Determining Cellular and Molecular Targets of The *Yersinia* Tyrosine Phosphatase YopH During Animal Infection

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I dedicate this thesis to my mom, dad, and family and to my closest friends.

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Abstract

The gram-negative enteric pathogen *Yersinia pseudotuberculosis* employs a type III secretion system that is required to translocate effectors, called Yops (*Yersinia* outer proteins), which are necessary for virulence. We identified the host cells in the Peyer's patches, mesenteric lymph nodes and spleen targeted for injection by YopH during oral-gastric route of infection (Chapter II). Using a fluorescence-based assay that employs a membrane permeable dye, CCF2-AM, that is a substrate for a YopH–βlactamase fusion protein, we identify the cell types targeted by using fluorescent antibodies specific for various cell markers. YopH preferentially targeted macrophages, neutrophils and dendritic cells in the spleen, Peyer's patches and mesenteric lymph nodes. In contrast, when spleen cells are harvested and then infected with Yersinia expressing H-TEM all cell types were equally susceptible to injection of YopH. When we analyzed the co-localization of bacteria in different organs such as Peyer's patches and spleen the bacteria localized preferentially with macrophages and neutrophils, in contrast in the mesenteric lymph nodes yersinia also localized with the same cells but also in B and T cells areas.

Interestingly, when the architecture of the organ was destroyed, *Yersinia pseudotuberculosis* preferentially targeted macrophages and neutrophils in the spleen at low multiplicity of infection (MOI) *ex vivo.*

In isolated splenocytes, YadA and Invasin increased the number of all cells types with translocated Yops, but professional phagocytes were still preferentially translocated with Yops in the absence of these adhesins. Together these results indicate that *Y. pseudotuberculosis* discriminates among cells it encounters

during infection and selectively delivers Yops to phagocytes while refraining from translocation to other cell types.

In addition, we were interested in analyzing the molecular targets of YopH in neutrophils during murine infection (Chapter III). YopH is a tyrosine phosphatase that inhibits a number of molecular processes such as ROS production, Ca+ flux and phagocytosis in tissue culture models. To identify the molecular targets of YopH we incubated splenocytes with CCF2-AM. Neutrophils translocated with YopH were sorted using a neutrophil fluorescently labeled antibody. Neutrophils were lysed and the molecular targets of YopH were determined using phospho tyrosine antibodies by Western blot. SLP-76 pathway was identified as a target of YopH during infection. Slp-76 responds to and transmits intracellular signaling once the receptor is engaged by its ligand. In addition, YopH translocation leads to a reduction of IL-10 production during infection. To our knowledge, we have identified for first time cellular targets of Type III secretion during murine infection.

Chapter I

1. Chapter I: Introduction

1.1 The Yersinia genus

Yersinia is a genus of bacteria in the family Enterobacteriaceae [1]. The *Yersinia* genus is composed of 11 species: *Yersinia pseudotuberculosis* (*Yptb*), *Yersinia pestis, Yersinia enterocolitica, Yersinia aldovae, Yersinia bercovieri, Yersinia frederiksenii, Yersinia intermedia, Yersinia kristensenii, Yersinia mollaretii, Yersinia rohdei* and *Yersinia ruckeri* [2]. *Yersinia* are Gram-negative non-sporing rods that can grow at temperatures from 4^{o}C to 40^{o}C, although their optimal temperature for growth ranges from 30^{o}C to 34^{o}C [3]. Yersinia are facultative anaerobes [4]. All *Yersinia* spp. are capable of growing on McConkey plates, are oxidase-negative, catalase-positive and can ferment glucose [5].

1.2 The Yersinia pathogens

There are three *Yersinia* species pathogenic to humans: *Y. pestis*, *Yptb* and *Yersinia enterocolitica* [6]. Both *Yptb* and *Y. enterocolitica* are flagellated and motile at 26^{o}C but become non-motile at 37^{o}C. In contrast *Y.pestis* is encapsulated and non-motile [7].

Y. pestis, the causative agent of bubonic and pneumonic plague, has been responsible for millions of deaths around the world and has caused several pandemics [8]. *Y. pestis* is transmitted from rodents to humans by the bite of fleas infected with bacteria; in contrast *Yptb* and *enterocolitica* are both food-borne pathogens [4].

These three species have common features that include a 70-kb virulence plasmid and tropism for the lymph nodes [9]. Many genes important for causing

disease have been identified on the chromosome and on the virulence plasmid. The 70-kb virulence plasmid encodes for the TTSS and the effectors called *Yersinia* Outer Proteins (Yops) [9-10]. The virulence plasmid has different names depending on the species: specifically, plasmid *Yersinia* Virulence (pYV) in *Y. enterocolitica*, plasmid Calcium Dependency (pCD) in *Y. pestis* and plasmid pIB1 in *Yptb* [11].

1.2.1 *Yersinia pestis*

Y. pestis is an extremely pathogenic species that evolved from an ancestral *Yptb* strain 1500–20,000 years ago, and the first known pandemic of plague occurred in the 6th and 7th centuries [12]. *Y. pestis* contains two additional plasmids important for bacterial survival in, and transmission by fleas as was as few extra chromosomal genes since its divergence from *Yptb* [13], suggesting that the majority' of *Y. pestis* virulence traits were passed on directly from *Yptb*.

One plasmid named Pesticin, Coagulase and Plasminogen activator (pPCP1) encodes the plasminogen activator (Pla), critical for virulence through the subcutaneous route, the bacteriocin pesticin and the pesticin immunity protein [14]. The pesticin activity has not been associated with virulence of *Y. pestis* [15]. Pla proteolytically cleaves plasminogen into plasmin, a potent serine protease. *pla* mutants cause a local infection at the site of inoculation but are not able to

1.1 Virulence determinants or functions involved in molecular evolution of *Y. pestis*

Function and protein name	Gene identifier(s)	Description	Status in organism:	
			Yptb	*Y. pestis*
Colonization and dissemination				
Inv	YPO1793	Invasin	Present	Inactivated
YadA	YPCD1.87c	Adhesin and invasin	Present	Inactivated
Pla	YPPCP1.07	Plasminogen activator promoting bacterial in vivo dissemination	Absent	Present
Elimination of host immune response				
YadA	YPCD1.87c	Serum resistance	Present	Inactivated
F1 capsule	YPMT1.81c to YPMT1.84	Resistance to phagocytosis	Absent	Present
O-antigen genes		Lack of O antigen is essential for Pla function	Present	Inactivated
Other virulence determinants				
Ypf	YPO2272 to YPO2281	Filamentous prophage	Absent	Present
Y. pestis-specific chromosomal regions				
	YPO0387 to YPO0396	Hypothetical proteins	Absent	Present
	YPO2087 to YPO2093	Prophage proteins	Absent	Present
Y. pseudotuberculosis-specific chromosome regions				
LpxL	YPTB2046	Loss of LpxL enables *Y. pestis* to evade LPS-induced inflammation	Present	Absent
R1	YPTB0872 to YPTB0878	Methionine salvage pathway required for virulence of *Yptb*	Present	Absent
ORF3 and ORF4	YPTB1495 and YPTB3368	Hypothetical proteins essential for viability of *Yptb*	Present	Absent
ORF2	YPTB1058	Putative pseudouridylate synthase necessary for optimal growth of *Yptb*	Present	Absent
R3	YPTB2193 to YPTB2201	Hypothetical proteins necessary for optimal growth of *Yptb*	Present	Absent

Modified from Dongsheng Zhou, and Ruifu Yang , AIA , 2009

spread systemically [16-17]. Presumably, Pla a potent serine protease enhances the invasiveness of *Y. pestis* by perturbing the host tissue barriers [14]. Pla also cleaves the C3 protein of the complement system and as a result it interferes with the complement activation and reduces the levels of chemokines at the site of infection. As a result, fewer inflammatory cells are recruited to the site of infection after a subcutaneous injection of mice with a *Y. pestis* (Pla+) [14].

The second plasmid, plasmid Murine Toxin 1 (pMT1) encodes a toxin and the capsular protein F1, which are important in the transmission of the disease. *Yersinia* murine toxin (Ymt) is highly lethal for mice and is necessary for *Y. pestis* for survival in fleas [18].

Y. pestis strains can be classified into four biovars based on the capability to ferment glycerol and arabinose and to reduce nitrate: *antiqua* (glycerol positive, arabinose positive, and nitrate positive), *mediaevalis* (nitrate negative, glycerol positive and arabinose positive), *orientalis* (glycerol negative, arabinose positive, and nitrate positive), and *microtus* (arabinose negative, nitrate negative and glycerol positive) In-frame deletion in *glpD* gene results in the glycerol-negative characteristic of biovar *orientalis* strains [19].

A method used to investigate the degree of homogeneity of *Y. pestis* is using ribotyping. Ribotyping classifies bacteria upon differences in rRNA, using the rRNA gene restriction patterns and hybridization with a 16S-23S rRNA probe from *Escherichia coli* [20]. The combination of the EcoRI and EcoRV patterns results in the elucidation of 16 ribotypes for *Y. pestis*. Two of them B and O ribotypes comprise the majority of strains (65.7%) isolated in a period of 72

years, while the remaining ribotypes represent less than 5 percent each [21]. A correlation was established between biovars and ribotypes: strains of biovar Orientalis were of ribotypes A to G, isolates of biovar Antiqua were of ribotypes F to O, and strains of biovar Medievalis were of ribotypes O and P [21].

1.2.3 Enteric *Yersinia* sp.

Yptb has a number of genes that no longer function in *Y. pestis* (CO92) that have been inactivated by frameshifts or deletions [13]. Those genes appear to be closely connected to flea-borne transmission and increased virulence of *Y. pestis Y. pestis* has as many as 30 copies of an insertion element named IS*100* both within the chromosome and on all three plasmids [13]. One example of a gene been disrupted is invasin (Inv). Its inactivation abrogates the ability of *Y. pestis* to invade epithelial cells [13] (TABLE 1.1).

Yptb and *Y. enterocolitica* lineages separated between 0.4 and 1.9 million years ago [12]. *Yptb* infections in humans are acquired by the ingestion of contaminated food products. The clinical manifestations are fever, abdominal pain, but diarrhea is uncharacteristic. Pathogenicity has been ascribed to a number of virulence factors, including the Yops, that are translocated into the host cell by a type III secretion system, the high pathogenicity island (HPI) and the adhesion molecules Inv and YadA [13]. Enteric *Yersinia* spp. colonize the gastrointestinal tract and can cross the intestinal barrier and colonize the Peyer's patches and the mesenteric lymph nodes. After oral inoculation both species can

spread systemically and colonize liver, spleen and even lungs causing a lethal infection in mice [22-25].

Y. enterocolitica strains can be isolated from a wide range of vertebrate hosts, including domesticated animals and sylvatic animals [26]. Disease outbreaks have been seen in small mammals [26-27]. Isolates of *Y. enterocolitica* strains that are pathogenic to humans generally do not cause disease in animals and vice versa [28]. *Y. enterocolitica* is classified into biotypes, based on biochemical properties such as daesculin hydrolysis, salicin fermentation and pyrazinamidase activity [29]. Six biotypes have been characterized. Biotypes 2, 3, and 4 are most prevalent in humans [30].

Serotypes are based on O-antigen (O-Ag) (lipopolysaccharide) and H-antigen (flagellum) antigens. 60 serotypes have been isolated [31]. The serotypes that cause disease to humans are O:3, O:5,27, O:8, O:9, and O:13 [32]. . Serogroup O:9 *Yersinia* stable toxin^{+} (Yst1^{+}) *Y. enterocolitica* results in diarrhea and death in rabbits orally infected [33]. *Y. enterocolitica* biotype 4 serogroup O:3, the strain most frequently associated with human illness, commonly colonizes the lymphoid in pigs, which are to be though to be the natural reservoir [26]. *Y. enterocolitica* O:3 can be found in butcher shops and supermarkets pork food [34]. Despite the fact that serogroup O:3 is nontoxigenic (Yst1^{-}) this strain is also capable of producing diarrhea in mice [35]. A non-invasive biotype 1A, serogroup O:6 strain was found from a child with diarrhea. This strain produces a heat stable enterotoxin (Yst II) [36]. Yst II antigenically related to Yst but differs in its DNA sequence [36].

In terms of its disease distribution in the world *Y. enterocolitica* is much more common in northern Europe, Canada, and Japan [37]. The vast majority of strains isolated in Canada and Europe are O:3 and O:9 serotypes [38]. The O:3 serotype is common in Japan [39].

Yptb strains are separated into serogroups (O:1-O:15) based mostly on antigenic differences in the O-Ag [54]. Genes necessary for the O-Ag biosynthesis are arranged in a gene cluster flanked by the *hemH* and *gsk* genes in the *Yptb* chromosome [11]. The O-Ag gene clusters of 21 strains of *Yptb* representing all identified serotypes fall into discrete groups reflecting the differences in the chemical composition of the O-Ags [40]. Serotypes O:1-O:5 are associated with animal disease while others not [41].

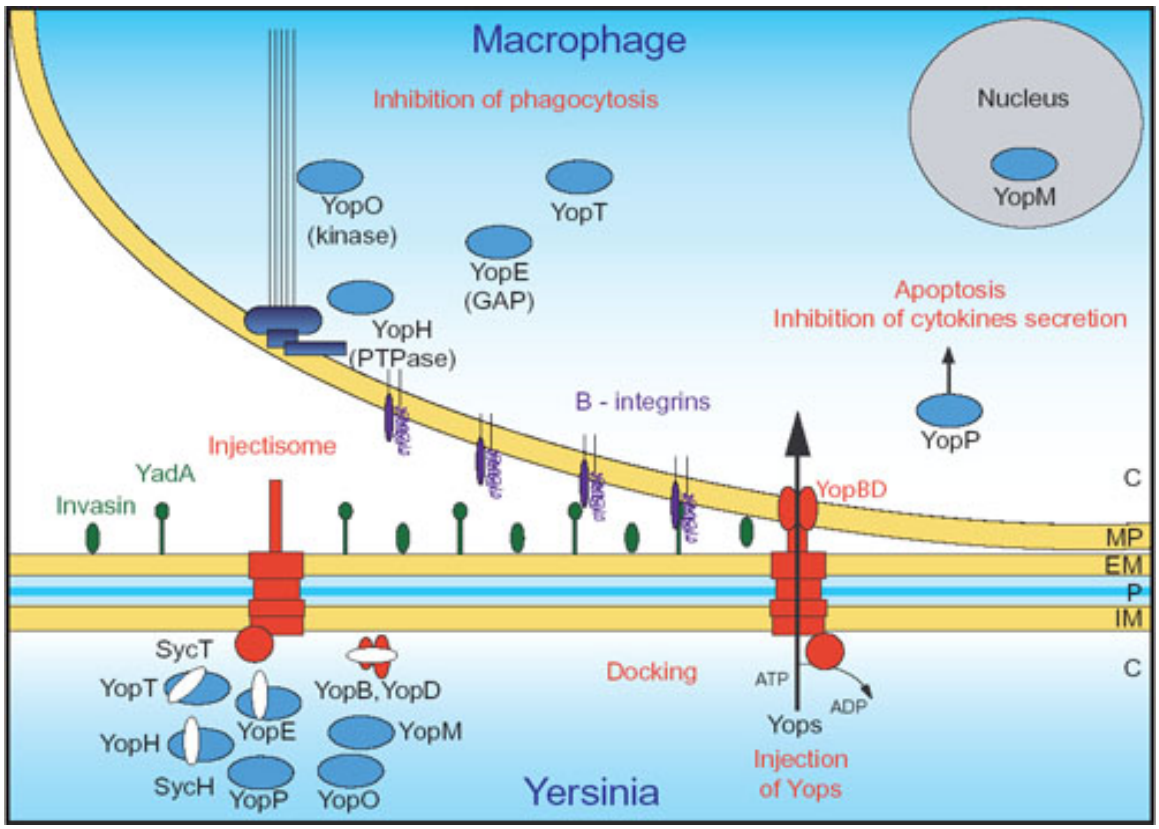
1.3 Type III Secretion system and Yersinia outer proteins

The type III secretion system and the Yops are encoded on a 70 Kb virulence plasmid [9]. This virulence plasmid is critical for *Yersinia* pathogenicity [9]. When grown at 37^{o}C and prior to contact with eukaryotic cells, *Yersinia* form structures at their surface which resemble syringe-like structures [9-10]. These structures, named the Ysc injectisome, are protein pumps that cross the peptidoglycan layer and the two bacterial membranes, are topped with a needle-like structure sticking outside the microorganism [9]. It is thought that the injectisome serves as a hollow tube that allows proteins to pass through the two membranes and the peptidoglycan layer [10]. The length of the needle is genetically controlled [42] and is formed by the polymerization of YscF

monomers. The tip of the YscF needle is capped by the low-calcium-response V or V antigen (LcrV) that forms a pentameric structure [43-44]. Upon contact with a eukaryotic cell, the bacterium inserts the translocon into the plasma membrane [44]. The translocon consists of LrcV and two pore forming proteins, YopB and YopD that make the pore through which the Yops are delivered. LcrV is necessary for pore formation but is not an integral membrane protein itself [44].

Most of the *Yersinia spp.* translocate six Yops ,YopE, YopH, YopM , YopT, YopO (YpkA) and,YopJ(YopP) into host cells [9]. The function of the Yops is to interfere with the signaling transduction cascades of mammalian cells and prevent the cell from responding effectively to bacterial infection [9]. Four of the identified (YopH, YopE, YopT, and YopO/YpkA) effectors disrupt the cytoskeleton dynamics and prevent phagocytocis [9] (FIG 1).

My thesis focuses on identifying the cells targeted by the TTSS during animal infection and determining the function of YopH in one of those targeted cell types. During the introduction, I will discuss the function of YopH and will review other *Yersinia* host factors required for translocation. Additionally, I will review which are the likely cell types encountered by *Yersinia,* as well as the cells targeted by other TTSS in different pathogens.



Biol Chem ,2010

Fig.1.1 *Yersinia* model for translocation of Yops.

Yersinia translocates Yops (bue) into the cytoplasm using a needle –like structure called injectisome. Tight contact with the host cell is required and is mediated by bacterial adhesins (green) e.g. YadA and Inv that bind to mammalian integrins (purple). Translocation of Yops is dependent on the presence of two pore-forming proteins YopB and YopD. Overall functions of Yops are inhibition of phagocytosis, ROS production, cytokine production and other cellular processes to effectively downregulate the immune response.

1.4 YopH

1.4.1 Molecular structure of YopH

The C-terminal catalytic domain of YopH is extremely homologous to eukaryotic PTPase enzymes [45]. All PTPases including YopH bear a highly conserved active site motif C(X)5R (PTP signature motif), that use a general catalytic mechanism [46]. The critical cysteine from PTP signature motif, located at position 403 in YopH, when mutated abrogates its enzymatic activity [46]. This catalytically inactive variant of YopH can still bind substrates of YopH and it has been used to trap host cell substrates of YopH in association with YopH [47]. The amino-terminal, non-catalytic domain of YopH has two functions. It is crucial for YopH secretion and binding of the YopH specific chaperone SycH [48]; and it also bind to several catalytic domain to substrates in eukaryotic cells [45].

The crystal structure of residues 1–129 of YopH from *Yptb* has been solved [45]. The segment required for secretion (residues 2–17) is the α1-helix, while the translocation domain (18–71) consists of the β1-strand. Residues 35–49 connect the β1-strand to α2 [45]. These residues are part of the region crucial for binding to SycH. Additionally, even though YopH displays ligand specificity comparable to that of the SH2 (Src Homology 2) domain, crucial for recognition of phosphorylated tyrosine residues on the other protein, the N-terminal amino acid sequence or tertiary structure does not have any resemblance with identified SH2 domains, usually found in eukaryotic tyrosine phosphatases [45] .

Four mutations (Q11R, V31G, A33D, and N34D) are known to have an effect on binding of the YopH N-terminal portion one of the known yopH target,

Cas however, these mutations do not alter the binding of YopH to its chaperone SycH [48]. Interestingly, addition of V31G substitution into substrate-trapping YopH reduces the ability of YopH to bind to focal adhesion p130Cas and to localize to focal adhesions in epithelial cells [48]. Furthermore, the V31G mutation decreases the ability of YopH to dephosphorylate target proteins in these cells. All these data suggest that the substrate- and SycH-binding activities of the YopH N-terminal domain can be separated and that the substrate function is critical for detection and dephosphorylation of substrates by YopH [48]. These mutations do not interfere with YopH translocation [48] but an observation I made in the lab is that these mutants had an overall reduction of YopH secretion compared to the WT suggesting that these mutants might have a regulatory defect.

Strains with either Q11R or V31G mutations in *Yptb* are not attenuated in a lung model [49]. In addition, the V31G mutant survives as well as WT in competition experiments in mice infected intravenously with equal dose of the mutant and WT *Yptb* [50]. These two observations together support the idea that pCas130 binding to YopH is not required for mice colonization.

1.4.2 Cell culture phenotype of YopH

In culture cells, YopH is a potent tyrosine phosphatase that interferes not only with phagocytosis but also with ROS production, calcium flux and cytokine secretion in a vast array of tissue culture cells in *Yptb* [51-54]. When translocated into macrophages, YopH dephosphorylates p130Cas and disrupts focal adhesions molecules [55].

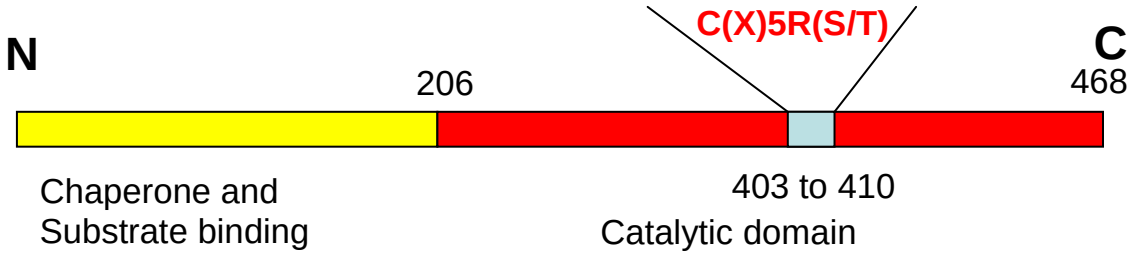


Fig.1.2 Protein Structure of Tyrosine Phophatase YopH

YopH also dephosphorylates the Fyn-binding protein Fyb and SKAP-HOM in macrophages [56-57]. These proteins interact and become tyrosine phosphorylated as a consequence of macrophage adhesion [57]. Interestingly, it has been shown that a central part of YopH interacts with the Fyb C-terminus in a phosphotyrosine-independent manner and that the N-terminal binding region of YopH is required for dephosphorylation of Fyb, inhibition of phagocytosis, and cytotoxicity of macrophages [56]. Using a YopH substrate trapping protein, it has been shown that Paxillin, a signal transduction adaptor protein, is enriched at focal adhesion sites in HeLa cells [58]. Paxillin is co-immunoprecipitated with YopH and dephosphorylated by YopH when lysates from HeLa cells infected with *Yptb* expressing YopH are subjected to immunoprecipitation with an anti-paxillin antibody [59].

Treatment of T cells with a recombinant membrane-permeable YopH leads to a drastic reduction in intracellular tyrosine phosphorylation and downregularion of T cell activation [60]. Lymphocyte-specific protein tyrosine kinase (Lck) was specifically precipitated by a substrate-trapping YopH mutant and Lck is inactivated at tyrosine residue that enhances cell activation when phosphorylated in T cells, thus leading to a total loss of Lck activity and, as a result, any tyrosine phosphorylation of downstream effector proteins [60]. Also in T cells, YopH targets the adaptor proteins which serve as a linker for activation of T cells (LAT) and SH2-domain–containing leukocyte protein of 76 kD (SLP-76), both of which are crucial for T cell antigen receptor (TCR) signaling [52]. Using HEK293 cell line it has been proposed that YopH interacts with other several

molecules, such as Gab1, Gab2, p85, and Vav but only Fyb, Lck, or PI 3 Kinase p85 are dephosphorylated by YopH [61].

YopH is also thought to inhibit inflammatory responses in macrophages in *Y. enterocolitica*, since YopH downregulates the production of the monocyte chemoattractant protein 1 (MCP1), a chemokine implicated in the recruitment of professional phagocytes to the sites of infection [62]. The mechanism of action has been proposed recently, where YopH dephosphorylates PI3 Kinase p85 blocking the PI3K/Akt pathway that induces MCP-1 production in HEK293 cells [61]. Similarly, it has been shown T cells are inhibited by YopH in their ability to produce cytokines, and B cells are incapable of upregulating surface expression of the costimulatory molecule, B7.2, after T cell mediated antigenic stimulation [63].

Below I discuss these targets of YopH in more detail.

1.4.3 SKAP-HOM

A tyrosine-phosphorylated protein p55 was immunoprecipitated using a substrate trapping form of YopH (YopHC403S) in the macrophage cell line J774A [57]. This protein also displays a decrease tyrosine phosphorylation in the presence of YopH and was purified and identified by mass spectrometry as SKAP-HOM suggesting that YopH disrupts the focal adhesion complex by a direct interaction with SKAP-HOM [57].

Focal adhesions are multi-molecular complexes at sites of integrin binding [64]. These complexes can transducer extracellular signals [64]. SKAP-HOM (also known as SKAP55R, RA70 and PRAP) is a cytosolic adaptor protein of

molecular weight 55 kDa [65]. SKAP-HOM displays homology to the adaptor protein SKAP55 (Src Kinase Associated Protein of 55 kDa) present in T cells [65]. The protein structure is composed of an amino (N)-terminal coiled-coil domain that is proceeded by a Pleckstrin homology domain (PH), two tyrosine-based signaling motifs (TBSMs) and a carboxyl (C)-terminal SH3 (Src-homology domain 3) domain [65]. SKAP-HOM is ubiquitously expressed in many cell types [66]. In macrophages SKAP-HOM becomes tyrosine phosphorylated after engagement of integrins by fibronectin (a ligand for β1 integrins) or ICAM-1(a ligand for β2 integrins) and is important for cell adhesion [67].

1.4.4 P130Cas

Interaction of the YopH N-terminal Domain with Tyrosine phosphorylated p130Cas was demonstrated in the Yeast Two-hybrid System [48]. In addition, the presence of p130Cas and another protein important for adhesion FAK was confirmed by immunoprecipitation with YopHC403A when blotting with anti-p130Cas and anti-FAK antibodies. The lack of FAK and p130Cas in the IP with WT YopH indicates that the interaction of YopH with these proteins is necessary for them to be phosphorylated to interact stably with YopH [68].

p130Cas (Crk-associated substrate) is an important Src substrate also localized at sites of cell adhesion to the extracellular matrix [69]. p130Cas is an scaffold protein that has many protein-protein interaction domains as well as an N-terminal SH3 domain, a “substrate domain” in the internal region of the protein and a Src-binding domain near the C terminus [70]. The “substrate domain” is characterized by 15 Tyr-X-X-Pro (YxxP) motifs that characterize the main sites of

tyrosine phosphorylation [69]. Tyrosine phosphorylation of substrate domain YxxP tyrosines creates docking sites for recruitment of SH2-containing signaling effectors [71-72]. Recruitment of Crk adaptor proteins to the substrate domain sites has been implicated in promoting Rac activation and cell movement [72].

Src binds to p130Cas by two separate mechanisms: first by direct binding, where the Src SH3 domain binds to the subtrate binding domain [73]. The second mechanism is by indirect binding, where the Src SH2 domain binds to a FAK family kinase that is bound to the p130Cas SH3 domain [73]. These two mechanisms of action contribute to phosphorylation of p130Cas substrate domain. The p130Cas SH3 domain also interacts with phosphotyrosine phosphatases PTP1B and PTP-PEST indicating that this domain can act as a molecular switch regulating both phosphorylation and dephosphorylation of substrate domains [69].

p130Cas tyrosine phosphorylation occurs in response to stimulation of a broad diversity of cell surface receptors such as receptor G-protein-coupled receptors, tyrosine kinases, ICAMs ,etc [70-72].

1.4.5 FAK

Focal adhesion kinase (FAK) of molecular weight 125-kDa is involved in a focal adhesion complexes [74]. FAK is an essential protein since its absence causes lethality in mice. The N terminus domain of the protein Fak has an autophosphorylation site that binds to the cytoplasmic domain of β1 integrin [75]. At the C terminus FAK binds to several molecules, including paxillin, Cas, and some other proteins that have SH2 domains [76]. The extracellular domain of

integrin receptor binds to the extracellular matrix inducing integrin clustering and recruitment of FAK. This kinase is subsequently phosphorylated and FAK associates with Src. This association of FAK with Src plays an important role in adhesion and migration [77]. The Cas-FAK binding increases phosphorylation of Cas, which causes recruitment of Crk which causes activation of small GTPase Rac1 to stimulate cell migration [78].

1.4.6 Paxillin

Upon integrin engagement Paxillin is tyrosine-phosphorylated by FAK and Src, creating binding sites for the adapter protein Crk which (via association with CAS) is crucial for transduction of outside-in signals that leads to cell motility and activation of several transcription factors that leads to a change in gene profile [79]. Paxillin has a number of motifs that mediate protein-protein interactions, including leucine-rich repeat sequences that begin with a leucine (L) and aspartate (D) (LD domain), two contiguous zinc finger domains, separated by a two-amino acid residue hydrophobic linker (LIM domains), an SH3 domain-binding site and SH2 domain-binding sites [76, 79]. These domains serve as docking sites for cytoskeletal proteins, kinases, GTPase activating proteins and other adaptor molecules that bring additional proteins into complex with paxillin [76]. Thus paxillin itself serves as a scaffold protein to recruit molecules to a particular cellular compartment, the focal adhesions, and to coordinate downstream signaling. Paxillin plays a key role coordinating signaling that regulates to motilyty and cell spreading [76, 79].

Paxillin was dephosphorylated by YopH in Hela cells by YopH. Paxillin was found in HeLa lysates before and after precipitation of YopHC403S using an anti-Paxillin antibody and was detected in the precipitate, suggesting that Paxillin paxillin interacts specifically with full-length YopHC403S in HeLa cells [58] .

1.4.7 LAT

LAT is an adaptor molecule associated with the plasma membrane in T, NK and mast cells [80]. In T cells, LAT plays a key role in TCR signaling. Upon activation of TCR receptor, Zap-70 phosphorylates Y171 and Y191 tyrosine residues of LAT within SH2 binding motifs, thus making LAT available for docking of downstream signaling targets e.g. Grb2, Gads/SLP-76, PLCγ1 and PI3 kinase [81-82].

1.4.8 Slp-76

SLP76 is found in cells in the hematopoietic lineage; specifically, SLP76 is expressed by platelets, macrophages, natural killer (NK) cells, developing B cells, neutrophils and mast cells [83]. Slp-76 has three main domains: an amino (N)-terminal acidic region with three tyrosine phosphorylation motifs (Y112, Y128 and Y145), a central proline-rich domain and a carboxy (C)-terminal SH2 domain [83]. The amino acids Y112 and Y128 mediate binding to VAV1, NCK and the p85 subunit of PI3K, while Y145 binds ITK [83]. The central proline-rich domain mediates binding of LCK and PLCγ1. The C-terminal SH3 domain binds Gads and C-terminal SH2 domain binds ADAP and HPK1 [83].

SLP76 was first identified as a target of the tyrosine kinases that are required for T cell signaling upon activation of T cell receptors (TCR). Slp 76 is a

scaffold protein that recruits other signaling molecules to induce downstream activation of TCRs [84-85]. Association of the TCR with a peptide antigen-bound MHC complex allows the interaction of CD4 and CD8 with MHC class II and class I molecules, consequently recruits the associated LCK to the area of the TCR/CD3 complex [86]. Upon TCR/CD3 engagement LCK is the first protein kinase that gets phosphorylated [87]. As a result of LCK phosphorylation immunoreceptor tyrosine-based activation motifs (ITAMs) present in each of the CD3 proteins of the TCR complex gets phosphorylated [83]. Phosphorylated ITAMs recruit and activate ZAP70. Zap70 phosphorylates SLP76 and LAT [83]. LAT is constitutively localized to the plasma membrane [88]. Phosphorylation of LAT recruits SLP76 to the cell membrane, where the two adaptors nucleate a multimolecular complex that contains several signalling proteins, including PLCγ1, VAV, NCK, ITK, ADAP, LCK and HPK1 [83]. When Slp-76 is overexpressed in Jurkat cells the protein plays a positive role in regulating T cell activation [83].

TCR engagement in SLP76-deficient Jurkat cells shows that PLCγ1 phosphorylation is decreased and as a consequence calcium flux is inhibited. Interestingly, phosphorylation of ZAP70, LAT and ITK are unaffected [83].

Slp76, an adaptor molecule, plays a critical role in Fc receptor and integrin signaling in neutrophils [89]. Activation of both signals leads to tyrosine phosphorylation and cytoplasmic relocalization of Slp76. Upon stimulation of Fc receptor or integrins in neutrophils lacking Slp76, ROS production was

significantly decreased [89], suggesting that SLP76 is important for keeping the bactericidal functions of neutrophils.

It has been shown that inhibition of an upstream effector of Slp76, Syk leads to a decrease of IL-10 in neutrophils [90]. It has been also demostrated that IL10 production is tightly regulated with ROS production and Calcium flux in a Syk dependent manner [91]. Syk has not been shown to be a target of YopH in neutrophils but previously, its homolog Zap70 has shown to be target of YopH in T cells [60].

Lat and Slp76 were specifically immunoprecipitated with the substrate trapping form of YopH in Jurkat cells (*yopHC403A-HA) Yptb* following activation of T cells with the CD3-specific antibody. This suggests that phosphorylated LAT and SLP-76 in activated T cells are primarily targeted by YopH. In contrast, neither adhesion FAK, paxillin,or p130cas which are primary targets of YopH in macrophages and epithelial cells were found co-precipitated with the substrate trapping form of YopH [52].

1.4.9 Lck

Lymphocyte-specific protein tyrosine kinase **(**Lck) is a 56 kDa protein found in T cells [92]. Lck plays an important role in signaling through the TCR receptor, when the T cell receptor is engaged by the particular antigen presented by MHC [93]. It binds to the cytoplasmic domains of the T helper and cytotoxic T cells co-receptors on CD4 and CD8 T cells, respectively [93]. Lck phosphorylates the ITAMs of the CD3 [87, 92]. After this ZAP-70 binds to them, Lck then phosphorylates and activates ZAP-70, which in turn phosphorylates LAT [94].

The tyrosine phosphorylation cascade started by Lck leads to intracellular mobilization of a calcium and activation of important signaling cascades within the lymphocyte [94]. For example Ras-MEK-ERK pathway that changes the gene expression (e.g IL-2) in the cell by activating NFAT, NF-κB, and AP-1 transcription factors [95].

The N-terminal tail of Lck is hydrophobically modified (palmitoylated and myristoylated), these modifications allow the protein to anchor the cell membrane [96]. In addition, the protein has a SH3 domain, a SH2 domain and in the C-terminal part lays the tyrosine kinase domain [97]. The two major tyrosine phosphorylation sites are Y394 and Y505 [98]. Y349 is an autophosphorylation site and is linked to activation of Lck [98]. Y505 is phosphorylated by Csk, which inhibits Lck function [98]. In resting T cells, Lck is constitutively inhibited by Csk phosphorylation on Y505 [99]. Lck is also inhibited by SHP-1 dephosphorylation on tyrosine 394 [100]. Lck can also be inhibited by Cbl ubiquitin ligase that is part of the degradation machinery of the cell [101]. Lck therefore may play a role as an activator or as an inhibitor of T cell activation. Lck phosphorylates ZAP-70 that induces phosphorylatyon of SLP-76, IL-2 receptor, Protein kinase C, ITK, PLC, SHC, RasGAP, Cbl, Vav1, and PI3K [85].

YopH dephosphorylates Lck at Tyr-394 as a result YopH blocks TCR signaling at its most receptor-proximal step in T cells [60]. Using a substrate

TABLE

1.2 Targets of YopH in tissue culture

Protein targets(kDa)	cell types	phenotype
SKAP-HOM(40)	macrophages	cell adhesion
p130Cas(130)	macrophages	cell adhesion
Paxillin(68)	epithelial cells	cell adhesion
FAK (125)	epithelial cells	cell adhesion
Slp-76 (76)	T cells	T cell activation
LAT (38)	T cells	T cell activation
Lck (50-60)	T cells	T cell activation
?	Neutrophils	Oxidative burst

trapping form of YopH in (YopH-D356A) the investigators showed that Lck – YopH interaction it was more likely to be direct [60].

1.5 Other Yersinia Outer Protein (Yops)

This thesis work focuses primarily on the effectors YopH and YopE. Since the work done in Chapter 2 can be applied to other Yops as well, I will review YopE and the rest of the Yops in this introduction.

1.5.1 YopE

YopE is a cytotoxic effector that disrupts the actin microfilament structure of cultured HeLa cells [9]. YopE is a 219 amino acid protein [102]. The first 15 amino acids of the N-terminal domain are required for secretion of YopE [103]. Translocation into eukaryotic cells, requires the N-terminal 50 amino acid residues of YopE when translocation is measured by cyclase activity of YopE1-50-CyaA fusion protein [104]. In our studies we fused the first 100 amino acids of YopE to the β-lactamase gene to assess for translocation of Yops using a CCF2-AM β-lactamase sytem (see details in material and methods in Chapter II)

YopE is a GTPase activating proteins (GAP) that accelerates GTP hydrolysis of small GTPases [9]. The GAP activity of YopE is necessary for virulence because a catalytically inactive mutant, *YopER144A*, is attenuated during infection animal infection [105]. Purified YopE targets small GTPases Rac1, RhoA,Rac2 and Cdc42 while in tissue culture cells, YopE inactivates RhoA, Rac1, Rac2, RhoG but not Cdc42 [105-107].

In mice infected intravenously *with Y. pestis* carrying a YopE-beta lactamase fusion protein, lactamase activity is detected in macrophages,

neutrophils and dendritic cells [108]. This result suggests that YopE functions in these cell types during infection.

YopE is important for enteropathogenic *Yersinia* species to colonize small intestine, Peyer's patches (PP), mesenteric lymph node (MLN) and spleen in mice [24]. Two mutants of YopE with different antiphagocytic, ROS-inhibitory and cell-rounding activities demonstrate that both phagocytosis and ROS production are essential for colonization in the spleen, while those YopE activities were less required for Peyer's patch colonization [105] .

1.5.2 YopM

While YopM has an essential role in pathogenesis since mutants of YopM are attenuated during oral infection, its function has until now not been determined [9]. YopM contains a leucine-rich repeat motifs (LRR) that might be important for protein-protein interactions. YopM can be found in the cytoplasm and in the nucleus [9]. A possible role of YopM has been proposed of controlling cell cycle and cell growth in mouse macrophages after transcriptional analysis [109], but data of another group contradicts these findings since YopM did not affect host cell transcription [110].

In previous experiments it was shown that YopM interacts with α-thrombin and inhibits platelet aggregation, but again these activities do not appear to be important during animal infection [111].

PRK2 and RSK1 have been shown to interact with YopM [112]. YopM stimulates the activity of both kinases *in vitro* [113]. YopM activation of RSK1 is independent of activity of YopJ on the MAPK pathway [113]. In addition, YopM is

also necessary for *Yersinia*-induced changes in RSK1 mobility in infected macrophage cells [113] .

Using an oral route of infection with *Yptb* a *yopM* mutant was attenuated in systemic spread, specifically in spleen, and it was less virulent in mice. Furthermore, *Yptb* mutants expressing YopM proteins that are unable to interact with either RSK1or PRK2 were also attenuated for virulence, suggesting that these interactions may be crucial for *Yptb* to cause disease [112]. Similarly, our lab has showed that C-terminal tail of YopM required for RSK1 interaction is crucial for colonization as *yopM* mutants that failed to interact with RSK1 had less bacterial burden in the tissues [114] .

Depetion of Gr1+ cells in mice resulted in an increase of survival of a *ΔyopM* in both the liver and spleen. In contrast, depletion of DC and macrophages inhibited growth of both WT and mutant strains, suggesting that YopM targets Gr1+cell functions [115].

1.5.3 YopJ

YopJ has several mechanisms to evade inflammatory responses. In tissue culture, this protease is necessary for the inhibition of MAPK and NF-kappa-B signaling [9]. YopJ prevents the activation of latter by inhibiting phosphorylation of I-kappa-B [116]. YopJ also blocks the LPS receptor (IL1-beta receptor), downmodulating the inflammatory response. YopJ blocks LPS-induced clonal expansion of immune cells [117]. YopJ also triggers activation of CREB which mediates transcriptional responses to various extracellular signals [118].

YopJ can function as an acetyltransferase, using acetyl–coenzyme A (CoA) to modify the important serine and threonine residues in the activation loop of MAPKK6 and by this means blocking phosphorylation thereby preventing the activation of signaling molecules [119]. Recently it was proposed that the presence of inositol hexakisphosphate is an activating host factor of YopJ [120].

YopJ also induces program cell death (apoptosis) in cells infected with *Yersinia* [121]. YopJ (in *Y. pestis* and *Yptb* and YopP in *Y.enterocolitica*) causes apoptosis in macrophages by cleaveage of pro-apoptotic molecule BID and the activation of caspase 9, 3 and 7 [122]. In contrast human neutrophils are resistant to apoptosis by YopJ and or YopP [123]. In addition, a strong neutrophils ROS production in response to pYV- *Yersinia* was associated with an increase in apoptosis of neutrophils. This suggests that *Yersinia* inhibition of PMN ROS production is critical for *Yersinia* to escape of the innate immune response due in part to a reduction in neutrophil cell death [123]. At the same time, it has been shown that *Y. pestis* can survive inside pig neutrophils and survival was dependent on PhoP and PhoQ that conferred resistance to the proteolytic action human PMN granule extracts [124].

Some substrates for YopJ are highly conserved ubiquitin like molecules [125]. YopJ stalls some post-translation modification of proteins, preventing addition of ubiquitin to these proteins [125]. The presence of YopJ decreases ubiquitination of TRAF3 and TRAF6 to prevent or remove the K63-polymerized ubiquitin conjugates necessary for signal transduction [126].

In animal infections, as a result of YopJ deletion a twofold decrease in the number of apoptotic immune cells was reported in lymphoid tissues. In addition, three fold increase in tumor necrosis factor alpha (TFF-α) levels in the blood was detected but did not affect virulence, colonization or spread to systemic sites [127].

1.5.4 YopO

YpkA/YopO is a serine/threonine protein kinase implicated in host actin cytoskeletal rearrangements and in inhibition of phagocytosis [128]. At a molecular level, YopO can be subdivided into four functional domains. The N-terminal domain of YopO plays a role as the chaperone-binding site and mediates plasma membrane localization after translocation into host cells [129] [130]. It is flanked by a serine/threonine kinase domain shown to phosphorylate the G protein subunit $G\alpha_q$. The C-terminal region of YopO contains a GDI-like domain able to bind Rac and Rho *in vitro*, this observation has been also supported by the crystal structure [131]. At the end of YopO structure there is short coronin homology domain that binds G-actin and promotes YopO kinase activity [132].

Two small GTPases Rac-1 and RhoA, has been shown to directly interact with YopO in a yeast two-hybrid assay and by immunoprecipitation *in vitro*. This binding is independent of phosphorylation of YopO and and also independent whether Rac-1 and RhoA are bound to GTP or GDP, in other words it is independent on their activation state [133].

N-terminal membrane-association region in conjunction with the GDI-like domain distinctively targets the Rac-mediated phagocytic pathway. Furthermore, it has been shown that the anti-phagocytic activity of YopO works by targeting GDI-free Rac1 at the cell membrane [132]. It is been also shown that YopO is able to distinctively block FcγR-mediated phagocytosis but has no major effect on CR3-dependent internalization [132].

The function of YopO in virulence in murine model of infection is not well elucidated. There is some data that suggest that YopO plays a role in animal virulence. For instance, *Yptb* expressing a mutant that lacks the Rho-GDI-like domain and the actin binding domain, or a strain expressing YopO point mutant that is unable to bind to Rho proteins are attenuated in an oral infection [131]. Likewise, *Yptb* strain expressing a deletion mutant in the kinase activity is also attenuated orally [134]. On the other hand there, deletion of YopO in *Yptb* has no effect on virulence in mice measured by percent survival after oral infection. *ΔyopO* strain of *Yptb* colonizes Peyer's patches, ileum, cecum, MLN, colon, and spleen after oral infection at the same levels that WT [24, 135], suggesting that the presence of a YopO impaired in its function is more deleterious for *Yersinia* that the absence of YopO during animal infection.

1.5.5 YopT

YopT is a cysteine protease and is conserved in the three pathogenic *Yersinia spp*. YopT recognizes prenylated Rho GTPases (RhoA, Rac and Cdc42) in the eukaryotic cell cleaving proteolytically the prenylated cysteine on the

GTPases, as a consequence they detach from the host membrane. Consequently, YopT causes the disruption of the actin cytoskeleton and contributes to the inhibition of phagocytosis of the pathogen [136]. YopT contribution during animal infection is discussed below

1.6 Redundancy or synergistic activities of Yops in tissue culture

Yersinia have evolved mechanisms in which the Yops target different host cell proteins, but with a very similar outcome in host cells with the ultimate objective of counteracting efficient killing propierties of professional phagocytes [9, 43-44]. For example, analyses of infected dendritic cells showed that YopE, YopH, and YopT work together to quickly damage the actin cytoskeleton of dendritic cells [137] .In addition, microscopic analyses and gentamicin killing assays showed that the maximum decrease of bacterial uptake was achieved by *Yersinia* mutant strains translocating only a single YopH or YopE indicating that these Yops enable *Yersinia* to inhibit the phagocytic function of dendritic cells [137].

Infection of HeLa cells with a strain expressing either YopE or YopT showed that YopE had a stronger antiphagocytic activity than YopT. The presence of YopE robustly inhibited activation of JNK, ERK and NFkappaB, and prevented production of IL-8; but YopT moderately inhibited these responses. In contrast, pore formation was inhibited equally by YopE or YopT. In conclusion, YopE is a powerful inhibitor of infection-induced signalling cascades, and YopT can partially compensate for the loss of YopE in cell culture assays [138].

1.7 Redundancy and synergistic activities of Yptb Yops during animal infection

YopE and YopT downregulate Rho GTPases by different mechanisms [44]. Strains carrying YopE in presence or absence of YopT colonize spleens at similar levels, suggesting that YopT is not necessary for virulence. In contrast, spleen colonization by strains lacking both YopE and YopT strains is significantly reduced. A strain lacking YopE but expressing YopT colonizes spleen at levels comparable to those of YopE expressing strain that lacks YopT strain, arguing that YopT can promote virulence in the absence of YopE [138].

While no single Yop was absolutely necessary for colonization or persistence in intestinal tissues in single-strain infections, the lack of both YopH and YopE together nearly eliminated colonization in all tissues, indicating either that these two Yops have some synergistic or redundant activities for colonization [24]. In competition infections with wild-type *Yptb*, the presence of wild-type bacteria interfered with the ability of the YopH *and* YopE mutants to colonize many organs [24].

In lung model developed in our lab using *Yptb,* a *ΔyopH* null mutant failed to grow to wild type levels after intranasal inoculation, but deletions of any other single Yops were not attenuated this lung infection in mice. In contrast, four double mutants of ΔH plus ΔO, ΔE, ΔJ or ΔM were more attenuated in colonization in the lungs than the ΔyopH strain suggesting that each of these Yops contributes to colonization in the absence of YopH [49].

1.8 Yersinia adhesins: YadA and Invasin

Enteropathogenic *Yersiniae* traverse the mucosal membrane of the small intestine, through the M cells in the ileum, and colonizing Peyer's patches and mesenteric lymph nodes (MLN) [22, 24-25]. A number of mechanisms that allow *Yersinia* adhesion *Yersinia* to mammalian cell membranes have been described [139-142]. The adhesins YadA and Inv of *Yptb* and *Y. enterocolitica* have been implicated in the initial adhesive events between the bacterium and the mammalian cells and as a result can control host cell signaling pathways to the advantage of the *Yersinia* [44].

1.8.1 YadA

The YadA (41–44 kDa) protein is a key adhesin of *Yptb and Y. enterocolitica,* interestingly YadA is inactive in *Y. pestis* and it is present as a pseudogene. YadA participates in adhesion to eukaryotic cells by binding to extracellular matrix proteins (ECM) such as fibronectin, laminin and collagen [143]. Antibodies against fibronectin and β_1 integrins reduced invasion, suggesting that invasion occurs via ECM establishing a link connecting YadA and the mammalian cell β_1 integrin receptors [144]. Remarkably, YadA from *Yptb* and *Y. enterocolitica* have different affinity properties to their ligands. For example *Yptb* has more affinity to fibronectin than laminin or collagen, while the opposite is true for *Y. enterocolitica* [144].

Structural analysis of YadA shows that the adhesin has of a collagen binding head domain critical for binding to collagen. In the N terminus novel consensus sequence motif, NSVAIG-S is present and it occurs eight times in the

N-terminal collagen binding head of YadA [145]. These sequences are critical for collagen binding, since mutations in these motifs reduced collagen binding almost completely. YadA also comprises a coiled-coil stalk and a membrane anchor at the C terminus [146]. YadA form oligomers with molecular weight of 160 to 250 kDa, depending on the *Yersinia* species and the serotype [140]. Mutants of YadA with shorter or longer structures can still mediate attachment efficiently to the mammalian cells, but they are defective in translocation of Yops [140]. This defect can be abolished by expressing simultaneously shorter or longer needles of TTSS , made of YscF protein [147].

The *yadA* gene is encoded on virulence plasmid pIB1 [148]. Two of the *Yersinia* outer proteins YopE and YopH, which participate actively in antagonizing the uptake procces cell are coordinately controlled together with YadA [149] by VirF, a positive transcriptional activator protein at 37℃ [150]. The peak of expression of *yadA* is achieved in exponential-phase at 37℃, where expression of *inv* is repressed, suggesting that YadA is the dominant adhesin that mediates intimate attachment and colonization of host tissue after the passage of bacteria through epithelial barrier [151]. These observations suggest that YadA and not Inv will promote binding to neutrophils, dendritic cells and macrophages allowing an efficient translocation of Yops to prevent uptake and killing by professional phagocytes. YadA promotes both bacterial adhesion and invasion totally independently of Inv and its expression neither affect its expression nor activity [151]. *yadA* is regulated in response to a number of

factors, such as temperature, ion and nutrient availability, and density during growth [151].

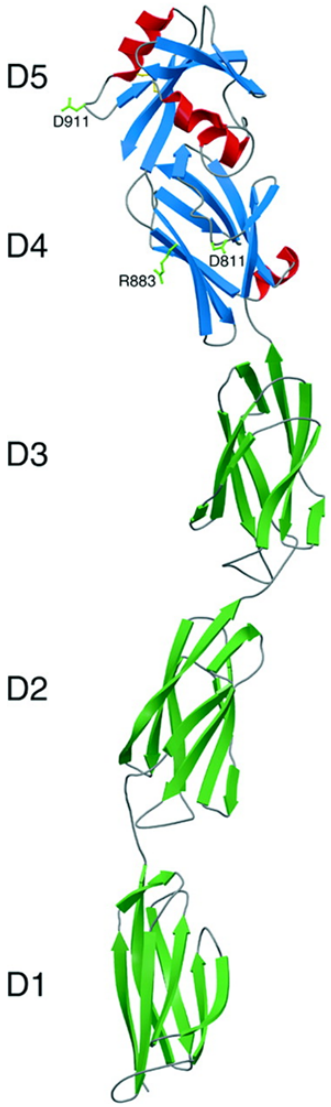
In addition to it role as cell adhesin, YadA seems to play a role in evasion of the innate system since it mediates serum resistance [152]. YadA of *Y. enterocolitica* heavily covers the bacterial surface by forming a capsule-like envelope (18nm) which hides the LPS and protect the bacteria from defensin lysis and complement killing [151]

Moreover, *Y*. *enterocolitica* YadA binds to C4b-binding protein (C4bp). Studies show that YadA acts as a C4bp receptor. C4bp bound to *Y. enterocolitica* participates in the factor I-mediated degradation of C4b, suggesting *Yersinia* uses YadA to promote evasion of complement-mediated killing in the host [153].

The contribution of YadA during animal infection is still contradictory. YadA is a virulence factor of Y. enterocolitica, on the other hand, there is work suggesting that YadA seems to be not necessary for the virulence of *Yptb* measured by LD50 [148, 154]. My data in chapter II shows that YadA and Inv are important for colonization in the Peyer's patches.

1.8.2 Invasin

Invasin is an adhesion molecule that promotes binding and internalization of *Yersinia* into eukaryotic cells [155]. The *inv* gene is located in the chromosome and encodes Invasin (Inv), an outer membrane protein of 103 kDa molecular weight. Inv binds to β_1 integrins on the apical surface of M-cells, thus facilitating



Hamburger *et al*. 1999

Fig.1.3 Ribbon diagram of the structure of *Y. pseudotuberculosis*.
Residues implicated in integrin binding are green. The α-helical regions in D5 and a 3_{10}helix in D4 are red. The disulfide bond in D5 is yellow, and β strands are blue (D4 and D5) or green (D1 through D3).

competent translocation of *Yersinia* across the intestinal mucosa into the Peyer's patches [156].

Inv was first identified in 1985 when the gene was cloned and its protein expressed in *E. coli*. When expressed, Inv was sufficient to convert a non-invasive strain of *E*. *coli* into an organism capable of invading Hep-2 cells [157].

Structural analysis of Inv show that the N-terminal region is anchored in the outer membrane, while the C-terminal portion is surface exposed and is comprised of five globular domains predominantly β-stranded with similarities to tandem fibronectin type III domains (FIG.1.3) [158]. The two most external C-terminal domains are critical for cell invasion and binding to β_1 integrins [159-160]. In addition, the second domain, sticking out from the outer membrane, has the ability to mediate Inv oligomerization and drastically increases bacterial uptake [161].It has been demonstrated that long O-antigen chains inhibits of *Yptb* invasion function, presumably by sterically blocking invasion access to the cells [162].

Even though a substantial amount of effort has been done to elucidate the function of Inv during bacterial infection, less is understood concerning the regulation of *inv* expression. Studies have established that at neutral pH *inv* is maximally expressed at 23℃ but almost none is detected at 37 ℃ *in vitro*; on the other hand, in *Y. enterocolitica*, as the pH is decreased to mimic that of the distal small intestine, *inv* expression at 37 ℃ increases to a level analogous to th at seen in cultures grown at 23 ℃ [163]. At both 23 ℃ and 37 ℃, maximal expression of Inv is observed in late exponential to early stationary phase

culture. In addition, *Y. enterocolitica* growing in Peyer's patches have been shown to express Inv [164] .

Mutation of *inv* gene has shown that Inv is not critical for infection in a murine model when LD50 is measured [148], but *inv* mutants showed a decrease in colonization in Peyer's patches [165]. These observation suggests that Inv is important throughout the early steps of bacterial infection through binding of Inv to β_1 integrins to penetrate the M cells and colonize the intestinal lymphoid tissues [156].

1.9 Overview of professional phagocytes

The three main function of the immune system is self versus non-self discrimination, to protect the organism from invading pathogens, and to get rid of modified or altered cells (e.g. cancer cells) [166]. Cells of the immune system are made in the bone marrow (BM) and they include myeloid lineage (basophils, neutrophils, eosinophils, macrophages and dendritic cells) and lymphoid lineage (T and B lymphocytes and Natural Killer). The myeloid progenitor (stem cells) differentiate into erythrocytes, neutrophils, platelets, monocytes, macrophages and dendritic cells while the lymphoid progenitor gives rise to the B and T cells and NK cells [167]. T cell development occurs in the thymus and therefore the precursor T cells migrate from the bone marrow to the thymus to undergo differentiation into two distinct types of T cells, the CD4+ T helper cell and the CD8+ pre-cytotoxic T cell [167]. The initial thymocytes do not express either CD4 or CD8. As they develop, they mature mature to $CD4^+$ or $CD8^+$ cells and then

they are released to peripheral tissues. Two class of T helper cells are formed in the thymus the TH1 cells, which help the pre-cytotoxic T cells to differentiate into cytotoxic T cells (CD8+), and TH2 cells, which help B lymphocytes to differentiate into plasma cells that release antibodies [168]. Because pathogens may replicate intracellularly (some bacteria, viruses and and parasites) or extracellularly (many bacteria including *Yersinia sp*., fungi and parasites), multiple mechanism of the immune system has evolved multiple mechanisms to recognize and neutralize these pathogens [169].

When a bacterial pathogen enters the host, the local cells sense the invader and begin a immune response to control the patogen. When just few bacteria invade the local responses can successfully control the pathogens. Antigen presenting cells (APCs) such as macrophages and dendritic cells can signal the host the presence of infection through the recognition of pathogen associated molecular patterns (PAMP)[170]. These molecular patterns can be referred to as molecular motifs conserved and shared by a certain group of microorganisms [171]. The best example of PAMP is LPS that is recognized by Toll-like receptors. Besides LPS, TLRs can recognize a variety of bacterial cell-wall lipoproteins, as well as fungal-wall elements and bacterial and viral nucleic acids [172].

The blood contains a variety of cell types such as neutrophils, lymphocytes, and monocytes, Neutrophils constitutes more than 50% of the white cells in the blood. Neutrophils, dendritic cells, monocytes and macrophages clear pathogens by phagocytosis [173]. When bacteria enter the host, if previous

immunity had occurred, pathogens are usually covered with host antibodies and fragments of complement [174] though they can also engulf particles in a opsonization independent fashion [175] (see below). There are a number of different receptors on professional phagocytes that help phagocytosis by recognizing the opsonized proteins on the surface of the bacteria. Two well known examples are receptors for complement and Fc receptors [175].

1.9.1 Neutrophils

Neutrophils are key mediators of the innate immune response to pathogens [176]. The ability of neutrophils to effectively kill pathogens is required for maintaining homeostasis in the host [176]. Neutrophils are capable of beginning the processes of phagocytosis, degranulation, and NADPH oxidase-dependent killing without new protein synthesis. However, early studies have shown that active transcription and protein synthesis are required for maintaining the full ability of neutrophils to kill pathogens either by phagocytosis or related bactericidal activity [177-178].

The transcriptional regulation of neutrophil development is mediated by several transcription factors and repressors [179]. For instance, PU.1, C/EBPα, RAR, c-MYB and CBF are implicated in early granulopoiesis which occurs primarily within bone marrow; while C/EBPε, PU.1, SP1, CDP, HOXA10 GFI-1 and signal transducer and activator of transcription proteins (STAT1, STAT3, STAT5) are implicated in final steps of neutrophil differentiation [179].

Contact of PMNs with the proinflammatory cytokines IFN-γ and GM-CSF leads to activation of STAT transcription members, whereas the proinflammatory

cytokines TNF-α and IL-1β induce NFκB [180]. In addition, Platelet-activating factor, fMLF, LPS, ROS, phagocytosis, and apoptosis also promote NFκB activation. NFκB activates synthesis of IL-1α/β, TNF-α, IL-8, IL-12, CCL4, CCL20, and CXCL1 in neutrophils [179].

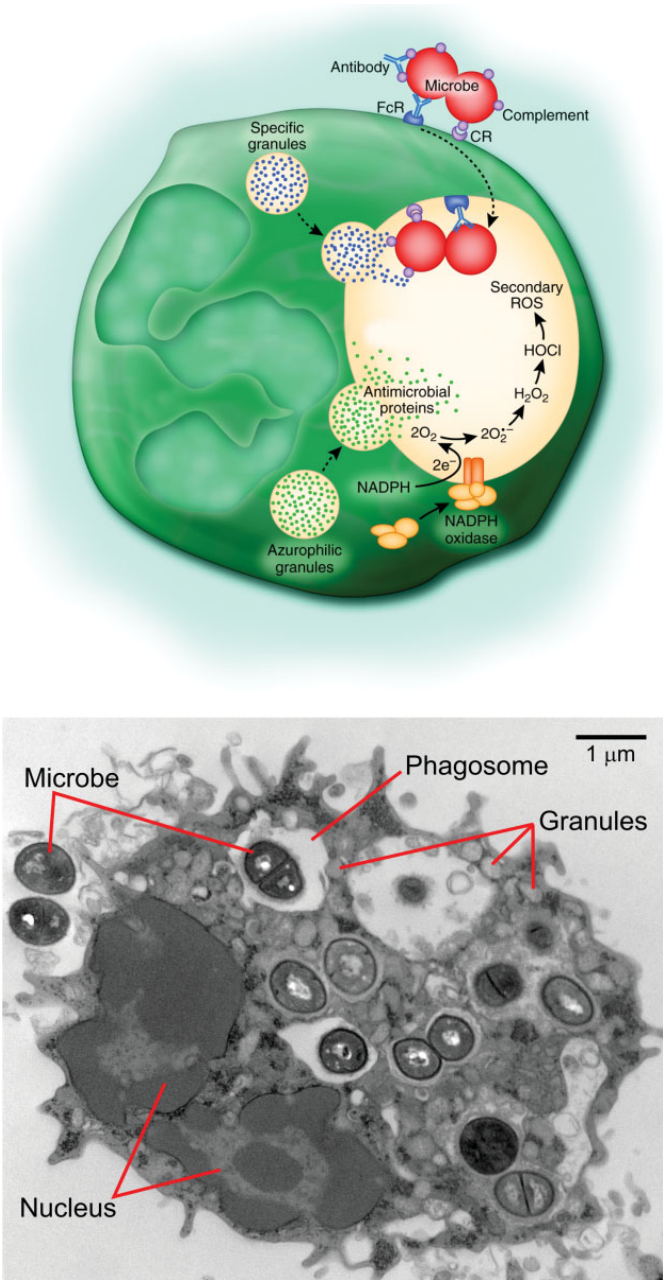
Following neutrophil release from bone marrow, they extravasate from blood vessels to the site of infection in response to chemokine signals [181]. Migrating neutrophils roll along the endothelial surface interacting with L-selectins that are constitutively expressed on neutrophils [182]. In the presence of inflammatory mediators, adherence rapidly switches to a high-affinity interaction that is mediated by activation of β_2-integrins on neutrophils and endothelial cell intracellular ICAM-1 and ICAM-2 [183]. Once they bound tightly, some neutrophil molecules on the membrane such as CD31, CD54, CD44, and CD47, assist migration into tissues [183]. Neutrophil movement is also controlled by chemoattractants secreted by the host during inflammation and pathogen byproducts. Il-8 is one of the most powerful neutrophil chemoattractant. IL-8 is also secreted by endothelial cells, monocytes, macrophages, mast cells, epithelial cells, keratinocytes and fibroblasts in response to infection [184].

Antibodies bound to the surface of microbes are recognized by neutrophil receptors specific for the Fc-region of antibody [176] (TABLE 1.3). Furthermore, opsonized bacteria with complement are bound effectively to neutrophils surface

TABLE.

1.3 Fc receptors

Receptor name	Principal antibody ligand	Affinity for ligand	Effect following binding to antibody
FcγRI (CD64)	IgG1 and IgG3	High (Kd ~ 10^{-9} M)	Phagocytosis Cell activation Activation of respiratory burst Induction of microbe killing
FcγRIIA (CD32)	IgG	Low (Kd > 10^{-7} M)	Phagocytosis
FcγRIIB2 (CD32)	IgG	Low (Kd > 10^{-7} M)	Phagocytosis Inhibition of cell activity
FcγRIIIB (CD16b)	IgG	Low (Kd > 10^{-6} M)	Induction of microbe killing
FcεRI	IgE	High (Kd ~ 10^{-10} M)	Degranulation
FcεRII (CD23)	IgE	Low (Kd > 10^{-7} M)	Possible adhesion molecule
FcαRI (CD89)	IgA	Low (Kd > 10^{-6} M)	Phagocytosis Induction of microbe killing



Scott D. Kobayashi and Frank R. DeLeo, 2009

Fig.1.4 Structure and bactericidal functions of neutrophils

receptors, such as CD35 (CR1), CD11b/CD18 (CR3) and CD11c/CD18 (CR4) [185].Ligation of these membrane-bound opsonin receptors initiates changes in the cytoskeleton that execute the physical process of phagocytosis. PMN phagocytosis starts a cascade of events that leads to activation of an array of neutrophil bactericidal mechanisms. Neutrophil antimicrobial activity is generated from two primary sources: by ROS production or by secreting proteases e.g., defensins [176].

The reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase of neutrophils plays an important role in curbing bacterial infections [179]. It produces superoxide by transferring electrons from NADPH similar to cellular respiration and coupling oxygen to produce free radical in form of superoxide. Superoxide can be made in phagosomes, which contain engulfed pathogens, or it can be released from the cell. Sequentially, superoxide forms hydrogen peroxide H_2O_2 generates reactive ROS. The molecular core of the NADPH oxidase complex is a cytochrome consisting of gp91^{phox} and p22^{phox} subunits and soluble components: small GTPase Rac2, p67^{phox}, p47^{phox}, and p40^{phox}. Upon activation, the soluble factors form a complex with the cytochrome and electrons are transferred from NADPH, across the membrane, and delivered to O_2 to generate O_2^- [179](FIG. 1.4)

Along with assembly of the NADPH oxidase, neutrophil phagocytosis also triggers degranulation, which involves fusion of cytoplasmic granules with the plasma or phagosome membrane [179]. There are many components within the

granules (lysosomes) which have bactericidal properities, defensins, bactericidal/permeability increasing protein (BPI), proteinase 3, lactoferrin, lysozyme and four members of the neutral serine protease family, including elastase, proteinase 3, azurocidin, and cathepsin G [179].

Increase in intracellular Ca^{2+} is granule exocytosis requires as well as ATP and GTP hydrolysis. Increases in intracellular Ca^{2+} alone are enough to stimulate the release of many of the granules in neutrophils [186]. The cytoskeleton undergoes profound changes to permit granules to reach inner surface of the cell membrane. This process of granule translocation and exocytosis requires activation and recruitment of many different signaling molecules, such as calmodulin, small GTPases. Several neutrophil receptors could activate increased Ca^{2+} levels, including G protein-coupled receptors, such as the formyl peptide receptor and chemokine receptors such as CXCR1, Fc receptors and integrin receptor [186], indicating that neutrophil function through signal transduction pathways are calcium dependent.

1.9.2 Monocytes

Monocytes are found in the blood, bone marrow, and spleen [187]. They differentiate into resident macrophages and dendritic cells under normal states, and in response to a pathogen or inflammation signals, monocytes can travel rapidly to sites of infection in the tissues and also differentiate into macrophages and dendritic cells to eliminate the pathogens [187] .

Monocytes represent immune effector cells, equipped with chemokine receptors and adhesion receptors that mediate migration from blood to tissues during infection [187]. They produce inflammatory cytokines and take up pathogens and toxic molecules. They can also differentiate into inflammatory DCs or macrophages during inflammation. Migration to tissues and differentiation to inflammatory DCs and macrophages are likely determined by the inflammatory milieu and pathogen-associated pattern-recognition receptors they encounter [188].

1.9.3 Macrophages

Macrophages are resident phagocytic cells in lymphoid and nonlymphoid tissues. Macrophages play a dual role in the innate immune response by clearance of apoptotic cells of the body and foreign pathogens [187]. Macrophages carry on the surface a number of pathogen-recognition receptors that allow them to efficiently engulf pathogens by phagocytosis [189]. On the other hand, they also play an important a role as crucial bridging with adaptive immune response. They can present antigens loaded to the MHC class receptors to helper T cells to induce antibody response or they can be targeted when infected by a pathogen by cytotoxic T cells as a result they die by apoptosis[190]. Macrophages can engulf pathogens that have been coated with immunoglobulins (opsonization), but macrophages also have the ability to phagocytose particles in the absence of immunoglobulin or complement coating [175].

Macrophage development starts in the bone marrow with $5x10^9$ monocytes being made and release into the bloodstream every day in humans.

After a few hours in the blood monocytes populate their target tissues [191]. Monocytes sense the local environment and differentiate accordingly into specific and heterogeneous cells, such as liver macrophages or alveolar macrophage of the lung, and in macrophages of connective tissue, etc [191-192].

Resident macrophages secrete a variety of proinflammatory cytokines, which triggers activation of immune response against invading bacteria [193]. Some of these macrophages are thought to be polarized toward an M1 phenotype. M1 macrophages are typically characterized by high levels of pro-inflammatory cytokines IL-12,IL-23, TNF-α and low expression the anti-immflamatory cytokine IL-10, produce ROS, express high levels MHCII molecules, and display antimicrobial functions. M1 cells are part of Th1 responses and mediate clearance of intracellular pathogens and tumor cells. These types of macrophages can induce extensive tissue damage [194]. Other resident macrophages called M2 macrophages, show increased phagocytic activity and produce high levels of IL-10. In general, these cells participate in polarized Th2 responses, facilitate extracellular pathogen clearance, reduce inflammation and promote tissue [195].

1.9.4 Dendritc cells

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) critical for induction of T-cell responses to control pathogens [196]. DCs are also necessary for the maintenance of tolerance to self-antigens by inhibition of potentially damaging T-cell responses [197]. DCs are present in small amounts mostly in the skin and the inner lining of the nose and internal organs such as

lungs, stomach and intestines. Undifferentiated DC are also present also in the blood [198].

Dendritic cells phagocytose potential pathogens, engulf, kill and process them into peptides. Those particles or antigens are exposed onto MHC II molecules for presentation to T cells. In order to stimulate pathogen-specific T cells successfully, DC undergoes maturation that not only includes upregulation of MHCII molecules on their surface, but also upregulation of co-stimulatory molecules and secretion of pro-inflammatory cytokines [170, 187]. Partially mature DCs express CC-chemokine receptor 7 (CCR7), allowing them to migrate from skin, nose, lungs, stomach and intestines to the lymphoid tissues. Once there, DCs interact with naive T cells, where they present the antigens to these cells to initiate the adaptive immune response [199].

Another way to stimulate DC maturation is by recognition of Pathogen-associated molecular patterns, or PAMP, such as bacterial cell wall components and DNA-RNA from bacteria and viruses through Toll-like receptors (TLRs) present on the DC [170].

Signals from other immune cells also contribute to DC maturation [200]. For example, natural killer (NK) cells are capable of inducing DC maturation through the production of tumour necrosis factor-α (TNF-α) as a result, this type of interaction enables DCs to achieve an antigen-specific T-cell responses that are that will successfully control the pathogen [201].

1.10 Identifying host cell types targeting by TTSS using CCF2-AM β-lactamase system during animal infection.

CCF2-AM, a β-lactamase substrate, has been used to detect bacterial type III reporter translocation into mammalian cells [108, 202-203]. A reporter is usually a β-lactamase protein fused to a translocation signal of a type three effector encoded in the chromosome. Cells translocated with the reporter are incubated with CCF2-AM. CCF2-AM contains ester moieties, which allows its diffusion through the plasma membrane [108]. Excitation of CCF2-AM results in green fluorescence emission in intact CCF2-AM. B-lactamase cleaves CCF2-AM establishing blue fluorescence emission, thus its activity can be detected and correlated with translocation of type three effectors by flow cytometry or visualized under fluorescence microscope [204].

1.10.1 *Yersinia spp*

Recently the CCF2-AM –β-lactamase system was used to identify host cell targeted by bacterial effector proteins during animal infection, specifically during plague infection. YopE-β-lactamase or YopM-β-lactamase hybrids and fluorescent staining of live cells were used to identity host cells targeted for translocation in animal infected with *Y. pestis*. Dendritic cells, macrophages, and neutrophils were injected most often, while B and T lymphocytes were hardly ever selected [108]. Therefore, this suggests that *Y. pestis* disables these cell populations to defeat host immune responses during infection [108].

This experimental system was also employed in *Y. enterocolitica* to determine the host cells targeted by the TTSS. In this particular study, the

authors used YopE-β-lactamase as a reporter. Yop translocation was detected in 13% of macrophages 11% of dendritic cells, 5% neutrophils, 2.3% of B cells, and 2.6% of T lymphocytes. Then they analyzed specifically the B cell subpopulations and determined that particularly follicular B cells were preferentially translocated with Yop. B cells injested with Yops were significantly activated compared to cells without Yops. Activation was assessed by the presence of CD69 on their surface. The authors also showed that neutrophils, dendritic cells, and macrophages were preferentially translocated with Yops, suggesting a distinct cellular tropism of Y*. enterocolitica* [203].

1.10.2 *Salmonella*

Salmonella encodes two TTSS in two pathogenicity islands SPI-1 and SPI-2 [205]. They encode for virulence factors necessary for Salmonella infection in animal hosts. Geddes and colleagues investigated the cell types targeted by the TTSS in *Salmonella* [202]. Similarly to the system employed in *Yersinia*, translational fusions between the β-lactamase reporter and a broad array of TTSS effectors secreted in this case via SPI-1, SPI-2, or both were constructed. Translocation of the fusion protein into a host cell was also determined by cleavage of a CCF2-AM. Interestingly, translocation of all six effectors tested could be observed in tissue culture. On the other hand, only effectors secreted by SPI-2 were detected in spleen cells when mice were infected intraperitoneally. Host cells targeted by *Salmonella* were identified using fluorescently labeled antibodies by flow cytometry. The targeted cells were lymphocytes, neutrophils, monocytes, and dendritic cells, but not mature

macrophages. Additionally, to investigate bacterial replication in these various cell types, mice were infected with *Salmonella* expressing red fluorescent protein. Bacteria was found in each of the cell targeted in previous experiment; nevertheless, most viable bacteria were present in neutrophils where *Salmonella* exhibited high preference, suggesting that *Salmonella* specifically target neutrophils most probably to downregulate their bactericidal functions, thus promoting intracellular survival and colonization in the animal [205].

1.11 Final remarks

In this introduction, I have tried to convey much of what is known about the activities of the T3SS effectors of Yersinia pseudotuberculosis and how they subvert the immune system. In my thesis work I have shown that certain cell types are more readily targeted by *Yptb* during an animal infection model and have discussed why this may be the case. Furthermore, using a combination of powerful techniques, we have identified a molecular pathway targeted by a translocated effector of *Yptb* during a mouse infection model.

CHAPTER II

2. Chapter II : The presence of professional phagocytes dictates the number of host cells targeted for Yop translocation during infection.

2.1 Abstract

Type III secretion systems deliver effector proteins from gram-negative bacterial pathogens into host cells, where they disarm host defenses, allowing the pathogens to establish infection. Although *Yersinia pseudotuberculosis* delivers its effector proteins, called Yops, into numerous cell types grown in culture, we show that during infection *Y. pseudotuberculosis* selectively targets Yops to professional phagocytes in Peyer's patches, mesenteric lymph nodes and spleen, although it co-localizes with B and T cells as well as professional phagocytes. Strikingly, in the absence of neutrophils, the number of cells with translocated Yops was significantly reduced although the bacterial loads were similar, indicating that *Y. pseudotuberculosis* did not arbitrarily deliver Yops to the available cells. Using isolated splenocytes, selective binding and selective targeting to professional phagocytes when bacteria were limiting was also observed, indicating that tissue architecture was not required for the tropism for professional phagocytes. In isolated splenocytes, YadA and Invasin increased the number of all cells types with translocated Yops, but professional phagocytes were still preferentially translocated with Yops in the absence of these adhesins. Together these results indicate that *Y. pseudotuberculosis* discriminates among cells it encounters during infection and selectively delivers Yops to phagocytes while refraining from translocation to other cell types.

2.2 Introduction

Type III secretion systems (TTSS) are multi-component export machines found in gram-negative bacterial pathogens that deliver effector proteins from the bacterial cytosol into host cells [206]. Once inside host cells, effector proteins modulate cellular functions, thus enabling the bacteria to inactivate host defenses and establish replication niches [207]. A plasmid-encoded TTSS is found in three pathogenic *Yersinia* species, which include the two enteric *Yersinia* pathogens, *Y. pseudotuberculosis* (*Yptb*) and *Y. enterocolitica* (*Ye*), and the causative agent of bubonic and pneumonic plague, *Y. pestis* [208-209]. These highly homologous secretion systems deliver at least 6 effector proteins, called Yops, into cells and are required to cause significant disease in humans and other mammals [9]. *Yersinia* spp colonize many tissues in mice including the Peyer's patches (PP), mesenteric lymph nodes (MLN), spleen, liver and lungs, depending on the route of infection [23, 165, 210]. During the initial steps of infection, *Yersinia* are intracellular as the enteric pathogens pass through M cells lining the intestinal wall [156, 211] and *Y. pestis* initially resides in macrophages after subcutaneous inoculation [212]. However, in general, the vast majority of *yersiniae* is found extracellularly during tissue infection [25, 213-214]. As extracellular pathogens, *yersiniae* could potentially deliver Yops into all of the host cells they encounter. In fact, *Yersinia* does effectively deliver Yops into many different cell types infected in culture including epithelial cells, macrophages, dendritic cells, T cells and neutrophils [63, 103, 215-217]

Yop delivery requires that *yersiniae* bind to mammalian cells [215, 218]. Several bacterial proteins, including Invasin and YadA, mediate *Yptb* binding and Yop delivery to host cells [218]. These two adhesins are not expressed in *Y. pestis* [219]. Invasin binds to β_1-containing integrins, causing activation of Src kinases, which in turn enhances Yop translocation into cells [218]. The interaction of YadA with mammalian cells is mediated indirectly through β1 integrins, by YadA binding to collagen or fibronectin which then bind β1-containing integrins [220]. In addition, opsonization of *Yersinia* by complement or antibody is sufficient to permit binding to macrophages and neutrophils and Yop delivery [215, 221]. It is clear that bacterial-encoded adhesins are important for infection in animals [144, 165]; however, it is unknown whether bacterial ligands, Fc and/or complement play a role in host cell binding and Yop delivery during animal infection. The fact that the receptors for Invasin and YadA, β1-containing integrins, are found on a large number of cells, including epithelial cells, professional phagocytes and B and T cells [222] explains, in part, why Yops are translocated into numerous cell types grown in culture and could mean that *Yptb* targets many cell types for Yop translocation during infection of tissues.

Most effector Yops have profound effects on cells grown in culture [63, 103, 215-217] and each Yop is important for tissue colonization and/or full virulence in mouse infections [24, 223-224]. However, it is not clear whether the effects observed in cultured cells correspond to their role in tissues during animal infections. Identifying host cells that are targeted for Yop translocation

during infection is one approach towards understanding how each Yop participates in dismantling host defenses. Cells with translocated Yops after intravenous infection with *Y. pestis* or *Ye* were identified, using fusions of the N terminus of Yops with TEM [108, 203]. TEM is a truncated version of β-lactamase, which has enzymatic activity that can be detected by cleavage of a fluorescence substrate [225]. *Y. pestis* and *Ye* delivered Yops to macrophages, neutrophils and dendritic cells in the spleen; in addition, Ye also delivered Yops to B cells [108, 203]. However, when *Y. pestis* or *Ye* were incubated with isolated splenocytes this specificity was not observed. These observations raise the question of whether the specific cell-type targeting during infection was due to *Yersinia* selectively co-localizing with these cells.

Using the TEM technology to identify host cells that are targeted by *Yptb* after oral infection, we find that *Yptb* had a pronounced specificity for Yop delivery into phagocytic cells, particularly neutrophils, in the PP, MLN and spleen. This specificity was not due solely to co-localization with *Yptb* during infection because phagocyte-specific targeting was retained in the absence of tissue architecture. Furthermore, in the absence of neutrophils, overall levels of translocation were severely reduced. Therefore, inherent properties of phagocytic cells interacting with *Yptb* promote Yop delivery during infection.

2.3 Results

2.3.1 Translocated YopH is enriched in neutrophils, macrophages and dendritic cell populations in PP, MLN and spleen after oral infection

To understand how the *Yptb* Yops circumvent host defenses, it is essential to identify the immune cell and/or other cells targeted by *Yptb* for Yop translocation during infection. To identify immune cells targeted by *Yptb* during infection, we generated a *Yptb* strain expressing a chimeric protein, HTEM, which contains the N-terminal secretion and translocation domain of the effector protein YopH fused to the reporter protein TEM. Chimeric proteins containing the first 100 amino acids of a Yop fused to another protein will generally be translocated into host cells [103, 108]. The strain, WT-HTEM, expressed, exported, and translocated both WT YopH and HTEM, and was as virulent as *Yptb* (Fig 2.1 and unpublished data). TEM, a β-lactamase, cleaves the membrane permeable dye CCF2-AM and changes its fluorescence from green to blue [226], thus enabling identification of mammalian cells containing HTEM by their blue fluorescence. In contrast, when cells were infected with a translocation defective mutant expressing HTEM, Δ*yopB*-HTEM,

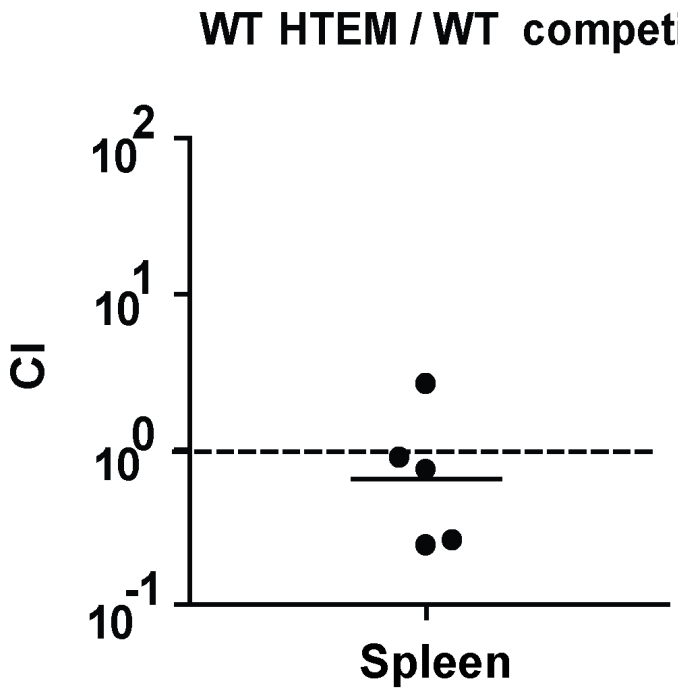


Fig 2.1 Colonization of *Yptb* WT-HTEM strain in competition WT *Yptb* 5 days post oral infection.

Eight week old female BALB/c mice were orally inoculated with 2x109 CFU of an equal mixture of WT IP2666 and WT-HTEM strain. Bacteria from spleens were plated on L plates and replica plated on L plates containing kanamycin to determine the ratio of WT to WT-HTEM (which is Kanamycin resistant) in the spleen. The competitive index (CI) was calculated as follows: CI = (number of WT-HTEM colonies/number of wt colonies)output /(number of WT-HTEM colonies/number of WT colonies)input. Black circles represent the CI from individual mice.

a mutant lacking a critical component of the translocon, cells remain green because the chimeric protein is not translocated into cells (Fig 2.2).

To identify immune cells into which HTEM has been translocated, mice were orogastrically infected with WT-HTEM and infection was allowed to proceed for 5 days. An oral-gastric route of infection was used because *Yptb* is a natural enteric pathogen, and its ability to disseminate from the GI tract to the PP, MLN and spleen permitted analysis of multiple tissues after oral infection [227]. At day 5 post-infection, mice were sacrificed, the PP, MLN and spleen were harvested, and single-cell suspensions were generated in the presence of antibiotics to halt any Yop translocation post-harvest (see experimental procedures). Cells were incubated with CCF2-AM, which freely diffuses through mammalian plasma membranes and is then modified by esterases, trapping CCF2-AM inside the cell [225]. Cells were then fluorescently labeled with the following antibodies to identify the indicated cell type: α-GR1 and α-CD11b to distinguish neutrophils ($GR1^+CD11b^+$) from macrophages ($CD11b^+GR1^-$), α-CD11c (primarily dendritic cells), α-B220 (primarily B cells), α-CD4 (primarily T helper cells), or α-CD8 (primarily cytotoxic T cells). Five days post-infection, the total number of neutrophils ($GR1^+CD11b^+$) and macrophages (GR1+CD11b+) increased 4-12 fold in the PP, MLN, and spleen compared to their levels in uninfected tissues (Table 2.1), while the level

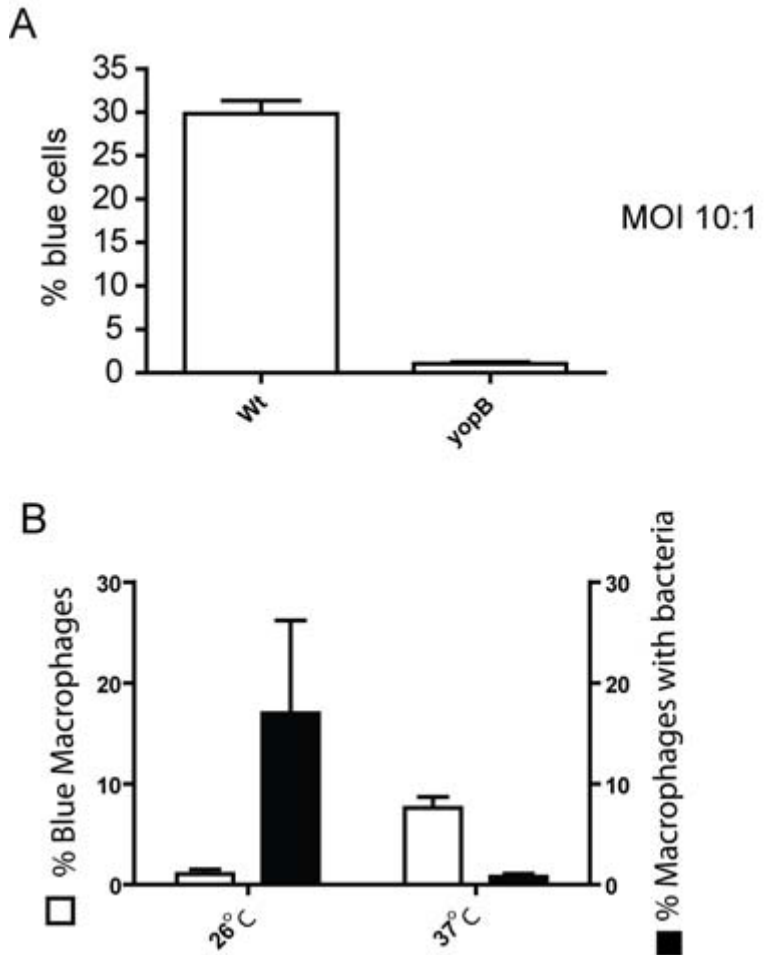


Fig 2.2 Translocation of Yops into splenocytes by WT-HTEM and *ΔyopB*-HTEM.

A. Splenocytes were harvested and infected with WT-HTEM or *ΔyopB*-HTEM at an MOI of 10 for 1 hour. The percentage of blue cells in splenocytes infected with WT-HTEM or ΔyopB-HTEM were plotted. The data shown are the average and SEM of at least three experiments. **B.** RAW264.7 macrophages seeded at 1x10^6/well in 6 well plates were infected at 37℃ with WT-HTEM grown at 26℃ or 37℃ in low calcium media at an MOI of 10. Gentamicin was added after 20 minutes and the cells were incubated for an additional hour. Two wells each were analyzed for the % of blue macrophages and the number of gentamicin resistant (i.e. internalized) *Yptb*. The % blue macrophages were plotted in the open bars and the % macrophages containing *Yptb* (assuming each *Yptb* was in a different macrophage) was plotted in the black bars. The data are the average from one experiment repeated in triplicate.

of B, T, and dendritic cells remained relatively constant (Table 2.1). Two to four percent of the cells in the PP and 1-3% of the cells in the MLN and spleen were blue as determined by flow cytometry following infection with WT-HTEM (Fig 2.3 A & Table 2.2). As observed with Marketon et al, a fraction of the tissue cell suspensions were not green (Fig 1A) indicating that they are dead because CCF2-AM is retained only in live cells [108]. In general, 10–30% of the neutrophils, macrophages and dendritic cells in the PP, MLN and spleen were blue, demonstrating that HTEM was readily translocated into these cell types (Fig 2.3 B, D & F). In contrast, less than 5% of the B and T cells contained HTEM.

We next determined whether *Yptb* targeted specific cell types more frequently than others. To do this, the percentage of each specific cell type in a tissue (Fig 2.3 C, E and G, left bars) was compared to the percentage of that cell type in the HTEM-containing (blue cells) population (Fig 2.3 C, E, & G, right bars). These percentages were compared to determine whether a type of cell was over or under-represented in the blue cell population compared to its representation in the tissue. Neutrophils were significantly enriched in the blue cell population of each organ (14-, 7-, and 8-fold in the PP, MLN, and spleen, respectively) compared their percentage in these organs. In addition, dendritic cells were significantly enriched in the blue cell population in the PP and spleen while macrophages were significantly enriched in the MLN (Fig 2.3 C, E, & G). In

TABLE

2.1 Percentage Cell Type found in uninfected and infected tissues[a]

	Log CFU	PMNs[b]	Macrophage[c]	Dendritic cells[d]	B cells[e]	T helper[f]	Cytotoxic T cells[g]
PP		0.38±0.09	0.86±0.14	1.17±0.23	48.5±16.2	11±1.4	7.00 ±4.24
Infected PP	5.6±0.3	4.63±3.01	7.0±0.6	1.49±0.76	38.7±11.4	7.8±2.5	4.40± 3.17
MLN		0.13±0.02	0.69±0.11	1.38±0.82	27.2±3.3	18.00 [h]	12.96 [h]
Infected MLN	5.0±0.6	4.84±1.0	6.32±3.0	3.0±0.54	38.6±3.45	12.9±2.15	3.0± 2.0
Spleen		0.54±0.16	1.05±0.18	1.92±0.78	34.1±2.7	19.5±5.3	9. 36±2.42
Infected Spleen	4.8±0.4	2.0±.5	4.0±2.0	1.0±0.5	39.5±5.0	12.3±3.0	10 .44±3.75

[a] All data were collected from 4 independent experiments unless indicated
[b] Defined as $GR1^+CD11b^+$
[c] Defined as $CD11b^+GR1^-$
[d] Defined as $CD11c^+$
[e] Defined as $B220^+$
[f] Defined as $CD4^+$
[g] Defined as $CD8^+$
[h] Numbers are from 1 experiment

TABLE

2.2 Percentage Blue cells in infected tissues[a]

PP	3.16±0.92
MLN	1.85±0.91
Spleen	1.87±1.26

[a] Data is from at least 4 independent experiments

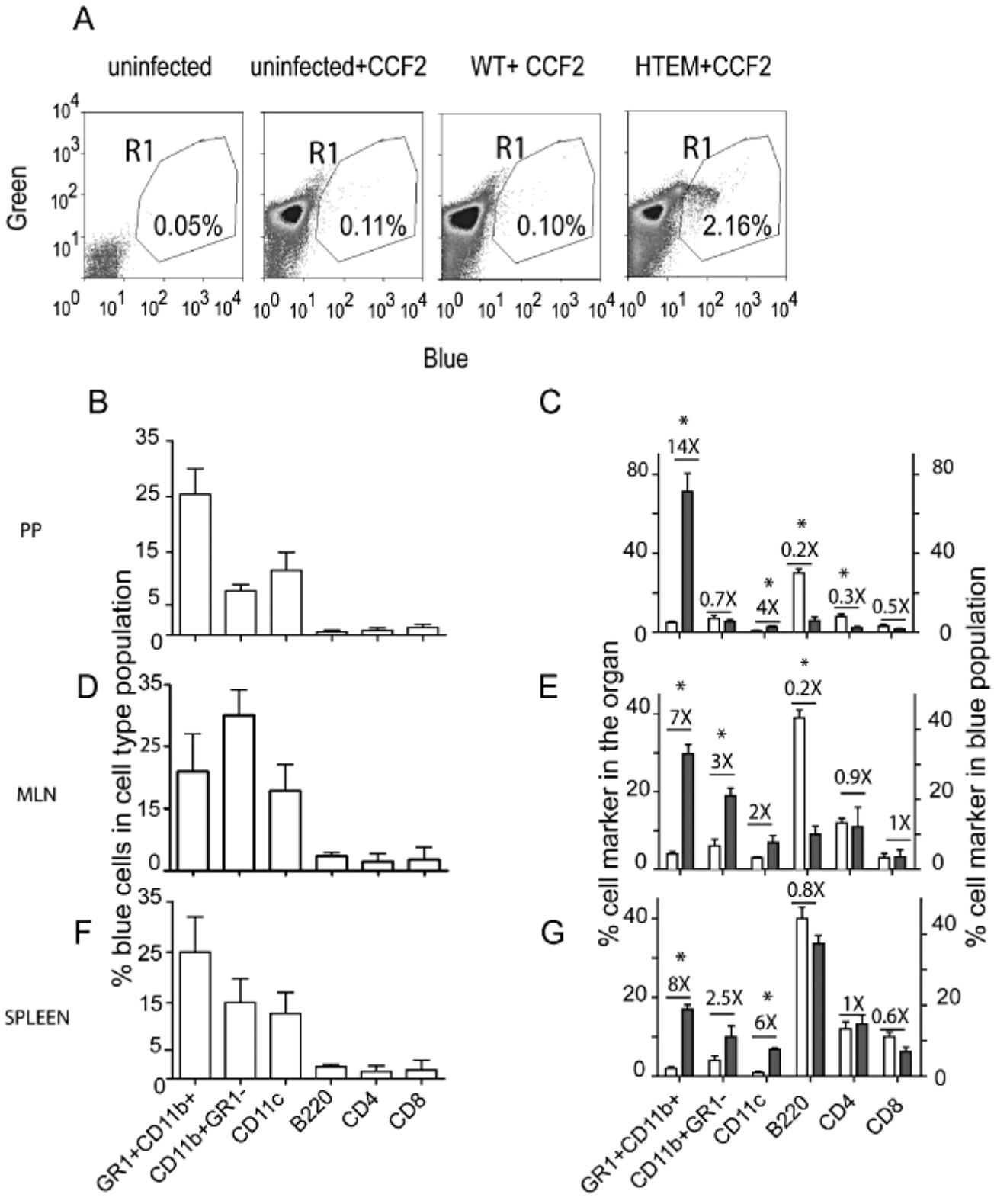

A
uninfected
uninfected+CCF2
WT+ CCF2
HTEM+CCF2
R1
0.05%
0.11%
0.10%
2.16%
Green
Blue
B
C
D
E
F
G
PP
MLN
SPLEEN
% blue cells in cell type population
% cell marker in the organ
% cell marker in blue population
14X
0.7X
4X
0.2X
0.3X
0.5X
7X
3X
2X
0.2X
0.9X
1X
8X
2.5X
6X
0.8X
1X
0.6X
GR1+CD11b+
CD11b+GR1-
CD11c
B220
CD4
CD8

Fig. 2.3 Neutrophils, macrophages, and dendritic cells are preferentially translocated with YopH during *Yptb* infection.

Mice were orogastically infected with $2x10^9$ CFU of WT-HTEM. Day 5 post-infection tissues were harvested and filtered to generate a single cell suspension. Cells were incubated with CCF2-AM and labeled with antibodies to the indicated cell surface marker(s). Fluorescence intensity was analyzed by flow cytometry. **(A)** Detection of green and blue cells by flow cytometry in PP of uninfected mice or mice infected with WT or WT-HTEM. The $blue^+$ cells are gated (R1) with the percentage of $blue^+$ cells indicated in the gate. **(B, D, F)** The percentage of $blue^+$ cells in the cell type indicated on the x-axis in (**B**) PP, **(D)** MLN, and (**F)** spleen. **(C, E, G)** The percentage of a cell type present in the entire organ (white bars) and the percentage of specific cell type present in the $blue^+$ population (black bars) in the (**C**) PP, **(E)** MLN, and (**G)** spleen. The fold enrichment of each cell type in the $blue^+$ population compared to total of that cell type in the whole organ is indicated above each set of bars. The experiment was repeated 4 times and the bars are the average + SEM from all the experiments. The asterisk indicates that there was a significant difference in the number of the indicated cell type in the $blue^+$ population compared to that same cell type in the whole organ based on a unpaired, two tailed, t test ($P<0.05$). Note the Y-axis in (**C**) differs from that of (**E**) and (**G**).

contrast to translocation of professional phagocytes, HTEM-positive B cells were significantly underrepresented in the PP and MLN and HTEM-positive CD4-T cells were significantly underrepresented in the PP (Fig 2.3 C & E).

To determine whether the apparent enhanced targeting to professional phagocytes and the apparent reduced targeting to B and T cells was due to killing of B and T cells by *Yptb*, we determined whether any specific cell types were preferentially killed during *Yptb* infection. Suspended splenocytes were infected at an MOI of 20 for 1, 2, 4 or 18 hours, after infection cells were stained with fluorescent antibodies to specific cell type markers and propidium iodine to identify dead cells. No enhanced cell death was observed among any particular cell type between 1-4 hours indicating that *Yptb* did not kill any specific cell types during this time period (Fig 2.4). As expected [122], 60-70% of the macrophages and neutrophils were dying 18 hours after infection. This data supports the idea that the selectivity of translocation towards professional phagocytes was not due to killing of other targeted cells. In fact, since *Yptb* causes death of macrophages in the MLN and spleen after infection [122], our observed enrichment of targeting of macrophages could be an underestimate of the actual amount of *Yptb* targeting.

2.3.2 YopE is translocated into the same neutrophils as HTEM during infection

During infection of mice, it is unknown whether *Yptb* injects more than one Yop into the same cell or distributes Yops to different cells. We investigated whether other Yops were injected into the same cells that had been translocated

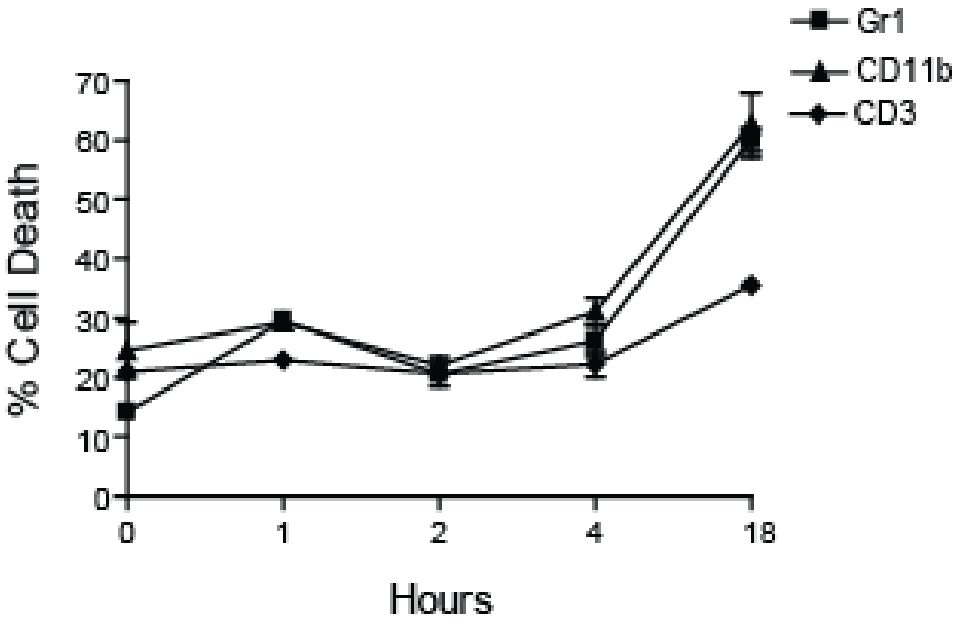


Fig 2.4 Splenocyte cell survival after infection with *Yptb.*

Splenocytes were infected with *Yptb* at an MOI of 20 for 1, 2, 4 or 18 hours and stained with Propidium iodine (PI) to identify dead cells. Cells were fluorescently labeled with the following antibodies to identify specific cell type undergoing cell death: α-GR1-PE (); α-CD11b-PE (); α-CD3-PE(). The cell death was calculated as follows: (percentage of PI+ antibody+)/(% antibody+ in the total splenocytes). The experiment was repeated 2 times and the average cell death is shown.

with HTEM. Mice were infected orally with WT-HTEM and five days post-infection, spleens were harvested and filtered to generate a single-cell suspension. Splenocytes were incubated with CCF2-AM and labeled with α-Gr1. Three populations of $GR1^+$ cells, $blue^{neg}$, $blue^{lo}$, and $blue^{hi}$ were gated based on their levels of blue and green fluorescence and sorted by flow cytometry (Fig 2.5 A). Equal numbers of $Gr1^+$ cells from each population were assessed for both HTEM and YopE translocation by Western blot. As expected, HTEM was found in the blue cell population. YopE was also found in the blue cells, indicating that at least two Yops are translocated into the same cells during infection (Fig 2B). The YopE detected in the western is unlikely to come from lysed bacteria because the buffer used to lyse cells does not release YopE from bacteria (Fig 2.6) and ([217]. In summary, these results demonstrate that both HTEM and YopE are translocated into the same neutrophils and suggest that other Yops may be delivered within the same cells during infection.

A comparable amount of HTEM and YopE were found in both the $blue^{hi}$ and $blue^{lo}$ cell populations. Since neutrophils, macrophages, and dendritic cells were significantly overrepresented in the $blue^{hi}$ population (Fig 2.3 C, E & G), the $blue^{lo}$ cell population was analyzed to determine whether these cells were similarly enriched. Neutrophils, macrophages and dendritic cells were also overrepresented in the $blue^{lo}$ population, while B and T cells remained underrepresented (Fig 2.7). Previous work has shown that *Yptb* can be found close to B and T cells in the MLN, suggesting that its close proximity to B and T cells does not result in Yop translocation [25].

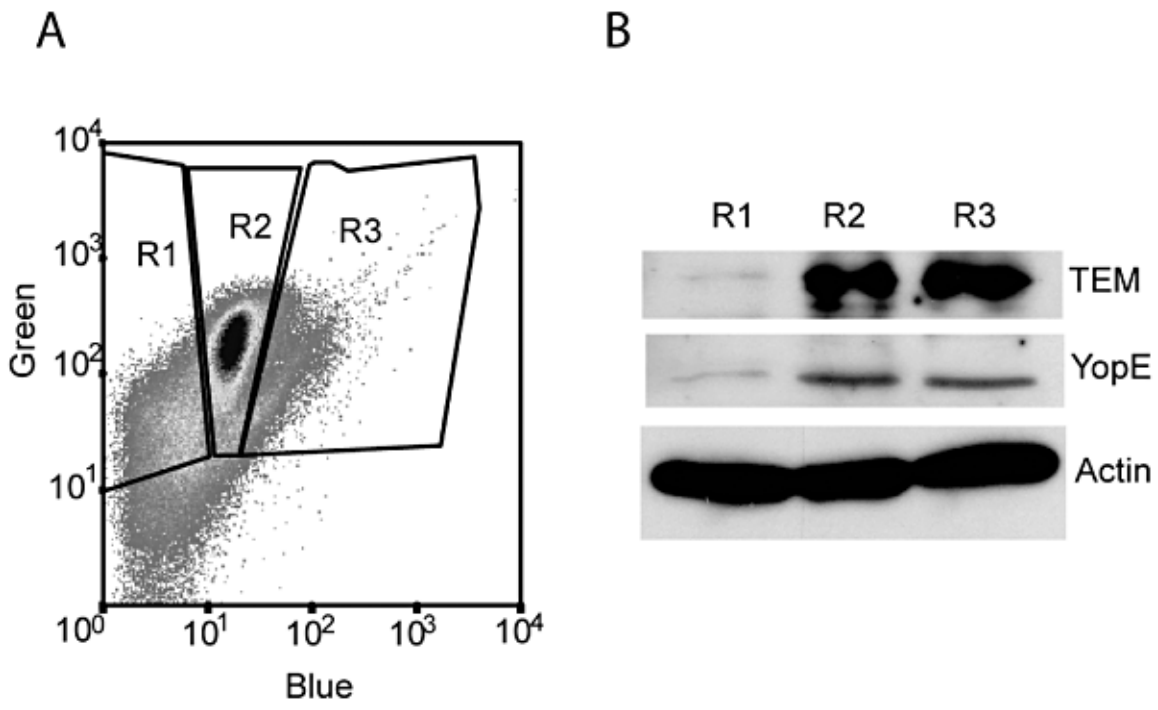


Fig. 2.5 HTEM and YopE are translocated into the same cells in the spleen during infection.

Four mice were infected orogastrically with WT-HTEM and five days post-infection, spleens were harvested, pooled, and labeled with CCF2-AM and antibody to GR1. **(A)** Gr1+ cells were sorted based on their blue/green fluorescence into $blue^{neg}/green^{+}$ (R1), $blue^{low}/green^{+}$ (R2), and $blue^{high}/green^{+}$ (R3) populations. **B**. Western blot of $2x10^5$ $GR1^{+}$ cells per gate. The lanes were loaded as follows, R1 $blue^{neg}$ (uncleaved CCF2-AM); R2 $blue^{low}$ (some CCF2-AM conversion); R3 $blue^{high}$. Blots were probed with antibody to TEM, YopE and actin, which was used as a loading control. The blot is representative of three independent experiments.

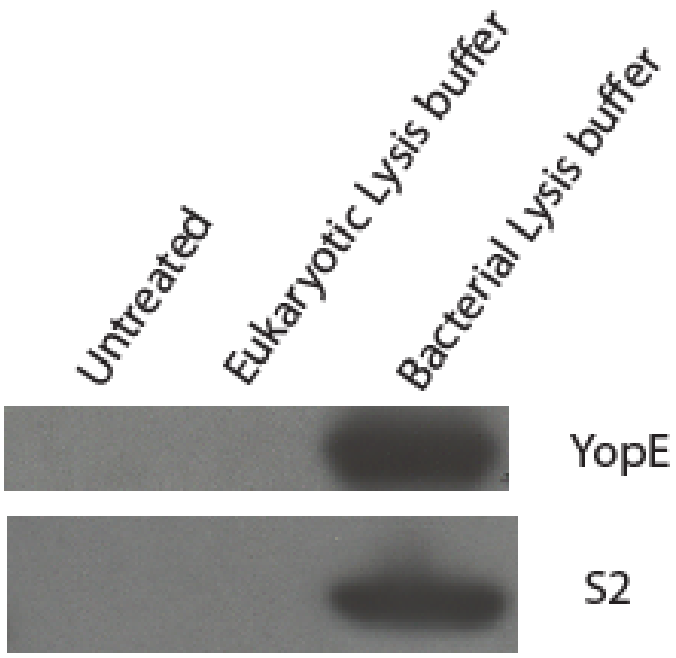


Fig 2.6. Western blot of YopE and S2 in supernatants of *Yptb* after incubation with eukaryotic and bacterial lysis buffers.

2x10^8 *Yptb* grown at 37℃ washed with PBS and then incubated wit h PBS, 50 µl of eukaryotic lysis buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/ml each of aprotinin, leupeptin, and pepstatin) for 20 min with gentle rocking at 4℃) as described in Experimental Procedures or 50ul of bacterial lysis buffer containing 4% SDS. The bacteria were briefly centrifuged and the supernatants run on SDS-PAGE, transferred to a membrane and analyzed by western with antisera to either YopE or S2.

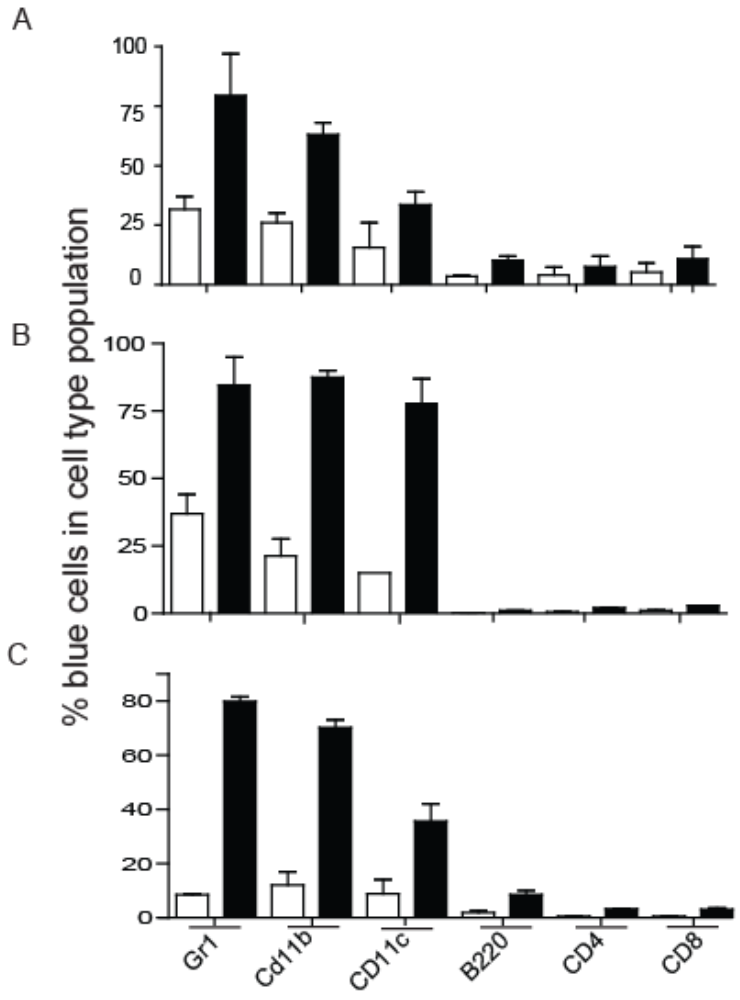


Fig 2.7. Neutrophils, macrophages, and dendritic cells are also preferentially translocated with HTEM in the bluelow/green+ during *Yptb* infection.

Mice were orogastrically infected with 2x109 CFU of WT-HTEM. Day 5 post-infection tissues were harvested and filtered to generate a single cell suspension. Cells were incubated with CCF2-AM and labeled with antibodies to the indicated cell surface marker(s). Fluorescence intensity was analyzed by flow cytometry. **(A, B, C)** The percentage of bluehigh/green+ cells (white bars) and the percentage of bluelow/green+ (black bars) in the cell type indicated on the x-axis are plotted (**A**) PP, **(B)** MLN, and (**C)** spleen. The experiment was repeated 4 times and the bars are the average + SEM from all the experiments.

2.3.4 *Yptb* co-localizes with neutrophils, macrophages, B and T cells in lymph nodes.

The strong preference for translocation of Yops into neutrophils, macrophages, and dendritic cells by *Yptb* could be due to several factors, including preferred co-localization of *Yptb* with these cells types. To investigate whether *Yptb* were localized predominantly in areas rich in professional phagocytes, immunohistochemical analyses of PP, MLN, and spleens from infected mice were performed. Tissues were stained with an α-*Yptb* antibody to detect *Yptb* and were counterstained with hematoxylin. The location of *Yptb* with respect to areas of inflammation, which included neutrophils, B and/or T cell rich areas, or boundary zones between germinal centers and areas of inflammatory was determined (Fig 3.8). Histological analysis of the PP indicates that the majority (over 60%) of *Yptb* microcolonies were found in areas of inflammation (Fig 3A-D, O), which included many neutrophils and macrophages as well as cellular debris. A significant minority (35%) of the microcolonies were found in B and T cell areas despite the observation that very few B and T cells were translocated with HTEM (Fig 2.3 B-C). Likewise in the MLN, a majority of *Yptb* microcolonies were found either in areas of inflammation or boundary zones in the spleen, the location of bacteria correlated more closely with the cell types that contain HTEM. The fact that fewer numbers of B and T cells were injected with HTEM (Fig 2.3) despite their close proximity to *Yptb* (Fig 2.8) suggests that *Yptb* was less proficient in translocating Yops into B and T cells than into professional phagocytes in tissue infection.

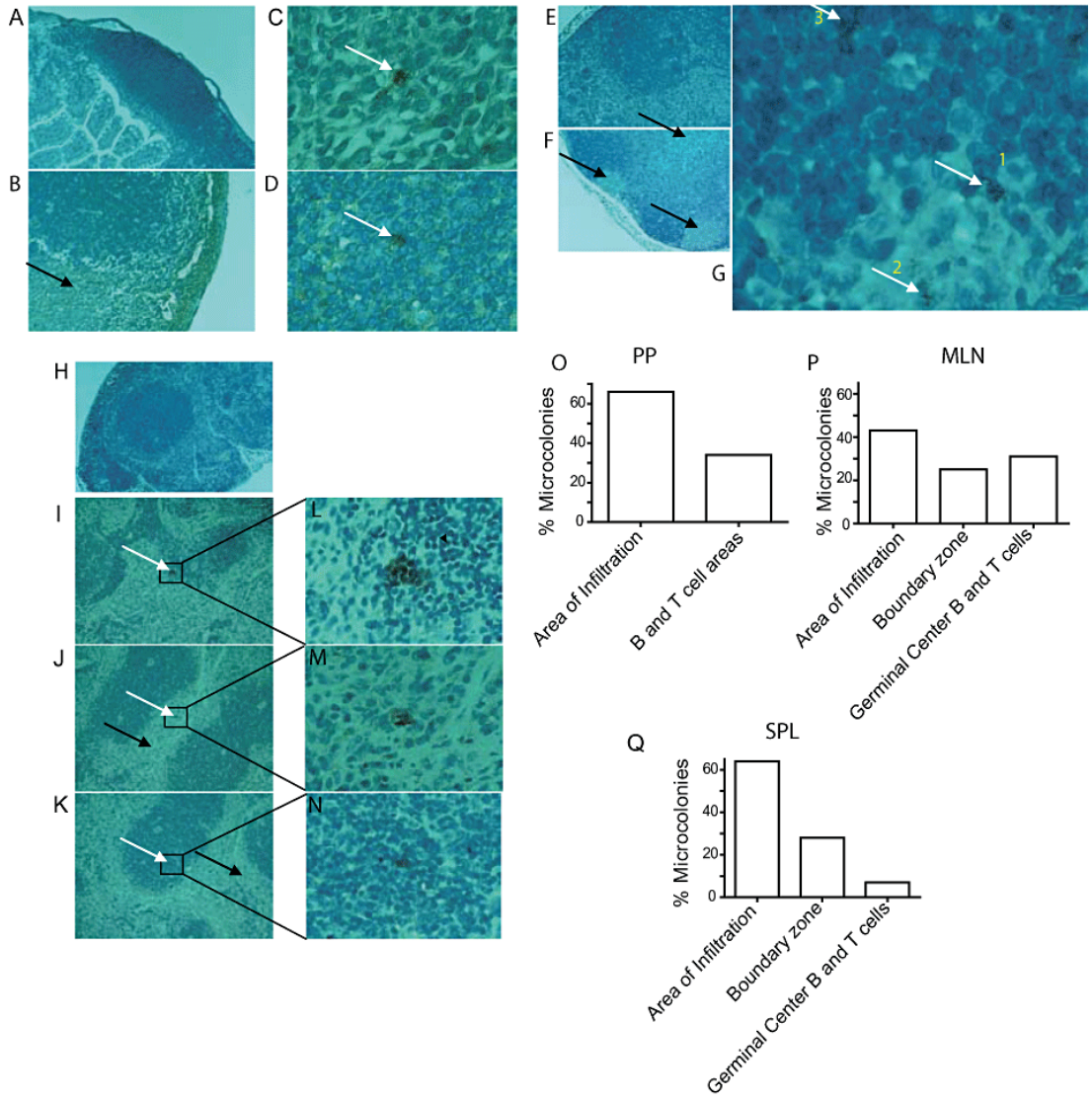


Fig.2.8 Localization of *Yptb* in the PP, MLN and spleen.

Immunohistochemical analysis of (**A-D)** PP, **(E-G)** MLN, and (**H-N)** spleens after 5 days infection with $2x10^9$ CFU WT-HTEM. Sections were stained with α-*Yptb* and hemotoxylin to identify *Yptb* and distinguish B and T cells from areas of inflammation. The location of *Yptb* was detected by 3,3'-diaminobenzidine which produces brown staining (indicated by white arrows). Black arrows indicate areas of inflammation. **(A, E, H)** Uninfected tissues (10X); (**B-C, F-G, I-J, L-M)** areas of inflammation in infected tissues (10X and 60X); (**C, G arrow 3, M)** colonies detected in areas of infiltration (60X); (**D, G arrow 1, N)** colonies detected in B and T cell areas (60X); **(G arrow 2, L)** colonies detected at boundary zone (60X). **(O-P)** Quantification of microcolonies is areas of inflammation, B and T cell zones, and boundary zones in (**O)** PP, **(P)** MLN, and **(Q)** spleens from 5 mice; 1-2 sections/mouse were analyzed and scored by 3 independent viewers.

2.3.5 Neutrophil depletion and suppression of inflammation reduces the total amount of translocated Yops.

To investigate the hypothesis that *Yptb* selectively translocates Yops into neutrophils rather than B and T cells in lymph tissues, we tested whether the number of cells targeted for Yop translocation changed when neutrophils were depleted. One day prior to infection with WT-HTEM and two days post-infection, mice were injected intraperitoneally with an isotype control antibody or the monoclonal antibody RB6-8C5, which binds to $Gr1^+$ cells and causes their depletion. Three days post-infection, the levels of $Gr1^+$ cells in the PP were reduced by 75-95% compared to the levels in mice treated with the isotype control antibody as ascertained by flow cytometry (Fig 2.9 A-C). Strikingly, the percentage of total cells containing HTEM was also reduced by 80-90% in neutropenic mice compared to mice receiving the isotype control antibody (Fig 2.9 C). The colonization levels were similar in both cohorts of mice (Fig 2.9 D), indicating that the reduction in translocation was not due to a decrease in bacterial load in the RB6-8C5 treated mice. Further analysis of the specific cell types targeted after treatment with RB6-8C5 indicated that most of the blue cell population was comprised of the remaining neutrophils (data not shown). Only the PP were analyzed because the bacterial loads in spleens were not comparable as the R6B-8C5 treated mice had detectable bacteria while the control mice were not colonized. The R6B-8C5-treated and infected mice did not survive when the infection was allowed to proceed longer than 3 days hindering any analysis at day 5. In summary, when fewer neutrophils were

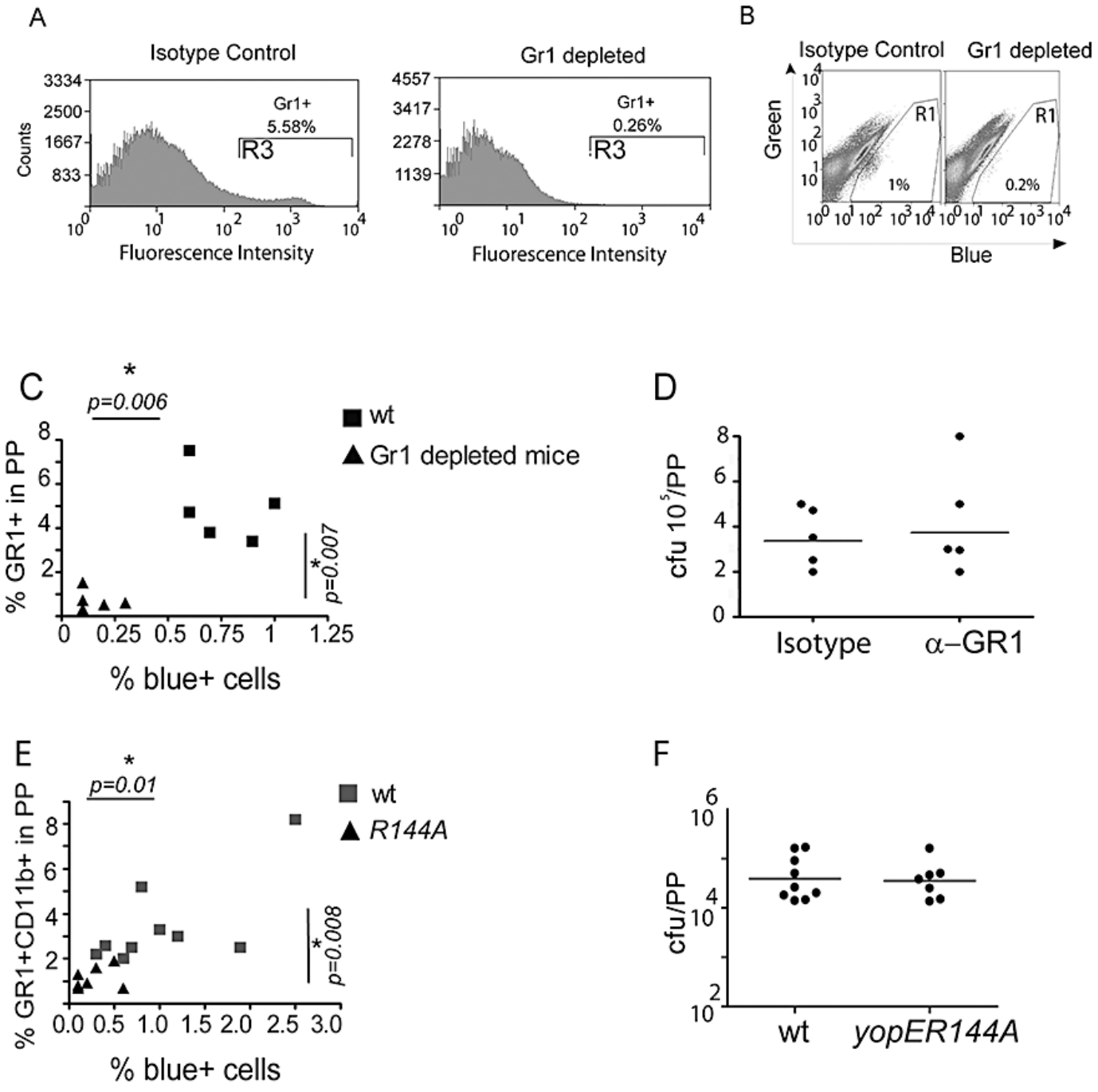

A
Isotype Control
Gr1 depleted
Counts
Gr1+
5.58%
Gr1+
0.26%
R3
Fluorescence Intensity
B
Isotype Control
Gr1 depleted
Green
R1
1%
0.2%
Blue
C
p=0.006
p=0.007
wt
Gr1 depleted mice
% GR1+ in PP
% blue+ cells
D
cfu 10^5/PP
Isotype
α–GR1
E
p=0.01
p=0.008
wt
R144A
% GR1+CD11b+ in PP
% blue+ cells
F
cfu/PP
wt
yopER144A

Fig. 2.9 Neutrophil depletion and suppression of inflammation reduces the total amount of translocated Yops.

(A-D) Mice were injected intraperitoneally with an isotype control (α-IgG2bκ) or the RB6-85C (α-GR1) rat monoclonal antibodies 1 day prior to and 2 days after oral infection with WT-HTEM. Three days post-infection, mice were sacrificed, the PP harvested and the number of blue$^+$ and GR1$^+$ cells were quantified. (**A)** Percent GR1+ cells in PP of an isotype control treated and an RB6-8C5 treated mouse by flow cytometry. The percentage of GR1+ cells (gated in R3) is indicated on the plots. (**B)** Percent blue$^+$ cells from PP (gated in R1) of isotype control treated mice and RB6-8C5 treated mice. **(C)** The percentage of blue$^+$ cells (x-axis) were plotted versus the percentage of GR1$^+$ cells (y-axis) in the PP from mice treated with the isotype control antibody (squares) and mice treated with α-GR1 (triangles). The experiment was repeated twice and data from all the mice are shown. Significant differences between both the GR1+ cells and the number of blue$^+$ cells from the two groups of mice were determined by an unpaired, two tailed t test. Both the percentage of GR1+ cells and the percentage of blue$^+$ cells in each group of mice were statistically different (unpaired, two tailed, t test; * indicates $P<0.01$) (**D**) Bacterial colonization in PP of the isotope control or RB6-8C5-treated mice shown as CFU/gm PP**.** Each dot represents one mouse; the bar represents the geometric mean. There was no statistical difference in the bacterial load between the two populations of mice by an unpaired, two tailed, t test, P=0.54. **(E-F)** Mice were intragastically infected with $2x10^9$ YPIII-HTEM or YPIII*yopER144A*-HTEM and the number of blue$^+$ and Gr1$^+$ cells were quantified by flow cytometry. 5 days post-infection PP were harvested. (**E**) The percentage of Gr1$^+$CD11b$^+$ cells and blue$^+$ cells in the PP were plotted from mice infected with YPIII-HTEM (squares) or YPIII*yopER144A* (triangles). The experiment was repeated three times and data from all mice are shown. Significant differences between the GR1+ and the blue$^+$ cells between the two groups of mice were determined by an unpaired, two tailed t test. (**F**) Bacterial colonization in mice infected with YPIII or YPIII*yopER144A*-HTEM shown as CFU/gm PP. There was no statistical difference in bacterial load by an unpaired two tailed t test P=0.59

present in the PP, fewer numbers of cells were targeted overall despite similar bacterial loads indicating that *Yptb* refrained from translocating Yops.

To further test the hypothesis that *Yptb* selectively targets professional phagocytes during infection, we exploited our previous observation that infection with a Δ*yopE* mutant fails to recruit neutrophils to the PP at day 5 post-infection despite colonizing PP at levels equivalent to WT bacteria [23] (Fig 2.9 F). This experiment allows us another means to functionally reduce neutrophil levels in *Yptb*-infected tissues. YPIII WT-HTEM or an *Yptb* strain expressing a catalytically inactive mutant of YopE and HTEM, YPIII *yopER144A*-HTEM, were used to infect mice. Five days post-infection, PP were harvested and assessed for the number of neutrophils, the number of blue cells, and the number of bacteria in the tissues (Fig 2.9 E-F). Mice infected with YPIII *yopER144A*-HTEM had lower levels of neutrophils and fewer HTEM-containing cells than mice infected with YPIII WT-HTEM, but comparable bacterial loads. Together, this experiment and the neutrophil depletion experiments suggest that *Yptb* does not indiscriminately translocate Yops into whatever cells are present, but rather selectively translocates Yops into neutrophils.

2.3.6 Professional phagocytes in spleen cell suspensions are preferentially translocated by *Yptb* at low multiplicity of infection.

We next analyzed whether the tissue microenvironment played a role in target specificity or whether there are inherent properties of professional phagocytes that permit *Yptb* to selectively translocate Yops into these cells. Spleens were harvested from uninfected mice and filtered to disrupt all tissue

architecture and generate a single-cell suspension. Splenocytes were incubated with CCF2-AM, infected at either a high MOI (20:1) or a low MOI (1:1) (without spinning the bacteria with the cells), labeled with different antibodies, and then analyzed by flow cytometry. A high MOI was used to determine whether all cell types tested could be translocated with HTEM while a low MOI was used to determine whether specific cells were preferentially targeted for HTEM translocation when bacteria were limited. At a high MOI, all cell types tested were translocated with HTEM (Fig 2.10 A), indicating that Yop translocation can occur in all splenocytes when the number of bacteria-host cell interactions were frequent, which is consistent with previous results [108]. Furthermore, no translocation specificity was observed as all cells were susceptible to translocation (Fig 2.10 B). In contrast, at a low MOI, neutrophils, macrophages and dendritic cells were preferentially targeted by *Yptb* for HTEM

translocation by 25-, 3-, and 5-fold, respectively, when compared to the total amount of these cell types in the spleen (Fig 5C-D). Furthermore, B cells were selectively excluded from translocation by *Yptb*. These results with splenocyte cell suspensions mirror those obtained during infection of mice (Fig 2.3 C, E & G) and show that intrinsic properties of neutrophils, macrophages and dendritic cells can dictate selective targeting of Yop translocation into these cells. Furthermore, they support the idea that co-localization during tissue infection is insufficient for cell-type specific targeting.

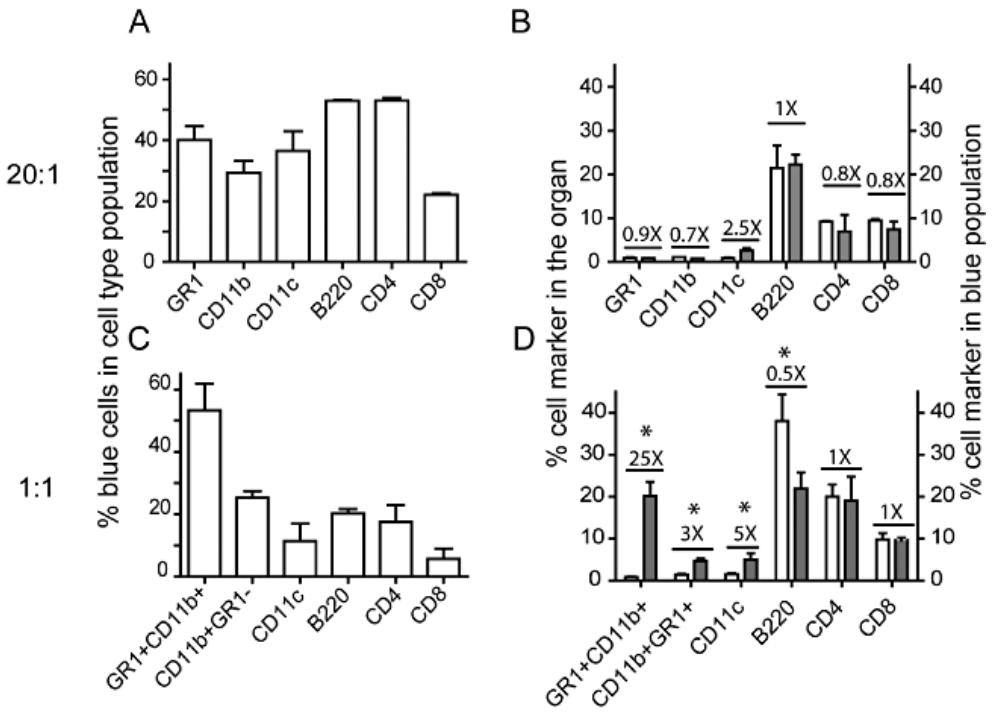
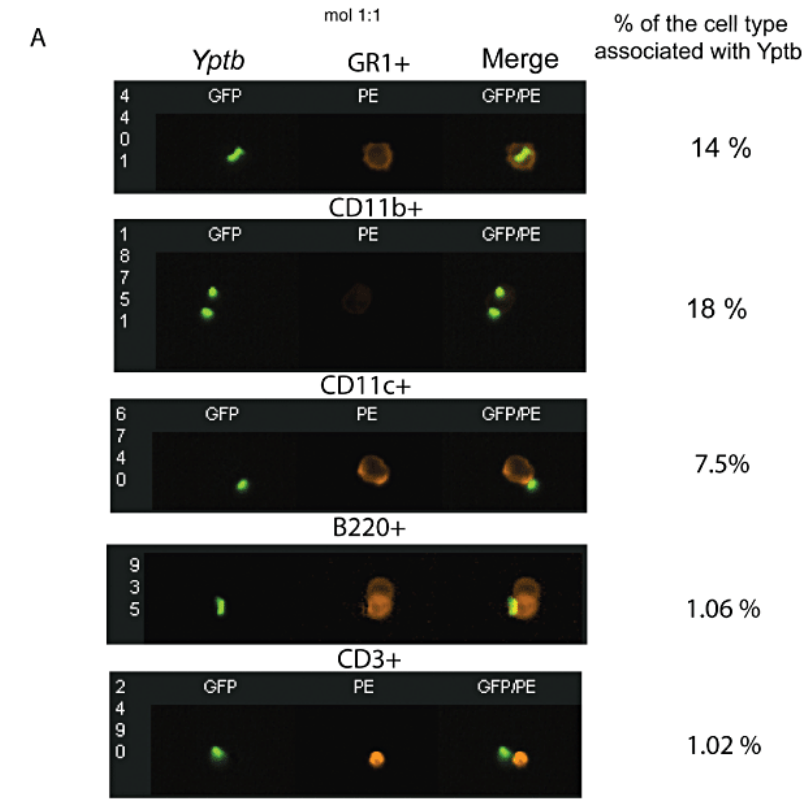


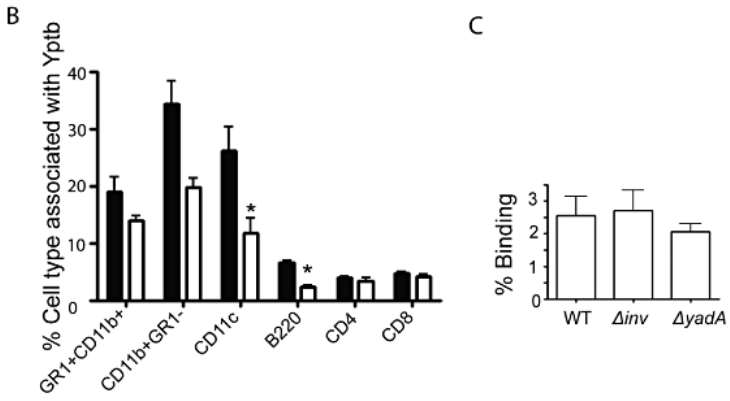
Fig. 2.10 WT-HTEM preferentially targets macrophages, neutrophils and dendritic cells from splenocytes at a low, but not high MOI.

Splenocytes were infected with WT-HTEM *(***A, B)** at a MOI of 20:1 or (**C, D)** at a MOI 1:1, incubated with CCF2-AM, and then distinguished by cell type by antibody and flow cytometry. The number of blue cells of a particular cell type was quantified and the number of each cell type in the spleen was quantified. (**A, C)** The percentage of blue cells (y axis) in the cell type indicated on the x-axis. (**B, D)** The percentage of each cell type present in the entire organ (white bars, y-axis on left) compared to the percentage of each cell type present in the blue^{+} population (black bars, y-axis on right). The fold enrichment of each cell type in the blue^{+} population when compared to the same cell type in the organ is denoted on top of each pair of bars. The experiment was repeated 4 times and the average + SEM are plotted. The asterisk indicates that there was a significant difference in the percentage of a specific cell type in the blue^{+} cells compared to the entire organ (unpaired, two tailed t test $P<0.05$).

2.3.7 Yptb binds preferentially to neutrophils and macrophages using YadA.

Translocation of Yops is dependant upon *Yptb* binding to cells [218]. Preferential binding of *Yptb* to neutrophils, macrophages and dendritic cells could determine the specificity of translocation to these cells and so we tested whether *Yptb* preferentially bound to these cells. *Yptb* expressing GFP was incubated with splenocytes, and cells were then labeled with different fluorescent antibodies to identify the cell types associated with GFP-*Yptb* by flow imaging and flow cytometry (Fig 2.11 A&B-white bars). To rule out the possibility that *Yptb* was associating with professional phagocytes because professional phagocytes chemotaxed towards and/or phagocytosed *Yptb*, cytochalasin D was added to the splenocytes to inhibit these activities. *Yptb* preferentially bound to neutrophils, macrophages and dendritic cells compared to B and T cells (Fig 2.11 A&B, white bars) in the absence of chemotaxis and/or phagocytosis. To test whether more professional phagocytes associated with *Yptb* when ability to chemotax and phagocytose bacteria was present, the numbers of different splenocytes that associated with *Yptb* was determined by flow cytometry (Fig 2.11 B, black bars). An increase in binding of GFP-*Yptb* to neutrophils, macrophages and dendritic cells was observed in the absence of the inhibitor (Fig 2.11 B, black bars), indicating that chemotaxis and/or phagocytosis enhanced the association of these cell types with *Yptb*. Nonetheless, even in the presence of cytochalasin D, *Yptb* preferentially attached to neutrophils, macrophages and dendritic cells.


A
mol 1:1
% of the cell type associated with Yptb
Yptb
GR1+
Merge
GFP
PE
GFP/PE
14 %
CD11b+
18 %
CD11c+
7.5%
B220+
1.06 %
CD3+
1.02 %


B
% Cell type associated with Yptb
GR1+CD11b+
CD11b+GR1-
CD11c
B220
CD4
CD8
C
% Binding
WT
Δinv
ΔyadA

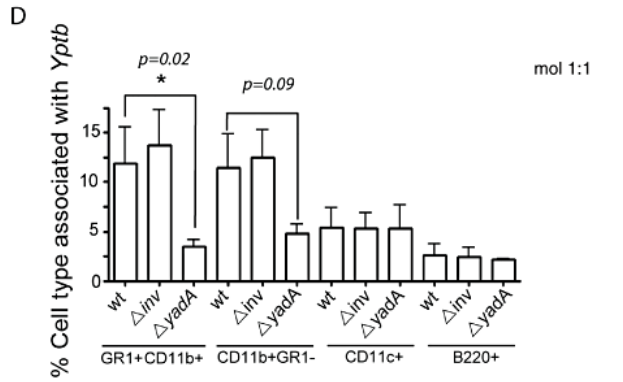

D
% Cell type associated with Yptb
p=0.02
p=0.09
mol 1:1
wt
Δinv
ΔyadA
GR1+CD11b+
CD11b+GR1-
CD11c+
B220+

Fig. 2.11 *Yptb* binds preferentially to professional phagocytes cells at low MOI.

(A) A single cell suspension of splenocytes was treated with 2 μM of cytochalasin D and then infected with WT IP2666 expressing GFP at a MOI of 1:1 for 1 hour. Splenocytes were labeled with antibodies to different cell markers and the binding of *Yptb* to specific cell types was analyzed by ImageStream. The experiment was done twice and the average number of a particular cell type associated with *Yptb* is shown. **(B)** A single cell suspension of splenocytes was treated with 2 μM of cytochalasin D (white bars) or left untreated (black bars) and then infected with WT IP2666 expressing GFP at a MOI of 1:1 for 1 hour. Splenocytes were labeled with antibodies to different cell markers and the binding of *Yptb* to specific cell types was analyzed by flow cytometry. The experiment was repeated 3 times and the average + SEM are graphed. The asterisk indicates significant differences between cytochalasin D treated and untreated cells as determined by t test ($P<0.05$). **(C-D)** Cells were treated with 2μM of cytochalasin D and infected with WT, *Δinv*, or *ΔyadA* expressing GFP at MOI 1:1 for 1 hour. **(C)** The percent cells bound to GFP-expressing bacteria was determined by fluorescence intensity in the FITC channel of the total splenocyte population. **(D)** The percentage of specific cell types in spleens bound by WT, Δ*inv* and Δ*yadA*. The experiment was repeated 4 times and the average + SEM are graphed. The asterisk indicates significant differences in the association of the indicated cell type by WT versus Δ*yadA* infected cells as determined by t test ($P<0.05$).

We next analyzed the role of two bacterial adhesins in directing specific binding to neutrophils and macrophages. Binding by Invasin and YadA to cells containing β1-integrins, which include professional phagocytes, as well as B cells and T cells, facilitates Yop delivery [218]. The presence of YadA and Invasin in *Yptb* grown at 37℃ was confirmed by western blot analysis (Fig 2.12). The binding of Δ*yadA* and Δ*inv* expressing GFP to splenocytes was tested in the presence of cytochalasin D by flow cytometry. No differences in the total amount of binding to splenocytes were detected among WT, Δ*inv* or Δ*yadA* (Fig 2.12 C). However, when specific cell types were analyzed for their ability to bind to GFP-Δ*inv* or GFP-Δ*yadA* (Fig 2.12 D), the Δ*yadA* mutant bound significantly less to neutrophils than GFP-*Yptb*, while binding to B cells and dendritic cells remained unchanged (Fig. 2.12 D). Since neutrophils are only approximately 0.54% of all splenocytes (Table 1), the 4-fold reduction in binding to neutrophils was not detectable in the total splenocytes (Fig 2.11 C). In the absence of Invasin, no difference was observed in binding to any cell type analyzed (Fig 2.11 D). These results demonstrate that YadA plays a critical role in selective association of *Yptb* with neutrophils whereas Invasin does not.

2.3.8 yadA and *invasin* mutants have reduced translocation into splenocytes compared to WT, but still target professional phagocytes for translocation

Yop translocation depends on both the binding of *Yptb* to host cells and activation of signal-transduction cascades within these cells, the latter of which is triggered by ligand-host cell receptor binding [218]. The Δ*yadA* mutant bound

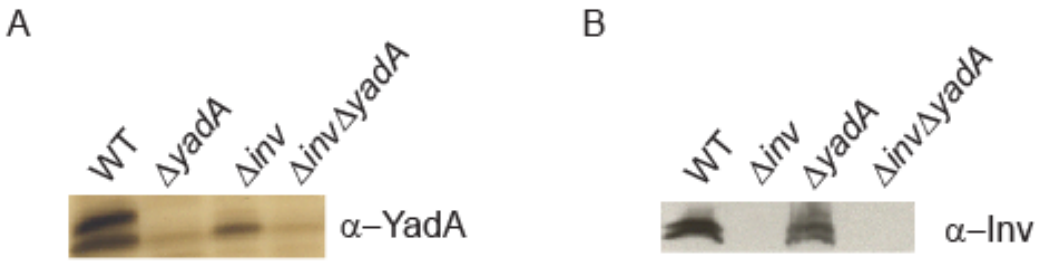


Fig. 2.12 Western blot of Invasin and YadA in WT, *Δinv, ΔyadA* and *ΔinvΔyadA.*

Bacteria were grown at 26℃ for 2 hours and then grown at 37℃ for 2 hours in 2xYT. Bacteria were lysed in SDS sample buffer and proteins were separated on SDS-PAGE, transferred to membrane and probed with (**A**) antibody to YadA (Santa Cruz) or (**B**) antibody to Invasin (gift from Dr. Isberg).

inefficiently to neutrophils (Fig. 2.11) and binding of invasin triggers signal transduction cascades that enhance translocation [218]. We tested whether∆*yadA*-HTEM, ∆*inv*-HTEM and/or ∆inv∆yadA-HTEM targeted fewer splenocytes for translocation using flow cytometry (Fig 2.13 A-B). Fewer splenocytes contained HTEM when infected with ∆*yopB*-HTEM, *Δinv-HTEM*, *ΔyadA-HTEM* or ∆*inv*∆*yadA*-HTEM compared to WT-HTEM (Fig 2.13 A-B and Fig 2.2). These data suggest that the individual adhesins play an important role in facilitating interactions with splenocytes and increase the number of cells targeted for translocation.

We next assessed whether specific cell types were targeted less frequently by specific adhesin mutants or if all cell types were reduced for targeting after infection with the different adhesin mutants. The number of neutrophils, macrophages, dendritic cells and B cells targeted for translocation by the single adhesin mutants, ∆*inv*-HTEM and ∆*yadA*-HTEM was significantly reduced compared to WT-HTEM (Fig 2.13 C). While the translocation into these cells was not significantly reduced after infection with the ∆*inv*∆yadA double mutant, this is likely due to the observation that 2 out of 6 experiments had very high levels of translocation after infection with the ∆*inv*∆*yadA* while 4/6 had lower

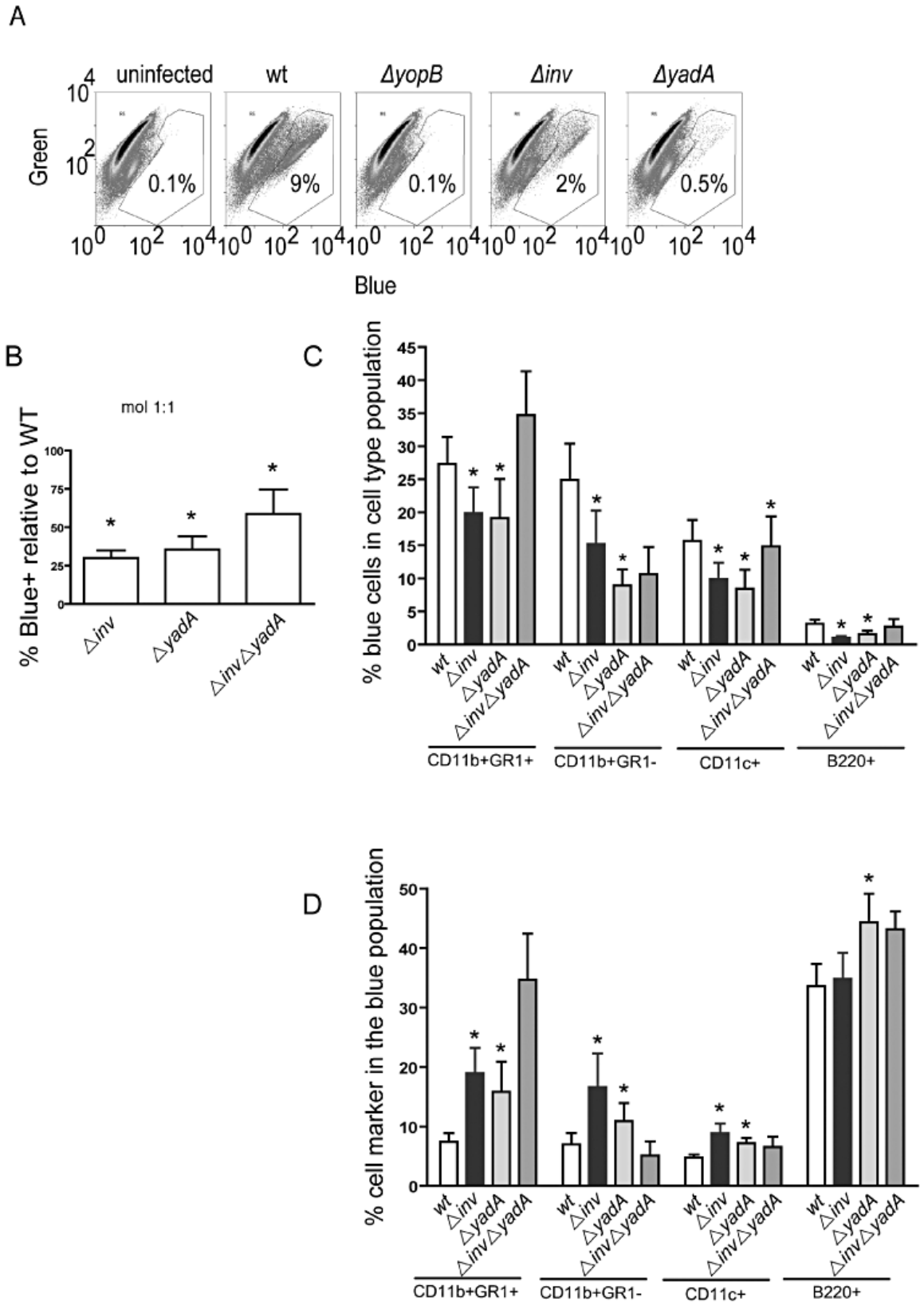

A
uninfected
wt
ΔyopB
Δinv
ΔyadA
Green
Blue
0.1%
9%
0.1%
2%
0.5%
B
mol 1:1
% Blue+ relative to WT
Δinv
ΔyadA
Δinv ΔyadA
C
% blue cells in cell type population
wt
Δinv
ΔyadA
Δinv ΔyadA
CD11b+GR1+
CD11b+GR1-
CD11c+
B220+
D
% cell marker in the blue population

Fig.2.13 *ΔyadA* and *Δinv* translocate HTEM into fewer numbers of splenocytes.

Splenocytes were left uninfected or infected with WT-HTEM, Δ*yopB*-HTEM, Δ*inv*-HTEM, or Δ*yadA*-HTEM at an MOI of 1:1, incubated with CCF2-AM and antibodies to distinguish particular cell types. (**A**) The percentage of blue^{+} cells was determined by flow cytometry**. (B)** The relative percent of blue+ cells by setting WT-HTEM to 100% and normalizing the percentage of blue cells of the HTEM mutant strain to WT-HTEM. Experiments with the Δ*inv*-HTEM, Δ*yadA*-HTEM and Δ*inv*Δ*yadA*-HTEM were repeated 9, 13 and 6 times, respectively. Differences were determined by using a paired t test with the WT-HTEM sample. **(C)** The percentage of blue^{+} cells in the indicated cell type after infection with Δ*inv*-HTEM, Δ*yadA*-HTEM or Δ*inv*Δ*yadA*-HTEM was compared to infection with WT-HTEM. **(D)** The percentage of a cell marker in the blue cell population after infection with Δ*inv*-HTEM, Δ*yadA*-HTEM or Δ*inv*ΔyadA-HTEM was compared to infection with WT-HTEM. (**C-D**) The bars represent the average + SEM of at least 8, 12 and 6 experiments for Δinv-HTEM, ΔyadA-HTEM and ΔinvΔyadA-HTEM analyzed with the indicated markers. Asterisk indicates significant differences between the number of blue cells in the WT-HTEM infected population versus the adhesin mutant population as determined by paired, t test ($P<0.05$).

levels than WT. These results indicate that YadA and Invasin interact with a variety of different cell types to promote translocation. Furthermore, these results combined with our previous results (Fig 2.11 C) indicate that YadA specifically promotes binding to neutrophils and plays an additional role to facilitate translocation when *Yptb* associates with other cells. When an MOI of 40 was used, all types of splenocytes were translocated with Yops; however again more professional phagocytes were targeted than B and T cells (Fig 2.14).

To determine whether the adhesion mutants targeted a different spectrum of cell types compared to WT, the percentage of each cell type in the translocated population was analyzed. This analysis should indicate whether either of these adhesins is critical for Yop translocation into a specific cell type. Surprisingly, neutrophils, macrophages and dendritic cells had enhanced translocation in the absence of either Invasin or YadA compared to WT (Fig 2.13 D). This result indicates that while the overall numbers of professional phagocytes with translocated Yops was reduced (Fig 2.13 B&C), *Yptb* still interacted preferentially with professional phagocytes compared to other cell types in splenocytes.

In these translocation assays, phagocytosis could not be prevented because disruption of actin polymerization also blocks translocation [218] and our unpublished data. Thus, it is possible that translocation of HTEM from *Yptb* may be occurring from within the phagosomes of professional phagocytes after *Yptb*

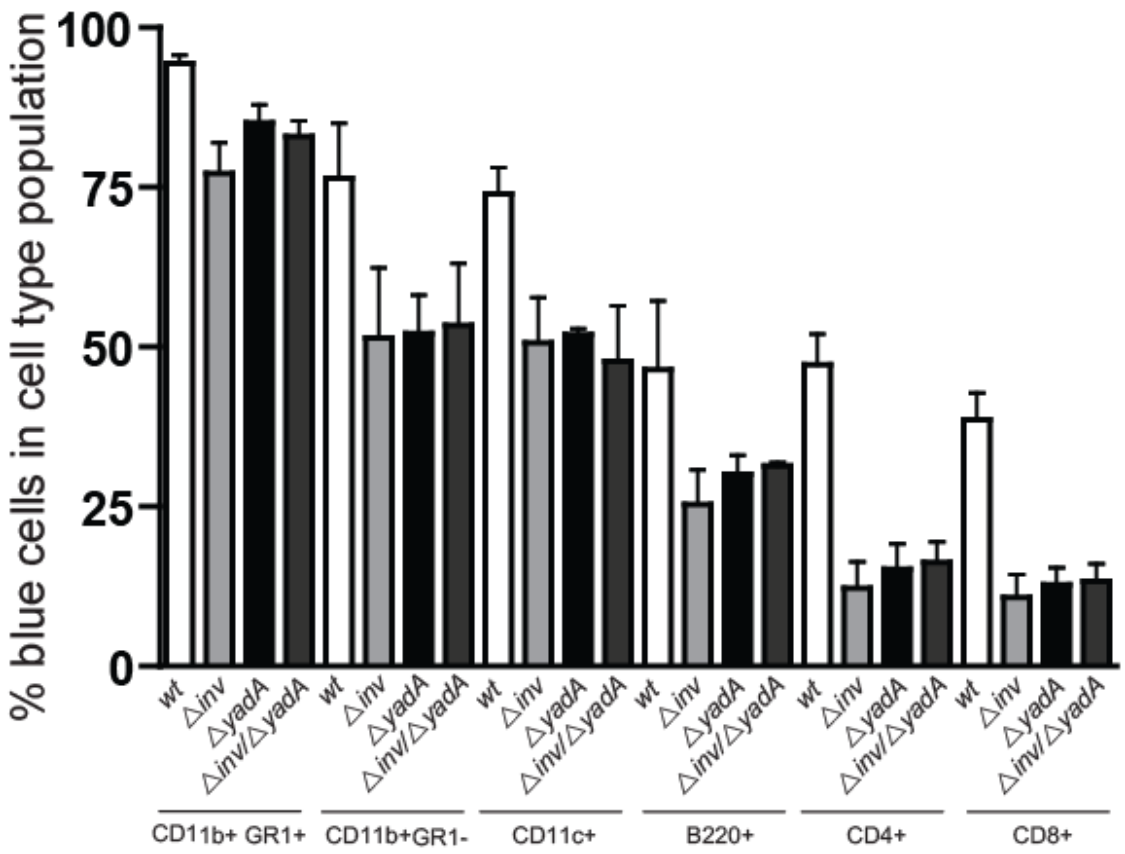


Fig 2.14 Translocation into splenocytes after infection at MOI 40:1 with WT-HTEM, *Δinv*-HTEM, *ΔyadA*-HTEM and *ΔinvΔyadA*-HTEM.

The number of blue cells in the indicated cell type was determined by flow cytometry after infection of splenocytes with WT-HTEM, Δinv-HTEM, ΔyadA-HTEM and ΔinvΔyadA-HTEM at MOI of 40.

has been internalized. In fact, recent studies support the idea that some Yop translocation can occur after phagocytosis of *Yptb* [228]. To investigate this possibility, WT-HTEM was grown at 26℃ or 37℃, conditi ons which promote or block invasion of phagocytes, respectively, and RAW264.7 macrophages were infected at an MOI of 10. At 26℃, *Yptb* expresses adhesins, but not the TTSS, so more bacteria are internalized whereas at 37℃ both adhesins and TTSS are expressed, so the Yops are rapidly delivered to phagocytes preventing phagocytosis. After 20 minutes, gentamicin was added to kill extracellular bacteria. Infection was allowed to proceed for an additional hour to permit the internalized *Yptb* to potentially express the TTSS and translocate HTEM. Afterwards, one cohort of cells used to measure HTEM translocation and the second was analyzed to determine the numbers of internalized bacteria. Importantly, 10 fold more macrophages were blue than contained internalized *Yptb* after infection with *Yptb* grown at 37℃ (Fig 2.2 B). These results indicate that HTEM was most frequently translocated from extracellular bacteria when the bacteria were grown at 37℃. In contrast, more cells contained *Yptb* than were blue after infection with *Yptb* grown at 26℃. Although the presence of *Yptb* in the blue cells was not tested, these results may indicate that some internalized *Yptb* may translocate Yops.

2.3.9 Δ*yadA* and Δ*inv* mutants colonize the Peyer's patches poorly after oral infection, but Δ*inv* translocate Yops into professional phagocytes.

To determine whether YadA and/or Invasin are critical for targeting neutrophils or macrophages during infection, mice were infected orogastrically with WT-HTEM, Δ*yadA*-HTEM, Δ*inv*-HTEM or Δ*inv*Δ*yadA*-HTEM and the numbers of bacteria, blue cells, and neutrophils in the PP were determined at three and five days post-infection (Fig 2.15 A-C). We found significantly fewer bacteria, fewer blue cells and fewer neutrophils in the PP of mice infected with Δ*yadA*-HTEM, Δ*inv*-HTEM and Δ*yadA*Δ*inv*-HTEM compared to WT-HTEM at both days. Therefore, it was impossible to conclude whether the reduced number of translocated neutrophils was due to the reduced bacterial load, the reduced numbers of neutrophils or an inability of the mutants to target these cells for translocation. However, fortuitously at day 5 post-infection, the PP of three mice infected with Δ*inv*-HTEM had bacterial levels that were within the lower range of mice infected with WT-HTEM (Fig 2.15 A). The number of blue cells and the types of cells targeted for Yop translocation in these PP were compared to three mice infected with WT-HTEM that had similar bacterial loads (Fig 2.15 D-G). The 3 mice in each cohort had similar numbers of blue cells and similar numbers of neutrophils (Fig 2.15 D). In addition, the number of neutrophils and macrophages translocated with Yops were comparable (Fig 2.15 F & G). These preliminary results suggest that Invasin is not solely responsible for the targeted translocation of HTEM into professional phagocytes during infection of PP.

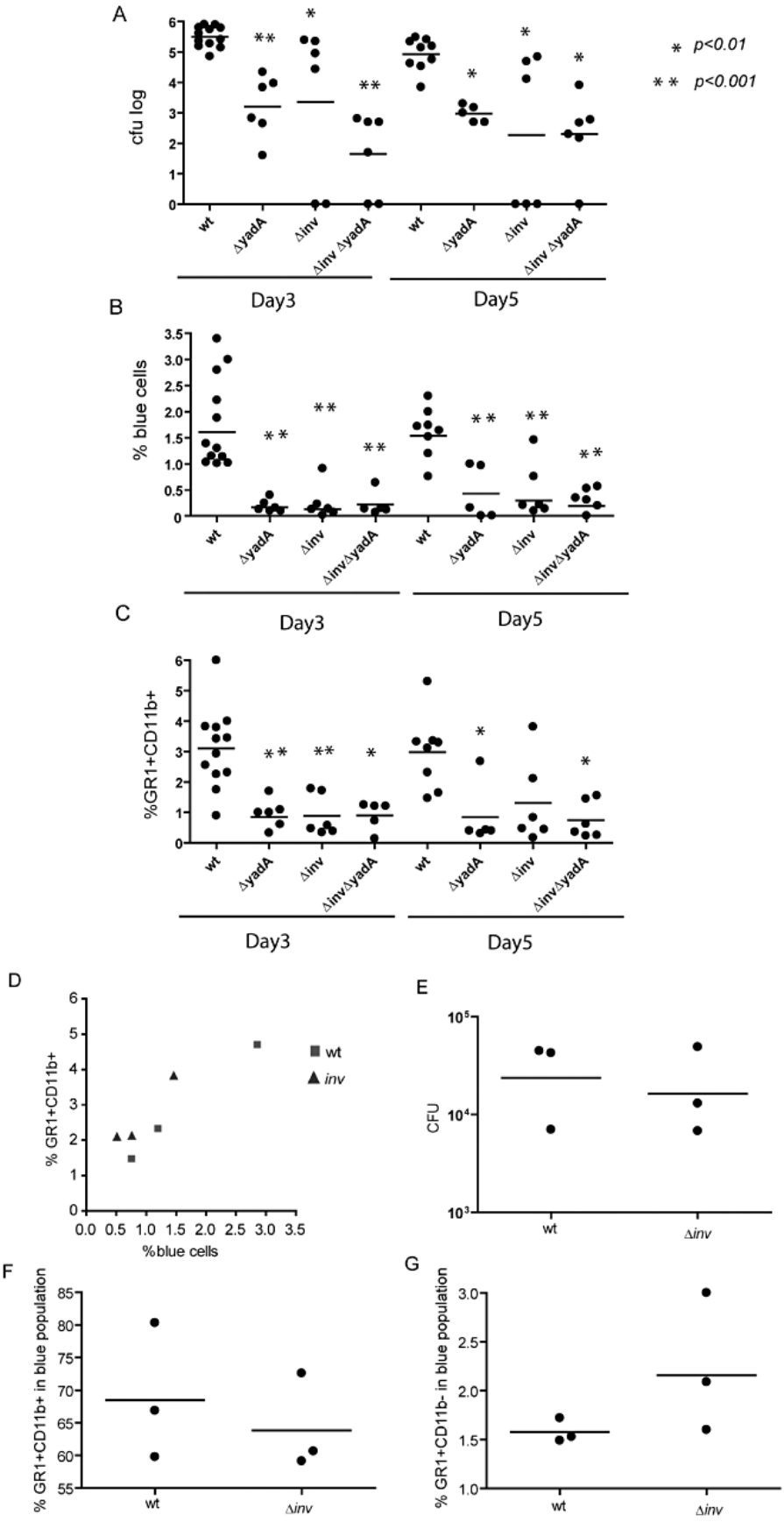

A
cfu log
* p<0.01
** p<0.001
wt
ΔyadA
Δinv
Δinv ΔyadA
Day3
Day5
B
% blue cells
wt
ΔyadA
Δinv
ΔinvΔyadA
Day3
Day5
C
%GR1+CD11b+
wt
ΔyadA
Δinv
ΔinvΔyadA
Day3
Day5
D
% GR1+CD11b+
%blue cells
wt
inv
E
CFU
wt
Δinv
F
% GR1+CD11b+ in blue population
wt
Δinv
G
% GR1+CD11b- in blue population
wt
Δinv

Fig. 2.15 YadA and Invasin are critical for colonization of Peyer's patches.

Seven to nine week old female BALB/c mice were infected orally with $2x10^9$ CFU of WT-HTEM, *ΔyadA-HTEM,* Δ*inv-HTEM* or Δ*inv*Δ*yadA*-HTEM and sacrificed at 3 or 5 days post infection. Each dot represents one mouse; the bars represent the average. **(A)** At each time point PP were harvested, a cell suspension was generated and plated for CFU. **(B)** Cells were incubated with CCF2-AM and the percentage of blue cells in PP was determined by flow cytometry at each time point **(C)** Cells were labeled with α-Gr1-PeCy5 and α-Cd11b-PeCy7 to determine the percentage of neutrophils by flow cytometry. Each dot represents data from an individual mouse. Bars indicate the geometric mean in (**A)** and average in (**B)** and (**C)**. The experiment was repeated three-five times and all the data was combined and analyzed using ANOVA with Tukey-Kamer multiple comparison. * indicates P value < 0.01 and ** indicates P value < 0.001. (**D**) The percentage of $blue^+$ cells (x-axis) were plotted versus the percentage of $GR1^+CD11b^+$ cells (y-axis) in the PP from mice colonized with comparable numbers of either WT-HTEM (squares) or *Δinv*-HTEM (triangles). (**E**) Bacterial colonization in PP of the WT-HTEM and Δinv-HTEM mice used in panels D, F and G**.** Each dot represents one mouse; the bar represents the geometric mean. There were no significant differences. **(F-G)** The percentage of Gr1+CD11b+ (**F**) or GR1+CD11b- (**G**) cells in the blue cell population in the PP after infection with either WT or Δ*inv*-HTEM. There were no significant differences.

Since it had previously been reported that a *yadA* mutant can reach the PP during early stages of infection but does not survive [144, 211], mice were infected with WT-HTEM and ΔyadA-HTEM for 6 hours, 1 day or 2 days. The number of bacteria in the PP, the number of blue cells and the number of neutrophils were counted to determine whether at earlier time points comparable numbers of WT-HTEM and Δ*yadA*-HTEM were detected (Fig 2.16). Consistent with previous results [144, 211], comparable bacterial loads were detected in the PP at 6 hours post-infection, but by day 1, most mice infected with WT-HTEM had higher bacterial loads than mice infected with ΔyadA-HTEM. Unfortunately, the number of blue cells at 6 hours were too low to evaluate whether the distribution of cells targeted by WT-HTEM versus ΔyadA-HTEM were different (Fig 2.16). These results demonstrate that YadA plays a critical role in the GI tract during infection, but whether YadA facilitates targeting of Yops to neutrophils or other cell during tissue infection cannot be evaluated due to low levels of overall colonization and translocation.

2.4 Discussion

After oral infection, pathogenic *Yptb* replicates primarily extracellularly in many organs and must counteract bactericidal actions of resident and incoming cells [25, 213-214]}. During infection of cultured cells, *Yersinia* translocates Yops into many different cell

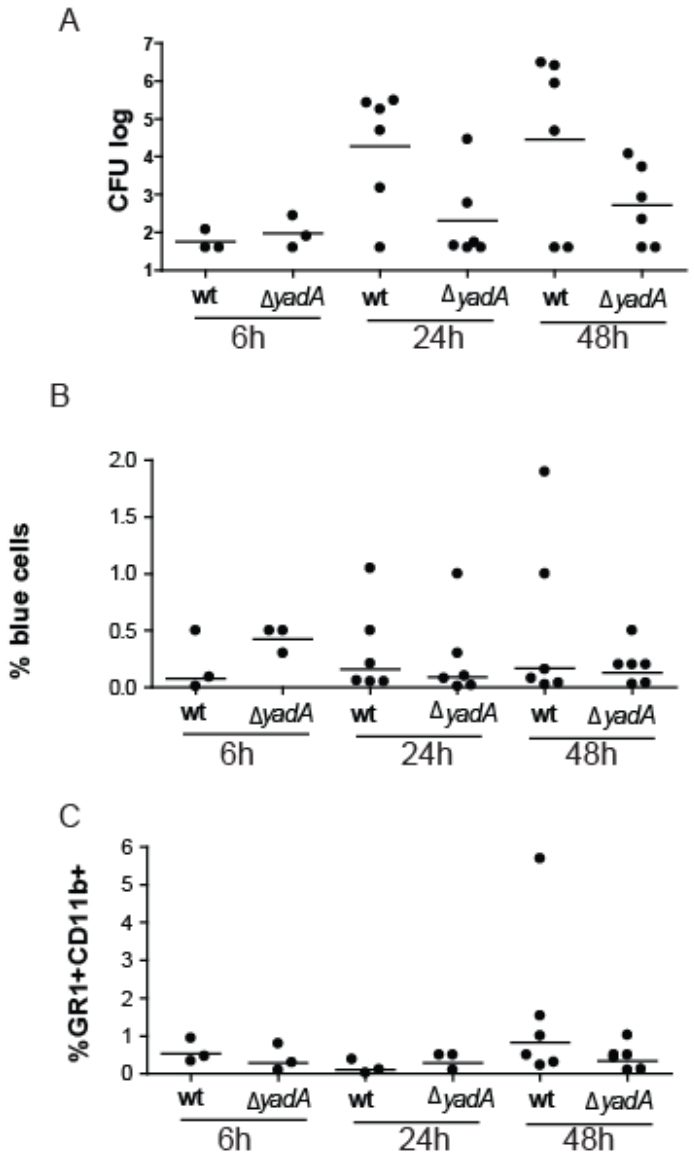


Fig 2.16 YadA is a key virulent factor for *Yptb* survival in the PP.

Eight week old female BALB/c mice were infected orally with 2x109 CFU with WT-IP2666 or *ΔyadA* and sacrificed at 6h, 24h, and 48h hours post infection. **(A)** At each time point PP were harvested, a cell suspension was generated and plated for CFU. **(B)** Cells were incubated with CCF2-AM and the percentage of blue cells in PP was determined by flow cytometry at each time point **(C)** Cells were labeled with α-Gr1-PeCy5 and α-Cd11b-PeCy7 to distinguish the neutrophil population by flow cytometry. The percentage of neutrophils in the PP was determined. Each dot represents data from an individual mouse. Bars indicate the geometric mean in (**A)** and average in (**B)** and (**C)**. The experiment was repeated twice and data from all mice are shown.

types including epithelial cells, macrophages, B cells, T cells, and dendritic cells [63, 103, 215-217]. Therefore, it seemed plausible that *Yptb* translocates Yops into all cells found in infected tissues, especially given that these cells express receptors capable of binding to *Yptb* adhesins [151, 160, 215, 229]. In contrast, here we demonstrate that after oral infection *Yptb* selectively targets neutrophils, and to a lesser extent macrophages and dendritic cells in the PP, MLN and spleen for Yop delivery. Moreover, in lymph nodes *Yptb* discriminates against B cells for translocation. Finally, in the absence of the preferred cellular targets, overall levels of Yop translocation were significantly reduced demonstrating that interactions between *Yptb* and specific cell types during infection must determine when and whether Yops are translocated.

Several lines of evidence indicate that *Yptb* specifically targets professional phagocytes because of inherent properties between these cells and *Yptb* rather than targeting phagocytes because phagocytes are their closest neighboring cells during tissue infection. First, in neutrophil-depleted mice or under tissue infection conditions where fewer neutrophils migrated to lymph nodes, *Yptb* targeted significantly fewer cells for translocation in the PP despite the fact that the bacterial load was similar. Consistent with this observation, mice lacking TNFR displayed increased levels of professional phagocytes as well as an increase in the level of total blue cells during infection with *Ye* [203]. Second, very few B and T cells were translocated with Yops in the lymph nodes, although *Yptb* co-localized with B and T cells in lymph nodes [25]. Finally, the marked preference for translocation of Yops into professional phagocytes and the

discrimination against B cells was recapitulated when the tissue architecture was disrupted and splenic cell homogenates were infected at low MOI. In contrast at high MOI, all cell types in the spleen were targeted by *Yptb,* suggesting that all cell types are capable of being translocated with Yops. Together, these results show that the inherent properties of the interaction between *Yptb* with certain host cells cause preferential translocation into those cells during infection, rather than the specificity of Yop translocation being driven merely by proximity.

Several features of *Yptb* and professional phagocytes could result in the selective targeting of professional phagocytes by *Yptb* during infection and in splenocytes suspensions. These features could include binding, the ability to chemotax, activation state of the bound cell, activation of specific signal-transduction cascades, and/or plasma membrane domains that favor insertion of the translocon. Since translocation requires binding to cells [218], the receptors on innate immune cells may recognize *Yptb* better than receptors on B and T cells. For instance, during the course of infection *Yptb* may become coated with complement or Fc and thus *Yptb* interactions with cells containing complement receptor and/or Fc receptor may be favored. Previous work with cultured cells has demonstrated that coating *Yersinia* with complement or Fc is sufficient to induce Yop translocation into cells with complement receptor or Fc receptor [215, 221]. Alternatively, *Yptb*-specific ligands may dictate the bacteria's association with neutrophils, macrophages and dendritic cells. In fact, our observation that Δ*yadA* binds to significantly fewer neutrophils from isolated

splenocytes than WT *Yptb* supports the idea that some *Yptb* ligands can promote interactions with particular host cells. While analysis of the few mice that were colonized by a Δ*inv* mutant indicated that the invasin mutant retained the ability to target neutrophils and macrophages as well as WT *Yptb* in the PP, *Yptb* may use YadA and/or other bacterial ligands to promote translocation to neutrophils, macrophages and/or dendritic cells during infection.

Several studies have examined the role of YadA in promoting *Yptb* dissemination from the GI tract to PP, MLN and spleen [144, 211]. In a ligated loop model of infection, a Δ*yadA* mutant rapidly penetrated the PPs; however, 48 hours post-oral inoculation Δ*yadA* was attenuated for growth in the PP [144, 211]. These results indicate that YadA is not needed for penetration and initial colonization of the PP, but YadA is required for survival within the PP. Our data and others is consistent with the idea that the Δ*yadA* mutant cannot target resident neutrophils and therefore is eliminated [144, 211]. Although invasin did not appear to influence binding to specific cell types, it is important to note that the IP2666 strain of *Yptb* expresses very little invasin compared to many other *Yptb* strains [214] and unpublished data. Thus a deletion in invasin in IP2666 may have less of an impact in cell binding and translocation than in other *Yptb* strains.

The activation state of the cells or the nature of the cell surface components may contribute to the efficiency of Yop translocation. Recently, Mejia et al showed that an increase in the activation of Src kinases after integrin stimulation correlated with an increase of Yop translocation in HeLa cells [218].

Perhaps cells that have recently migrated to tissues or have become activated are consequently more primed for translocation by *Yptb*. It remains to be determined whether specific subsets of dendritic cells, macrophages and T and B cells are targeted by *Yptb*. In fact, *Y. enterocolitica* targeted Yop predominantly to a subset of B cells, follicular B cells [203]. Another potential means of regulating translocation is that different cell membranes may be more or less conducive towards insertion of the translocon. Work in *Shigella* has indicated that translocon components are preferentially secreted in the presence of membranes rich with sphingomyelin and cholesterol and bind in regions rich in cholesterol [230-231]. While a similar phenotype has not been demonstrated with *Yptb*, it is possible that the translocon can more easily insert into the membranes of professional phagoctes than into B and T cells.

An intriguing question is whether or not Yop translocation occurs after phagocytosis by professional phagocytes during infection. Over 95% of *Yptb* is extracellular in the MLN and spleen after infection [25, 213] and we showed that when *Yptb* were grown at 37℃, 10 fold more macrophages were de tected that had translocated Yops than that contained internalized *Yptb*. Combined these results suggest that at least some macrophages in tissues are likely to be translocated with Yops from extracellular *Yptb*. However, we cannot exclude the possibility that during infection some *Yptb* is first internalized by professional phagocytes and then translocates HTEM or that some *Yptb* translocate Yops while they are being engulfed by phagocytes, with the result that a professional phagocyte has both internalized *Yptb* and translocated Yops. Distinguishing

between these two possibilities during tissue infection is technically challenging. Nonetheless, we think it plausible that some fraction of professional phagocytes with translocated Yops may contain *Yptb*. Understanding the fate of these two populations will provide insights into whether internalized *Yptb* alters host defenses and enhances bacterial growth during infection.

Previous studies have examined the splenic cell types targeted by *Y. pestis* and *Ye* after intravenous infection [108, 203]. Interestingly, both the enteric *Yersinia* and, *Y. pestis* targeted professional phagocytes, despite the fact that they do not share many of the same adhesins. Specifically, *Y. pestis* lacks YadA and invasin, and expresses some unique adhesins and some which are shared among *Yersinia* spp [188, 210]. Given the similar cell tropism for translocation, it is possible that some of the shared adhesins function during infection to direct Yop translocation into professional phagocytes. Alternatively, these three species may have functionally redundant adhesins. *Yptb*, *Y. pestis* and *Ye* targeted all splenocyte suspensions when infected at high MOI but as no studies were done with a lower MOI with *Y. pestis* or *Ye*, it is unclear if this selectivity is apparent under conditions where bacteria are limiting [108, 203]. One notable difference between the enterics and *Y. pestis* was that B cells were not discriminated against translocation in the spleens by the enteric *Yersinia* spp, but were discriminated by *Y. pestis*. This difference could be due to the difference in species, the location of the bacteria, and/or rate cell death of specific cell types. An important difference between the two studies with the enteric *Yersinia* spp is that after oral infection with *Ye* not enough bacteria were found in the PP to

detect blue cells [203]. In contrast, we found that PP had the highest levels of colonization and greatest number of blue cells after oral infection with *Yptb.* The molecular basis for this difference could be that the *Yptb* strains, IP2666 and YPIII, used in our studies might deliver Yops more efficiently into the targeted cells during infection. Supporting this idea is that observation that the level of colonization of the spleen after oral infection with *Yptb* was 100x less than that of *Ye* [203] suggesting that Yops are delivered more efficiently in our model. Additional differences between our model and that with *Ye* were the strain of mice used (C57Bl/6 versus BALB/c) and that mice infected with *Ye* were also given the immunosuppressant, desferrioxamine [203]. The immunosuppressant may have altered the physiology of the cells such that they were less receptive to Yop delivery. Nonetheless, it is striking that despite the fact that the overall levels of bacteria were 100x higher in the *Ye* and *Y. pestis* infected mice, the cellular tropism for Yop delivery was similar among all species.

This study is the first to examine the different types of cells with translocated Yops in multiple tissues after oral infection. Interestingly, there were differences in the cell types that were both enriched and/or underrepresented among each tissue. For example, CD4 T cells were underrepresented for targeting in the PP, but were not underrepresented in the MLN and spleens while B cells were underrepresented in both the PP and MLN but not the spleen. On the other hand, dendritic cells were enriched for targeting in the PP and spleen, while macrophages were enriched for targeting in the MLN, but not the PP. As of yet, the reasons for these different tropisms is unclear, but further

investigation into the subset of dendritic, macrophages, B and T cells might reveal that the differences are due to subsets of cells found in each tissue. For instance, the ability of specific subsets cells to chemotax towards bacteria in different tissues may explain, in part, both the tropism for professional phagocytes and the differences in the cells targeted in different tissues.

Using the TEM technology to identify intracellular niches and regulation of effector proteins, *Salmonella* was found to reside inside neutrophils after intraperitoneal infection [202]. Furthermore, several *Salmonella* effector proteins were translocated at different times in infection demonstrating that there was a hierarchy of effector protein translocation during infection. While YopH and YopE were both translocated into the same neutrophils in the spleen at day 5 post-infection, it remains to be determined whether all effector Yops are translocated continually throughout the course of infection and/or whether all Yops enter each individual cell.

In conclusion, despite the fact that *Yptb* readily translocates Yops into a variety of cell types in culture, *Yptb* demonstrates selective translocation into specific cell types during infection. Future experiments determining what cellular and bacterial factors are critical for the tropism to neutrophils, macrophages and dendritic cells, and how the Yops have altered the physiology of those cells during infection will be essential to understanding how *Yersinia* dismantles the immune response.

2.5.1 Construction of strains and plasmids

TABLE

2.3 *Yersinia* strains used in this study

Strain	Relevant characteristics	Reference
JM-102	IP2666	Joan-Miquel Balada Llasat, 2006.
JM-417	YPIII	Mecsas 2001
YED-08	IP2666 HTEM	This study
MLF-122	IP2666 *Δinv*	This study
YED-35	IP2666 *Δinv* HTEM	This study
YED-32	IP2666 *Δinv* GFP	This study
YED-37	IP2666 *ΔyadA*	This study
YED-38	IP2666 *ΔyadA* GFP	This study
YED-39	IP2666 *ΔyadA* HTEM	This study
CC-120	IP2666 ∆*yopB* HTEM	This study
WS-396	YPIII HTEM	This study
WS-397	YPIII *yopER144A* HTEM	This study
CC-151	DH5αλpir pRS47s-HTEM	This study
JM-549	DH5αλ pRK600	Segura *et al.,*2001

TABLE

3.4 List of primers

Primer name	Primer Sequence
YopH 5’	GAG ACA GGA TTC GCA TGC CAG TAT TAC CAT CTG TTC CCG C
YopH 3’	GAG ACA GCG GCC GCC AAG TAC ACT ATT ACC ATT GCC GAC
TEM 1 5’	GAG ACA GCG GCC GCC ACC CAG AAA CGC TGG TG
TEM1 3’	GAG ACA GAG CTC GCA TGC TGA GTA AAC TTG GTC TGA CAGT
YadA p1	GTA CCG TCG ACC ATC CGG TTT GAG GTG AGG
YadA p2	TTT GTG CATG CGA CCT GCT GAC AAA CGA GGA
YadA p 3	AGT TCG CAT GCA CAA AGG TTT AGC CAG TTC AGC
YadA p 4	GAT CCG AGC TCC GTA GCA AAT ATC GGA GAG ATT G
Inv 1	ACG CGT CGA CAA GGC AAC CAT CAG GAT TAA T
Inv 2	CGC GCA TGC AAA CTC ACT GAT TGG CTG GAA
Inv 3	CGC GCA TGC TAC TTG GCG TTC CCG CTC TGT
Inv 4	CGG GAG CTC ATC CAA GGC TGG GTA TTG CAA

To generate the YopH-TEM (HTEM) fusion construct, the TEM1 portion of β-lactamase was amplified from pBR322 using the primers TEM1-5' and TEM1-3' (Table 4) with PCR. The TEM1 fragment was ligated into pSR47s (Merriam et al., 1997) to generate pSR47s-TEM1. The DNA encoding the promoter and the first one hundred amino acids of *yopH* was amplified from IP2666 by PCR using YopH5' and YopH3' primers (Table 4). The YopH100 PCR product was ligated into pSR47s-TEM1 to generate pSR47s-HTEM.

The HTEM gene was introduced into *Yptb* strains, including WT IP2666, Δ*inv*, Δ*yadA*, WT YPIII and YPIII*yopER144A*, (Table 3.4) by conjugation to generate a single cross-over strain which expresses HTEM and YopH. Strains were analyzed by SDS-PAGE and western blot analysis for secretion of HTEM, YopH and all other effector Yops.

To generate Δ*inv* strain, primer pairs, Inv1 with Inv2 and Inv3 with Inv4 (Table 3.4), were used to amplify the flanking regions of *invasin*. The PCR fragments generated were digested with SphI, purified and ligated together. The ligated fragment was amplified using primers Inv1 and Inv4, purified, digested with SacI and SalI and ligated into the suicide vector pCVD422 and transform into SY327λpir. The resulting plasmid, pCVD442-invKO, was used to generate *Δinv* by conjugation as described in (Logsdon and Mecsas, 2003)

To generate Δ*yadA* deletion, primer pairs YadA1 with YadA2 and YadA3 with YadA4 (Table 3.4) were used to amplify flanking regions of YadA using PCR. The fragments generated were purified and combined in another PCR reaction using primers YadA1 and YadA4 (Table 4.4). The resulting fragment

was cloned into the PCR suicide vector pCVD422 to generate pCVD442-yadAKO and transformed into SY327λpir. Δ*yadA* strains was then tranconjugated into an IP2666 strain as described in (Logsdon and Mecsas, 2003).

To generate the ΔyadAΔinv mutant, pCVD442-yadAKO was transconjugated into Δinv as described above.. The absence of adhesins, YadA and Inv, in the Δ*yadA* and Δ*inv* strains were confirmed by Western blot (Fig 2.12).

2.5.2 CCF2-AM conversion assays after murine infections

Mice were infected as previously described [24] with the following modifications. BALB/c mice (NCI) were infected intragastrically with $2x10^9$ CFU of WT-HTEM unless otherwise is indicated. Infections were allowed to proceed for 5 days, except in the experiments involving neutrophil depletion, where infections were allowed to proceed for 3 days.

To generate a single cell suspension from cells in the PP, MLN and/or spleen, these tissues were harvested aseptically into 5 ml HBSS containing $MgCl_2$ and $CaCl_2$ (Cellgro). All tissues were pressed through a 70μm cell strainer (Falcon) and 100ug/ml gentamicin was added to kill bacteria and preventing Yop translocation for all subsequent steps. Cells were transferred to a 15 ml tube and spun at 340xg for 5 minutes. Prior to straining the spleens, spleens (but not PP or MLN) were perfused with 80U/ml collagenase (Roche) and incubated for 30 minutes at 37℃ to liberate dendritic cells and elimi nate auto-fluorescence. Collagenase activity was halted by the addition of 2ml HBSS lacking $MgCl_2$ and $CaCl_2$ (Cellgro) and supplemented with 1mM EDTA. Cells from spleens were resuspended in 10 mL BD Pharm Lyse™ solution (Pharmagen) to lyse

erythrocytes and immediately spun at 340xg for 5 min. Cells from PP, MLN and spleens were washed twice with PBS and then MLN and spleens were resuspended in 3 ml RPMI+10% FBS and PP were resuspended in 1mL of RPMI+10% FBS.

To label single cell suspensions, the cells were incubated with 1µg/ml CCF2-AM compound (Invitrogen) for 2 hours at 30℃ in the presence of 1.5mM probenecid (Sigma) and 100µg/ml gentamicin (Sigma). 100 µL of cells were aliquoted into a 96-well plate and blocked with 50µl of a 1:200 dilution of Mouse BD Fc Block™ (BD) for 10 minutes at 4℃. Cells were incuba ted in 50µl of FACS buffer (PBS+1% FBS+0.02% NaAzide) containing fluorescent antibodies to GR-1-PE-Cy5 (eBiocience), CD11b-PE-Cy5 (eBiocience), CD11b-PE-Cy7 (eBiocience) CD11c-PECy5 (eBiocience), B220-PeCy5 (BD), CD4-PECy5 (BD) and/or CD8-PECy5 (BD) at dilutions of 1:75 for 30 minutes at 4℃. Samples were washed twice in FACS buffer, centrifuged at 340xg, resuspended in 100µl in FACS buffer and analyzed on an LSRII (Becton Dickson) FACS machine. $2X10^5$ cells were acquired per sample and data were analyzed using Summit v4.3 software. Cells from tissues that not were incubated with CCF2-AM and/or antibodies as well as cells from uninfected tissues or tissues infected with WT were used as negative controls

The Institutional Animal Care and Use Committee of Tufts University approved all animal procedures.

2.5.3 CCF2-AM conversion assays in splenocyte suspensions

Spleens from uninfected, 6-8 week old BALB/c mice were harvested in RPMI+10%FBS in a 6-well plate, treated with collagenase and a cell suspension was generated as described above. Bacteria were grown overnight in LB and diluted 1:40 in 2xYT supplemented with 20mM sodium oxalate and 20mM $MgCl_2$. The bacteria were grown with aeration at 26℃ for 2 hours and then shifted to 37℃ for 2 hours prior to infection of splenocytes. Cells were infected with WT-HTEM or mutant strains at the indicated MOI for 1hr at 37°C. Infected splenocytes were labeled with CCF2-AM and prepared for flow cytometry with antibodies as described above.

2.5.4 Yptb adherence assays to splenocyte suspensions.

A 1mL aliquot splenocyte cell suspension from an uninfected spleen was infected with *Yptb* strains expressing GFP for an hour in a 24-well plate at 37℃ with no spinning. *Yptb* expressing GFP were grown as described above and the media was supplemented with 10 μg/ml chloramphenicol. Cells were labeled with antibodies and analyzed on ImageStream (Amnis) with IDEAS analytical software or an LSRII (Becton Dickson) FACS with Summit v4.3 software as described above.

2.5.5 Immunohistochemistry

The location of *Yptb* was determined as described [25] with the following modifications. PP, MLN, and spleen, from uninfected mice or mice infected intragastrically with 2×10^9 *Yptb* were processed, embedded in paraffin, cut in 8 μm sections and stained as described [25]. Samples were scored blindly by at least two investigators using a Nikon Eclipse TE2000-U microscope.

2.5.6 YopE and HTEM Translocation

Splenocytes from infected mice were treated with CCF2-AM and labeled with GR1-PE-Cy5 antibody (eBiocience 15-5931-81). Cells were sorted in the MoFLo FACS sorter (Cytomation, Fort Collins, CO,) and $2x10^5$ $blue^{hi}$-GR1+, $2x10^5$ $blue^{lo}$-GR1+ and $2x10^5$ $blue^{neg}$-GR1+ cells were collected. Cells from each population were lysed with 50 µl of eukaryotic lysis buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 5 µg/ml each of aprotinin, leupeptin, and pepstatin) for 20 min with gentle rocking at 4℃ and processed as described [217]. These lysis conditions have been shown to lyse the plasma cell membrane, but not bacteria membranes as shown in Fig S4 and [217].

2.5.7 Granulocyte Depletion

Granulocyte depletion experiments were performed as described [23] with the following modifications. Mice were injected with the antibodies 1 day prior to and 2 days after oral inoculation with $8x10^8$ *Yptb* YopHTEM. Day 3 post infection PP were harvested and single cell suspension was generated. 10µL of the 5ml cell suspension was plated on L plates containing kanamycin (50 µg/mL) to determine the bacterial burden in the tissue. Cells were labeled with CCF2-AM and with GR-1-PE-Cy5 antibody as described above. Fluorescent intensities of labeled cells were detected by flow cytometry using BD LSR II System. Data were analyzed using Summit v4.3 software. The experiment was performed twice and data from both experiments are shown.

CHAPTER III

3. Chapter III: Identifying molecular targets of YopH in neutrophils during animal infection

3.1 Abstract

The gram-negative enteric pathogen *Yersinia pseudotuberculosis* employs a type 3 secretion system (TTSS) that is required to translocate critical virulence factors, called Yops, into mammalian cells. We have previously determined that the primary cellular targets of the TTSS during mouse infection are professional phagocytes. We used the CCF2-AM β lactamase system to distinguish translocated cells from non-translocated cells after animal infection with WT and *ΔyopH* mutants. Specifically, to determine which proteins were dephosphorylated in the presence of YopH, a tyrosine phosphatase, cells lysates were probe with phosphotyrosine antibodies by Western blot. SLP-76 was identified as a target of YopH during infection. Slp-76 responds to and transmits intracellular signaling once integrin or Fc recptors are engaged [89]. In addition, we tested Paxillin, another known YopH target, and Syk an upstream effector of Slp-76 but their phosphorylation state was not affected by YopH. We also showed that YopH translocation into neutrophils led to a reduction of IL-10 production during infection. We have identified for first time a cellular target of Type III secretion during murine infection.

3.2 Introduction

Polymorphonuclear neutrophils (PMNs) kill invading bacteria using mechanisms such as production of reactive oxygen species (ROS), phagocytosis

and degranulation [232-235]. PMNs migrate from the blood to peripheral tissues in response to pathogens or tissue damage. Neutrophils are one of the first lines of defense against pathogens. They secrete cytokines and chemokines to elicite the immune response recruiting additional immune cells to the infected areas and activating resident immune such as monocytes, macrophages and dendritic cells [236]. Recently, it has been shown that upon bacterial infection, and in contrast to macrophages and monocytes, neutrophils secrete low amounts of pro inflammatory cytokines (IL-12 or INFγ); on the other hand, neutrophils secrete high amounts of the anti-inflammatory cytokine IL-10, suggesting that neutrophils contribute poorly to inflammatory responses [90]

Yersinia pseudotuberculosis (Yptb) has mechanisms to counteract killing by professional phagocytes. One important mechanism involves type three secretion system (TTSS) and effector proteins, called Yersinia outer proteins (Yops) [9]. Yops are translocated through the TTSS into host cells where they disrupt normal cellular functions [9]. One of the Yops translocated into the eukaryotic cell is YopH, a tyrosine phosphatase that has homology with a typical sequence of eukaryotic protein tyrosine phosphatases (PTPases) [68]. It was this similarity that first identified YopH as a tyrosine phosphatase [95, 237]. We showed previously that *Yptb* preferentially translocated YopH into neutrophils, macrophages and dendritic cells when mice were infected with *Yptb* [238]. Furthermore, when neutrophil depleted mice were infected with wild type *Yptb*, the overall levels of Yop translocation were drastically reduced [238]. From these

observations, we hypothesize that *Yersinia* selectively translocates YopH into neutrophils to inhibit their functions and permit *Yersinia* survival.

Using cell culture models, several potential YopH molecular substrates have been identified in epithelial cells, macrophages, dendritic cells and T cells. For instance, Paxillin, a signal transduction adaptor protein which is localized to focal adhesions, is dephosphorylated when HeLa cells are infected with *Yersinia* expressing YopH [58]. In macrophages [68], YopH dephosphorylates p130Cas, FAK, ADAP and SKAP-HOM [57, 61, 68]. p130Cas interacts with FAK and both are involved in an integrin mediated signalling cascade crucial for focal adhesion. ADAP and Skap-HOM are adaptor molecules necessary for recruitment of downstream effectors after integrin signaling. Slp76, an adaptor molecule, was identified as a target of YopH in T cells [89]. YopH also targets additional tyrosine kinases in T cells that are important in signaling through T cell receptor (TCR) such as LAT, Lck and Zap70. Although these proteins are not present in neutrophils, a homolog of Zap70, Syk, signals to Slp-76 in neutrophils and shares 93% amino acid identity with Zap70 so it may be a target of YopH [60]. In addition, similar to TCR signaling, the FcγR signaling depends on phosphorylation of ITAMs localize within the Fcγ chain by Src family members Lyn and/or Fyn, Hgr, and Fgr followed by recruitment of Syk in neutrophils to these phosphorylated motifs. All of these proteins (Paxillin, p130Cas, FAK, ADAP, SKAP-HOM, Syk, Slp-76) found in neutrophils may be targets of YopH during infection.

By isolating neutrophils translocated with Yops from mice infected with WT or *ΔyopH Yptb* strains, we have identified a protein dephosphorylated by YopH during murine infection.

3.3 Results

3.3.1 YopH dephosphorylates a ~70KD protein in splenic neutrophils and in neutrophils translocated with YopH during animal infection.

To understand the biological consequences of YopH in neutrophils during animal infection [238], we investigated the specific proteins that are dephosphorylated by YopH in neutrophils during murine infection. To identify tyrosine phosphorylated proteins that were dephosphorylated in splenic neutrophils after infection with WT *Yptb* but not with ΔyopH *Yptb* strains, isolated splenocytes were infected with *Yptb* strains expressing a chimeric protein of the translocation signal sequence of YopE fused to a portion of β-lactamase (TEM) which translocated a protein called ETEM. WT-ETEM or ΔyopH –ETEM infected splenocytes were incubated with CCF2-AM to detect ETEM translocation using cleavage of CCF2-AM and its subsequent change in its fluorescence as a read-out for translocation. CCF2-AM is a membrane diffusible substrate of TEM. Splenocytes incubated with CCF2-AM fluoresce green (uncleaved CCF2-AM) when excited with UV light. On the other hand splenocytes fluoresce blue (cleaved CCF2-AM) when they have been translocated TEM. Splenocytes were labeled with CCF2-AM and α-PE-Cy5-Gr1 and α-PE-Cy7-CD11b antibodies. Two populations of $GR1^+$ $CD11b^+$ cells, $blue^{neg}$, and $blue^{hi}$, were gated and sorted. Then $Gr1^+$

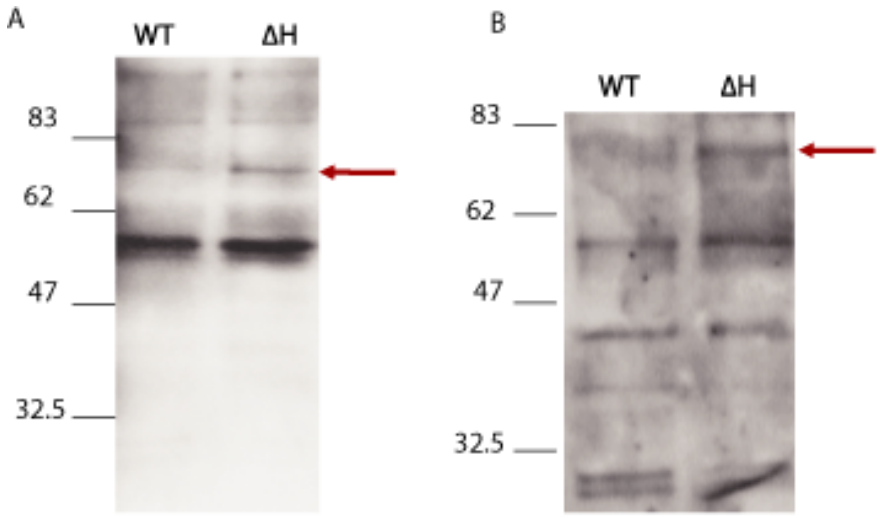


Fig.3.1 YopH dephosphorylates a ~70KD species in infected splenic neutrophils and after animal infection.

A. Splenocytes were infected at MOI 10:1 for an hour either with WT-ETEM or *ΔyopH*-ETEM strains. Infected splenocytes were labeled with CCF2-AM and prepared for flow cytometry by labeled with PE-Cy5-Gr1^{+} and PE-Cy7-CD11b^{+} antibodies. Blue neutrophils infected with WT or *ΔyopH* were isolated by flow cytometry, lysed and probed for phospho-tyrosine residues with an α-tyrophosphatase antibody (4G10). **B.** Mice were infected intravenously either with WT-ETEM (500 cfu) or *ΔyopH*-ETEM (2000 cfu) and five days post-infection, spleens were harvested. Splenocytes were incubated with CCF2-AM and labeled with PE-Cy5-Gr1^{+} and PE-Cy7-CD11b^{+} antibodies. Blue neutrophils were isolated by FACS, lysed and probed for phospho-tyrosine residues.

$CD11b^+$ cells from blue population were assessed for tyrosine phosphorylation using an α-p Tyr antibody by western blot (FIG.3.1A). A distinct band was observed around 70 kDa in neutrophils infected with ΔyopH-ETEM that was absent in neutrophils infected with WT-ETEM. This suggests that YopH interferes with the function of this protein.

Next we determined whether this 70 kDa band and/or other bands were detected in neutrophils isolated from mice intravenously infected with either WT-ETEM or *ΔyopH*-ETEM. Five days post-infection, spleens were harvested and translocated neutrophils were isolated and run on a western blot. Again, a 70 kDa phosphorylated protein was observed during infection with *ΔyopH* -ETEM which was absent in the presence of YopH (WT-ETEM) (FIG.3.1 B). No other differences were detected in these blots.

3.3.2 Identification of the 70 kDa band.

Using cell culture models, a number of YopH targets have been identified such as SKAP-HOM, p130Cas, Paxillin (68 KDa), FyB, Slp-76 and Zap-70 [52, 56-57, 60]. Based on the molecular weight of the YopH-dependent tyrosine phosphorylated band found in FIG. 3.1 (~70 kDa), we investigated Paxillin (68 KDa), Slp-76 (76 KDa) and/or Syk (72 KDa) as possible targets of YopH in neutrophils [52, 58, 60]

We first tested whether YopH-dependent dephosphorylation of Paxillin occurred in neutrophils after infection of isolated splenocytes or in mice. We

isolated translocated splenic neutrophils either from infected splenocytes or from neutrophils isolated from spleens day five post infection either with the WT-ETEM or *ΔyopH*-ETEM strains. Using an α-phospho-paxillin (Tyr118), dephosphorylation of paxillin was not detected in either isolated infected splenic neutrophils (FIG.3.2 A) or in splenic neutrophils after after intravenous infection (FIG.3.2 B)

To confirm that we could detect dephosphorylation of Paxillin in epithelial cells by YopH as was previously reported [58], HEp-2 cells were infected either with WT-ETEM or *ΔyopH*-ETEM strains for 1hr. Cells were then lysed with sample buffer and proteins were separated by SDS-PAGE, transfered to a membrane and probed with antibody to either phospho-Paxillin, total Paxillin or Rho-GDI (as a loading control). Paxillin was indeed dephosphorylated in a YopH dependent manner in epithelial cells grow in tissue culture, since we observed a decrease in phosphorylation in cells infected with WT when compared to cells infected with *ΔyopH* strain (FIG.3.2 C). Combine these data suggest that even though the effect of YopH in tissue culture cells is evident, YopH does not target one of the key phosphorylated residues of Paxillin in isolated splenocytes or during mouse infection.

Another potential target, Slp-76 plays a vital role in signaling through Fc receptor, integrin and C-Type Lectin-Like Receptors (C-TLR) in neutrophils. Mice were infected with WT-ETEM or *ΔyopH*-ETEM to determine whether Slp-76 was dephosphorylated in a YopH dependent manner during murine infection (FIG.3.2 D). Day five post infection the level of phospho -Slp76 in neutrophils translocated

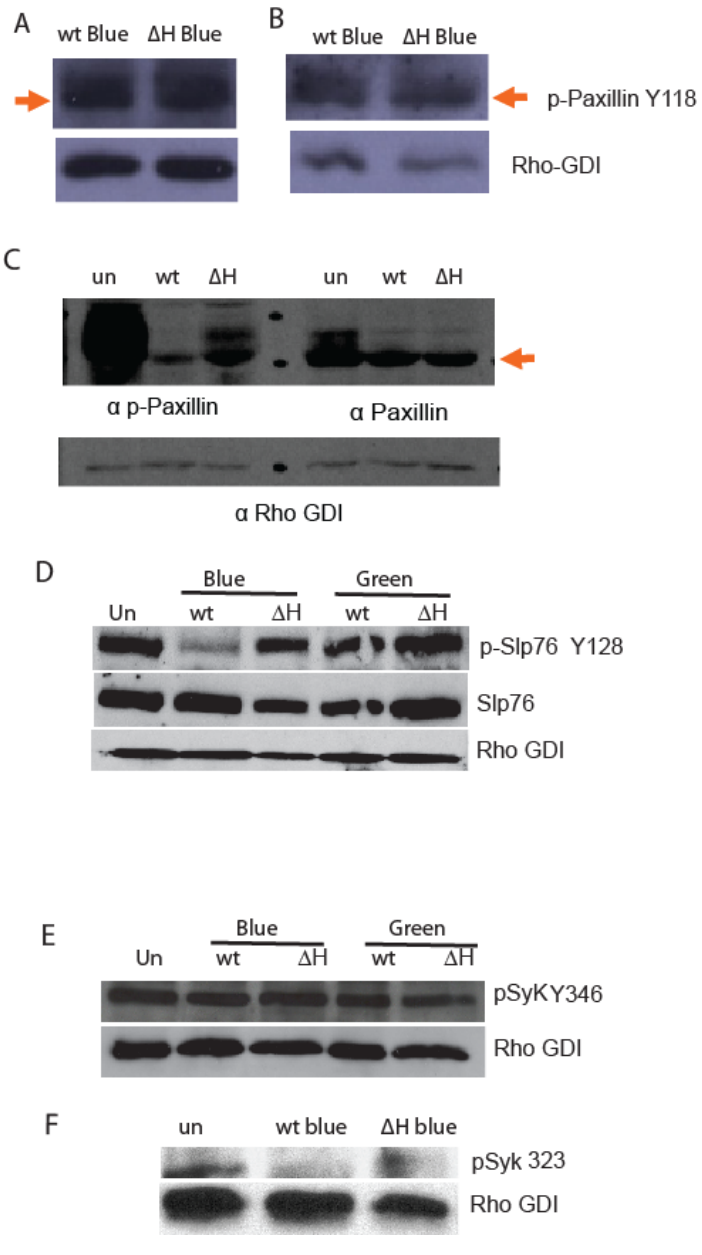

A
wt Blue
ΔH Blue
B
wt Blue
ΔH Blue
p-Paxillin Y118
Rho-GDI
C
un
wt
ΔH
un
wt
ΔH
α p-Paxillin
α Paxillin
α Rho GDI
D
Blue
Green
Un
wt
ΔH
wt
ΔH
p-Slp76 Y128
Slp76
Rho GDI
E
Blue
Green
Un
wt
ΔH
wt
ΔH
pSyKY346
Rho GDI
F
un
wt blue
ΔH blue
pSyk 323
Rho GDI

Fig. 3.2 Molecular targets of YopH during animal infection.

(A) Isolated splenocytes were infected either with WT-ETEM or *ΔyopH*-ETEM strains. Infected splenocytes were labeled with CCF2-AM and prepared for flow cytometry to isolate neutrophils labeled with PE-Cy5-$Gr1^+$ and PE-Cy7-$CD11b^+$ antibodies. Neutrophil were lysed, transferred to a membrane and probed against phospho-Paxillin and Rho-GDI antibodies. **(B)** Blue neutrophils from infected mice with WT or *ΔyopH* were sorted, lysed and probed for phospho- Paxillin .**(C)** HEp-2 cells were infected either with WT-ETEM or *ΔyopH*-ETEM. Cells were lysed with sample buffer and proteins were separated by SDS_PAGE, transfer to a membrane and probed with α-phospho-Paxillin, total Paxillin and Rho-GDI as a loading control. Spleens from infected animals with WT-ETM or *ΔyopH*-ETEM were labeled with CCF2-AM and prepared for flow cytometry to isolate neutrophils. Neutrophils from infected mice with WT or *ΔyopH* were sorted, lysed and probed for **(D)** phospho- Slp-76 , **(E-F)** Syk.

with ETEM from mice infected with WT-ETEM was drastically reduced compared to the level in neutrophils translocated with ETEM from the Δ*yopH* strain (ΔH blue) (FIG.3.2 A). This result indicates that the Slp76 pathway is a targeted by YopH during murine infection.

Syk (72 kDa) plays a role in transmitting signals from a variety of cell surface receptors including Fc receptor, integrins and C-TLR receptor in neutrophils to Slp-76. Zap-70, a homolog of Syk is a target of YopH in T cells [60]. We investigated whether dephosphorylation of Slp-76 by YopH due to dephosphorylation of Syk. To test this, mice were infected with WT-ETEM or *ΔyopH*-ETEM. Five day post infection, spleens were harvested, treated with CCF2-AM and blue and green neutrophils were sorted and lysed and the lysate were analyzed by Western blot. The presence of phospho-Syk at Y346 a site that enhances phosphorylation of downstream effectors and at Y323, a site that negatively regulates receptor signaling in these cells was determined by immunoblotting. The level of phospho-Syk in neutrophils translocated with all the Yops (WT Blue) was comparable to the level of neutrophils translocated with Δ*yopH* strain (ΔH blue) (FIG.3.2 E-F), indicating that Y352 and Y323 residues of Syk are not targets of YopH.

Preliminary data suggests that residue Slp-76 tyrosine 112 is also dephosphorylated in a YopH dependent manner during animal infection (FIG. 3.3)

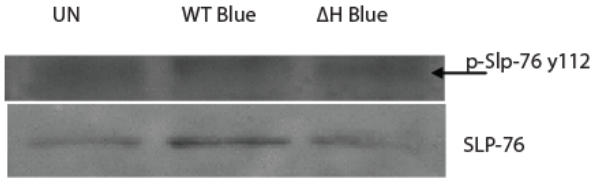


FIG.3.3 Preliminary data indicate that Y112 residue is dephosphorylated by YopH

Isolated splenocytes were infected either with WT-ETEM or *ΔyopH*-ETEM strains. Infected splenocytes were labeled with CCF2-AM and prepared for flow cytometry to isolate neutrophils labeled with PE-Cy5-$Gr1^+$ and PE-Cy7-$CD11b^+$ antibodies. Neutrophil were lysed, transferred to a membrane and probed against phospho-Paxillin and Rho-GDI antibodies. Spleens from infected animals with WT-ETM or *ΔyopH*-ETEM were labeled with CCF2-AM and prepared for flow cytometry to isolate neutrophils. Neutrophils from infected mice with WT or *ΔyopH* were sorted, lysed and probed for phospho- Slp-76 (Y112), and Slp76 antibodies

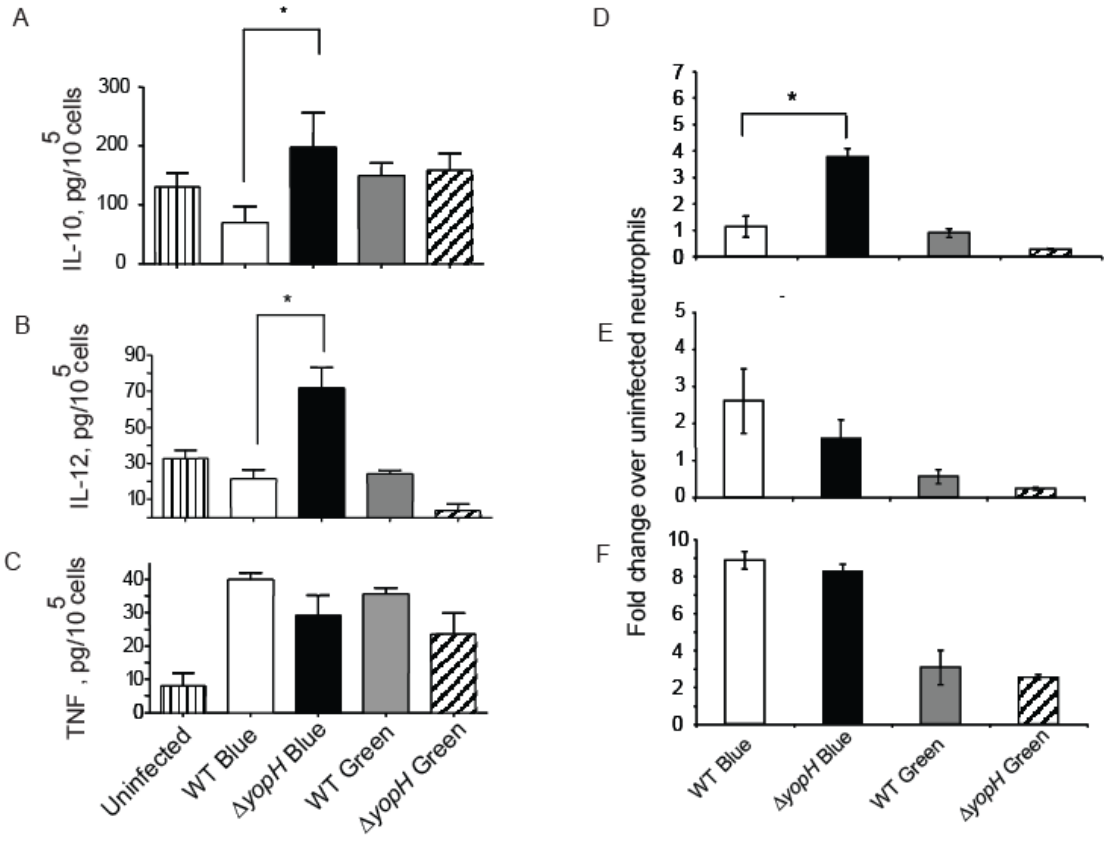

A
B
C
D
E
F
IL-10, pg/10^5 cells
IL-12, pg/10^5 cells
TNF , pg/10^5 cells
Fold change over uninfected neutrophils
Uninfected
WT Blue
ΔyopH Blue
WT Green
ΔyopH Green
*

Fig.3.4 IL-10 and IL-12 production are inhibited by YopH during animal infection.

Mice were infected intravenously either with WT-ETEM or *ΔyopH*-ETEM. Five days p.i, spleens were collected **A-C.** Cytokines levels (A. IL-10, B. IL-12, C.TNF-α) were detected in isolated $Gr1^{+}CD11b^{+}$ cells in the presence brefeldin A by CBA (see material and methods).The bar graphs shown are representative of at least 3 experiments; in each experiment five mice were pooled. **D-F.** Trancripts levels of cytokines (D. IL-10, E. IL-12, F.TNF-α) from neutrophils ($Ly6G^{+}$) of infected animals. The bar graphs shown are representative of at least 3 experiments, each experiment represents an individual mouse. One-way ANOVA for all groups significant, *P < 0.05

3.3.3 IL-10 and Il-12 production are inhibited by YopH during animal infection

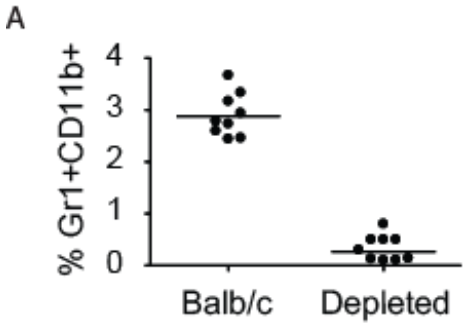
YopH is known to inhibit calcium flux in human neutrophils, thus inhibiting ROS production [53]. In murine macrophages IL-10 production is dependent on ROS production and activation of Syk, an upstream effector of Slp-76 [91]. Additionally, neutrophils that lack Slp-76 after Fc receptor stimulation exhibit a decrease in calcium flux and ROS production [53, 239]. Thus, we hypothesized that YopH targets ROS production and concomitantly blocks IL-10 production in neutrophils during animal infection. Therefore, we tested whether IL-10 levels were altered in translocated neutrophils after murine infection. To investigate this hypothesis, mice were infected intravenously either with WT-ETEM or *ΔyopH*-ETEM. Five days post-infection, spleens were harvested, treated with CCF2-AM and Brefeldin A to prevent cytokine release from all cells. Neutrophils were labeled with GR1-PE Cy5 and CD11b-PE Cy7 antibodies, and blue and green $GR1^+$ $CD11b^+$ populations were collected to analyze for intracellular cytokine levels using BD™ Cytometric Bead Array (CBA) (see material and methods). Strikingly, IL-10 and Il-12 levels were reduced in cells translocated infected with YopH but were increased in neutrophils after infection with *ΔyopH*-ETEM (FIG 3.4 A, B). Since neutrophils exposed to different pathogens secrete Il-10 but not IL-12 [90], we hypothesize that the observed decrease in IL-12 was due to the presence of inflammatory monocytes in the sorted population which are also $GR1^+CDllb^+$ [90].

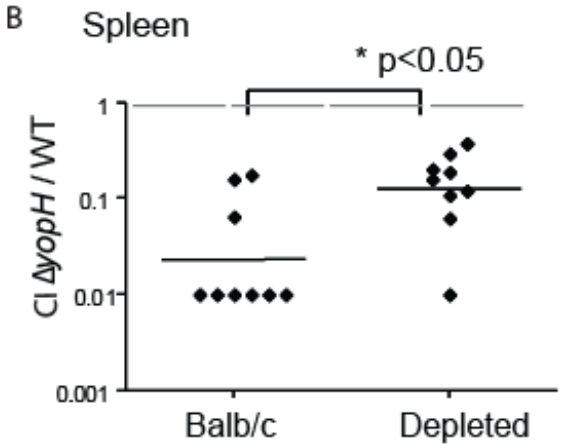
To determine whether IL-10 transcript levels were also low in neutrophils translocated with YopH, we isolated mRNA from translocated (blue) and non-

translocated (green) neutrophils. In these experiments, we used $Ly6G^+$ cell to isolate neutrophils from inflammatory monocytes which are $Ly6G^-$. IL-10 transcripts level correlated with our findings at the protein levels, (FIG.3.4 D). We also observed that IL-12 mRNA levels were not affected by YopH activity in these neutrophils (FIG.3.4 E), supporting the hypothesis that the increase in protein levels in the $Gr1^+CD11b^+$ cells may have reflected levels detected in inflammatory monocytes. The protein and transcript levels of TNF-γ was not affected by the presence of YopH during animal infection (FIG. 3.4 C and F)

3.3.4 Depletion of neutrophils permits growth *ΔyopH* mutant during co-infection with wild type *Yptb* in murine infection.

To determine whether YopH plays a key role in inactivating neutrophils during *Yptb* infection, Balb/c were depleted of neutrophils and injected intravenously with an equal mixture WT-Kan and *ΔyopH* in a competition experiment. Three days post infection, the levels of $Ly6G^+$ cells in the spleen were reduced by 70%-90% as measured by flow cytometry (FIG.3.5 A). As shown in Figure 3.5 B-C, the *ΔyopH* strain grew better in the spleens and livers relative to the WT strain in the neutrophil depleted mice than in control mice. This result indicates that function of YopH is important in neutrophils for bacterial to survive in these organs. The colonization levels measured by total CFU in these organs were similar in both groups of mice (data not shown), suggesting that at this time point, depletion did not lead to more bacterial growth.


A
% Gr1+CD11b+
4
3
2
1
0
Balb/c
Depleted


B
Spleen
* p<0.05
CI ΔyopH / WT
1
0.1
0.01
0.001
Balb/c
Depleted

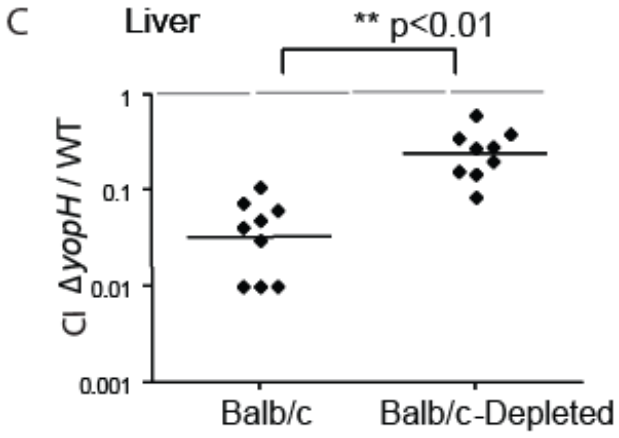

C
Liver
** p<0.01
CI ΔyopH / WT
1
0.1
0.01
0.001
Balb/c
Balb/c-Depleted

Fig.3.5 Colonization of *Yptb* ΔyopH strain in competition WT *Yptb* 5 days post i.v inoculation.

BALB/c mice were infected intravenously with an equal mixture of WT-Kan IP2666 and *ΔyopH* IP2666. A second group of BALB/c mice were injected with the antibody (1A8) 1 day prior to and 2 days after intravenous inoculation of an equal mixture of WT IP2666 and *ΔyopH* IP2666. **A.** Percentage of Gr1+CD11b+ cells in untreated mice and mice treated with Ly6G antibody. Black circles represent the percentage of neutrophils from individual mice. Bacteria from spleens and livers were plated on L plates and replica plated or patched on L plates containing kanamycin to determine the ratio of *ΔyopH* to WT-Kan in the **(B)** spleens and **(C)** livers. The competitive index (C.I.) was calculated as follows: C.I. = (*ΔyopH* / WT-Kan output ratio)/(*ΔyopH* / WT-Kan input ratio). Black diamonds represent the CI from individual mice. Statistically significant differences (*) were determined by unpaired Student *t* test (*$P < 0.05$ or **$P < 0.01$).

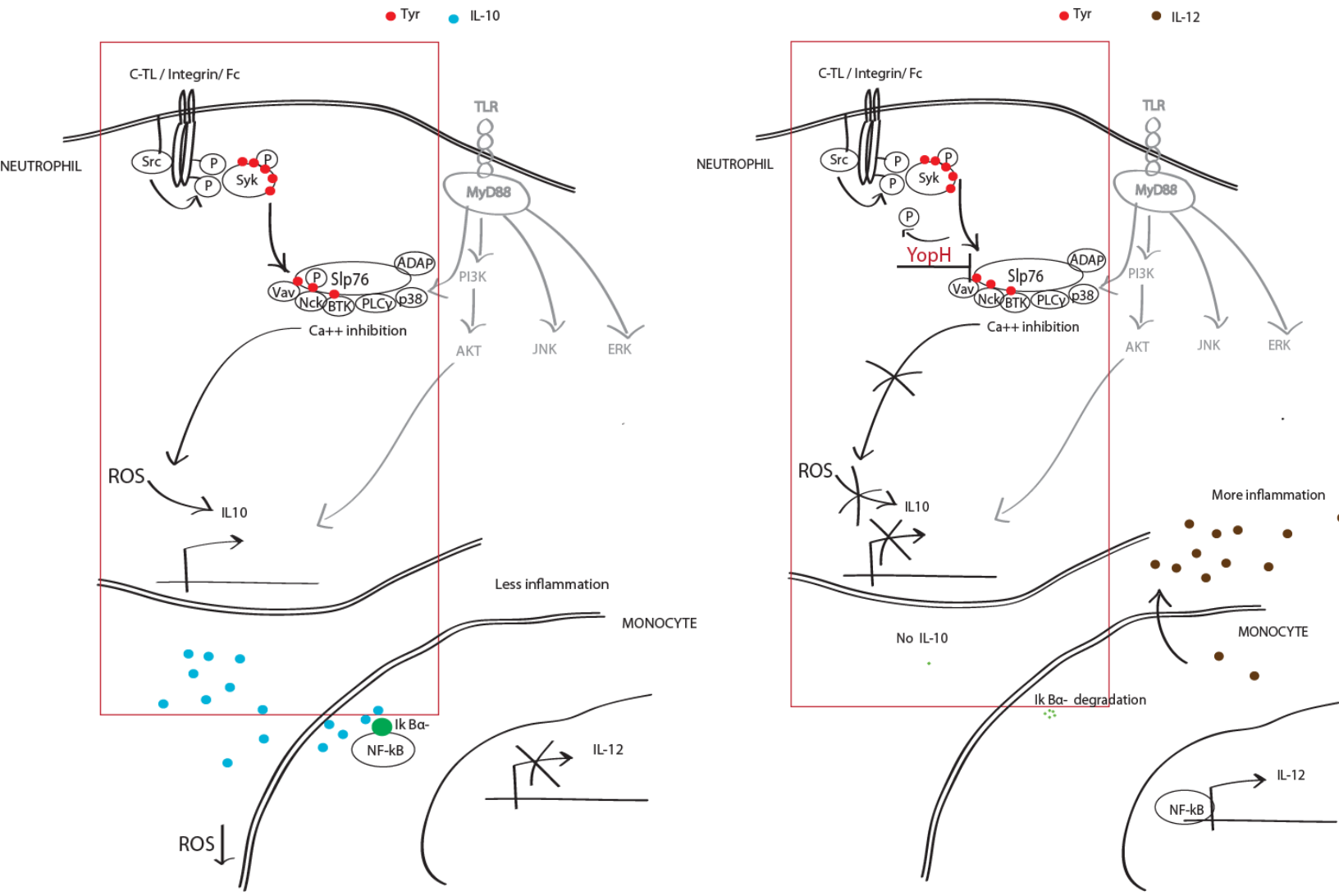


Fig.3.6 Model of YopH action in neutrophils

3.4 Discussion

Until now it has been technically difficult to identify the molecular targets of T3SS bacterial effectors in specific cells during animal infection since there were no good methods for isolating the cells translocated with effectors. Previously, we and others have shown that during murine infection neutrophils are selectively targeted for translocation of Yops [108, 203, 238], suggesting that the function ofYops in neutrophils is critical for successful colonization. Potential functions of neutrophils that YopH could target include phagocytosis, ROS production and/or degranulation by neutrophils. This work identifies a molecular target of a Type III secretion effector in neutrophils during mouse infection. Specifically, the Slp-76 pathway in neutrophils is a pathway that is targeted and inactivated by YopH during animal infection. Slp-76 is known to be important for proper Ca+ mobilization in neutrophils and crucial in production of ROS [89]. Furthermore, in this work we show evidence that supports the idea that YopH targets neutrophils bactericidal functions of ROS production and degranulation because in the absence of neutrophils *Yptb* lacking YopH does better in competition with the wild type strain during animal infection. Since the growth of the *ΔyopH Yptb* strain is only partially compensated the lack of neutrophils, we suggest that YopH has additional functions in other cell types.

What bactericidal functions of neutrophils are inhibited by YopH? Upon engagement of Fc or integrin receptor with bacterial ligands, a signaling phosphorylation cascade occurs, leading to phagocytosis, cell spreading, production of reactive oxygen species (ROS), and secretion of anti-inflamatory

cytokines in neutrophils [89-90]. This signal transduction cascade activates phospholipases, protein kinases and leads to a transient induction of intracellular calcium [53]. In this work, we identify a signal transduction pathway that is targeted and inactivated by YopH. Specifically, we show YopH dependent dephosphorylation of Slp76 during animal infection. We tested an upstream effector of Slp-76, Syk but we did not detect an effect of YopH on Syk suggesting that YopH directly targets Slp-76. However, there are several caveats to this conclusion. First we tested two Syk tyrosine phosphorylated residues, Y346, known for enhancing phosphorylation and activation of phospholipase C-gamma [240] and, Y323, a negative regulatory phosphorylation site within the SH2-kinase linker region in Syk Y346 [101]. There are other Syk tyrosine phosphorylation sites that need to be tested that are also required for phosphorylation of SLP-76 to before concluding that Syk is not a target of YopH. For example, there are two other conserved tyrosines besides Y346 in the linker region of both Syk (Y317, Y342) and ZAP70. Interestingly, it has beeen shown that Syk Y342 phosphorylation is required in FcεRI-mediated signaling in mast cells. The data showed that a Y342F mutant was considerably impaired in Fc receptor-induced phosphorylation of several signaling molecules, including SLP-76 leading to a defect in histamine release [241].

Neutrophil signaling downstream of integrin and Fc receptors is a field of active investigation; however, it is well established that proper signaling is dependent on Slp-76 function [89]. Once those receptors are engaged outside – in signaling is triggered leading to fully integrin activation facilitating cell

spreading, calcium flux, ROS production and degranulation [242]. Those functions are greatly impaired in neutrophils that lack Slp-76 [89]. Since YopH dephosphorylates Slp-76, we would expect downstream functions are impaired. In fact, YopH distinctively blocks an immediate-early Ca2+ signal in human neutrophils which consequently affects ROS production [53]. Previously, our lab has shown the importance of YopH function since a strain lacking YopH is attenuated during animal infection [24].

In T cells the role of Slp-76 has been extensively studied [83]. Slp76 plays an important role for signaling downstream of immunoreceptors and integrin. It has been shown that phosphorylation of LAT recruits SLP-76 to the plasma membrane, where the two adaptors recruit a complex that contains numerous signaling proteins that includes PLCgamma1, VAV, NCK, ITK, ADAP and HPK1 building a key signaling complex[83]. Interestingly, our lab has evidence that nucleation of Slp-76 complex is disrupted by YopH in Jurkat cells leading to failure to spread on CD3 coated slides (unpublished results), suggesting that misslocalization of the Slp-76 pathway components caused by YopH could lead also to a faulty signaling in neutrophils.

Mechanistically, we hypothesize that dephosphorylation of tyrosine residues 112 and 128 of Slp-76 by YopH, critical residues for activation of downstream effectors Vav, NCK and PLCγ in T cells [243] disrupts signaling that leads to inhibition of degranulation and a reduction of ROS production in neutrophils during animal infection. In fact, defects in SLP-76$^{-/-}$ neutrophil

functions are associated with defects in activation of downstream effectors of Slp-76, such as Vav, PLCγ2, and p38. Interestingly, in SLP-76$^{-/-}$ neutrophils activation of Syk is comparable to that observed in wild-type neutrophils [89]. These data are consistent with the known role of SLP-76 downstream of Zap-70 in T cells [244] and suggest a critical role of SLP-76 as a scaffold protein that nucleates many critical components for successful signaling in neutrophils. We need to investigate the effect of YopH on these downstream effectors during animal infection in neutrophils.

Additionally, we show that IL-10 production is inhibited in neutrophils translocated with YopH during infection. Recently, a group showed that zymosan treated macrophages generated ROS which in turn stimulated secretion of IL-10 and these was dependent on Syk activation [91]. While the mechanism by which ROS activate production of the anti-inflammatory IL-10 remains to be clarified, we speculate that neutrophil secreted IL-10 will inhibit production of free radicals [245] and diminish local inflammatory damage by other cells. In fact, there are data in macrophages that supports these hypotheses, suggesting that addition of IL-10 inhibits IκB-α degradation. As a result of this inhibition, IL10 suppresses NF-κB activation and inhibits ROS production in response to LPS stimulation [245]. Thus, suppression of NF-κB activity by secreted IL-10 from neutrophils is a plausible mechanism for regulating inflammatory responses in other cell types. During *Yptb* infection, we showed that YopH suppresses IL-10 production in neutrophils. Furthermore, IL-10 production had a profound effect in inhibiting IL-12, a cytokine regulated by NFκB, presumably in monocytes that were not

intoxicated with Yops, suggesting direct and indirect effects of YopH on cytokine production during animal infection. See model FIG. 3.6

In summary, we demonstrate that YopH function in neutrophils is critical for survival of *Yptb* and YopH targets a Slp-76 signal-transduction pathway. Inactivation of this pathway disrupts essential function such as calcium flux, ROS production and the production of anti-inflammatory cytokine IL-10, allowing *Yersinia* successfully to establish an animal infection.

3.5 Experimental Procedures

3.5.1 CCF2-AM conversion assays after murine infections and neutrophil sorting

6- to 8-week-old BALB/c mice (NCI) were challenged intravenously with 500 cfu of WT-ETEM and 2000 cfu of *ΔyopH*-ETEM unless otherwise indicated. Infections were allowed to proceed for 5 days, except in the experiments involving neutrophil depletion, where infections were stopped after 3 days. We followed the standard protocol previously published to generate a single-cell suspension from spleen and to label splenocytes with CCF2-AM and fluorescently antibodies. GR-1-PE-Cy5 (eBiocience15-5931-81), CD11b-PE-Cy7 (eBiocience 25-5931-81) or Ly6-G (BD Pharmingen 560601) antibodies were used at dilutions of 1:75 for 30 min at 4℃. Cells wer e sorted in the MoFLo FACS sorter (Cytomation, Fort Collins, CO). At least 1×10^5 blue^{hi}-neutrophils, and

1×10^5 green^{hi}-neutrophils cells were collected from mice infected with wild type or *ΔyopH*. Uninfected green^{hi}-neutrophils cells were also collected as controls.

3.5.2 CCF2-AM conversion assays in splenocyte suspensions

Isolation and infection of splenocytes as well labeling with CCF2-AM and preparation for sorting with antibodies against neutrophils were done as previously described by Durand *et al.*2010

3.5.3 Antibodies used in Western blot

Anti-Phosphotyrosine, 4G10 Platinum (Millipore *Cat.* #05-1050), Phospho-Paxillin (Tyr118) antibody (Cell Signaling Cat. #2541), Paxillin antibody (Cell Signaling Cat #2542), SLP-76 Antibody(Cell Signaling Cat ##4958) phospho-SLP-76 (pY113 pY128) antibodies (BD Pharmingen Cat #558367 and 558388), Phospho-Zap-70(Tyr319)/ Syk(Tyr352) antibody(Cell Signaling Cat. #2701), Phospho-Syk (Tyr323) Antibody (Cell Signaling Cat. #2715), Rho GDI (A-20) (Santa cruz Cat. # sc-360)

3.5.4 Cytokine detection

6- to 8-week-old BALB/c mice were infected intravenously either with WT-ETEM or *ΔyopH*-ETEM. Five days p.i, spleens were collected and Gr1$^+$CD11b$^+$ cells were isolated as previously described [238] with a following modification: cell isolation was performed in the presence of brefeldin A (3.0 ug/ml) to enhance detection of mouse intracellular cytokines. Gr1$^+$CD11b$^+$ were lysed with a eukaryotic lysis buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40) in the presence of protease inhibitors (Sigma P8340) and the tyrosine

phosphatase inhibitor, sodium orthovanadate (Na_3VO_4) (Sigma S6508) at 100µM final concentration and the inhibitor of serine/threonine, sodium fluoride (NaF) at 20mM final concentration (Sigma S7920) for 30 minutes at 4^oC. Supernatants were collected and cytokine levels were analyzed by BD™ Cytometric Bead Array (CBA) following manufacturer's instructions (BD Cat. No.552364). Cytokines levels were analyzed using a FACS LSR II using BD DIVA software (BD Bioscience).

3.5.5 Real Time PCR

RNA was isolated using Tri-Reagent (Molecular Research Centre, Inc. Cincinnati, OH) following the manufacturer instructions. Briefly, samples were homogenized in 1 ml of Tri-Reagent and incubated for 5 min at RT. RNA was extracted by adding 0.2 ml of chloroform and centrifuging the samples at 10,000 rpm for 15 min at 4 ^oC. RNA was precipitated with 0.5 ml of isopropanol and resuspended in H_2O.

One µg of RNA was transcribed to cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Branchburg, NJ). The RNA was mixed with 5 µl of 10X buffer, 10 µl of 20 mM $MgCl_2$, 10 µl of dNTPs, 1 µl of random hexamers, 1 µl of reverse transcriptase and H_2O to a final volume of 50 µl. The PCR profile used to transcribe the samples required an incubation step performed 25 ^oC for 10 min, followed by the reverse transcription step done at 48^oC for 30 min, and an inactivation step performed at 95 ^oC for 5 min.

Reverse-transcribed cDNA was amplified with published primer sets for mouse IL-10 (**Fw** GGT TGC CAA GCC TTA TCG GA) (**Rv** ACC TGC TCC ACT

GCC TTG CT), IL-12p35 (**Fw** CTT AGC CAG TCC CGA AAC CT) (**Rv** TTG GTC CCG TGT GAT GTC T) , TNF-a (**Fw** CAT CTT CTC AAA ATT CGA GTG ACA A) (**Rv** TGG GAG TAG ACA AGG TAC AAC CC) and GAPDH(Fw TGT AGA CCA TGT AGT TGA GGT CA) (**Rv** AGG TCG GTG TGA ACG GAT TTG) using SYBR Green PCR master mix (Applied Biosystems) and an ABI PRISM 7900HT detection system (Applied Biosystems) following the manufacturer's instructions. Fold induction of mRNA was determined from the threshold cycle (C_t) values normalized for GAPDH expression and then normalized to the value derived from the naive controls. mRNA transcripts levels were compared by one-way ANOVA followed by Tukey's multiple comparison test of the means.

3.5.6 Granulocyte depletion and competition experiments

Granulocyte depletion experiments were performed as described (Logsdon and Mecsas, 2006 [238]) with the following modifications. Mice were intraperitoneally injected with 50 µg of Ly6G antibody (1A8) one day prior to and two days after intravenous inoculation with equal mixture of IP2666 WT-Kan resistant or IP2666 *ΔyopH*- Kan sensitive (~1000 total bacteria) [49]. Day 3 post infection spleens and livers from control and depleted mice were harvested and single-cell suspensions were generated. Serial dilutions were plated on L plates (50 µg ml^{-1}) to determine the bacterial burden in the tissues. Colonies were patched or replica plated onto L-plates and kanamycin plates to determine the ratio of *ΔyopH*(Kan^s) to wild-type (Kan^r) bacteria in each tissue (output). Data for the competition experiments are expressed as a competitive index (C.I.) as follows: C.I. = (mutant/wild-type output ratio)/(mutant/wild-type input ratio)

4. Chapter IV: Future Directions

This work is the first to have examined what are the cell types in various tissues to be targeted by Yops after an oral infection with *Yersinia*. We show that during infection *Yptb* selectively targets Yops to professional phagocytes in Peyer's patches, mesenteric lymph nodes and spleen. We have investigated different hypotheses in Chapter II, such as whether the location of *Yptb* with specific cells types or the expression of bacterial adhesions account for the specificity in translocation into the mammalian cell. I discovered that *Yptb* recognizes specific cell types and therefore the co-localization of Yptb with a specific cell type was not responsible for translocation to specific cell types. However, molecular basis for the distinct cell tropism for translocation remains unclear.

In addition to understanding the molecular basis for the cell tropism, there are other questions that this work raised and should be addressed. For example, what is the fate of bacteria that are surrounded by neutrophils? There are several possible scenarios. One very speculative idea is that the bacteria associated with neutrophils may have died due to killing properties of neutrophils yet they could have facilitated infection by inactivating normal signaling mechanisms from these cells to other cells in the immune system. Alternatively, some of these bacteria may successful resist neutrophils killing properties and are able to survive and divide. We would like to investigate whether *Yptb* resistant to reactive oxygen species or granule killing are able to create a niche and multiply in areas of neutrophil influx. We speculate that neutrophils could

secrete factors that allow *Yptb* to replicate once *Yptb* has inhibited ROS production, cytokine secretion, granule killing and phagocytosis through Yops activities. These are very challenging experiments to perform during a mouse infection, and perhaps the best method would be do perform live in vivo imaging of an infection and track the movement and fate of both neutrophils and bacteria.

Finally, since bacteria are also associated with areas of B and T cells and we showed that these cell types are not preferentially translocated with Yops in mesenteric lymph nodes, we would like to investigate whether the T3SS is active or inactive when bacteria are associated with certain cell types in different tissues during animal infection. To do so we suggest using a YopE antibody to detect the presence of translocated Yops into cells located in areas of neutrophil influx or in B and T cell areas using immunofluorescence microscopy. Alternatively, laser capture microdissection would allow us to determine the levels of expression of the TTSS system in different microenvironments.

Below a list of experiments that we propose that will help to understand *Yptb* tropism for certain cell types and to unravel in more details the molecular mechanisms of YopH action.

Co-localization of *Yptb* with eukaryotic cells by immunofluorescence

We did a relatively crude analysis of the cell types within mammalian tissues co-localized with *Yptb*. A more exhaustive analysis should be done. In the future, I suggest the use of immunofluorescence to perform colocalization studies of *Yptb* with the target mammalian cells. One could use antibodies to stain for

certain cell types of the infected tissue and look for co-localization with bacteria expressing a fluorescent protein. A YopE antibody could be use to assess for T3SS functionality when surrounded by neutrophils or localized with B and T cell areas. We expect that neutrophils that are in contact with bacteria should have YopE internalized. In contrast, B and T cells that are in close proximity with bacteria should not have YopE translocated, suggesting that the T3SS is either inactive or not present when bacteria are associated with lymphocytes.

Identify other *Yptb* adhesins required for translocation

Several bacterial proteins, including Invasin and YadA, mediate *Yptb* binding and Yop delivery to host cells. We analyzed the contribution of these two adhesins in binding and delivering of Yops into certain cell types during animal infection (Chapter II). However, other adhesins such as Ail and pH 6 antigen (Psa), might contribute delivering Yops into professional phagocytes. These adhesins have been implicated in playing a role in Yop delivery [1] and it is clear that bacterial-encoded adhesins are important for infection in animals [2-4] However, whether adhesins and/or host-cell opsonins and their receptors participate in host cell binding and Yop delivery during animal infection still remains unclear.

Determine which specific cell receptors are critical for preferential Yops translocation in to phagocytes. There are several strategies that we can use

to determine which cell receptors are critical for preferential delivery of Yops into host cells.

a) Using knockout mice for specific receptor on professional phagocytes

When incubated in a mixed population of cell types from spleens, *Yptb* binds preferentially to macrophages, dendritic cells and neutrophils (Chapter II), suggesting that *Yptb* binds to receptors found predominantly on professional phagocytes leading to a preferential targeting of Yops to these cells. To determine receptors that are critical for binding to *Yptb*, knockout mice lacking particular receptors found specifically on phagocytes will be tested to determine whether Yops are translocated into these cells during infection. Examples of such receptors are complement receptor 3, Fc receptors and toll-like receptors (TLRs)

b) Genome-wide RNAi screen for host factors required for Yop translocation

Furthermore, we suggest identifying host factors required for Yop translocation using a high-throughput RNAi screen. To determine which eukaryotic factors are necessary for Yop translocation we will test the ability of a phagocytic cell line carrying a dsRNAs to translocate WT-HTEM. The siRNA library will target a wide range of cellular functions. Possible cellular hits will be those cells that give us a low blue vs green intensity ratio measured by CCF2-AM cleavage. After the primary screen, we suggest focusing on eukaryotic factors that affect translocation and are found on the plasma membrane.

Dissecting the subpopulations translocated with Yops during animal infection

Further investigation into the subset of dendritic, macrophages, B and T cells may show that the differences are due to subsets of cells found in each tissue. For example, the ability of a specific subset of cells to chemotax towards bacteria in different tissues may explain to some extent, both the tropism for professional phagocytes, and the differences in cells targeted in different tissues. Some studies analyzing subpopulations of targeted populations have been done in *Y. enterocolitica* and have specifically looked at the population of B cells that are translocated with Yops. Köberle and colleagues found that Yop injection was predominantly evident in follicular B cells and to a lesser extent in newly formed B cells (NFB) [5].

Determining whether other residues of Syk are targeted by *Yptb*

We tested two Syk tyrosine phosphorylated residues, Y346, and, Y323. In addition, there are other Syk tyrosine phosphorylation sites that need to be tested that are also required for phosphorylation of SLP-76. For instance, Syk Y342 phosphorylation has been shown to be required in FcεRI-mediated signaling in mast cells. The data showed that phosphorylation of Y342F mutant followed by Fc receptor stimulation, induced (of) several signaling molecules, including SLP-76 leading to a defect in histamine release [6].

Determining whether known downstream effectors of Slp-76 are targeted by *Yptb*

Newbrough and colleagues showed a rapid tyrosine phosphorylation of Vav in wild-type neutrophils following integrin ligation. This signal is distinctly decreased in SLP-76$^{-/-}$ neutrophils. The same was observed for PLCγ2 and p38 MAP kinase phosphorylation [7]. The opposite is seen in the case of Syk kinase [7]. Activation of Syk kinase in SLP-76$^{-/-}$ neutrophils was equal when compared to wild-type neutrophils, suggesting that Vav, PLCγ2 and p38 are downstream of Slp-76 and Syk is upstream. Using the same approach as suggested in Chapter III for the isolation of neutrophils, we suggest assessing the phosphorylation state of these proteins in the presense or absence of YopH during animal infection.

Determining the effect of YopH on NADPH oxidase complex and Slp-76 during animal infection

Our results show that YopH competes better with wild-type *Yptb* in neutropenic mice. YopH has been shown to inhibit calcium flux and oxidative burst in neutrophils. (YopH) This effector also interferes with NADPH oxidase assembly, but its effect disappears when the complex is already formed [8]. Furthermore, Slp-76 is necessary for activation of Vav, a Rac1 GEF. Rac is critical for NADPH oxidase complex formation and subsequently production of ROS. We speculate that YopH decreases ROS by targeting Slp76->Vav->Rac->NADPH->ROS. If indeed YopH targets this pathway, we speculate that YopH

mutant will do better in mice lacking gpphox91-/-, a protein necessary for NADPH oxidase assembly, during animal infection. We speculate that *Yersinia* will colonize better if we infect a Slp-76 knockout mouse (a gift/ in collaboration with from Koretzky) with a YopH mutant of *Yptb* compared to a colonization in a WT mouse. A positive result will suggest that targeting Slp-76 function by *Yptb* is important for colonization during animal infection. In fact, Tena Rolan, a member of our lab is testing which tyrosine residues, Tyr 113, Tyr 128 and Tyr 145, are critical for YopH targeting using mice that have mutation in these residues (gift of Koretzky)

Effect of cytokines in Yop translocation

We are interested in studying whether the presence of certain cytokines affects Yop injection into phagocytic cell lines or whole splenocytes. Specifically, we hypothesize that the activation state of a professional phagocyte may alter its ability to be targeted for Yop translocation. To do so I suggest incubating cells with different cytokines (e.g., Il-12, Il-10 and TNF-α) and infecting cells with WT HTEM *Yptb* in order to determine the differences in translocation using the CCF2-AM –β lactamase system.

Determining YopH inhibition of calcium flux and the Fc receptor-mediated oxidative burst

Andersson and colleagues investigated the effect of *Yptb* on Ca^{2+} flux in neutrophils. They found that *Yptb*, via YopH, distinctively inhibits a Ca^{2+} signal in human neutrophils [9]. We would like to repeat this result in murine neutrophils. The tyrosine phosphatase activity of YopH was also necessary for inhibition of the Fc Receptor-Mediated Oxidative Burst in Macrophages. I suggest testing YopH inhibition of oxidative burst in isolated neutrophils followed by stimulation of Fc receptors.

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