-mediated cell death, termed pyroptosis, which is a newly identified pathway of programmed cell death associated with an inflammatory response that is accompanied by plasma membrane permeability and nuclear condensation (Handa et al., 2007). The activation of caspase-1 in dying macrophages leads to a release of the pro-inflammatory cytokines IL-1β and IL-18, which contributes to the pro-inflammatory profile of shigellosis (Zychlinsky et al., 1994a; Sansonetti et al., 2000; Zychlinsky et al., 1992).

1.8.2. Control of the *Shigella*-induced inflammation

Shigella is not only able of inducing NF-κB activation in IEC, it is also able to dampen the resulting inflammation. So far, three effectors have been identified as being involved in this process:

- OspG, a protein kinase, binds to a number of ubiquitinylated ubiquitin-conjugating enzymes, including UbcH5. UbcH5 is a component of the Skp1-culin-F-box protein complex that promotes the ubiquitination of phosphorylated IκBα and its subsequent degradation. OspG interferes with the degradation of IκBα and therefore delays NF-κB activation (Kim et al., 2005). Accordingly, inactivation of OspG increases the inflammatory response of infected tissue resulting in higher destruction of the intestinal epithelium as shown in the rabbit ligated ileal loop model (Kim et al., 2005).

- IpaH belongs to a family of 10 effectors that are encoded on the VP and the chromosome. IpaH translocates into the nucleus where it interacts with the mRNA splicing factor U2AF resulting in a decreased expression of the chemokine and cytokine genes. Furthermore, IpaH acts as an ubiquitin ligase that interacts with the pheromone response signalling, in *Saccharomyces cerevisiae*, which promotes the proteasome-dependent destruction of the MAPKK Ste7 (Rohde et al., 2007). Moreover, recent publications have showed that IpaH, through the E3 ligase activity, dampens the NF-κB-mediated inflammatory response. IpaH interacts with NEMO/IKKγ and ABIN-1, an ubiquitin-binding adaptor protein, promoting ABIN-1-

dependent polyubiquitylation of NEMO. Consequently, polyubiquitylated NEMO undergoes proteasome-dependent degradation, which affects NF-κB activation. Accordingly, deletion mutants of *ipaH* cause a more severe inflammation and greater proinflammatory cytokine production than the wild type *Shigella* does. In the murine model of pulmonary infection, an infection with an *ipaH* mutant results in a 30-fold decrease in bacterial colonization (Ashida et al., 2010; Okuda et al., 2005).

- OspF, a phosphothreonine lyase, translocates into the host cell nucleus where it shows a specific phosphatase activity. OspF dephosphorylates and therefore inactivates MAPKs, such as ERK1/2, JNK and p38. MAPKs inactivation prevents the phosphorylation of the serine 10 residue of histone H3 that is required for the transcription of a subset of NF-κB regulated genes (Arbibe et al., 2007; Li et al., 2007). This leads to a down-regulation of the inflammatory response. Accordingly, upon infection of rabbit ligated ileal loops (Arbibe et al., 2007) and in the murine model of pulmonary infection (Kramer et al., 2007), the *ospF* mutant causes a more severe mucosal destruction and induced an increased recruitment of PMN cells than the wild type strain does.

1.8.3. Resolution of primary infection

1.8.3.1. Recruitment of PMNs

PMN play a major role during a *Shigella* infection. Their recruitment to the site of infection causes the massive tissue destruction implicated with shigellosis. PMNs are the only known cell type that efficiently kills *Shigella*, therefore they ultimately play a major role in bacterial clearance and a subsequent resolution of an infection. One reason why *Shigella* is neutralised by PMNs is that the bacteria are unable to escape from the phagocytic vacuoles of PMNs, unlike in macrophages or epithelial cells. PMNs degrade the virulence factors that are necessary for *Shigella* escape from phagocytic vacuoles, thus enabling PMN to effectively kill the bacteria (Weinrauch et al., 2002). IL-8 and IL-1β are both upregulated during an infection. Further, IL-8 is a potent chemoattractant for PMN leading to the massive PMN recruitment, which is a key characteristic of shigellosis. Blocking of IL-1β at the early stage of infection leads to lower inflammation, reduced recruitment of PMNs to the site of infection and

therefore results in a reduced invasion of bacteria (Sansonetti et al., 1995). PMNs, even at the cost of epithelial destruction, actively participate in the protection against bacterial translocation, mucosal diffusion, and systemic dissemination. Blocking of IL-8 inhibits recruitment and tissue damage through the PMN but results in an increased transepithelial translocation of the bacteria, as well as an overgrowth in the lamina propria and an increased passage into the mesenteric blood (Sansonetti et al., 1999). However, it is actually paradoxical that such response does not lead to the immediate eradication of the invading pathogen. A combination of *in vitro* and *in vivo* experiments (Perdomo et al., 1994) have established another paradigm, also called "fatal attraction", in which the early inflammation serves as a "Trojan horse" for invasive *Shigella*. Inflammation disrupts the permeability of the epithelial barrier via induced transmigration of inflammatory cells, particularly PMN, thereby allowing access of bacteria to the basolateral pole of IECs, which is permissive to bacterial entry (Germani and Sansonetti, 2006).

1.8.3.2. NK cells and non specific T cells

The *Shigella* survival and particular bacterial invasion into IECs, leads to an activation of the innate immune system and creates a particular cytokine and chemokine environment, characterized by the production of IL-1β, IL-18, IL-8 and TNF-α. Interestingly, IFN-γ is significantly reduced during the acute stage compared to that during the convalescent stage of infection, suggesting that the down-regulation might be induced by the bacteria (Raqib et al., 1997). However, IFN-γ is essential for the control of a *Shigella* infection and the recovering from a primary infection. For example, experimental data has showed that IFN-γ activated macrophages were able to kill intracellular *Shigella* (Way et al., 1998). Experiments with the murine model of pulmonary infection demonstrated a recruitment of T and NK cells to the site of infection which are responsible for the local IFN-γ production (Le-Barillec et al., 2005). T cells are also recruited to the rectal mucosa of infected patients during the acute phase of shigellosis (Islam and Christensson, 2000). These data demonstrate that both T cells and NK cells contribute to the early control of *Shigella* infection through amplification of an inflammatory response.

1.8.3.3. Monocytes and activated macrophages

Compared to resident macrophages *Shigella* cannot escape phagocytic vacuoles of monocytes and therefore is killed by this cell type. However, despite early bacterial death, *Shigella* impacts the monocyte response to bacterial phagocytosis. Compared to wild type *Shigella* strains, non virulent strains induced high level production of the proinflammatory cytokines IL-1β, IL-6 and TNF-α and a necrotic cell death, whereas phagocytosis of a virulent strain lead to a suppression of proinflammatory cytokine production and an apoptotic cell death (Hathaway et al., 2002). Inhibition of cytokine secretion by monocytes may represent an important virulence mechanism by which *Shigella* controls the inflammatory response (Phalipon and Sansonetti, 2003).

1.8.4. Current model of the physiopathology of *Shigella* infection

After an oral ingestion *Shigella* reaches the colon and rectum. There, *Shigella* crosses the epithelial layer of the intestinal barrier via M cells (Sansonetti et al., 1999). Resident macrophages, in the microfold-cell pocket of M cells, immediately uptake the passing bacteria and, in turn, are rapidly killed by *Shigella.* Macrophage death leads to a massive release of the proinflammatory cytokines (Zychlinsky et al., 1992). From dead macrophages, released *Shigella* reach the basolateral pole of IEC, where they induce their uptake. Once internalized in IEC *Shigella* rapidly lyse the membrane of the phago-lysosomel vacuole. Then, free in the cytoplasm, *Shigella* rapidly multiply (Clerc and Sansonetti, 1987). Intracellular motility mechanisms enable the bacteria to move inside the host cell and to spread from cell to cell (Bernardini et al., 1989). The release of proinflammatory chemo- and cytokines of infected tissue leads to a massive recruitment of PMNs to the site of infection. The transmigration of the recruited PMN destabilises the epithelial barrier and promotes further invasion by luminal bacteria (Figure. 1.8.4.). Although the recruitment of PMNs leads to the massive tissue destruction typical for shigellosis, they account for bacterial clearance.

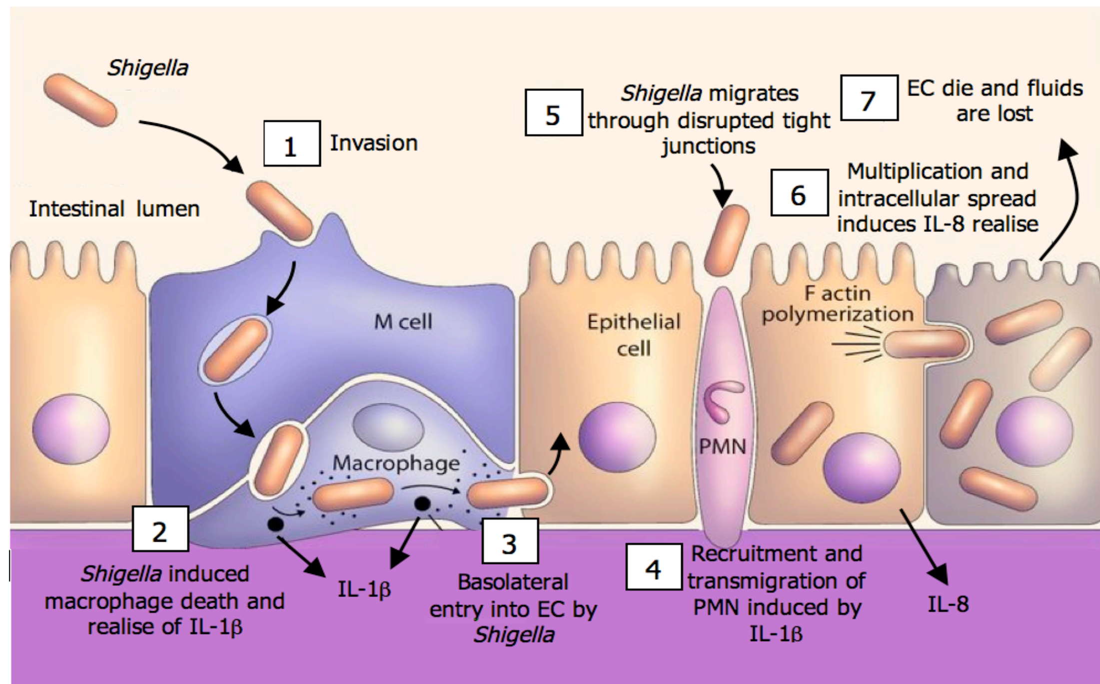


Figure 1.8.4: Simplified view of *Shigella* infection. *Shigella* crosses the epithelial layer via M cells. Resident macrophages are rapidly killed, then *Shigella* enters EC via the basolateral pole. Once free in the cytoplasm *Shigella* multiplies and spreads from cell to cell. Chemo- and cytokines release by infected tissue leads to PMN recruitment. Adapted from. "*Shigella* infection" Epidemiology of Infectious Diseases. © Johns Hopkins Bloomberg School of Public Health.

1.9. Adaptive immunity to *Shigella* infection

Shigella-specific immunity induced upon natural infection is characterised by the induction of a humoral response. Protective immunity arises only after several episodes of infection, is only of short duration, and seems to be poorly efficient in limiting re-infection, in particular in young children (Phalipon and Sansonetti, 2007). Cytokines and chemokines are key players in linking innate and adaptive immunity. The proinflammatory cytokine and chemokine environment that is induced during infection by Shigella (Pedron et al., 2003; Raqib et al., 1995; Trinchieri, 2003) is accompanied by the production of anti-inflammatory mediators such as IL-10 and TGF-β in order to limit tissue destruction and, therefore, avoid host death. However, IL-10 and TGF-β are potent immunosuppressive cytokines that impair the development of an efficient T_H1-type immunity (Taylor et al., 2006). Moreover, Shigella is able, via the expression of a type III-secreted bacterial effector, to directly inhibit the development of an IL-12/IFN-γ-mediated T_H1-type response (Gamelas-

Magalhaes, unpublished data). The acute inflammation induced by *Shigella* infection also causes a massive cell death in infected tissue. Indeed, apoptotic cell death, including macrophage, DC, T and B cells, in the lamina propria of infected patients is markedly upregulated at the acute stage of shigellosis (Raqib et al., 2002). Analyses of apoptotic cells in rabbit Peyer's patches revealed that about 40 % of T cells undergo apoptosis (Zychlinsky et al., 1996). Besides the direct effect of such massive cell death on immune cells, the impact of the acute inflammation on the development of the adaptive immunity is only poorly understood. So far, it is also not known if *Shigella* has developed abilities to directly impair T cell functions.

1.9.1. Humoral response

The humoral immune response against *Shigella* is characterised by the production of serum IgG and secretory IgA antibodies. Both are directed against LPS and some virulence plasmid encoded proteins (Jennison and Verma, 2004). The humoral immune response is the major component of protective immunity to shigellosis. Antibody mediated protection is species and serotype-specific, with no cross-protection against infection with *Shigella* strains from other species or serotypes, this further emphasises LPS as the major *Shigella* antigen. The overall importance of an antibody response to *Shigella* infection has been confirmed in a study which showed that the reduced and delayed humoral immune response of children in comparison to adult patients is the likely cause of the increased susceptibility of children to shigellosis (Raqib et al., 2002). The importance of anti-*Shigella*-LPS IgA was also seen in the protection of mice during an intranasal challenge with *Shigella* (Phalipon et al., 1995). Furthermore, anti-LPS IgG and IgA antibodies in the breast milk of mothers exposed to shigellosis appear to be responsible for a decreased severity of shigellosis in *Shigella*-infected infants (Clemens et al., 1986).

1.9.2. Cellular responses

1.9.2.1. Dendritic cells (DC)

DCs represent an important link between innate and adaptive immunity, as they are the main APCs. *Shigella* is able to down-regulate the expression of the chemokine CCL20 in IEC, resulting in a decreased recruitment of DCs to the lamina propria of infected tissue (Sperandio et al., 2008). Like in macrophages, infection of human monocyte-derived DCs also results in rapid IpaB-dependent cell death, followed by a release of IL-1β and IL-18. But, compared to macrophages, cytotoxicity can only be partially blocked by caspase-1 inhibitors, but is completely blocked with a pan-caspase inhibitor. This suggests a caspase-1-independent cytotoxic mechanism induced by *Shigella* in infected DCs. The rapid death of DCs during the early stages of shigellosis is likely to have adverse consequences for generation of an adaptive immunity (Hathaway et al., 2002; Raqib et al., 2002). It has been shown that the effector OspF could directly binds and dephosphorylates the MAPK kinases Erk ½ and p38 in DCs. This suggests that OspF could be, at least partially, involved in the apoptotic death of DCs and eventually resulting in the down-regulation of the host immune response (Kim et al., 2008). Furthermore, *Shigella* via OspF inhibits IL-12 production in DCs and therefore inhibits the induction of a T_H1 type environment (Gamelas-Magalhaes, unpublished data).

1.9.3. *Shigella*-specific T cell response

Shigella multiplies inside IECs and secretes effectors into the cytoplasm but it is also largely found in extracellular compartments. It is likely that this extracellular state of *Shigella* is responsible for the dominant role of humoral immunity observed in *Shigella*-infected hosts. However, even if *Shigella* is considered to be an intracellular pathogen with the ability to gain access to the cytosol of IECs and APCs, it has been impossible to demonstrate *in vivo* the priming of CD_8^+ T cells whereas CD_4^+ T cell priming has been reported (Sellge et al., 2010; Zwillich et al., 1989). It is hard to believe that *Shigella* did not evolve any mechanism to subvert lymphocyte function since T cell responses play an important role in the clearance of bacterial pathogens.

Indeed, priming of *Shigella*-specific T cell response and whether and how T cells contribute to protection remains still poor defined. PBMCs isolated from volunteers challenged with a *S. dysenteriae* 1 vaccine candidate have been shown to produce IFN-γ, but not IL-4 and IL-5, after Ag-specific stimulation (Samandari et al., 2000). Furthermore, an increased proportion of T cells expressing activation and memory markers and the expansion of defined TCR Vβ families have been reported in patients with natural infection (Islam et al., 1995; Islam et al., 1996). Recently, we have shown that *Shigella*-specific T_H17 T cells are the predominantly primed T cell subtype. *Shigella*-specific T_H1 T cells are only significantly induced upon secondary infection and specific T_H2 and CD_8^+ T cells are undetectable. The T_H17 T cells are primed in a MHC class II and IL-6-dependent, but in an IL-12/23p40-independent manner. The *Shigella*-specific T_H17 pool gives rise to an enhanced recall response up to 12 months after priming, suggesting the presence of a long-term memory state. The clearance of primary infection is impaired in the absence of T cells, but independently of IL-17A. However following re-infection, IL-17A produced by *Shigella*-specific T_H17 cells becomes important to ultimately restrict bacterial growth (Sellge et al., 2010).

1.10. Aim of the work

Shigella induces an acute inflammatory response leading to massive tissue destruction within a particular cytokine and chemokine environment. The innate immune response has been well studied. However, only little evidence indicates an impact of such an acute inflammation on the development of the adaptive immunity against *Shigella*. Nevertheless, to develop an adaptive immunity, several infections are required to mount a protective antibody response but this humoral protection is only of short-term duration. Whereas the humoral response has been investigated and is based on the production of anti-LPS-IgG and IgA antibodies, the cellular immune response against *Shigella* is only poorly understood. Our working hypothesis is that besides an indirect effect of the inflammatory response on the priming of the specific immunity, *Shigella* has the capacity to impair T cell functions by a direct cross-talk with these cells. Indeed, *Shigella* becomes in contact with T cells as soon as it crosses the intestinal barrier through M cells that are part of the epithelium covering the lymphoid follicles that are associated to the intestinal mucosa. These are also the inductive sites for the priming of local specific immune responses. In addition, several *Shigella* TTSA effectors target important signalling pathways in EC, they may target those pathways in cells of the adaptive immune response as well. It is therefore likely that *Shigella* has evolved strategies, thanks to the expression of those effectors, to manipulate the cells of the adaptive immunity it encounters after crossing the intestinal barrier.

This work aims a better understanding of *Shigella*-T cell cross-talks with the aspect of studying a direct interaction of *Shigella* with T lymphocytes.

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Bacterial strains

2.1.1.1. *Shigella* strains

Strain	Characteristics	Reference
M90T	*S. flexneri* 5a wild type strain	(Sansonetti et al., 1982)
SF 401	M90T-*ΔmxiD*	(Allaoui et al., 1993b)
SF 701	M90T-*ΔipgD*	(Allaoui et al., 1993a)
SF 623	M90T-*ΔipaA*	(Menard et al., 1993)
SF 620	M90T-*ΔipaB*	(Menard et al., 1993)
SF 621	M90T-*ΔipaC*	(Menard et al., 1993)
SBF1	M90T-*ΔipgB1*	(Hachani et al., 2008)
SF 945	M90T-*ΔicsB*	(Allaoui et al., 1992)
SC 560	M90T-*ΔicsA*	(Vasselon et al., 1991)
SF 1060	M90T-*ΔmxiE*	(Mavris et al., 2002)
PV 24e	M90T-*ΔospG*	Collection of the Unité PMM
SF 709	M90T-*ΔipgD*-pAB17	Collection of the Unité PMM
ΔospF	M90T-*ΔospF*	(Arbibe et al., 2007)
M90T-GFP	M90T-GFP	Collection of the Unité PMM
mxiD-GFP	M90T-*ΔmxiD* -GFP	Collection of the Unité PMM

2.1.1.2. *E. coli* strains

The *E. coli* strain DH5α, a derivate of *E. coli* K-12, was used for plasmid carrying.

2.1.2. Primary cells and cell lines

2.1.2.1. Primary cells

Human monocyte depleted Peripheral Blood Mononuclear Cells (PBMC) were provided by Daniel Scott-Algara, Unité des Régulations des Infections Rétrovirales, Institut Pasteur.

2.1.2.2. Cell lines

Jurkat, Clone E6-1 cells (ATCC® TIB-152™) were used as a human CD_4 T cell line (Schneider and Schwenk, 1977).

2.1.3. Antibodies

Antibody	Manufacturer
Anti-*Shigella*-LPS (SF5a-LPS)	Collection of the Unité PMM
Anti-human-CD28 (clone CD28.2)	Biolegend, Uithoorn, Netherlands
Anti-Phospho-ERM (#3141)	Cell Signal Technology, Danvers, USA
Anti-ERM (#3142)	Cell Signal Technology, Danvers, USA
Anti-Actin (A2006)	Sigma-Aldrich, Lyon, France
Anti-rabbit-Alexa Fluor® 647 (A21244)	Invitrogen, Cergy Pontoise, France
Anti-mouse-Cy3 (115-165-146)	Jackson Medicorp Inc, Montreal, Canada
Anti-rabbit-Alexa Fluor® 350 (A11046)	Invitrogen, Cergy Pontoise, France
Anti-rabbit GAR(IgG(H+L)/PO	Nordic Immunology, Tilburg, Netherlands

2.1.4. Plasmids

Plasmid	Characteristics	Reference
IpgD-GFP (pKN16)	GFP-tagged IpgD, Kan^R	(Niebuhr et al., 2002)
mut-GFP	GFP-tagged IpgD with Cys438 to Ser, Kan^R	(Niebuhr et al., 2002)
GFP	EGFP, Kan^R	(Niebuhr et al., 2002)
mRFP-PLCδ1PH	mRFP-tagged PH domain of PLCδ1, Amp^R	(van der Wal et al., 2001)

2.1.5. Kits

Kit	Manufacturer
Human CD_4^+ T cells isolation kit	Milteny Biotec, Bergisch Gladbach, Germany
Amaxa® Cell Line Nucleofector® Kit V	Lonza, Cologne, Germany
EndoFree Plasmid Maxi Kit	Quiagen, Courtaboeuf, France

2.1.6. Antibiotics

Antibiotic	Stock solution	Working concentrations	Manufacturer
Ampicillin	100 mg/ml (in H_2O)	100 µg/ml	Sigma-Aldrich, Lyon, France
Chloramphenicol	25 mg/ml (in EtOH)	25 µg/ml	Sigma-Aldrich, Lyon, France
Kanamycin	50 mg/ml (in H_2O)	50 µg/ml	Sigma-Aldrich, Lyon, France
Streptomycin	100 mg/ml (in H_2O)	100 µg/ml	Euromedex, Souffelweyersheim, France
Gentamicin	50 mg/ml (in H_2O)	50 µg/ml	Sigma-Aldrich, Lyon, France

2.1.7. Cell culture materials, media and additives

2.1.7.1. Cell culture materials

Material	Manufacturer
Round bottom 96 well plate	TPP, Trasadingen, Switzerland
12 well plates	TPP, Trasadingen, Switzerland
6 well plates	TPP, Trasadingen, Switzerland
Cell culture flask 75 cm^2	TPP, Trasadingen, Switzerland
Transwell® system (3421) 5,0 µm	Corning, Amsterdam, Netherlands
Coverslips 18 mm	Marienfeld, Lauda-Königshofen, Germany
Malassez chamber	Preciss, Strasbourg, France
Separation filters 30 µm	Milteny Biotec, Bergisch Gladbach, Germany

2.1.7.2. Cell culture media and additives

Material	Manufacturer
RPMI 1640 Gibco®	Invitrogen, Cergy Pontoise, France
Fetal Bovine Serum (FBS)	Biowest, Nuaillé, France
L-glutamine Gibco®	Invitrogen, Cergy Pontoise, France
Sodium pyrovate Gibco®	Invitrogen, Cergy Pontoise, France
HEPES-buffer Gibco®	Invitrogen, Cergy Pontoise, France
Pen Strep Gibco®	Invitrogen, Cergy Pontoise, France
CXCL12 (300-28)	Pepretech ,London, UK
D-PBS Gibco®	Invitrogen, Cergy Pontoise, France

2.1.8. Instruments

Hardware	Manufacturer
-20° Freezer	Liebherr, Colmar, France
Bacterial incubator	Jouan, St-Herblain, France
Bacterial shaker	Infors, Massy, France
Balance	Mettler Toledo, Viroflay, France
Cell culture hood	Thermo Scientific, Courtaboeuf, France
Cell culture incubator	Thermo Scientific, Courtaboeuf, France
Cell culture microscope	Leica, Nanterre, France
Centrifuge (3415)	Sigma-Aldrich, Lyon, France
Centrifuge (Sorvall RC5B)	Thermo Scientific, Courtaboeuf, France
Chemi-doc XRS	Bio-Rad, Marnes-la-Coquette, France
Confocal microscope (SP5)	Leica, Nanterre, France
Digital pH meter	VWR, Fontenay Sous Bois, France
Electrophoresis power supply	GE Healthcare, Saclay, France
FACSCalibur	Becton Dickinson, Le Pont-De-Claix, France
Fine balance	Mettler Toledo, Viroflay, France
Fridges	Liebherr, Colmar, France
Heat block	Falc, Treviglio, Italy
Inverted widefield microscope	Carl Zeiss, Nanterre, France
Multi-channel pipettes	Biohit, Bonnelles, France
Pipettes	Gilson, Middleton, USA
Spectrophotometer	Eppendorf, Paris, France
Table centrifuge	Eppendorf, Paris, France
Water bath	Thermo Scientific, Courtaboeuf, France

2.1.9. Chemical products and consumables

Chemical products, which are not listed below came from: Sigma-Aldrich (Lyon, France), Merck (Fontenay Sous Bois, France) or Prolab (Cheshire, UK). The consumables came from Becton Dickinson (Le Pont-De-Claix, France) Corning (Amsterdam, Netherlands) or Greiner (Courtaboeuf, France).

Product	Manufacturer
Acrylamide (30%)/Bisacrylamide (0.8%)	National diagnostics, Atlanta, USA
Trypticase	Becton Dickinson, Le Pont-De-Claix, France
Bacto-Agar	Becton Dickinson, Le Pont-De-Claix, France
Gel blotting paper (Whatman®)	Biometra, Goettingen, Germany
Nitrocellulose transfer membrane Protean pore size 0,45 µm	GE Healthcare, Saclay, France
ECL	Bio-Rad, Marnes-la-Coquette, France
Stripping buffer	Thermo Scientific, Courtaboeuf, France
Congo Red	Serva, Heidelberg, Germany

2.1.10. General buffers used

PBS (1x)

NaCl	137 mM
KCl	2.7 mM
Na_2HPO_4	10 mM
KH_2PO_4	2 mM
pH	7.4

TBS (1x)

Tris-HCl	20mM
NaCl	500mM
pH	7.4

TBS-T (1x)

Tris-HCl	20mM
NaCl	500mM
Tween 20	0.1%
pH	7.4

TE

Tris-HCl	10 mM
EDTA	1 mM
pH	8,0

2.1.11. Bacterial media used

Luria Bertrani broth

Yeast extract	5 g
Tryptone	10 g
Natriumchlorid	10g
H_2O qsp. 1 liter	

Trypticase soy broth

Commercial powder from Becton Dickinson Ref: 211768

- 30g
- H_2O qsp. 1l

Trypticase soy agar plates

Commercial powder from Becton Dickinson Ref: 236950

- 40g
- H_2O qsp. 1l

Congo red agar plates

Tryticase	3g	/100ml
Agar	1,5g	/100ml
Congo Red	0,01g	/100ml

H_2O qsp. 100 ml

2.2. Methods

2.2.1. Bacterial culture

Bacteria were grown at 37 °C for over night on Congo red agar plates. For infection, a Congo red positive *Shigella* colony was picked and inoculated into trypticase soy liquid medium and grown over night at 37 °C. Before infection of T cells, the bacteria were transferred into fresh trypticase soy liquid medium and grown to an OD_{600} = 0.6.

2.2.2. Bacterial concentration

Bacterial concentration was determined by measuring the optical density at a wavelength of 600 nm (OD_{600}). An OD_{600} of 1 corresponds to a concentration of 10^9 colony forming units (cfu) per ml.

2.2.3. Cell culture

Jurkat T cells, human PBMCs and human primary CD_4^+ T cells were cultured in RPMI medium supplemented with 10 % de-complemented foetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin. The cells were cultured in tissue culture flasks in an incubator at 37 °C with 5 % CO_2 and 100 % humidity. Tissue culture work was done under sterile conditions. Jurkat T cells were diluted every 3 days into fresh pre-warmed medium.

2.2.4. T Cell stimulation

T cells were stimulated by incubation for 3 days with 50 µg/ml phorbol 12-myristate 13-acetate (PMA). PMA stimulates T cells by a direct activation of protein kinase C (Kay, 1991).

2.2.5. Freezing and thawing of cells

For long term storage, Jurkat T cells were resuspended in RPMI with 10 % FCS and 10 % DMSO and aliquoted in 1 ml cryo-tubes. The cells were frozen for 24 - 72 h at -80 °C, before being placed in liquid nitrogen. When needed, the frozen cells were quickly thawed and resuspended in 75 ml tissue culture medium. The resuspended cells were centrifuged at 300 g for 10 min. The supernatant was discarded and the cells were subsequently resuspended in pre-warmed tissue culture media and further cultured in tissue culture flasks.

2.2.6. Cell counting

The cell concentration was determined by counting the cells under a light microscope using a Malassez counting chamber.

2.2.7. CD_4^+ T Cell isolation

Human CD_4^+ T cells were isolated from PBMCs by using the CD_4^+ T cells isolation kit from Milteny Biotec®. The human CD_4 T cells were isolated by depletion of non-CD_4^+ T cells (negative selection). Non-CD_4 cells were indirectly magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies, as the primary labelling reagent, and anti-biotin monoclonal antibodies conjugated to MicroBeads®, as the secondary labelling reagent. No washes were required in between both labelling steps. The magnetically labelled non-CD_4^+ cells were depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled CD_4^+ cells passed through the column. Cells were collected in a tube centrifuged for 10 min at 300 g and resuspended in RPMI medium containing 10 % FCS, 100 U/ml penicillin, 100 μg/ml streptomycin. Cells then were incubated as described above.

2.2.8. Statistical analysis

The t-test was used to assess whether the means of two groups were statistically different from each other. Two groups were assumed to be statistically different if the *p*-value was < 0.05. Statistically significant differences were indicated by asterisks: **p* < 0.05; ***p* < 0.01; ****p* < 0.001. The error bars represent the standard error of the mean (SEM).

2.2.9. T cell infection

For infection, T cells were washed in pre-warmed RPMI medium without FCS, then centrifugated for 10 min at 300 g. After counting T cells were seeded in round-bottomed 96 well plates at a concentration of 3×10^5 cells in 100 µl per well. The bacteria were also washed in RPMI medium without FCS after a centrifugation of 10 min at 4000 g. Depending on the MOI, bacteria were diluted in RPMI medium to a concentration of 6×10^7 bacteria/ml for a MOI of 10 or 6×10^8 bacteria/ml for a MOI of 100. T cells were infected by adding 50 µl of the corresponding bacteria solution to the cells and a following centrifugation step of 5 min at 300g. Immediately after centrifugation cells were incubated at 37 °C and 5 % CO_2. At desired time points the infection process was stopped by adding gentamicin at a final concentration of 50 µg/ml.

2.2.10. Cell transfection

Jurkat T cells were transfected by electroporation using the Amaxa® Cell Line Nucleofector® Kit V. The cells were electroporated with 10 µg DNA / 5×10^6 cells using the Nucleofector® Device electroporator with the Nucleofector® Program X-005. After electroporation, cells were incubated in complete medium (RPMI medium containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate and 10 % fetal bovine serum) overnight at 37°C.

2.2.11. Fluorescence activated cell sorter (FACS)

FACS is a powerful tool for the analysis, characterisation, and quantification of cell properties on a single cell level. A cell suspension is entrained in the centre of a narrow, rapidly flowing stream of liquid, which passes through a beam of light followed by an electronic detection apparatus where the fluorescent characteristic of each cell is measured. The properties measured include relative cell size and relative cell granularity, in addition to surface molecules or intracellular constituents stained with fluorescent dyes or labelled antibodies.

2.2.12. Gentamicin assay

To identify and quantify bacterial invasion into T cells a gentamicin assay was used. Gentamicin is an antibiotic that is not able to cross the cytoplasmic membrane of eukaryotic cells and therefore is not able to kill intracellular bacteria. T cells were cultured and infected as described above. Gentamicin was added 60 min after infection. At indicated time points cells were lysed with 0.5 % sodium desoxycholate. Dilutions of the final suspensions were made and plated on trypticase soy agar. Enumeration of the bacteria was performed after overnight incubation at 37 °C.

2.2.13. Quantification and characterisation of T cell death

It has been shown that the fluorescent dye propidium iodide (PI) enters exclusively damaged cells (Macklis and Madison, 1990). Therefore, PI was used to quantify cell death in infected T cells by FACS. T cells were grown and infected as described above. At various time points infected samples were transferred into FACS tubes and stored on ice. To quantify cell death samples were incubated for 1 min with PI (dilution 1:1000). The PI-positive cell population was then detected by FACS. For the characterisation of the cell death, cells were, in addition to PI, also stained with the apoptosis marker Annexin V for 15 min in the dark. Obtained data were evaluated by using FlowJoe® software.

2.2.14. Immunofluorescence staining for intracellular bacteria

Immunofluorescence staining (IF) was used to detect intracellular bacteria. T cells were grown and infected as described above, with the exception of the use GFP-expressing bacteria. One h after infection, T cells were fixed for 15 min with fresh 4% paraformaldehyde (PFA) in PBS, directly in round-bottomed 96 well plates. Samples were transferred on glass coverslips, precoated over night with 10 µg/ml poly-L-lysine at 4 °C, and centrifuged for 1 min at 300 g. The cells were then washed twice with PBS. Samples were first stained for 30 min with an anti-*Shigella*-LPS antibody (antibody dilution 1:100 in PBS) and then washed 3 times with PBS. The T cells were further incubated for 30 min with a corresponding fluorochrome-conjugated secondary antibody solution (antibody dilution 1:100 in PBS) followed by 3 times washing with PBS. The cells were permeabilised in 0.1 % Triton-100 for 5 min followed by 3 times washing with PBS. Cells were then incubated for 30 min with phalloidine-rhodamine followed by 3 times washing with PBS. The coverslips were mounted using Prolong-mounting medium. All incubation times were done in the dark and at room temperature. Immunofluorescence pictures were acquired either by inverted widefield (Carl Zeiss® Inc.) or a confocal (SP5, Leica®) microscopy under oil immersion. Images were captured and stored as LIF files.

2.2.15. Induction of polarisation and IF staining for polarised T cells

Transfected Jurkat T cells were starved for 1 h in serum free RPMI medium. 500 µl containing 5 x 10^5 cells were then transferred into a well of a 12-well plate with a coverslip, precoated with poly-L-lysine, at the bottom. Cells were allowed to settle for 20 min at 37 °C. To induce polarisation, SDF-1 at a final concentration of 200 ng/ml was added to into each well for 45 s at 37 °C and 5 % CO_2. Cells were fixed by the addition of ice-cold 4 % PFA after 15 min and samples were washed twice with PBS. Samples were blocked for 10 min with PBS with BSA (1 mg/ml). Samples were then incubated for 1 h with a primary anti-CD28 antibody (diluted 1:100 in PBS-BSA) and then washed 3 times in PBS. Samples were then incubated with a Cy3-conjugated secondary antibody (diluted 1:100 in PBS-BSA) followed by 3 times washing in PBS.

Cells were permeabilised for 5 min with 0.1 % Triton X-100. After permeabilisation samples were again blocked for 10 min with PBS-BSA. Primary anti-phospoERM antibodies were added for 1 h (diluted 1:100 in PBS-BSA) followed by 3 times rinsing in PBS. The Alexa 647-conjugated secondary (diluted 1:100 in PBS-BSA) was added for 1 h antibody followed by 3 rinses in PBS. Coverslips were mounted on glass slides using ProLong® mounting medium. All incubation times were done in the dark and at RT. Samples were examined by using confocal microscopy (SP5, Leica®).

2.2.16. Migration assay

Jurkat T cells were serum-starved in migration medium (RPMI containing 1 % BSA, 10 mM HEPES buffer, pH 6.9) for 3 h. Cells were then infected with bacteria for 30 min. followed by a washing step with migration medium with gentamicin (50 µg/ml) Migration assays were performed in transwell chambers with 5 µm polycarbonate membrane precoated with 20 µg/ml fibronectin on both sides of the filter over night at 4 °C. Human CXCL12 was diluted to a concentrations of 100 nm/ml in migration medium with gentamicin and added to the lower chamber of the transwells. Medium alone was added to cells left unstimulated. The membranes were placed on top, and 5 x 10^5 serum-starved cells were loaded into the upper chamber in 100 µl migration medium. The cells were allowed to migrate for 2.5 h at 37 °C in 5 % CO_2, and migrated cells were collected, counted, pelleted, and resuspended in ice-cold PBS for FACS analysis. Migration assay with transfected Jurkat T cell was performed as described above with the exception that the polycarbonate membranes were not precoated with fibronectin and gentamicin was not added to the migration medium. The migration index was calculated by dividing the number of migrated cells by the number of migrated cells without stimulation. For the migration assay with transfected cells only GFP-expressing cells were taken into account.

2.2.17. Phospholipid analysis

High-performance liquid chromatography or high-pressure liquid chromatography, (HPLC) is a form of column chromatography and is used to identify, and quantify compounds based on their idiosyncratic polarities and interactions with the column's stationary phase. Jurkat T cells were incubated in phosphate-free minimal essential medium (MEM) containing 1 mCi [^{32}P] orthophosphate/dish (200 mCi/ml) for 6 h before infection in order to reach the isotopic equilibrium. Cells were then infected for 30 min as described above. Lipids from infected cells were extracted, separated on thin-layer chromatography (TLC), scraped off, deacylated and analysed by HPLC. [^{32}P]-lipids separated by HPLC were identified using standards.

2.2.18. Preparation of whole cell lysates (WCL)

To obtain WCL the infected Jurkat T cells were centrifuged for 5 min at 300 g. Cells were lysed inside the round-bottomed 96 well plates with denaturing sample buffer (2x denaturing sample buffer contains 50 mM Tris-HCL pH 6.8, 5 % (v/v) 2-mercaptoethanol, 0,005 % (w/v) bromphenol blue, 4 % (w/v) SDS and 20 % (v/v) glycerol). To obtain WCL from transfected Jurkat T cells 10^6 cells were washed in cold PBS before being resuspended in 100 µl of denaturing sample buffer. Then cells extracts were heated at 100 °C for 10 min. The WCLs were then stored at -20 °C.

2.2.19. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is the method used to separate proteins by mass under denaturing conditions. Sodium dodecyl sulfate (SDS) is an anionic detergent that disrupts nearly all noncovalent interactions in native proteins and applies a negative charge to each protein. WCLs were loaded on polyacrylamide gels that are composed of two layers: a large pore polyacrylamide gel (5 %) stacking gel (pH 6.8) which insures a simultaneous entry of the proteins into the resolving gel and a (8 – 14 % acrylamide monomer) resolving gel (pH 8.8) which separates the proteins according to their size.

Running buffer 10X		Resolving gel buffer		Stacking gel buffer	
30,3 g	Tris	181,71 g	Tris	30,3 g	Tris
144,1 g	Glycine	4 g	SDS	2 g	SDS
10 g	SDS	H_2O qsp. 1 l		H_2O qsp. 500 ml	
H_2O qsp. 1 l		adjust pH 8,8		adjust pH 6,8	

Stacking gel buffer	Resolving gel buffer (8% Acrylamide)
4,5 ml H_2O	2,8 ml H_2O
650 µl Acrylamide (40 %)	1,9 µl Acrylamide (40 %)
1,25 ml Stacking gel buffer	5 ml Resolving gel buffer
12.5 µl TEMED	25 µl TEMED
25 µl APS	50 µl APS

2.2.20. Immunoblotting

The Western blot (alternatively, immunoblot) is a method used to detect specific proteins in a given sample of tissue homogenate or extract using antibodies specific to the target protein. For this purpose SDS-PAGE gels were electroblotted at 100 mA (for one gel) for 1 h to nitrocellulose transfer membrane using a "semi dry Blot system". Therefore, the gel was assembled in direct contact with the nitrocellulose membrane flanked by gel blotting papers wetted with transfer buffer. This "sandwich" was placed between two graphite electrode plates. To check the efficiency of the transfer, a Ponceau S fixative dye solution was used to stain the membranes which were washed afterwards with deionised water. For Western blot analysis the membranes were incubated in blocking buffer for 1 h at RT or over night at 4 °C on a shaker. Subsequently the membranes were transferred into a 50 ml Falcon® tube and incubated for 1 h at RT or over night at 4 °C on a tube rolling advice with an appropriate dilution of the primary antibody in TBS/BSA 0.5 %

directed against the protein of interest. The membranes were washed three times with for 10 min with washing buffer. The appropriate peroxidase-conjugated (HRP) secondary antibody was diluted in TBS/BSA 0.5%, and added to the membrane. The nitrocellulose membranes were subsequently incubated for 1 h at RT on a shaker. After washing the membranes three times with washing buffer, each time for 10 min the antigen-antibody complexes were detected using the BioRad® Substrate ECL kit and autoradiography according to the manufacturer's instructions.

Transfer buffer	Blocking buffer		Washing buffer	
25 mM Tris-HCL pH 6,8	1x	TBS	1x	TBS
192 mM Glycine	0,1% (v/v)	Tween 20	0,1% (v/v)	Tween 20
20% (v/v) Methanol	5% (w/v)	skimmed milk powder		

2.2.21. Immunoblot stripping

The removal of primary and secondary antibodies from a membrane is possible, so that the membrane can be relabelled with alternative antibodies. The nitrocellulose membrane was firstly incubated in distilled H_2O for 10 min. Then the membrane was incubated in BioRad® stripping buffer for 30 min followed by an additional 10 min incubation in distilled H_2O. After stripping the membrane should be incubated in blocking buffer for 1 h at RT or overnight at 4 °C on a shaker. Then the membrane can be re-probed as described above.

2.2.22. Plasmid DNA Purification

E. coli DH5α strains containing the eukaryotic expression vectors were grown in LB-medium, containing the corresponding antibiotic, over night at 37 °C. The purification of the plasmidic DNA, used for the transfection, was performed by using the EndoFree Plasmid Maxi Kit® from Quiagen. Purification protocol was performed according to the manufacturer's instructions.

Results

3. Results

3.1. *Shigella* induces T cell death

Shigella is able to induce cell death in macrophages and dendritic cells (Hathaway et al., 2002; Zychlinsky et al., 1992). To investigate whether *Shigella* has an impact on T cell survival, we infected Jurkat T cells, a human T cell line, with either the *Shigella* wild type strain M90T or with the non-invasive *Shigella* mutant ΔmxiD. The infection was stopped after 1h by adding gentamicine and propidium iodide (PI) was used to monitor cell death; the percentage of dead cells was then analyzed by fluorescence activated cell sorting (FACS). We observed that upon contact with the wild type strain M90T, but not with the non-invasive mutant ΔmxiD, Jurkat cells showed a significant increase in cell death that was dependent on the multiplicity of infection (MOI). An average of 10 % of cell death was detected for the MOI of 10 (10 bacteria for 1 Jurkat cell) and of 30 % for the MOI 100 (100 bacteria for 1 Jurkat cell) at 4h post-infection (Figure 3.1. A). The kinetics of the cell death was then analysed for the MOI of 100 from 1 – 4 h. *Shigella*-induced cell death started from 2h post infection and then reached a plateau at 4 h post infection (Figure 3.1. B). To characterise the nature of the *Shigella*-induced cell the markers Annexin V and PI were used. As necrotic or late apoptotic cells are positive for both markers, only a combination of these markers and kinetics gives the possibility to discriminate whether an apoptotic or a necrotic cell death occurs. Upon an infection of Jurkat T cells with the wild type *Shigella* strain M90T with a MOI of 100 for 1 to 4 h, we observed that the cells undergo a necrotic cell death, as we could only measure an increase in PI or PI/Annexin V positive cells but not in only Annexin V positive cells at any time point (Figure 3.1. C).

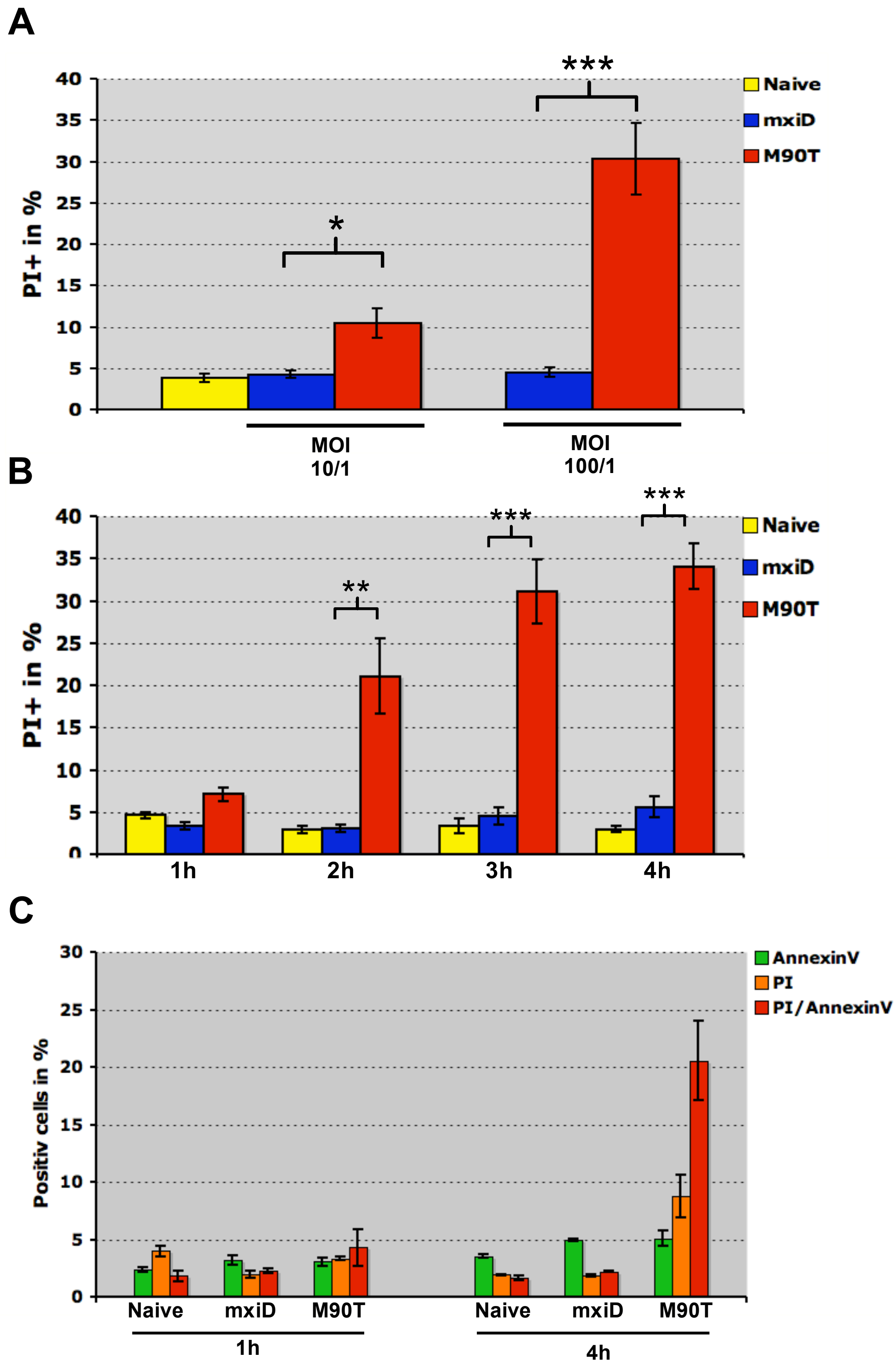


Figure 3.1. *Shigella* induces cell death in Jurkat cells.

Jurkat T cells were infected with the wild type *Shigella* strain M90T or the non-invasive mutant ΔmxiD. **(A)** *Shigella* induced cell death observed at 4 h post infection. **(B)** Kinetics of the induced cell death after an infection with a MOI of 100. **(C)** Annexin V and PI staining of Jurkat T cells infected with a MOI of 100.

3.2. *Shigella*-induced cell death is TTS dependent

A functional type III secretion apparatus (TTSA) is necessary to induce cell death in macrophages and dendritic cells (Hathaway et al., 2002; Zychlinsky et al., 1992). To investigate the requirement of a functional TTSA for the observed cell death, Jurkat T cell were infected with the wild type strain M90T and with the *Shigella* mutants impaired in the functionality of the TTSA. The *Shigella* mutant *ΔmxiD* does not assemble a complete needle. The translocon mutants *ΔipaB* and *ΔipaC* are unable to inject effectors into the host cell cytoplasm. The mutant *ΔipaB* is hyper secretive whereas secretion in the mutant *ΔipaC* is blocked. The mutant *ΔmxiE* does not secrete the mxiE-regulated effectors. The other mutants tested are the single effector mutants *ΔipaA, ΔospF, ΔospG, ΔicsB, ΔicsA, ΔipgD* and *ΔipgB1*. Jurkat T cells were infected with a MOI of 100 and PI-positive cells were analysed at 4 h post infection by FACS. The Jurkat T cells undergo cell death when infected with either M90T or with mutants, which are still endowed with a complete and functional TTSA. Indeed the mutants *ΔmxiE, ΔospF, ΔospG, ΔicsB, ΔicsA, ΔipgD* and *ΔipgB1* showed a similar level of cell death as compared to M90T. The mutant *ΔipaA* showed a 3-fold reduction of cell death compared to M90T but was still significantly higher as compared to *ΔmxiD*. *ΔipaB* and *ΔipaC* did not show an increased rate of cell death compared to *ΔmxiD* (Figure 3.2.).

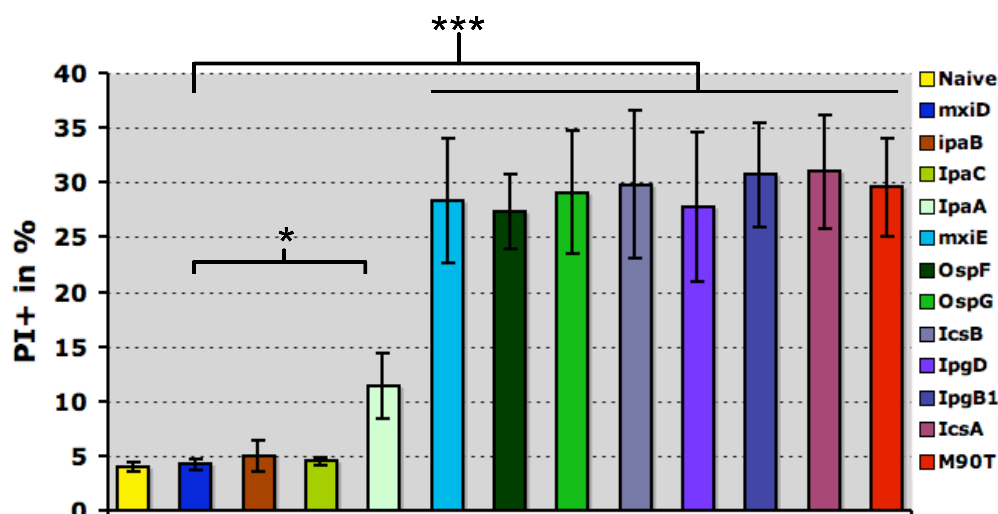


Figure 3.2. *Shigella*-induced cell death in Jurkat T cells is TTSA dependent.
Jurkat T cells infected with the wild type *Shigella* strain M90T or different TTSA mutants.

3.3. *Shigella* induced T cell death is favoured by an intimate contact of the bacteria with the cells

As the *Shigella* induced Jurkat T cell death is dependent on a functional TTSA, we investigated if it would also depend on a direct contact of the bacteria with the cells. To monitor a contact of the bacteria with the Jurkat T cells we infected the cells with either M90T or ΔmxiD expressing a green fluorescent protein (GFP). GFP positive cells together with the dead cell marker PI was then analysed by FACS. Cells were infected with the MOIs of 10 and 100 for 1 to 4 h and samples were taken each hour. In cells infected with *ΔmxiD*-GFP with a MOI of 100 we observed one population in which all cells were GFP positive whereas the one infected with an MOI of 10 showed a GFP negative and a GFP positive population after 1 h of infection. Surprisingly in cells infected with the wild type strain M90T-GFP the appearance of an additional population of GFP positive cells appeared which was higher in intensity than in the one infected with *ΔmxiD*-GFP. The GFP high positive population resembled 30% of the cells infected with the MOI of 100 and 10 % with the MOI of 10. While the GFP intensity in cells infected with M90T-GFP with the MOI of 100 did not change during time, the intensity of the GFP high positive cell population in cells infected with M90T-GFP with the MOI of 10 still increased with time. Interestingly, the GFP high positive cell population in with M90T-GFP infected cells favoured to undergo cell death as indicated by the increase of the PI positive cell staining in this population. Furthermore, as the infection was stopped after 1 h, the GFP high positive cell population was constant over time and only started to decrease at 4 h post infection in the samples infected with the MOI of 100 (Figure 3.3.).

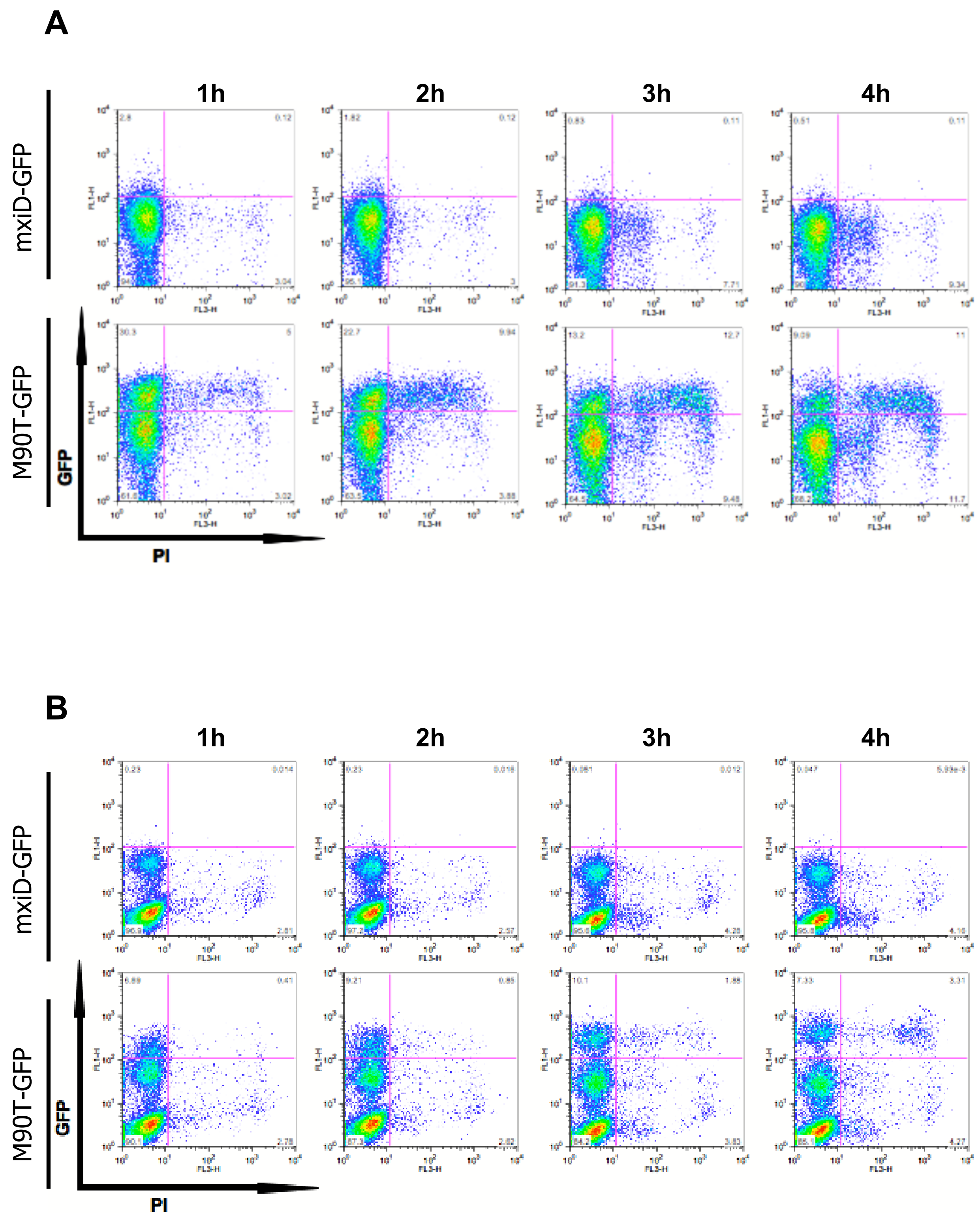


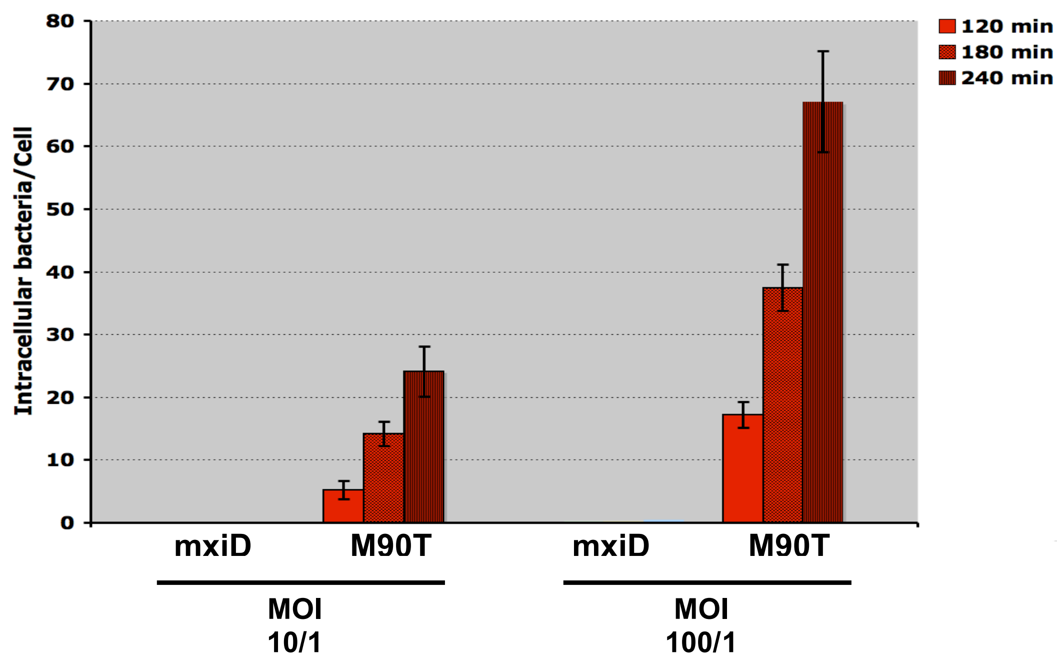
Figure 3.3. *Shigella* induced T cell death is favoured by an intimate contact of the bacteria with the cells

Jurkat cells infected with the wild type *Shigella* strain M90T-GFP or ΔmxiD-GFP with a MOI of 100 **(A)** and 10 **(B)**.

3.4. *Shigella* invades T cells

Next we investigated the ability of *Shigella* to invade T cells, as we wondered if the observed GFP-high positive cell population in Jurkat T cells infected with M90T-GFP could be due to intracellular *Shigella*. Two different approaches were used: 1. A gentamicin assay in which extracellular but not intracellular bacteria are killed upon adding gentamicin, an antibiotic that is not able to cross the plasma membrane of eukaryotic cells. Jurkat T cells were infected with the wild type strain M90T and the non-invasive mutant *ΔmxiD*. For the infection two different MOIs were used, the MOI of 100 and the MOI of 10. We observed that the *Shigella* wild type strain M90T was able to invade Jurkat T cells, but not the mutant *ΔmxiD*. We further could show that the wild type strain M90T was not only able to invade Jurkat T cells it was also able to proliferate inside those cells, as seen with the increase in the number of intracellular bacteria per living cell over time (Figure 3.4. A). To calculate the ratio between bacteria and living cells, FACS in combination with a PI staining was used to discriminate dead from living cells. 2. Immunofluorescent staining was used to visualise intracellular *Shigella*. For this, Jurkat T cells were infected with GFP-expressing *Shigella* strains. Samples were stained with an anti-Shigella LPS antibody to visualise extracellular bacteria. We monitored intracellular M90T inside the Jurkat T cells but not the non-invasive mutant ΔmxiD 1 h after infection (Figure 3.4. B). By counting Jurkat T cells with containing intracellular bacteria, we further showed that an average of 90 % of the cells infected with M90T-GFP at a MOI of 100 harboured intracellular bacteria whereas only 15 % of cells infected with the MOI of 10 did.

A



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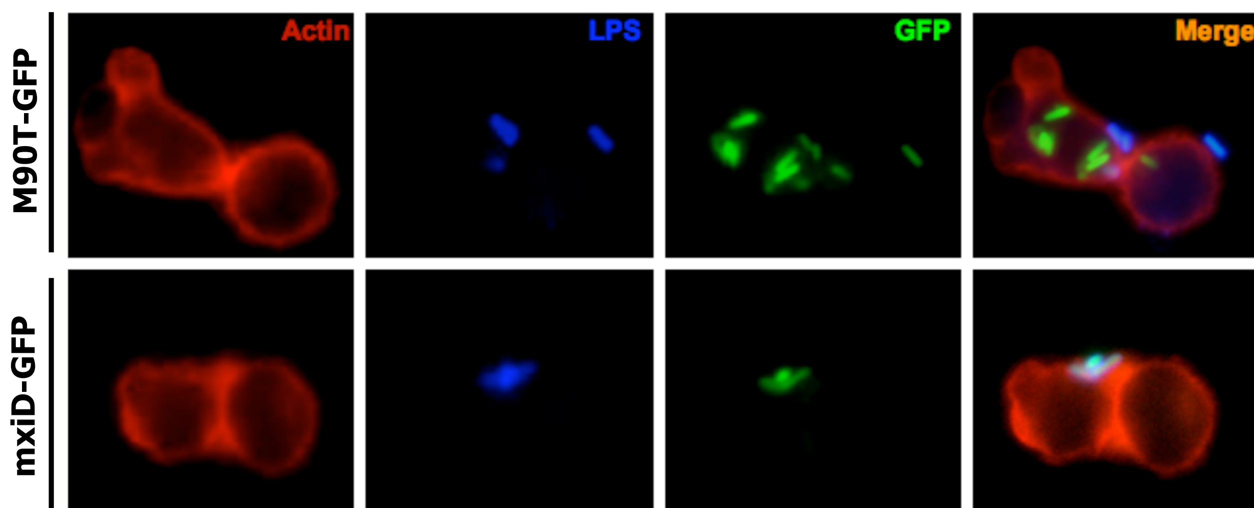

Figure 3.4. *Shigella* invades Jurkat T cells.

Jurkat cells infected with the wild type *Shigella* strain M90T or the mutant *ΔmxiD* **(A)** Gentamicine assay shows that increasing cfu counts M90T invades and proliferates inside Jurkat T cells. **(B)** Immunofluorescent staining of Jurkat T cells infected with a MOI of 100. After 1 h cells were fixed and stained for actin (red) extracellular bacteria (blue) and total bacteria (green).

3.5. *Shigella* inhibits T cell migration

As *Shigella* invades Jurkat T cells we investigated if this could have an impact on T cell function. A critical aspect of T cell function is migration; indeed lymphocytes are among the most motile cells (Kehrl, 2006). We therefore analysed whether T cell migration was impaired upon contact of T cells with *Shigella*. Jurkat T cells express the CXCR4 chemokine receptor (Peacock and Jirik, 1999) and migrate rapidly in response to a gradient of the CXCR4 ligand CXCL12. A classical migration assay was performed using a trans-well-system (Ottoson et al., 2001). In order to avoid a different amount of cell death between Jurkat T cells infected with the wild type strain M90T and the mutant *ΔmxiD*, we modified our infection protocol by reducing the time of infection to 30 min and by using only the low MOI of 10. Through these modifications no difference in cell death was observed between wild type and mutant during the time of the experiment (data not shown). Jurkat T cell migration was analysed by counting the T cells in the lower well of a trans-well-system that had migrated towards the CXCL12 added into the lower chamber. We showed that Jurkat T cells, infected with the wild type strain M90T, displayed a significant reduction of 30 % in migration; this was not observed with the TTSA mutant *ΔmxiD* (Figure 3.5. A). This reduced migratory response was not due to a difference in the chemokine receptor CXCR4 expression at the surface of the cells, as no difference in the expression level was measured (data not shown). By using the M90T-GFP strain in combination with analysing the two wells of the trans-well-system by FACS and immunofluorescent staining, we observed that the GFP high positive Jurkat T cell population resembles highly infected cells. Further, this cell population seen in the upper well was not detected in the lower well (Figure 3.5. B). Moreover, no intracellular bacteria were found inside migrating T cells. Interestingly, although a reduction of 30 % in migration was observed, only 10 % of the non migrated cells contained intracellular bacteria. We detected that ≈ 5 % of the infected cells had ≥ 4 intracellular bacteria and that ≈ 5 % had only 1 - 2 intracellular bacteria.

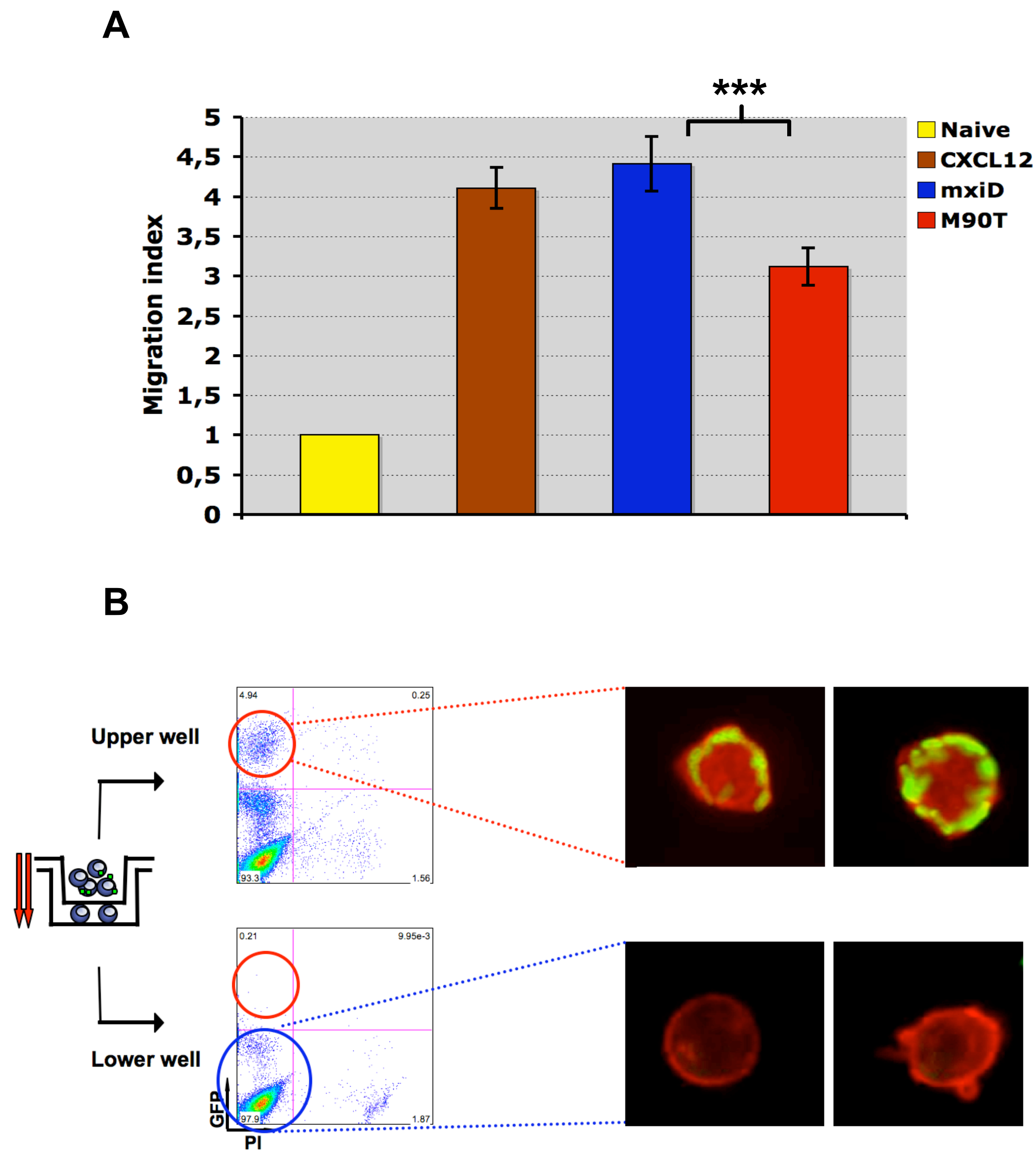

Figure 3.5. *Shigella* inhibits Jurkat T cell migration.

(A) Migration assay: Jurkat T cells infected with the wild type *Shigella* strain M90T (yellow): not infected, without chemokine; CXCL12 (brown): not infected, with chemokine; mxiD (blue): infected with ΔmxiD; M90T (red): infected with M90T. **(B)** Upper and lower wells of a trans-well-system analysed by FACS and immunofluorescence staining after infection of Jurkat T cells with M90T-GFP with a MOI of 10. GFP-high positive cells only found in the upper well (red circle).

3.6. *Shigella*-induced inhibition of T cell migration is dependent on the effector IpgD

To identify the *Shigella* effector(s) responsible for the inhibition of T cell migration, different mutants were tested in the migration assay. We identified one single effector mutant that did not show any inhibition in T cell migration after infection. This single TTS effector mutant did not express the TTS effector IpgD and induced a migration index equivalent to the one induced by the *ΔmxiD* mutant. Infection of Jurkat T cells with a *ΔipgD* mutant complemented with a plasmid expressing IpgD, showed a migration index like cells infected with the wild type M90T (Figure 3.6. A). The effector IpgD is a phosphoinositide 4-phosphatase that specifically dephosphorylates phosphatidylinositol-(4,5)-bisphosphate (PIP2) into phosphatidylinositol-(5)-monophosphate (PI(5)P). In ECs IpgD is responsible for dramatic morphological changes of the host cell, leading to a decrease in membrane tether force associated with membrane blebbing and actin filament remodelling. Although involved in the formation of the fully structured entry sides, IpgD is not involved in invasion (Niebuhr et al., 2002). To investigate if the effector IpgD had the capacity to inhibit migration alone or in combination with other effectors, we transfected Jurkat T cells with different constructs, expressing either IpgD-GFP, mut-GFP or GFP alone. Whereas IpgD-GFP transfected cells express a fully enzymatically active IpgD, mut-GFP transfected cells express, through a point mutation in the active site, only an enzymatic dead form of IpgD. The transfection efficiency, analysed by FACS, was of about 30 – 40 %, taking only GFP-positive and PI negative cells into account. The migration assay with the transfected Jurkat T cells showed a 50 % reduction in migration of cells transfected with IpgD-GFP as compared with cells transfected with mut-GFP. No significant differences in migration could be observed between GFP and mut-GFP transfected cells (Figure 3.6. B). The migration assay with transfected cells was analysed by FACS in combination with cell-counting of migrated T cells. For the calculation of the migration index only GFP-positive and PI-negative cells were taken in account.

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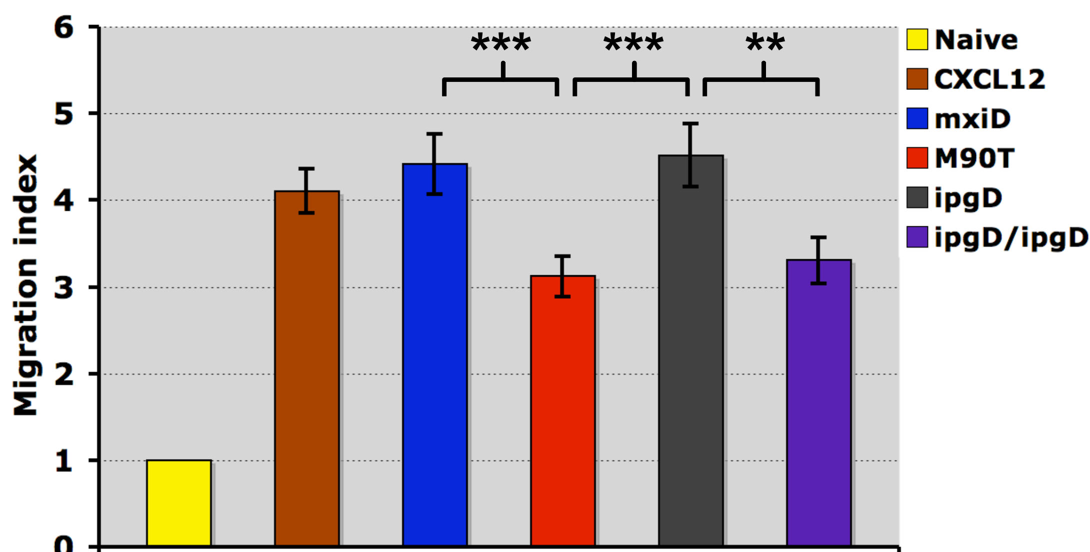

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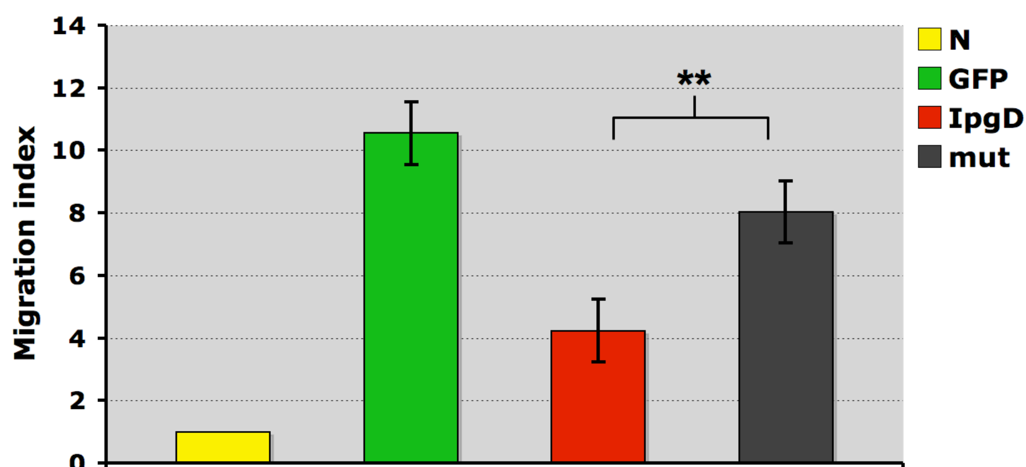


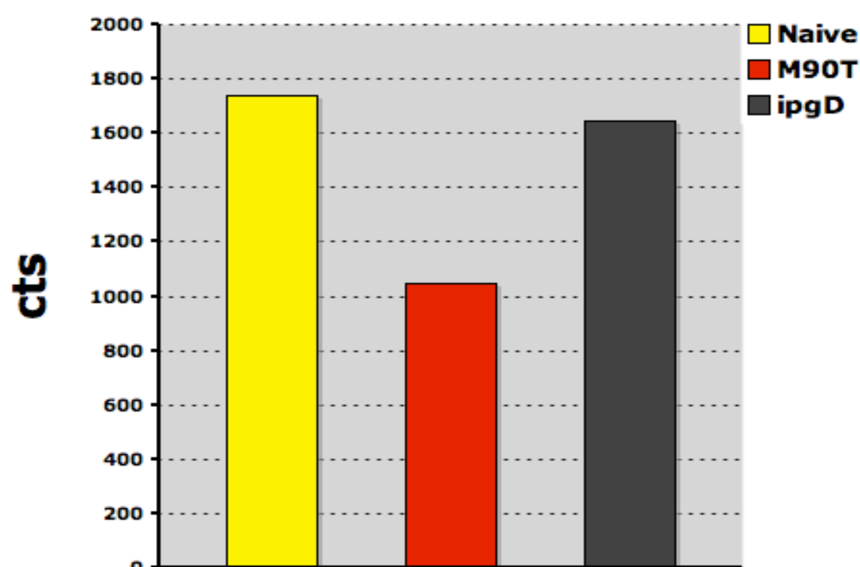
Figure 3.6. The TTS effector IpgD is responsible for the reduced T cell migration.

(A) Migration assay of Jurkat T cells infected with the wild type strain M90T, *ΔmxiD, ΔIpgD* or with the complemented *ΔipgD*. Naive (yellow): not infected, without chemokine; CXCL12 (brown): not infected, with chemokine; mxiD (blue): infected with *ΔmxiD*; M90T (red): infected with M90T. *ipgD* (dark grey): infected with *ΔipgD*. ipgD/ipgD (purple): infected with complemented *ΔIpgD*. **(B)** Migration assay of Jurkat cells transfected with either IpgD-GFP (red), mut-GFP (dark grey) or GFP (green). Naive cells (yellow) not transfected without chemokine.

3.7. IpgD hydrolyses PIP2 in Jurkat T cells upon infection with *Shigella*

Phosphoinositides have been implicated in regulation of cell polarity, motility and chemotaxis (Skwarek and Boulianne, 2009). IpgD is translocated directly into the host cell, where it functions as a phosphoinositide phosphatase that dephosphorylates PIP2 to generate PI(5)P (Niebuhr et al., 2002). Therefore, we investigated whether IpgD targets PIP2 in Jurkat T cells as it does in HeLa cells. Jurkat T cells were labelled with $^{32}P_i$ and infected with the wild-type strain M90T or the mutant *ΔipgD*. Samples were then analysed by HPLC to determine the level of PIP2 in the labelled and infected Jurkat T cells. The results showed that as soon as 30 min after contact between bacteria and host cells, the cellular [^{32}P]PIP2 level dropped dramatically in cells infected with the wild type strain M90T, as compared to non infected cells, but not in cells infected with the *ΔipgD* mutant (Figure 3.7. A). This demonstrates that IpgD also hydrolyses PIP2 in Jurkat T cells. We went on to monitor the effect of IpgD on the PIP2 level in the plasma membrane. To do so Jurkat T cells were co-transfected with either the IpgD-GFP or mut-GFP with a PLCδ1PH-RFP construct. The PLCδ1PH-RFP construct expresses the PH domain of phospholipase Cδ (PLC) that specifically binds to PIP2. Only after binding of PIP2 PLCδ1PH-RFP is able to interact with the plasma membrane and therefore can be used to detect PIP2 at the plasma membrane (Varnai and Balla, 1998). The double transfected cells were then analysed by confocal microscopy. The localisation of PLCδ1PH-RFP in cells transfected with mut-GFP was similar to that of the control. In contrast to cells transfected with IpgD-GFP, the localisation of PLCδ1PH-RFP at the plasma membrane was lost (Figure 3.7. B). The hydrolysis of PIP2 mediated by IpgD leads to a decrease of PIP2 in the plasma membrane.

A



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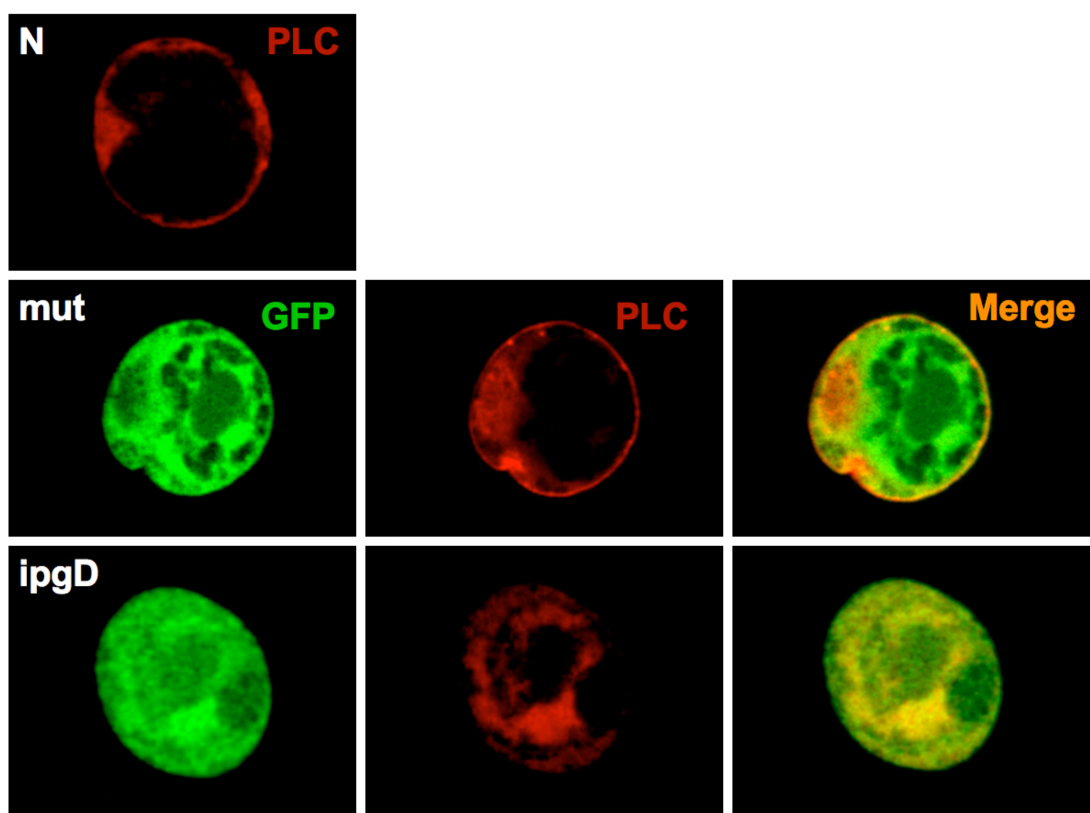


Figure 3.7. IpgD hydrolyses PIP2 in Jurkat T cells.

(A) Quantification of [^{32}P]PIP2 levels in infected Jurkat T cells. Naive (yellow): not infected; M90T (red) infected with M90T. ipgD (dark grey): infected with ΔipgD. **(B)** IpgD mediated hydrolysis of PIP2leads to decrease of the pool of PIP2 at the plasma membrane. Jurkat T cells transfected with: (N): PLCδ1PH-RFP only; (mut): PLCδ1PHGFP and mut-GFP; (ipgD) PLCδ1PHGFP and IpgD-GFP.

3.8. IpgD is responsible for ERM dephosphorylation in infected cells

A protein family which activity is closely linked to PIP2 is the ERM protein family. The ERM proteins connect the actin cytoskeleton with the plasma membrane, but only activated ERM proteins are able to do so (Charrin and Alcover, 2006). Moreover, the ERM protein activation is modulated during T cell polarization and migration, as, for example, ERM proteins get rapidly dephosphorylated upon contact with chemokines or adhesins. Activation of the ERM proteins occurs through two steps, first by binding of PIP2 and second by phosphorylation of a conserved threonine residue (Fievet et al., 2004). Since the TTS effector IpgD reduces the pool of PIP2 at the plasma membrane of infected Jurkat T cells, we analysed whether this reduction could have an impact on the pool of phosphorylated and therefore activated ERMs. To answer this question Jurkat T cells were infected with either the wild type strain M90T or the mutant *∆ipgD* and the ERM phosphorylation levels were assayed by Western blotting using an anti-phospho-ERM antibody. For the loading controls, anti-ERM and anti-actin antibodies were used. Interestingly, Jurkat T cells infected with the wild type strain M90T, but not with *∆ipgD,* showed a rapid and sustained reduction of the level of phosphorylated ERM proteins as compared with non-infected cells. The levels of total ERM and actin did not change (Figure 3.8. A). In Jurkat T cells transfected with the constructs IpgD-GFP and mut-GFP we confirmed that the effector IpgD was responsible for the reduced pool of phosphorylated ERM proteins, as also the, with IpgD-GFP, transfected cells showed a lower level of ERM phosphorylation, as compared with the non-transfected control or cells transfected with mut-GFP (Figure 3.8. B). These results indicate that the enzymatic activity of IpgD is responsible for the reduced level of ERM phosphorylation in T cells infected by *Shigella*.

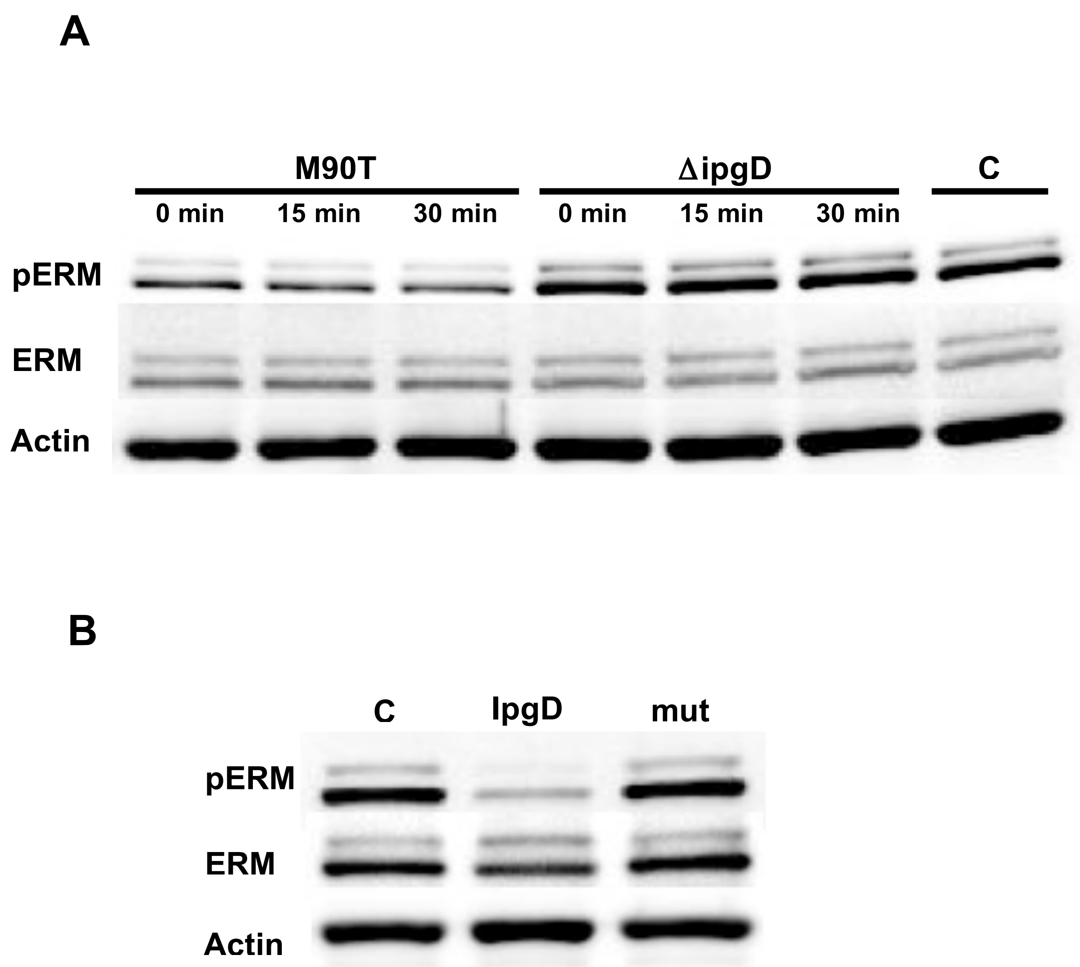


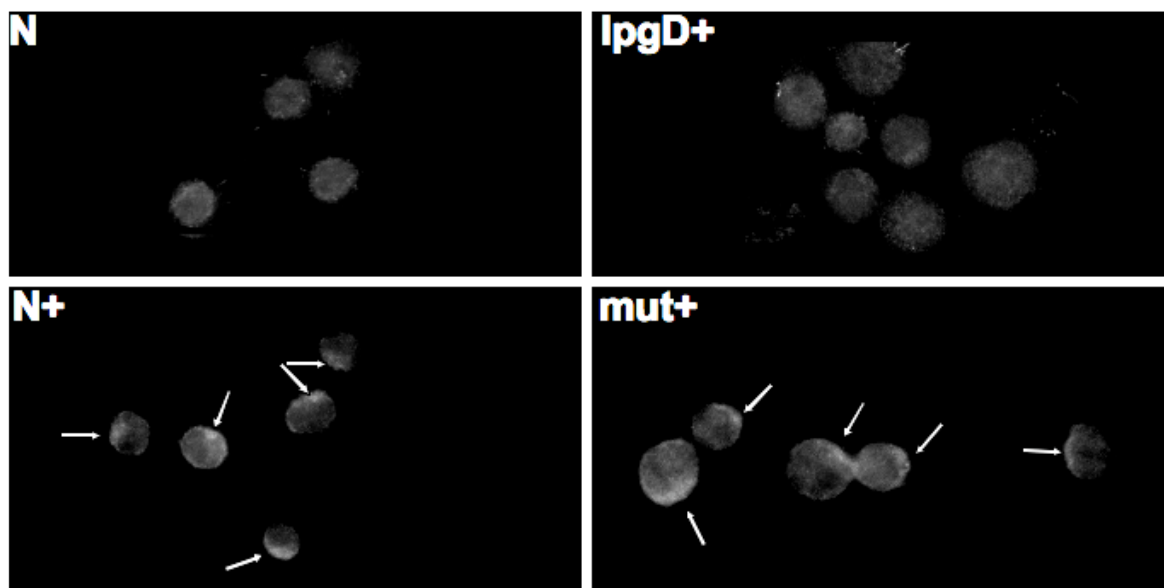
Figure 3.8. IpgD induces ERM dephosphorylation.

(A) Western blot of Jurkat T cells infected with the wild type *Shigella* strain M90T, the mutant *ΔipgD* or C not infected. **(B)** Western blot of Jurkat T cells transfected with either IpgD-GFP (IpgD), mut-GFP (mut) or not transfected (**C**). Blots were incubated with anti-phosphorylated-ERM (pERM), anti-total ERMs (ERM) and anti-actin (Actin) antibodies.

3.9. IpgD inhibits polar phospho-ERM localisation upon chemokine stimulation

An important step in T cell migration is the ability of T cells to polarize upon contact with chemokines. ERM proteins play a crucial role in T cell polarisation. T cells polarise in response to adhesins or chemokines and the formation of two poles are observed: a lamellopodial structured pole at the front edge and a posterior protrusion pole, called the uropod, at the rear edge. The ERM proteins are involved in the formation of this uropod (Allenspach et al., 2001). Since IpgD decreases the pool of phosphorylated ERM proteins, we wondered if this could have an impact on the phospho-ERM localisation upon chemokine stimulation. We transfected Jurkat T cells with IpgD-GFP or mut-GFP and then stimulated the transfected cells with the chemokine CXCL12 for 45 s. Cells then were stained with an anti-phospho-ERM and an anti-CD28 antibody to label the cell surface, transfected cells were detected as GFP positive cells. Jurkat T cells with phospho-ERM proteins localised to one side of the cell were counted by using wide field microscopy. For the quantification of localised all cells were counted as after fixation the GFP detection level in some transfected cells was too low to distinguish transfected from non-transfected cells. We observed that the accumulation of phosphorylated ERMs proteins to one pole of the cell, after CXCL12 stimulation, was reduced in cells transfected with IpgD-GFP as compared with cells transfected with mut-GFP or the control (Figure 3.9. A and C). We detected a 5-fold reduction of cells displaying an accumulation of phospho-ERM at one pole transfected with IpgD-GFP as compared with the control or with mut-GFP transfected cells (Figure 3.9. B).

A



B

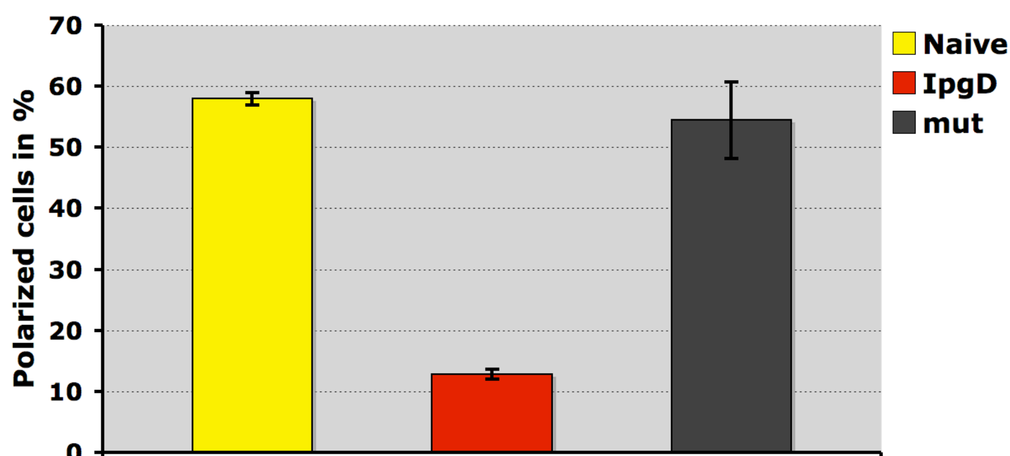


Figure 3.9. A+B. IpgD inhibits polar phospho-ERM localisation upon chemokine stimulation.

(A) Jurkat T cells, N: not transfected, not stimulated; N+: not transfected, stimulated with CXCL12; IpgD+: transfected with IpgD-GFP, stimulated with CXCL12; mut+: transfected with mut-GFP, stimulated with CXCL12. White arrows: phospho-ERM localisation to one pole of the cell after stimulation. **(B)** Quantification of a minimum of 150 cells per condition and experiment; Naive (yellow): IpgD (red) transfected with IpgD-GFP; mut (dark grey) transfected with mut-GFP.

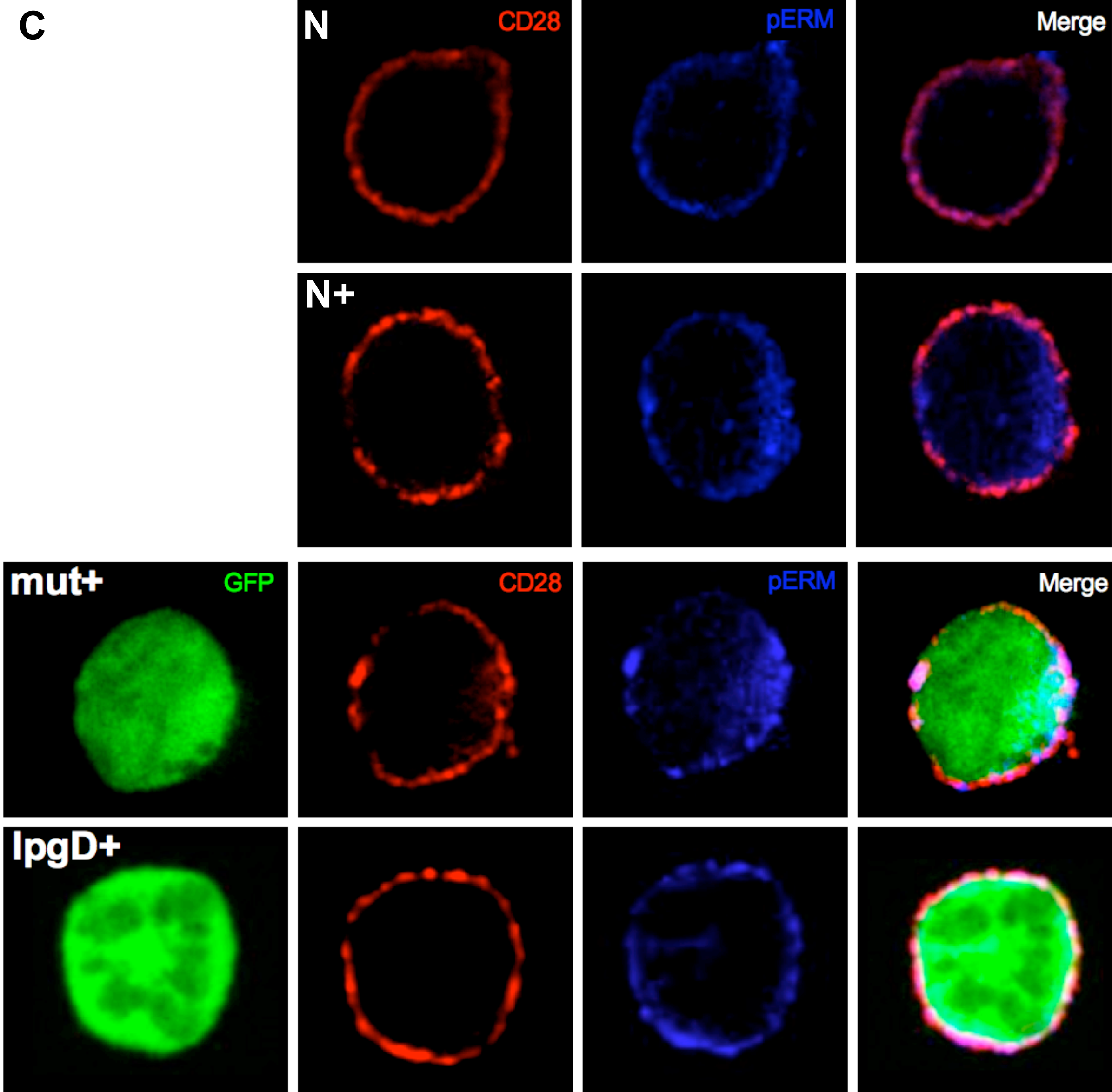


Figure 3.9. C. IpgD inhibits polar localisation of phospho-ERM upon chemokine stimulation.

(C) Jurkat T cells, N: not transfected, not stimulated; N+: not transfected, stimulated with CXCL12; IpgD+: transfected with IpgD-GFP, stimulated with CXCL12; mut+: transfected with mut-GFP, stimulated with CXCL12.

3.10. *Shigella* infects stimulated primary human T cells

Jurkat T cells are an immortal human T cell line and, as every cell line, they have advantages and disadvantages. The advantages of using cell lines are the convenience of supply, long-term culture and have uniformity and consistency in terms of responses. One of the disadvantages is that most of the immortalised cell lines can have mutations in some signalling pathways and can therefore react differently to certain stimuli as compared to what primary cells would do. For example Jurkat T cells are deficient in the protein expression of the two lipid phosphatases SH 2 domain containing inositol polyphosphate phosphatase (SHIP) and phosphatase and tensin homolog deleted on chromosome ten (PTEN) (Abraham and Weiss, 2004). To prove that our observations are not due to artefacts inherent to the Jurkat T cell line, we investigated the ability of *Shigella* to invade monocyte depleted peripheral blood mononuclear cells (PBMC) as they consists of ≈ 75 % T cells. Surprisingly, we could not observe either intracellular *Shigella* in the PBMCs nor could we detect the GFP high positive cell population we observed in the Jurkat T cells infected with M90T-GFP, even not in cells infected with a MOI of 100 (Figure 3.10. A). Jurkat T cells represent an active stage of T cells and as compared to naive primary T cells they proliferate and therefore have also higher metabolism. We wondered what would happen after PBMC stimulation with phorbol myristate acetate (PMA). PMA is a potent activator of the signal transduction enzyme protein kinase C (PKC) resulting in an activation of primary T cells. After infection of PMA stimulated PBMCs with *Shigella*, we observed intracellular bacteria in cells infected with M90T but not with the non-invasive mutant ∆mxiD (Figure 3.11.). Monitored by immune fluorescent staining, the number of infected PBMCs was 80 - 90 % for the MOI of 100 and 15 – 20 % for the MOI of 10, similar to the observations in Jurkat T cells. Furthermore, the GFP high positive cell population observed in Jurkat T cells infected with M90T-GFP could now be observed in stimulated PBMCs. Also this GFP high positive cell population favoured to undergo cell death, like shown in Jurkat T cells (Figure 3.12. A - B). To test the hypothesis that *Shigella* might not be able to invade primary cells but would still be able to inhibit migration, we infected primary human

CD_4^+ T cells purified out of PBMCs. However, we could not observe any inhibition in migration of human CD_4^+ T cells infected with the wild type strain M90T (Figure 3.10. B). But, as seen in the *Shigella*-invasion into stimulated PBMCs, a migration assay performed with *Shigella* infected stimulated human primary CD_4^+ T cells show a significant reduction in the migration of M90T and complemented *ΔipgD* infected cells but not in cells infected with the mutants *ΔmxiD* or *ΔipgD* (Figure 3.11 A). FACS analysis of the upper and lower compartments of the trans-well-system used for the migration assay showed, like in Jurkat T cells, that the GFP high positive cell population is unable to migrate towards the chemokine gradient (Figure 3.11. B). The results observed with human PBMCs and with purified activated human CD_4^+ T cells, are similar to what was observed in the human Jurkat T cell line. This suggests that the data obtained with the Jurkat T cell line are not specific for this cell line only and can be extrapolated to other human T cells subsets.

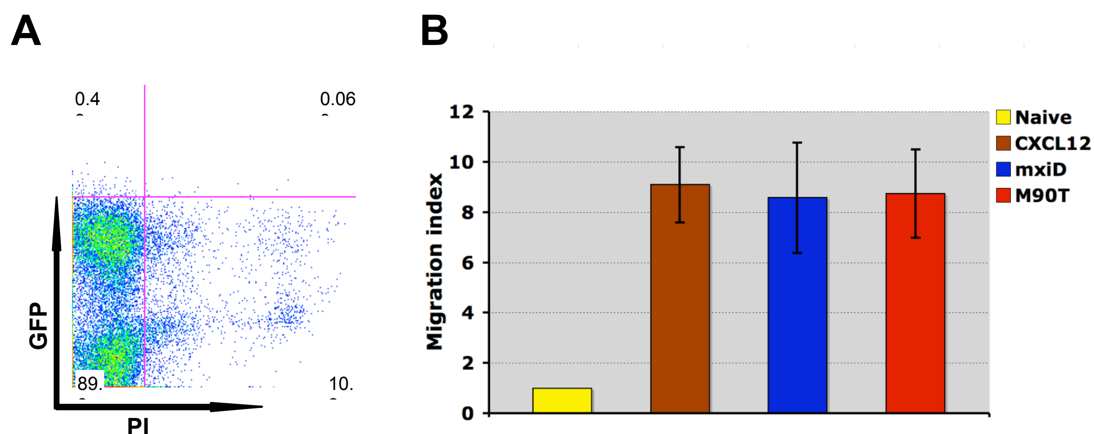


Figure 3.10. *Shigella* shows no impact in primary T cell migration.

(A) FACS analysis of PBMCs infected with M90T-GFP with a MOI of 100 **(B)** Migration assay: Naive human CD_4^+ T cells infected with the wild type strain M90T or with the mutant *ΔmxiD* with a MOI of 10. Naive (yellow): not infected, without chemokine; CXCL12 (brown): not infected, with chemokine; mxiD (blue): infected with ΔmxiD; M90T (red): infected with M90T.

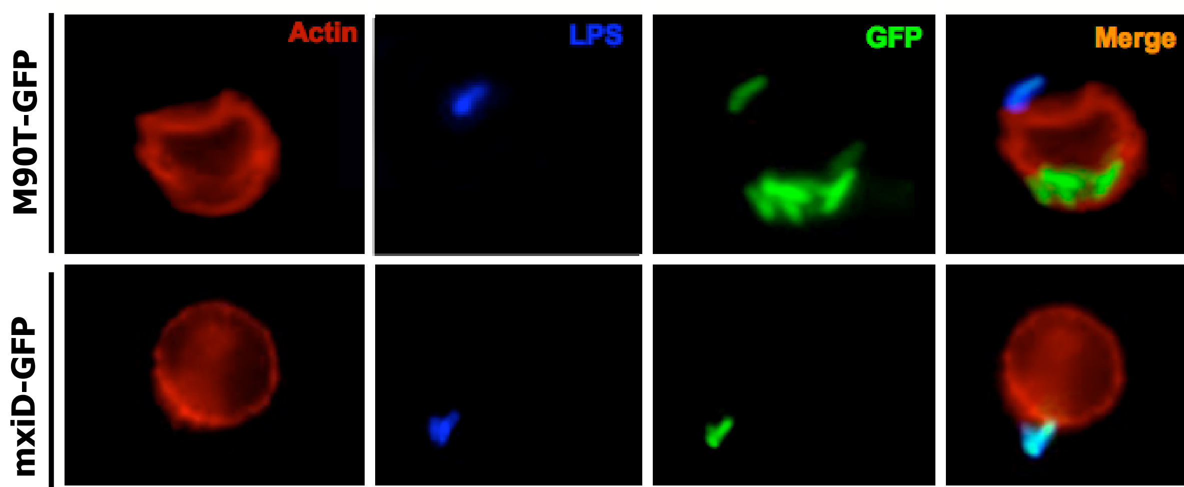
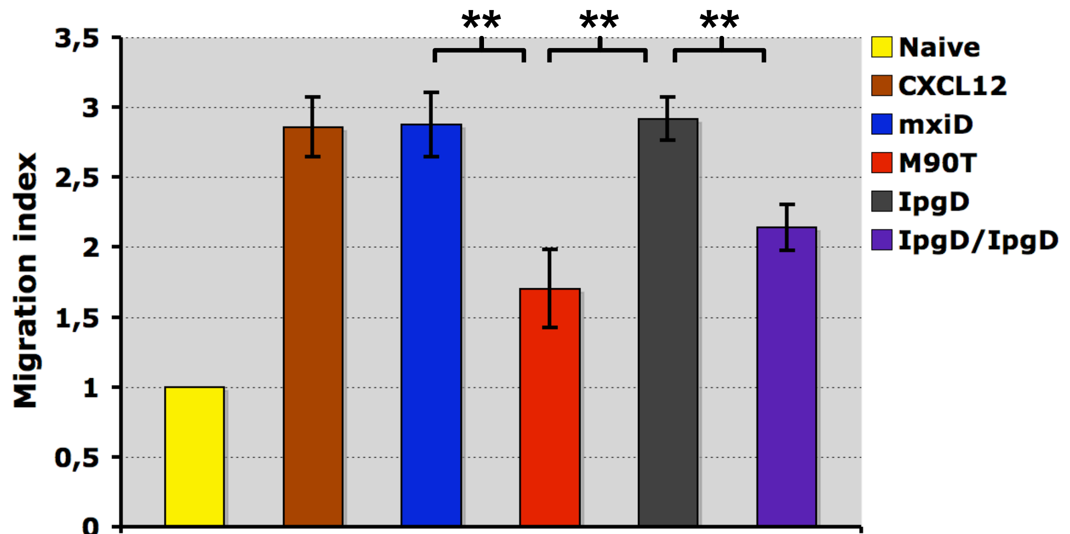


Figure 3.11. *Shigella* invades activated human PBMC.

Immunofluorescent staining of stimulated human PBMCs infected with the wild type *Shigella* strain M90T-GFP or ΔmxiD-GFP with a MOI of 100. After 1h cells were fixed and stained for actin (red) extracellular bacteria (blue) and total bacteria (green).

A



B

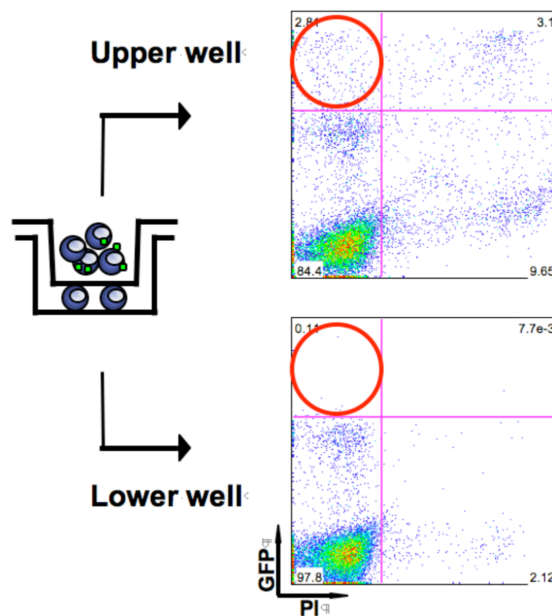


Figure 3.11. The TTS effector IpgD is responsible for the reduced T cell migration in activated human primary T cells.

(A) Migration assay of stimulated human CD_4^+ T cells infected with the wild type strain M90T, *ΔmxiD, ΔIpgD* or with the complemented *ΔipgD*. Naive (yellow): not infected, without chemokine; CXCL12 (brown): not infected, with chemokine; mxiD (blue): infected with *ΔmxiD*; M90T (red): infected with M90T. *ipgD* (dark grey): infected with *ΔipgD*. ipgD/ipgD (purple): infected with complemented *ΔIpgD*. **(B)** Upper and lower wells of a trans-well-system analyzed by FACS and immunofluorescence staining after infection of stimulated human CD_4^+ T cells with M90T-GFP with a MOI of 10. GFP-high positive cell only found in the upper well (red circle).

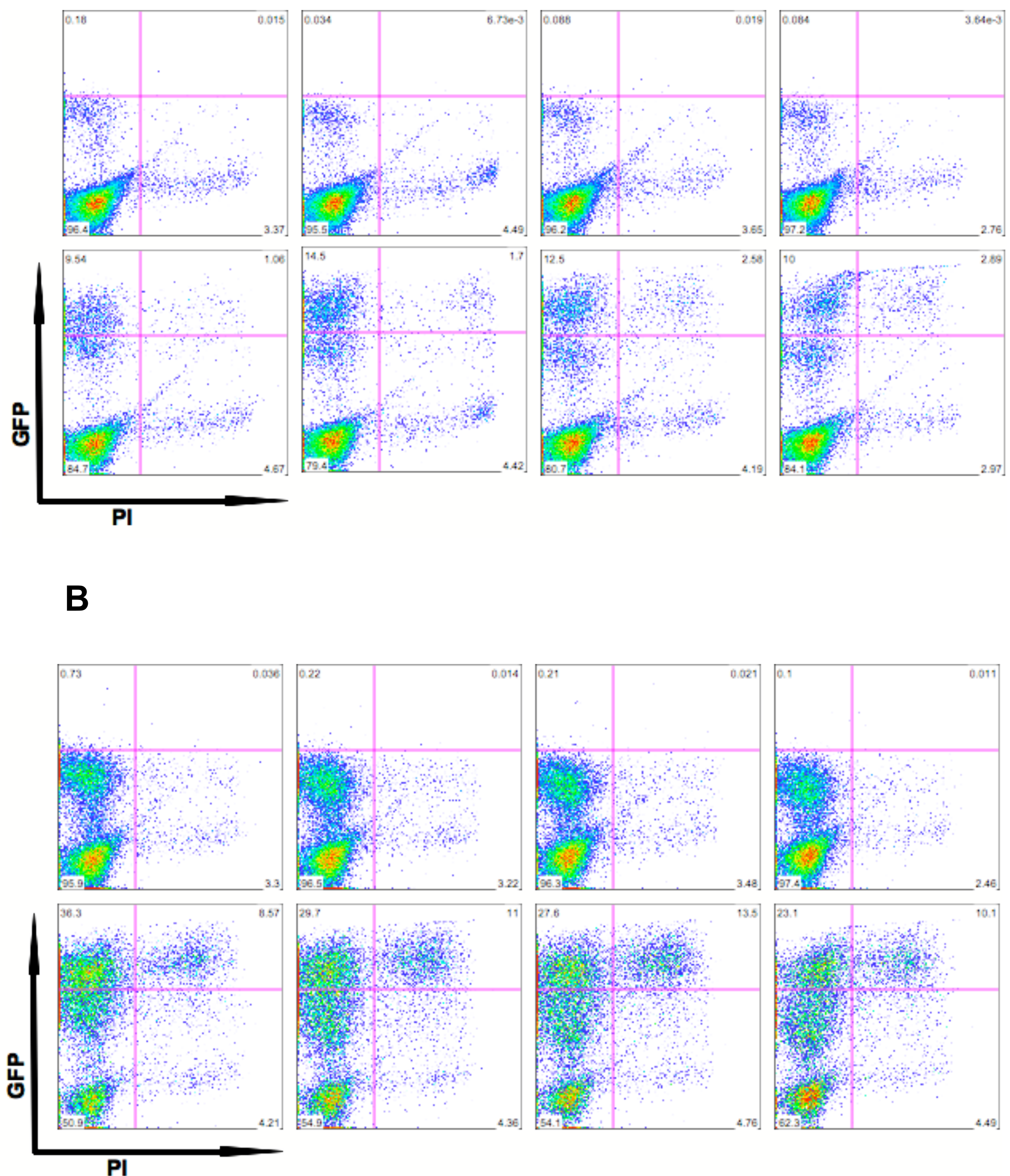


Figure 3.12. Increased cell death in PBMCs is contact dependent.

Stimulated human PBMCs infected with the wild type *Shigella* strain M90T-GFP or ΔmxiD-GFP with a MOI of 10 **(A)** and 100 **(B)**.

Discussion

4. Discussion

Suppression or evasion of host immune responses is necessary for pathogenic bacteria to establish an infection. Compared with the amount of research reporting interactions of *Shigella* with cells of the innate immune system, only little is known about the interaction of *Shigella* with cells of the adaptive immune system, particularly with lymphocytes. The natural protective immunity against *Shigella* arises after several episodes of infection is of short duration and, therefore, is poorly efficient in limiting re-infection, in particular among young children (Phalipon and Sansonetti, 2007). This suggests a manipulation of the adaptive immune response by *Shigella*, eventually through a direct interaction with cells of the adaptive immunity including lymphocytes. Indeed, sooner or later *Shigella* will face lymphocytes, as they are abundant in the gut. In a healthy human adult, the mucosal immune system contributes almost 80 % of all immunocytes. It is difficult to believe that *Shigella* did not evolve mechanisms to subvert lymphocyte function since those cells are crucial for the induction of an immune response upon infection. During lymphocyte response in the gut, mucosal homing "programs" control the traffic of naive, activated, and memory T lymphocytes from peripheral tissues to inductive tissue of the MALT, and finally to effector tissues of the lamina propria (LP) and epithelia. All these processes are tightly regulated. Targeting the equilibrium of this system could become beneficial for an intestinal pathogen, as it would bring disorder in the immune response against that pathogen. Patients with WHIM Syndrome (Wart, Hypogammaglobulinemia, Infection, and Myelokathexis syndrome) for example show an increased susceptibility to bacterial and viral infections. The WHIM Syndrome is caused by a function mutation in the CXC chemokine receptor 4 (CXCR4) resulting in a hyperactivity of the receptor (Kawai and Malech, 2009).

Shigella has developed abilities to interact with and manipulate the host immune response. Some examples are: the *Shigella*-induced down-regulation of antimicrobial peptide production by intestinal epithelial cells (Sperandio et al., 2008), the release of IL-1β through *Shigella*-induced macrophage apoptosis (Zychlinsky et al., 1992) or

the downregulation of the IFN-γ production in patients infected with *Shigella* at the acute phase of the disease (Raqib et al., 1997). All these data suggest that *Shigella* has evolved mechanisms to manipulate the host immune response in a particular direction to promote invasion into and survival of the bacterium in the host. This raises the hypothesis that *Shigella*, besides an indirect effect on the inflammatory response on the priming of the specific immunity, has the capacity to impair T cell functions by a direct cross-talk with this cells. We report here for the first time that *Shigella* is able, via the TTSA, to directly cross-talk with T lymphocytes.

We showed that *Shigella* has an impact on T cell survival. Indeed the *Shigella* wild type strain M90T, but not the non-invasive mutant *mxiD*, induced a MOI-dependent T lymphocyte death *in vitro*. The induced cell death was favoured by an intimate contact of the bacteria with the T lymphocytes, as clearly seen with the increased PI staining of the GFP-high positive cell population in T cells infected with GFP-expressing *Shigella*. *Yersinia pseudotuberculosis*, like *Shigella* a member of the family of *Enterobacteriaceae,* also induces T cell death. It has been shown that the high-affinity ligand for β1-integrins, the invasin (Inv) protein of *Y. pseudotuberculosis*, was responsible for the observed T cell death. The Inv protein causes an apoptotic/necrotic caspase-independent cell death by binding to the β1-integrins subunit CD29 (Arencibia et al., 2002). But whereas killed bacteria or the Inv protein alone are sufficient for inducing cell death in the case of *Yersinia* only live *Shigella* with an intact TTSA are able to induce T cell death, suggesting an active process triggered by *Shigella*. The T cell death induced by *Shigella* is further a necrotic caspase-independent cell death. A major consideration is whether the high MOI of 100 used for some experiments can be considered physiological, as to our knowledge the bacteria / T cell ratio that can be found in vivo during a *Shigella* infection is not known.

In addition to the results obtained with the mutants that are defective for invasion, the results obtained with the *ΔipaA* mutant point out the relationship between invasion and induced cell death. Indeed, with this mutant displaying a decreased

ability in cell invasion less cell death was shown to occur. We demonstrated that *Shigella* is not only able to invade T lymphocytes but is also able to proliferate inside those cells. It is quite likely that the GFP-high positive cell population in T cells infected with GFP-expressing *Shigella* reflects a high amount of intracellular bacteria. The percentage of highly infected T cells measured in immunofluorescence staining corresponds to the percentage of the GFP-high positive cell population measured by FACS. Therefore the increase in intensity of the GFP-high positive cell population over time in combination with the increased PI staining of those cells hardly suggest that the induced cell death is triggered by the replication of intracellular bacteria. Cell death eventually occurs through bacterial overgrowing of the host cell and/or by the limitation of nutrition caused by the intracellular bacteria. Geddes et al. detected intracellular *Salmonella* in purified spleen T and B cells after an intraperitoneal infection of mice, but compared to our result they did not report *Salmonella* invasion into T cells *in vitro*. Furthermore, in their experiments a *Salmonella*-pathogenicity-island 1 (SPI-1) *Salmonella* mutant was found inside T cells with the same frequency as wild type *Salmonella*. Knowing that SPI-1 is needed for invasion into non-professional phagocytic cells, this questions an active process induced by *Salmonella* (Bueno et al., 2010).

Invasion of non-phagocytic cells by *Shigella* involves the secretion and injection of effectors inside host cells. Therefore, the need of a functional TTSA for the invasion of T lymphocytes by *Shigella* indicates that the injection of effectors also occurs into those cells. Preliminary results indicate that there is no injection of *Shigella* effectors into primary human T cells but there is injection into PMA-stimulated primary human CD_4^+ T cells (data not shown). This is in agreement with the finding that *Shigella* was only invading PMA-stimulated primary CD_4^+ T cells, isolated from human PBMCs, but not non-stimulated primary human CD_4^+ T cells. Particularly interesting, in these preliminary results, was the finding that *Shigella* injects effectors without necessarily inducing an uptake into the cell. The data that *Shigella* is mainly interacting with stimulated primary T cells are especially interesting as intestinal epithelial T lymphocytes (IEL) are a phenotypically, developmentally, and functionally complex

population of cells, and whether IELs are resting T cells, activated T cells, or T cells that exist in some intermediate stage of activation has been debated for many years (Montufar-Solis et al., 2007). Subsequent studies showed that T cells are cytolytic upon isolation from the intestinal epithelium in the absence of overt stimulation and that most IELs express some markers of activated T cells (Klein, 1986). IELs, in both the small and large intestines, exist in a novel state of intermediate or partial T-cell activation. Therefore, IELs have been described as being 'activated yet resting' T cells (Shires et al., 2001). Therefore, it can be discussed if stimulated primary T cells represent more the T cell phenotype that is found in the MALT. It makes sense for *Shigella,* as an intestinal pathogen, to target those types of cells. Furthermore, in this way *Shigella* could eventually target *Shigella*-specific T cells that are primed during an infection and therefore subsequently inhibit an induction of an efficient adaptive immune response. We do not know how *Shigella* distinguishes between activated and non-activated T cells. But it is quite likely that the effectors IpaD, IpaB and IpaC are involved in this process. IpaD is located at the tip of the needle and it is believed to play an important role in target recognition and in promoting the membrane insertion of IpaB and IpaC (Espina et al., 2006; Sani et al., 2007). IpaB interacts with CD44 and it partitions during infection within specialised membrane microdomains enriched in cholesterol and sphingolipids, called rafts. Moreover, *Shigella* invasion is impaired after cholesterol depletion (Lafont et al., 2002). This leads to the idea that a change in the membrane composition of T lymphocytes, induced by an activation, could promote injection of effectors and a subsequent invasion of those cells. IpaD, IpaB and IpaC also interact with $\alpha_5\beta_1$ integrins (Watarai et al., 1996), which could be another possibility for *Shigella* to target activated T cells. Indeed, $\alpha_5\beta_1$ integrins appear in two forms, a non-activated "bent" and an activated "extended" form. $\alpha_5\beta_1$ integrins get rapidly activated upon lymphocyte stimulation (Kinashi, 2007). Like stimulated T cells Jurkat T cells express significant amounts of constitutively activated $\alpha_5\beta_1$ integrins (Seminario et al., 1998). This could eventually explain why *Shigella* invades stimulated primary human T cells and Jurkat T cells with the same efficiency. Therefore, *Shigella* could be eventually able to recognise the two forms of the $\alpha_5\beta_1$ integrins.

T cells are among the most motile cells in the human body and their trafficking into and out of lymphoid organs as well as into and out of inflammatory sites involves a high coordination and regulation level of adhesion molecules, chemoattractants, receptor expression levels and signalling pathway modulation (Kehrl, 2006). As *Shigella* injects effectors in ECs, which target important signalling pathways, we wondered if one of them could have an impact on T cell motility. We therefore investigated if *Shigella* invasion has an impact on T cell migration. Indeed, T cells infected with the wild type strain but not with the non-invasive mutant showed a significant reduction in their ability to migrate towards a chemokine gradient. Interestingly, the inhibition was not due to the T cell death discussed before, because a modified infection protocol showed no differences in cell death between the wild type strain and its non-invasive mutant. *Shigella*-invaded T cells lost their ability to migrate upon chemokine stimulation. Consistently no intracellular bacteria were detected inside T cells that were able to migrate in our migration assay. Interestingly only 10 % of the T cells were actually infected, but we observed a reduction of 30 % and 50 % of migrated T cells for Jurkat T cells and stimulated primary human T cells respectively. These data go in agreement with the already mentioned preliminary data showing an injection of effectors into T cells without inducing a subsequent uptake of the bacteria (data not shown). So far the injection of effectors has always been related with the induction of the uptake of *Shigella* into the cell cytoplasm. Therefore, this is a breakthrough finding as it is the first report showing a significant impact on cell functions directly related to the injection of *Shigella*-effectors without promoting bacterial uptake. Already the report of Sperandio et al. suggested an eventual injection of effectors through the apical side of ECs without promoting *Shigella*-invasion, but compared to their work we could demonstrate a direct link between the injection of effectors and the manipulation of cell functions in the absence of intracellular bacteria. Furthermore, these data point out that the injection of effectors might have, at least in T cells, a higher relevance for the manipulation of host cells than invasion does. Injection of effectors, in addition to possible invasion, might also increase the number of targeted cells and would have therefore a greater impact on the modulation of immune responses

during an infection. In this way *Shigella* might be able to manipulate and interact with more cell types than the ones reported so far. Therefore, more research needs to be done to determine all the different cell types that could be targeted by *Shigella* through the injection of effectors. Our data give rise for new strategies *Shigella* could have developed to manipulate host immune responses. The impairment of T cell migration could be part of the mechanisms leading to the fact that a primary infection with *Shigella* does not induce a protective immunity against *Shigella* and that several reinfections are needed to mount a protection (Phalipon and Sansonetti, 2003). We did not observe an inhibition in migration of non-stimulated primary T cells. Therefore further investigation is needed to see if *Shigella* is unable to interact with naive T cells, or if *Shigella* impairs other T cell functions than migration in non-stimulated T cells. For example, *Yersinia* is able to inhibit or alter T cell responses. The *Yersinia* effector YopH, a tyrosine phosphatase, facilitated this inhibition. Indeed, YopH specifically targets the adaptor proteins, linker for activation of T cells (LAT) and SH2 domain–containing leukocyte protein of 76 kD (SLP-76), which are crucial for TCR signalling. This leads to a suppression of T cell activation and IL-2 production (Gerke et al., 2005). In contrast to *Yersinia* we could not observe an impact on T cell activation or TCR signalling in *Shigella* infected T cells (data not shown). Interestingly *Yersinia* also discriminates between cell types and, although *Yersinia* delivers its effector proteins into numerous cell types in vitro, during an infection *Yersinia* selectively delivers effectors to phagocytes (Durand et al., 2010).

Other pathogens have been reported to manipulate T cell migration. Rossi Paccani et al., for example, reported that the adenylate cyclase toxin (CyaA), released by *Bordetella pertussis,* directly affects adaptive immune responses by catalysing cyclic AMP (cAMP) production in peripheral blood lymphocytes. A treatment with CyaA results in an impairment of T-lymphocyte activation and chemotaxis. Therefore the authors claimed that the pleiotropic activities of CyaA on T cells might contribute to the suppression of the development of pathogen-specific adaptive immune responses. This can be questioned however: compared to *Shigella,* a primary *B. pertussis* infection leads to a long lasting protective immunity; further, the authors

did not show any entry of CyaA into T cells. Another major consideration is whether the toxin concentrations used in this study can be considered physiological as CyaA concentrations that can be found in vivo during *B. pertussis* infection is not known. Another example is the HIV-1 protein Nef which inhibits T cell chemotaxis in response to the ligand SDF-1. The Nef protein down-modulates LFA-1 expression on T cells and therefore diminishes adhesion and polarization of T cells and, as a result, leads to a decreased migration across the endothelium (Park and He, 2009). Therefore it seems to be a good strategy for a pathogen to target the migratory capacity of T cells.

Continuing with the investigation of the molecular mechanism responsible for the reduced migration, we identified the *Shigella* effector IpgD as the main effector responsible for the inhibition. IpgD accounts for the inhibition of migration as observed with the reduced migration of IpgD-transfected T cells. Furthermore, the inhibition is due to the enzymatic activity of IpgD since an enzymatic dead variant of IpgD does not display any inhibitory effect on migration. Under the condition of no secretion, IpgD is stored in the bacterial cytoplasm associated with its specific chaperon IpgE and is only translocated through the TTSA upon contact with epithelial cells (Niebuhr et al., 2000). IpgD is a phosphoinositide 4-phosphatase that specifically dephosphorylates phosphatidylinositol-(4,5)-bisphosphate (PIP2) into phosphatidylinositol-(5)-monophosphate (PI(5)P). In epithelial cells, IpgD is responsible for dramatic morphological changes of the epithelial host cell, leading to a decrease in membrane tether force associated with membrane blebbing and actin filament remodelling (Niebuhr et al., 2002). Although involved in the formation of the fully structured entry sites, IpgD is not involved in invasion in IECs. Moreover, through the induction of the accumulation of PI(5)P, IpgD is involved in the activation of the host cell PI-3-kinase/Akt pathway. It is believed that this plays an important role in the host cell response for survival (Niebuhr et al., 2002). Like in epithelial cells, we showed a reduction of the PIP2 level in T cells, especially at the plasma membrane. Like in EC, IpgD is also not involved in the invasion into T cells, but compared to epithelial cells, where the *ΔipgD* mutant induces an increased cell

death (Pendaries et al., 2006), we did not observe any difference in survival of T cells infected with M90T or *ΔipgD*. Furthermore, preliminary data also showed no difference in the Akt phosphorylation of Jurkat T cells transfected with IpgD, as it is reported for EC (Niebuhr et al., 2002). But we observed a significant reduction in the ability of T cells to migrate towards a chemokine gradient. To determine if this reduction is due to a complete immobilisation or to a loss of directed movement will need further investigation.

The metabolism of phosphoinositides (PI) plays a key role in the regulation of receptor-mediated signal transduction, actin remodelling and membrane trafficking in eukaryotic cells (De Matteis and Godi, 2004; Gillooly et al., 2001; Martin, 2001). Affecting the concert of PIs seems to be a good strategy as many intracellular bacterial pathogens modulate and exploit PIs to ensure survival and efficient intracellular replication. Pathogens manipulate PIs by direct or indirect mechanisms. *Salmonella enterica, Mycobacterium tuberculosis* and some *Escherichia coli* evolved, like *Shigella,* effectors mimicking mammalian phosphatases. The *Salmonella* effector SopB for example shares similarity with mammalian PI 4- and 5-phosphate phosphatases and, *in vitro*, preferentially hydrolyses PI(3,4)P2, PI(3,5)P2 and PI(3,4,5)P3, into PI(3)P. SopB dephosphorylates PIP2 and soluble inositol polyphosphates (Marcus et al., 2001; Norris et al., 1998). SopB is thought to promote the uptake of *S. enterica* in two ways, involving the hydrolysis of PIP2 and the production of PI(3)P. SopB was found to diminish specifically the cortical PIP2 pool, thus destabilising cytoskeleton–plasma membrane interactions (Terebiznik et al., 2002). Enteropathogenic *E. coli* (EPEC) uses a TTS effector encoded by the LEE (locus of enterocyte effacement) pathogenicity island to prevent its uptake by macrophages (Goosney et al., 1999). EPEC blocks PI3K activity and accumulation of PI(3,4,5)P3 at contact sites of the bacteria with macrophages (Celli et al., 2001). Also *Mycobacterium tuberculosis* secretes PI phosphatases to keep low the levels of PI(3)P on *Mycobacterium*-containing vacuoles (MCVs). The lipid phosphatase SapM specifically dephosphorylates and removes PI(3)P from the phagosome, thus inhibiting fusion with late endosomes/lysosomes (Vergne et al., 2005). The secreted

M. tuberculosis protein phosphatase virulence factor MptpB shares an active-site signature with eukaryotic lipid phosphatases such as the PI 3-phosphatase PTEN. MptpB exhibits a triple-specificity phosphatase activity towards phosphotyrosine, phosphoserine/threonine as well as PI substrates (Beresford et al., 2007). This phosphatase shows a broad substrate specificity and readily dephosphorylates all mono-phosphorylated PIs (PI(3)P, PI(4)P and PI(5)P], as well as PI(3,5)P2 *in vitro*. Taken together, SapM and MptpB likely act in concert to deplete PI(3)P from MCVs. Viruses interact with PI as well: the Tat protein of HIV for example binds with a high affinity to PIP2, allowing Tat to perturb the PIP2-mediated recruitment of cellular proteins to the plasma membrane (Rayne et al., 2010). Our results raise a new role of bacterial effectors that by targeting the PI metabolism in inhibit T cell migration.

A protein family whose activity is closely related to the binding of PIP2 and which also play an important role in signal transduction and cell motility is the ERM protein family. The ERM family consists of Ezrin, Moesin and Radixin (Bretscher et al. 2002). The ERM proteins exist under two confirmations, a "dormant" state, in which the protein presents a head to tail folded conformation, and an "active" state, in which the protein is unfolded and fully capable to interact with the membrane components and the actin cytoskeleton. The activation of the ERM proteins occurs through two steps: first by binding of PIP2 to the FERM domain leading to a conformational change and therefore rendering the conserved threonine residue more accessible to phosphorylation and second by the phosphorylation of the now accessible conserved threonine residue in the C-terminal domain (Fievet et al., 2004). We showed that the reduced level of PIP2 induced by IpgD leads to a significant decrease in the amount of phosphorylated and therefore activated ERM proteins in T cells. The consequences of this effect of IpgD on lymphocytes could be that the low level of phosphorylated ERMs, disables the T cell to respond to chemokine stimulation, as ERM proteins rapidly get dephosphorylated after a ligand binding to a chemokine receptor. We can rule out an impact on the CXCR4 receptor expression level for the reduced or inhibited migration, since we did not detect any difference in its expression level in T cells infected with the wild type strain or the mutant ipgD (data not shown). Another

hypothesis could be that because of the low level of PIP2, host kinases, like LOK (Belkina et al., 2009), are unable to re-phosphorylate the ERM proteins due to the fact that the phosphorylation side is not longer accessible. If the low level of PIP2 is directly responsible for the ERM protein dephosphorylation or if it just inhibits ERM re-phosphorylation needs further investigation. Also further investigation needs to be done to determine if IpgD inhibits chemokine receptor signalling or if other mechanisms eventually are involved leading to the reduced migration, like, for example, chemokine receptor signalling.

After chemokine recognition, T cells start to polarise leading to the formation of two poles: a lamellopodia structure at the front edge and a posterior protrusion called the uropod at the back edge (Allenspach et al., 2001). Involved in forming the uropod, ERM proteins play a crucial role in cell polarisation during T cell migration. We showed that in T cells transfected with IpgD there is no localisation of the phosphorylated ERM proteins to one pole of the T cell upon chemokine stimulation. This also indicates the PIP2 involvement in the polarisation process of T cells. ERM proteins are particularly involved in the formation of the uropod and their activation is modulated during T cell polarization. The low level of PIP2 could lead to a permanent inactivation of the ERMs and the cell is therefore not able to form the uropod anymore. The ERM proteins connect actin filaments with the plasma membrane and are therefore important for actin rearrangements inside the cells. This could have an impact on the formation of an immunological synapse, which then therefore would inhibit an efficient T cell activation by APCs with all the consequences for the development of an adaptive immune response. If the low level of PIP2 inhibits ERM signalling or actin cytoskeleton rearrangement induced by the ERMs will need further investigations. *Shigella* is not the only pathogen that targets the ERM protein family. *Neisseria meningitidis* for example interferes with the transendothelial migration of leukocytes. Bacteria adhering to endothelial cells actively recruit ezrin, moesin, and ezrin binding adhesion molecules. These molecules no longer accumulate at sites of leukocyte–endothelial contact, preventing the formation of the endothelial docking structures required for proper leukocyte

diapedesis (Doulet et al., 2006). Also an infection of lymphocytes by HIV-1 induces a complex array of cortical rearrangements that lead to the formation of what has been termed the virological synapse (Belkina et al., 2009). On contact, GP120 on the surface of the virus binds CD_4 on the lymphocyte surface, recruiting this transmembrane protein and the associated protein CXC-chemokine receptor 4 (CXCR4) to the contact region. Concomitantly, there is a local activation and accumulation of moesin and ezrin. Small interfering RNA-mediated knockdown of ERMs strongly diminishes the ability of HIV 1 to enter and infect cells (Barrero-Villar et al., 2009; Kubo et al., 2008). Therefore targeting the ERM protein family might be a good strategy for a pathogen to establish an infection.

4.1. Conclusion

In this work we demonstrated that *Shigella* is directly cross-talking with T lymphocytes which has never before been reported. We showed that *Shigella* induces cell death in T cells upon contact with the wild type strain M90T. New was also the finding that the wild type *Shigella* strain M90T was able to invade and replicate in T cells. The need for a functional TTSA suggests that the induced cell death and invasion are an active processes induced by the bacteria. To our knowledge, we first demonstrated the involvement of a TTSA effector in the manipulation of T cell motility by targeting the pool of PIs. We identified the *Shigella* effector IpgD as the responsible effector for the observed inhibition. We showed that the effector IpgD was responsible for a reduction of intracellular PIP2 levels. It is quite likely that this manipulation of the pool of phosphoinositols is responsible for the migratory inhibition. We also showed a reduced level of phosphorylated ERM proteins in infected as well as in IpgD-transfected T cells. Furthermore, IpgD transfected T cells have a reduced ability to localise phosphorylated ERM at one pole of the cell upon chemokine stimulation, suggesting that IpgD is inhibiting T cell polarisation. The results obtained in this work lead to a better understanding of *Shigella*-host interaction. It also gives rise for new strategies *Shigella* could have developed to modify host immune responses during an infection. The inhibition of T

cell migration, by *Shigella,* could be part of the mechanisms responsible for why a primary infection with *Shigella* does not lead to a protective immunity against *Shigella* and why several reinfections are needed to mount a protection.

4.2. Outlook

The results on *Shigella* T cell cross-talk have been obtained using an *in vitro* system. Therefore it remains to be established *in vivo* whether the impact of IpgD on migration is relevant during infection and whether this effect has specifically evolved to contribute to the impairment of the adaptive immune response. First results using two-photon-microscopy underpinning an impact on of *Shigella* on T cell migration *in vivo*. *Shigella* might also have an impact, via IpgD, on T cell interaction with APCs as the ERM proteins are shown to be involved in the formation of the immunological synapse. There might also be the possibility that other *Shigella* effectors, which some of them have already been shown to interact with important signalling pathway in epithelial cells, to have an impact on T cell function. Interestingly, we observe invasion and an impact on migration of *Shigella* on stimulated but not in non-stimulated primary human T cells, therefore more research needs to be done on if *Shigella* is targeting naive primary T cells as it targets stimulated primary T cells. Preliminary results suggest only an injection of effectors in stimulated primary T cells, this could help for the identification of a *Shigella*-specific surface receptor which has not been identified so far. In total this would give rise of a new view on *Shigella* host interaction and would help understanding the host immune responses to *Shigella* infection.

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Appendix

6. Appendix

6.1. Abbreviations

Ag	Antigen
Akt	serine–threonine kinase Akt
AP-1	Activator protein-1
APC	Antigen-presenting cells
APS	Ammonium persulfate
ATP	Adenosintriphosphat
Bcl6	B-cell lymphoma 6 protein
BLT1	Leukotriene B4 receptor 1
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
CCL	Chemokine (C-C motif) ligand
CCL20	Chemokine (C-C motif) ligand 20
CCR	C-C chemokine receptor
CCR7	C-C chemokine receptor type 7
CD	Cluster of differentiation
C-ERMAD	C-terminal ERM-association domain
cfu	Colony forming units
CXCL	Chemokine (C-X-C motif) ligand
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCR4	CXC chemokine receptor 4
CXCR5	C-X-C chemokine receptor type 5
DAG	Diacylglycerol
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOCK	Dedicator of cytokinesis
DP2	Prostaglandin D2 receptor

E. coli	*Escherichia coli*
E3KARP	Type 3 kinase A regulatory protein
EBP50	Ezrin-radixin-moesin-binding phosphoprotein 50
ELMO	Engulfment and Cell Motility
ERK	Extracellular signal-regulated kinase
ERM	Ezrin, Radixin, Moesin
ETEC	Enterotoxigenic *Escherichia coli*
FACS	Fluorescence activated cell sorter
F-actin	Filamentous actin
FAE	Follicle-associated epithelium
FBS	Foetal bovine serum
FERM	Four.1-Ezrin-Radixin-Moesin domain
FoxP3	Forkhead box Protein 3
FYVE	Fab1, YOTB/ZK632.12, Vac1, and EEA1
g	Gram
g	Gravitational acceleration
GALT	Gut-associated lymphoid tissue
GFP	Green fluorescent protein
GPCR	G-protein-coupled receptors
GTP	Guanosintriphosphat
h	hour
hBD-3	Human β-defensin
HCL	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HEV	High endothelial venules
HMGB1	High-Mobility-Group-Protein B1
HPLC	High-pressure liquid chromatography
HRP	Horseradish peroxidase
IκBα	nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α
ICAM	Intercellular adhesion molecule
ICAM-1	Inter-cellular adhesion molecule 1

ICOS	Inducible co-stimulator
IcsA	intra- and intercellular spread
IEC	Intestinal epithelial cells
IF	Immunofluorescence staining
IFN-γ	Interferon γ
IFN-γ	Interferon γ
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IKK	IκB kinase
IL	Interleukin
IP3	Inositol triphosphate
Ipa	Invasion plasmid antigens
IpaA	Invasion plasmid antigen A
Ipg	Invasion plasmid gene
IpgC	Invasion plasmid gene C
IS	Immunological synapse
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
kb	Kilobase
kDa	Kilo Dalton
l	Litre
LAT	Linker of activatin of T cells
LFA-1	Lymphocyte function-associated antigen 1
LOK	Lymphocyte-oriented kinase
LP	Lamina propria
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
M cells	Microfold cells
mA	Milliampere
MALT	Mucosa-associated lymphoid tissue

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
mDia1	Mammalian diaphanous 1
MEM	Minimal essential medium
MHC	Major histocompatibility complex
min	Minute
ml	Millilitre
MLN	Mesenteric lymph nodes
mM	Millimolar
MOI	Multiplicity of infection
μg	Microgram
Mxi	Membrane expression of Ipa
NEMO	NFκB essential modulator
NFκB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NFAT	Nuclear factor of activated T-cells
NK cells	Natural killer cell
N-WASP	Neural Wiscott-Aldrich syndrome protein
OD	Optical density
p	*p*-value
p	Polymeric
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular motifs
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PGD2	Prostaglandin D2
PGN	Release peptidoglycan
PH	Pleckstrin homology
PI	Phosphoinositides
PI	Propidium iodide
PI(3,4)P2	Phosphatidylinositol 3,4-bisphosphate

PI3K	Phosphatidylinositol 3-kinase
PIP	Phosphatidylinositol
PIP1	Phosphatidylinositol 4-monophosphate
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKB	protein kinase B
PKC	protein kinase C
PLC	Phospholipase C
PLCθ	Phospholipase θ
PMA	phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear cells
PP	Peyer's patches
PRR	Pathogen-recognition receptors including
PSGL-1	P-selectin/P-selectin glycoprotein ligand-1
qsp	quantitat suficient per
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated kinase
RPMI	Roswell Park Memorial Institute
RT	Room temperature
S. flexneri	*Shigella flexneri*
S1P	Sphingosine 1-phosphate
SDF-1	Stromal cell-derived factor-1
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SHET	*Shigella* enterotoxin
Shh	Sonic hedgehog
sIgA	Secretory immunoglobulin A
SLO	Secondary lymphoid tissues
SLP-76	SH2 domain containing leukocyte protein of 76 kDa
Spa	Surface presentation of antigens protein

TBS	Tris buffered saline
T_{Cm}	Central-memory T cells
TCR	T cell receptor
T_{Em}	Effector-memory T cells
TEMED	Tetramethylethylenediamine
T_{FH}	Follicular helper T cells
TGF-β	Transforming Growth Factor β
T_H1	T helper cell type 1
T_H17	T helper cell type 17
T_H2	T helper cell type 2
TLC	Thin-layer chromatography
TNF-α	Tumornekrosefaktor α
T_{Reg}	Regulatory T cells
TREM	Triggering receptor expressed on myeloid cells
TRL	Toll-like receptors
TTSA	Type III secretion apparatus
U	Unit
VP	Virulence plasmid
WCL	Whole cell lysates
WHIM	Wart, Hypogammaglobulinemia, Infection, and Myelokathexis syndrome
ZAP-70	Zeta-chain-associated protein kinase 70

6.2. Publications, conferences and oral presentations

Publications

Gernot Sellge, Joao G. Magalhaes, **Christoph Konradt** Jörg H. Fritz, Wilmara Salgado-Pabon, Gerard Eberl, Antonio Bandeira, James P. Di Santo, Phillippe J. Sansonetti, Armelle Phalipon: Th17 cells are the dominant T cell subtype primed by *Shigella flexneri* mediating protective immunity.
J Immunol. 2010 Feb 15;184(4):2076-85.

Christoph Konradt, Elisabetta Frigimelica, Wilmara Katharina Nothelfer Salgado-Pabon, Jost Enninga, Bernard Payrastre, Philippe Sansonetti, and Armelle Phalipon: The *Shigella* TTS effector IpgD inhibits T cell migration. Manuscript **under preparation.**

Conferences

2009	Journées departementales (Departmental retreat), La Colle sur Loup, France
2007	Journées departementales (Departmental retreat), Le Croisic, France (Poster presentation)
2006	Journées departementales (Departmental retreat), Chatou, France (Poster presentation)
2006	Spetsai Summer School 2006, EMBO/FEBS/FEMS Lecture course Spetsai, Greece (Poster presentation)
2006	9th International Conference: DENDRITIC CELLS in Edinburgh, Scotland, Great Britain (Poster presentation)

Oral presentations

2009 Novartis Vaccines & Diagnostics s.r.l, Siena, Italy

Topic: Manipulation of the adaptive immune response by Shigella: Analysis of interactions with lymphocytes in vitro