The presence of professional phagocytes dictates the number of host cells targeted for Yop translocation during infection

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Summary

Type III secretion systems deliver effector proteins from Gram-negative bacterial pathogens into host cells, where they disarm host defences, 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 colocalizes 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 cell 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.

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 (Gophna et al., 2003). Once inside host cells, effector proteins modulate cellular functions, thus enabling the bacteria to inactivate host defences and establish replication niches (Mattoo et al., 2007). 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 (Gemski et al., 1980; Portnoy and Falkow, 1981). These highly homologous secretion systems deliver at least six effector proteins, called Yops, into cells and are required to cause significant disease in humans and other mammals (Cornelis, 2002). 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 (Pepe and Miller, 1993; Logsdon and Mecsas, 2006; Felek and Krukonis, 2009). During the initial steps of infection, Yersinia are intracellular as the enteric pathogens pass through M cells lining the intestinal wall (Marra and Isberg, 1997; Clark et al., 1998) and Y. pestis initially resides in macrophages after subcutaneous inoculation (Meyer, 1950). However, in general, the vast majority of yersiniae is found extracellularly during tissue infection (Simonet et al., 1990; Balada-Llasat and Mecsas, 2006; Bergman et al., 2009). 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 (Sory et al., 1995; Yao et al., 1999; Grosdent et al., 2002; Davis and Mecsas, 2007; Brodsky and Medzhitov, 2008).

Yop delivery requires that yersiniae bind to mammalian cells (Grosdent et al., 2002; Mejia et al., 2008). Several bacterial proteins, including Invasin and YadA, mediate Yptb binding and Yop delivery to host cells (Mejia et al., 2008). These two adhesins are not expressed in Y. pestis (Parkhill et al., 2001). Invasin binds to β1-containing...
integrins, causing activation of Src kinases, which in turn enhances Yop translocation into cells (Mejia et al., 2008). 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 (Tertti et al., 1992). In addition, opsonization of Yersinia by complement or antibody is sufficient to permit binding to macrophages and neutrophils and Yop delivery (Fallman et al., 1995; Grosdent et al., 2002). It is clear that bacterial-encoded adhesins are important for infection in animals (Pepe and Miller, 1993; Heise and Dersch, 2006); 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 (Brakebusch and Fassler, 2005) 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 (Sory et al., 1995; Yao et al., 1999; Grosdent et al., 2002; Davis and Mecsas, 2007; Brodsky and Medzhitov, 2008) and each Yop is important for tissue colonization and/or full virulence in mouse infections (Leung et al., 1990; Monack et al., 1998; Logsdon and Mecsas, 2003). 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 defences. Cells with translocated Yops after intravenous infection with Y. pestis or Ye were identified, using fusions of the N-terminus of Yops with TEM (Marketon et al., 2005; Koberle et al., 2009). TEM is a truncated version of β-lactamase, which has enzymatic activity that can be detected by cleavage of a fluorescence substrate (Zlokarnik et al., 1998). Y. pestis and Ye delivered Yops to macrophages, neutrophils and dendritic cells in the spleen; in addition, Ye also delivered Yops to B cells (Marketon et al., 2005; Koberle et al., 2009). 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 colocalizing 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 colocalization with Yptb during infection because phagocyte-specific target-
α-C11c (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- to 12-fold in the PP, MLN and spleen compared with their levels in uninfected tissues (Table 1), while the level of B, T and dendritic cells remained relatively constant (Table 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. 1A and Table S1). 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 is retained only in live cells (Marketon et al., 2005). 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. 1B, D and 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. 1C, E and G, left bars) was compared with the percentage of that cell type in the HTEM-containing (blue cells) population (Fig. 1C, E and 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 with 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. 1C, E and G). In contrast to translocation of professional phagocytes, HTEM-positive B cells were significantly under-represented in the PP and MLN and HTEM-positive CD4+T cells were significantly under-represented in the PP (Fig. 1C and 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 a multiplicity of infection (moi) of 20 for 1, 2, 4 or 18 h, 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 and 4 h indicating that Yptb did not kill any specific cell types during this time period (Fig. S3). As expected (Bergsbaken and Cookson, 2007), 60–70% of the macrophages and neutrophils were dying 18 h after infection. This data support 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 (Bergsbaken and Cookson, 2007), our observed enrichment of targeting of macrophages could be an underestimate of the actual amount of Yptb targeting.

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 with HTEM. Mice were infected orally with WT-HTEM and 5 days post infection, spleens were harvested and filtered to generate a single-cell suspension. Splenocytes were incubated with CCF2-AM and labelled

|                  | Log cfu | PMNs | Macrophage | Dendritic cells | B cells | T helper | Cytotoxic T cells |
|------------------|---------|------|------------|-----------------|---------|----------|------------------|
| 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    | 18.00⁰ | 12.96⁰ |
| 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 ± 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 four 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 one experiment.

PMNs, polymorphonuclears.

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with α−GR1. Three populations of GR1+ cells, blueneg, bluelo and bluehi, were gated based on their levels of blue and green fluorescence and sorted by flow cytometry (Fig. 2A). 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. S4 and Davis and Mecsas, 2007). 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^+^ and blue^−^ cell populations. Since neutrophils, macrophages and dendritic cells were significantly overrepresented in the blue^+^ population (Fig. 1C, E and G), the blue^−^ cell population was analysed to determine whether these cells were similarly enriched. Neutrophils, macrophages and dendritic cells were also overrepresented in the blue^−^ population, while B and T cells remained under-represented (Fig. S5). 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 (Balada-Llasat and Mecsas, 2006).

Yptb colocalizes 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 colocalization 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 haematoxylin. 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 centres and areas of inflammatory was determined (Fig. 3). Histological analysis of the PP indicates that the majority (over 60%) of Yptb microcolonies were found in areas of inflammation (Fig. 3A–D and 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. 1B and C). Likewise in the MLN, a majority of Yptb microcolonies were found either in areas of inflammation or boundary zones between germinal centres and areas of inflammation (Fig. 3E–G and P); however, 30% of the microcolonies were found in B and T cell-rich areas. In contrast, almost 90% of the microcolonies in the spleen were found in areas of inflammation and the boundary zones adjacent to areas of inflammation. Thus, 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. 1) despite their close proximity to Yptb (Fig. 3) suggests that Yptb was less proficient in translocating Yops into B and T cells than into professional phagocytes in tissue infection.

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-HETEM and 2 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 with the levels in mice treated with the isotype control antibody as ascertained by flow cytometry (Fig. 4A–C). Strikingly, the percentage of total cells containing HTEM was also reduced by 80–90% in neutropenic mice compared with mice receiving the isotype control antibody (Fig. 4D). The colonization levels were similar in both cohorts of mice (Fig. 4D), 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 analysed 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 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 ΔyopE mutant fails to recruit neutrophils to the PP at day 5 post infection despite colonizing PP at levels equivalent to WT bacteria (Logsdon and Mecsas, 2006) (Fig. 4F). This experiment allows us another means to functionally reduce neutrophil levels in Yptb-infected tissues. YPIII WT-HETEM or a Yptb strain expressing a catalytically inactive mutant of YopE and HTEM, YPIII yopER1444A-HETEM, 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. 4E and F). Mice infected with YPIII yopER1444A-HETEM 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.

Fig. 3. Localization of Yptb in the PP, MLN and spleen. A–N. Immunohistochemical analysis of (A–D) PP, (E–G) MLN and (H–N) spleens after 5 days of infection with 2 × 10^9 cfu WT-HTEM. Sections were stained with α-Yptb and haematoxylin 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 (10×); (B–C, F–G, I–J, L–M) areas of inflammation in infected tissues (10× and 60×); (C, G arrow 3, M) colonies detected in areas of infiltration (60×); (D, G arrow 1, N) colonies detected in B and T cell areas (60×); (G arrow 2, L) colonies detected at boundary zone (60×).

O–Q. Quantification of microcolonies in areas of inflammation, B and T cell zones, and boundary zones in (O) PP, (P) MLN and (Q) spleens from five mice; one to two sections per mouse were analysed and scored by three independent viewers.

Professional phagocytes in spleen cell suspensions are preferentially translocated by Yptb at low moi

We next analysed whether the tissue microenvironment played a role in target specificity or whether there are inherent properties of professional phagocytes that...
Neutrophil depletion and suppression of inflammation reduces the total amount of translocated Yops.

A–D. Mice were injected intraperitoneally with an isotype control (α-IgG2b) 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. Per cent 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. Per cent 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; *P < 0.01).

D. Bacterial colonization in PP of the isotope control or RB6-8C5-treated mice shown as cfu per gram of 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 and F. Mice were intragastrically infected with 2 ¥ 10^9 YPIII-HTEM or YPIIIfopER144A-HTEM and the number of blue+ and GR1+ cells were quantified by flow cytometry. Five 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 YPIIIfopER144A (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 YPIIIfopER144A-HTEM shown as cfu per gram of PP. There was no statistical difference in bacterial load by an unpaired, two-tailed, t-test, P = 0.59.
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), labelled with different antibodies, and then analysed 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. 5A), 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 (Marketon et al., 2005). Furthermore, no translocation specificity was observed as all cells were susceptible to translocation (Fig. 5B). 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 with the total amount of these cell types in the spleen (Fig. 5C and 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. 1C, E and 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 colocalization during tissue infection is insufficient for cell type-specific targeting.

Yptb binds preferentially to neutrophils and macrophages using YadA

Translocation of Yops is dependant upon Yptb binding to cells (Mejia et al., 2008). 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 labelled with different fluorescent antibodies to identify the cell types associated with GFP–Yptb by flow imaging and flow cytometry (Fig. 6A and 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...
Fig. 6. Yptb binds preferentially to professional phagocytes cells at low moi.
A. A single-cell suspension of splenocytes was treated with 2 μM cytochalasin D and then infected with WT IP2666 expressing GFP at an moi of 1:1 for 1 h. Splenocytes were labelled with antibodies to different cell markers and the binding of Yptb to specific cell types was analysed by ImageStream. The experiment was performed 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 cytochalasin D (white bars) or left untreated (black bars) and then infected with WT IP2666 expressing GFP at an moi of 1:1 for 1 h. Splenocytes were labelled with antibodies to different cell markers and the binding of Yptb to specific cell types was analysed by flow cytometry. The experiment was repeated three 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 and D. Cells were treated with 2 μM cytochalasin D and infected with WT, Δinv or ΔyadA expressing GFP at moi 1:1 for 1 h. 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, Δinv and ΔyadA. The experiment was repeated four times and the average ± SEM are graphed. The asterisk indicates significant differences in the association of the indicated cell type by WT versus ΔyadA-infected cells as determined by t-test (P < 0.05).
neutrophils, macrophages and dendritic cells compared with B and T cells (Fig. 6A and B, white bars) in the absence of chemotaxis and/or phagocytosis. To test whether more professional phagocytes associated with Yptb when their ability to chemotax and phagocytose bacteria was present, the number of different splenocytes that associated with Yptb was determined by flow cytometry (Fig. 6B, black bars). An increase in binding of GFP–Yptb to neutrophils, macrophages and dendritic cells was observed in the absence of the inhibitor (Fig. 6B, 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.

We next analysed 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 (Mejia et al., 2008). The presence of YadA and Invasin in Yptb grown at 37°C was confirmed by Western blot analysis (Fig. S6). The binding of ΔyadA and Δinv 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, Δinv or ΔyadA (Fig. 6C). However, when specific cell types were analysed for their ability to bind to GFP–Δinv or GFP–ΔyadA (Fig. 6D), the ΔyadA mutant bound significantly less to neutrophils than GFP–Yptb, while binding to B cells and dendritic cells remained unchanged (Fig. 6D). Since neutrophils are only approximately 0.54% of all splenocytes (Table 1), the fourfold reduction in binding to neutrophils was not detectable in the total splenocytes (Fig. 6C). In the absence of Invasin, no difference was observed in binding to any cell type analysed (Fig. 6D). These results demonstrate that YadA plays a critical role in selective association of Yptb with neutrophils whereas Invasin does not.

YadA and Invasin mutants have reduced translocation into splenocytes compared with 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 (Mejia et al., 2008). The ΔyadA mutant bound inefficiently to neutrophils (Fig. 6) and binding of invasin triggers signal transduction cascades that enhance translocation (Mejia et al., 2008). We tested whether ΔyadA-HTEM, Δinv-HTEM and/or ΔinvΔyadA-HTEM targeted fewer splenocytes for translocation using flow cytometry (Fig. 7A and B).

Fewer splenocytes contained HTEM when infected with ΔyopB-HTEM, Δinv-HTEM, ΔyadA-HTEM or ΔinvΔyadA-HTEM compared with WT-HTEM (Fig. 7A and B and Fig. S2A). 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, Δinv-HTEM and ΔyadA-HTEM was significantly reduced compared with WT-HTEM (Fig. 7C). While the translocation into these cells was not significantly reduced after infection with the ΔinvΔyadA double mutant, this is likely due to the observation that two out of six experiments had very high levels of translocation after infection with the ΔinvΔyadA while four out of six had lower 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. 6C) 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. S7).

To determine whether the adhesion mutants targeted a different spectrum of cell types compared with WT, the percentage of each cell type in the translocated population was analysed. 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 with WT (Fig. 7D). This result indicates that while the overall number of professional phagocytes with translocated Yops was reduced (Fig. 7B and C), Yptb still interacted preferentially with professional phagocytes compared with other cell types in splenocytes.

In these translocation assays, phagocytosis could not be prevented because disruption of actin polymerization also blocks translocation (Mejia et al., 2008 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 has been internalized. In fact, recent studies support the idea that some Yop translocation can occur after phagocytosis of Yptb (Zhang et al., 2008). To investigate this possibility, WT-HTEM was grown at 26°C or 37°C, conditions that promote or block invasion of phagocytes, respectively.
Fig. 7. \(\Delta\text{yadA}\) and \(\Delta\text{inv}\) translocate HTEM into fewer numbers of splenocytes. Splenocytes were left uninfected or infected with WT-HTEM, \(\Delta\text{yopB}\)-HTEM, \(\Delta\text{inv}\)-HTEM or \(\Delta\text{yadA}\)-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 \(\Delta\text{inv}\)-HTEM, \(\Delta\text{yadA}\)-HTEM and \(\Delta\text{inv}\Delta\text{yadA}\)-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 \(\Delta\text{inv}\)-HTEM, \(\Delta\text{yadA}\)-HTEM or \(\Delta\text{inv}\Delta\text{yadA}\)-HTEM was compared with infection with WT-HTEM.

D. The percentage of a cell marker in the blue cell population after infection with \(\Delta\text{inv}\)-HTEM, \(\Delta\text{yadA}\)-HTEM or \(\Delta\text{inv}\Delta\text{yadA}\)-HTEM was compared with infection with WT-HTEM.

C and D. The bars represent the average ± SEM of at least 8, 12 and 6 experiments for \(\Delta\text{inv}\)-HTEM, \(\Delta\text{yadA}\)-HTEM and \(\Delta\text{inv}\Delta\text{yadA}\)-HTEM analysed 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 (\(P < 0.05\)).
and RAW264.7 macrophages were infected at an moi of 10. At 26°C, Yptb expresses adhesins, but not the TTSS, so more bacteria are internalized whereas at 37°C both adhesins and TTSS are expressed, so the Yops are rapidly delivered to phagocytes preventing phagocytosis. After 20 min, 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 analysed 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°C (Fig. S2B). These results indicate that HTEM was most frequently translocated from extracellular bacteria when the bacteria were grown at 37°C. In contrast, more cells contained Yptb than were blue after infection with Yptb grown at 26°C. Although the presence of Yptb in the blue cells was not tested, these results are consistent with the idea that some internalized Yptb may translocate Yops.

\[\Delta\text{yadA} \text{and } \Delta\text{inv mutants colonize the PP poorly after oral infection, but } \Delta\text{inv translocate Yops into professional phagocytes}\]

To determine whether YadA and/or Invasin are critical for targeting neutrophils or macrophages during infection, mice were infected oro-gastro-intestinally with WT-HTEM, \(\Delta\text{yadA-HTEM}\), \(\Delta\text{inv-HTEM}\) or \(\Delta\text{inv}\Delta\text{yadA-HTEM}\) and the numbers of bacteria, blue cells and neutrophils in the PP were determined at 3 and 5 days post infection (Fig. 8A–C). We found significantly fewer bacteria, fewer blue cells and fewer neutrophils in the PP of mice infected with \(\Delta\text{yadA-HTEM}\), \(\Delta\text{inv-HTEM}\) and \(\Delta\text{yadA}\Delta\text{inv-HTEM}\) compared with WT-HTEM at both days. Therefore, it was impossible to conclude whether the reduced number of translocated neutrophils were 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 \(\Delta\text{inv-HTEM}\) had bacterial levels that were within the lower range of mice infected with WT-HTEM (Fig. 8A). The number of blue cells and the types of cells targeted for Yop translocation in these PP were compared with three mice infected with WT-HTEM that had similar bacterial loads (Fig. 8D–G). The three mice in each cohort had similar numbers of blue cells and similar numbers of neutrophils (Fig. 8D). In addition, the number of neutrophils and macrophages translocated with Yops were comparable (Fig. 8F and G). These preliminary results suggest that Invasin is not solely responsible for the targeted translocation of HTEM into professional phagocytes during infection of PP.

Since it had previously been reported that a \(\text{yadA}\) mutant can reach the PP during early stages of infection but does not survive (Marra and Isberg, 1997; Heise and Dersch, 2006), mice were infected with WT-HTEM and \(\Delta\text{yadA-HTEM}\) for 6 h, 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 \(\Delta\text{yadA-HTEM}\) were detected (Fig. S8). Consistent with previous results (Marra and Isberg, 1997; Heise and Dersch, 2006), comparable bacterial loads were detected in the PP at 6 h post infection, but by day 1, most mice infected with WT-HTEM had higher bacterial loads than mice infected with \(\Delta\text{yadA-HTEM}\). Unfortunately, the number of blue cells at 6 h was too low to evaluate whether the distribution of cells targeted by WT-HTEM versus \(\Delta\text{yadA-HTEM}\) were different (Fig. S8). 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.

**Discussion**

After oral infection, pathogenic Yptb replicates primarily extracellularly in many organs and must counteract bac-tericidal actions of resident and incoming cells (Simonet et al., 1990; Balada-Llasat and Mecsas, 2006; Bergman et al., 2009). During infection of cultured cells, *Yersinia* translocates Yops into many different cell types including epithelial cells, macrophages, B cells, T cells and dendritic cells (Sory et al., 1995; Yao et al., 1999; Grosdent et al., 2002; Davis and Mecsas, 2007; Brodsky and Medzhitov, 2008). 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 (Leong et al., 1990; Pettersson et al., 1996; Eitel and Dersch, 2002; Grosdent et al., 2002). 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 neighbouring cells during tissue infection. First, in
Fig. 8. YadA and Invasin are critical for colonization of Peyer’s patches. Seven- to nine-week-old female BALB/c mice were infected orally with \(2 \times 10^9\) cfu of WT-HTEM, \(\Delta\text{yadA}-\text{HTEM}\), \(\Delta\text{inv}-\text{HTEM}\) or \(\Delta\text{inv}\Delta\text{yadA}-\text{HTEM}\) 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 labelled with \(\alpha\text{-GR1-PeCy5}\) and \(\alpha\text{-Cd11b-PeCy7}\) to determine the percentage of neutrophils by flow cytometry.

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 \(\Delta\text{inv}-\text{HTEM}\) (triangles).

E. Bacterial colonization in PP of the WT-HTEM and \(\Delta\text{inv}-\text{HTEM}\) mice used in (D), (F) and (G). Each dot represents one mouse; the bar represents the geometric mean. There were no significant differences.

F and 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 \(\Delta\text{inv}-\text{HTEM}\). There were no significant differences.
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 (Koberle et al., 2009). Second, very few B and T cells were translocated with Yops in the lymph nodes, although Yptb colocalized with B and T cells in lymph nodes (Balada-Llasat and Mecsas, 2006). 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, the activation state of the bound cell, activation of specific signal-transduction cascades, and/or plasma membrane domains that favour insertion of the translocon. Since translocation requires binding to cells (Mejia et al., 2008), 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 favoured. 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 (Fallman et al., 1995; Grosdent et al., 2002). Alternatively, Yptb-specific ligands may dictate the bacteria’s association with neutrophils, macrophages and dendritic cells. In fact, our observation that Δyada 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 Δinv 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 (Marra and Isberg, 1997; Heise and Dersch, 2006). In a ligated loop model of infection, a Δyada mutant rapidly penetrated the PP; however, 48 h post oral inoculation Δyada was attenuated for growth in the PP (Marra and Isberg, 1997; Heise and Dersch, 2006). 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’ are consistent with the idea that the Δyada mutant cannot target resident neutrophils and therefore is eliminated (Marra and Isberg, 1997; Heise and Dersch, 2006). 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 with many other Yptb strains (Simonet and Falkow, 1992; S. Mohammadi and M. Higgins, unpubl. 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. (2008) showed that an increase in the activation of Src kinases after integrin stimulation correlated with an increase of Yop translocation in HeLa cells. 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, Ye targeted Yop predominantly to a subset of B cells, follicular B cells (Koberle et al., 2009). 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 (Hayward et al., 2005; Epler et al., 2009). 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 phagocytes 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 (Balada-Llasat and Mecsas, 2006; Bergman et al., 2009) and we showed that when Yptb were grown at 37°C, 10-fold more macrophages were detected 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 profes-
sional 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 defences and enhances bacterial growth during infection.

Previous studies have examined the splenic cell types targeted by Y. pestis and Ye after intravenous infection (Marketon et al., 2005; Koberle et al., 2009). 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, of some which are shared among Yersinia spp. (Forman et al., 2008; Felek and Krukonis, 2009). 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 performed with a lower moi with Y. pestis or Ye, it is unclear if this selectivity is apparent under conditions where bacteria are limiting (Marketon et al., 2005; Koberle et al., 2009). 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 (Koberle et al., 2009). 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 the observation that the level of colonization of the spleen after oral infection with Yptb was 100× less than that of Ye (Koberle et al., 2009) 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 (Koberle et al., 2009). 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 100× 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 under-represented among each tissue. For example, CD4 T cells were under-represented for targeting in the PP, but were not under-represented in the MLN and spleens while B cells were under-represented 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 are 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 (Geddes et al., 2007). 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.

Experimental procedures

Construction of strains and plasmids

Plasmids, strains and strain constructions are described in Supporting information (Appendix S1). Strains are listed in Table S2; primers are listed in Table S3. The absence of adhesins, YadA and Inv, in the ΔyadA and Δinv strains were confirmed by Western blot as described in Supporting information (Fig. S6).
Professional phagocytes increase Yop translocation

CCF2-AM conversion assays after murine infections

Mice were infected as previously described (Logsdon and Mecsas, 2003) with the following modifications. BALB/c mice (NCI) were infected intragastrically with 2×10^9 cfu of WT-HTEM unless otherwise 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 of HBSS containing MgCl_2 and CaCl_2 (Cellgro). All tissues were pressed through a 70 µm cell strainer (Falcon) and 100 µg ml^-1 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 340 g for 5 min. Prior to staining the spleens, spleens (but not PP or MLN) were perfused with 80 U ml^-1 collagenase (Roche) and incubated for 30 min at 37°C to liberate dendritic cells and eliminate autofluorescence. Collagenase activity was halted by the addition of 2 ml of HBSS lacking MgCl_2 and CaCl_2 (Cellgro) and supplemented with 1 mM EDTA. Cells from spleens were resuspended in 10 ml of BD Pharm Lyse™ solution (Pharmagen) to lyse erythrocytes and immediately spun at 340 g for 5 min. Cells from PP, MLN and spleens were washed twice with PBS and then MLN and spleens were resuspended in 3 ml of RPMI + 10% FBS and PP were resuspended in 1 ml of RPMI + 10% FBS.

To label single-cell suspensions, the cells were incubated with 1 µg ml^-1 CCF2-AM compound (Invitrogen) for 2 h at 30°C in the presence of 1.5 mM probenecid (Sigma) and 100 µg ml^-1 gentamicin (Sigma). One hundred microlitres of cells were aliquoted into a 96-well plate and blocked with 50 µl of a 1:200 dilution of Mouse BD Fc Block™ (BD) for 10 min at 4°C. Cells were incubated in 50 µl 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 min at 4°C. Samples were washed twice in FACS buffer, centrifuged at 340 g, resuspended in 100 µl in FACS buffer and analysed on an LSRII (Becton Dickson) FACS machine. A total of 2×10^5 cells were acquired per sample and data were analysed using Summit v4.3 software. Cells from tissues that were not 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.

Yptb adherence assays to splenocyte suspensions

A 1 ml 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°C with no spinning. Yptb expressing GFP were grown as described above and the medium was supplemented with 10 µg ml^-1 chloramphenicol. Cells were labelled with antibodies and analysed on ImageStream (Amnis) with IDEAS analytical software or an LSRII (Becton Dickson) FACS with Summit v4.3 software as described above.

Immunohistochemistry

The location of Yptb was determined as described (Balada-Llasat and Mecsas, 2006) 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 (Balada-Llasat and Mecsas, 2006). Samples were scored blindly by at least two investigators using a Nikon Eclipse TE2000-U microscope.

YopE and HTEM translocation

Splenocytes from infected mice were treated with CCF2-AM and labelled with GR1-PE-Cy5 antibody (eBiocience 15-5931-81). Cells were sorted in the MoFlo FACS sorter (Cytomation, Fort Collins, CO) and 2×10^5 blue^GFP-GR1^, 2×10^5 blue^GFP-GR1^ and 2×10^5 blue^GFP-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^-1 each of aprotinin, leupeptin and pepstatin) for 2 min with gentle rocking at 4°C and processed as described (Davis and Mecsas, 2007). These lysis conditions have been shown to lyse the plasma cell membrane, but not bacteria membranes as shown in Fig. S4 and Davis and Mecsas (2007).

Granulocyte depletion

Granulocyte depletion experiments were performed as described (Logsdon and Mecsas, 2006) with the following modifications. Mice were injected with the antibodies 1 day prior to and 2 days after oral inoculation with 8×10^8 Yptb YopHTEM. Day 3 post infection PP were harvested and single-cell suspension was generated. Ten microlitres of the 5 ml cell suspension was plated on L plates containing kanamycin (50 µg ml^-1) to determine the bacterial burden in the tissue. Cells were labelled with CCF2-AM and with GR-1-PE-Cy5 antibody as described above. Fluorescent intensities of labelled cells were detected by flow cytometry using BD LSR II System. Data were analysed using Summit v4.3 software. The experiment was performed twice and data from both experiments are shown.

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Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1. Supplementary experimental procedures.

Fig. S1. Colonization of Yptb WT-HEM strain in competition WT Yptb 5 days post oral infection. Eight-week-old female BALB/c mice were orally inoculated with 2 ¥ 10^5 cfu of an equal mixture of WT IP2666 and WT-HEM 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-HEM (which is kanamycin resistant) in the spleen. The competitive index (CI) was calculated as follows: CI = (number of WT-HEM colonies/number of WT colonies)WT/HEM/(number of WT-HEM colonies/number of WT colonies)WT. Black circles represent the CI from individual mice.

Fig. S2. Translocation of Yops into splenocytes by WT-HEM and ΔyopB-HEM. A. Splenocytes were harvested and infected with WT-HEM or ΔyopB-HEM at an moi of 10 for 1 h. The percentage of blue cells in splenocytes infected with WT-HEM or ΔyopB-HEM were plotted. The data shown are the average and SEM of at least three experiments.
B. RAW264.7 macrophages seeded at 1 ¥ 10^5 per well in six-well plates were infected at 37°C with WT-HEM grown at 26°C or 37°C in low-calcium media at an moi of 10. Gentamicin was added after 2 min and the cells were incubated for an additional hour. Two wells each were analysed 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.

Fig. S3. Splenocyte cell survival after infection with Yptb. Splenocytes were infected with Yptb at an moi of 20 for 1, 2, 4 or 18 h and stained with propidium iodine (PI) to identify dead cells. Cells were fluorescently labelled 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 twice and the average cell death is shown.

Fig. S4. Western blot of YopE and S2 in supernatants of Yptb after incubation with eukaryotic and bacterial lysis buffers. A total of 2 ¥ 10^7 Yptb grown at 37°C were washed with PBS and then incubated with 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^-1 each of aprotinin, leupeptin and pepstatin) for 2 min with gentle rocking at 4°C as described in Experimental procedures or 50 μl of bacterial lysis buffer containing 4% SDS. The bacteria were briefly centrifuged and the supernatants run on SDS-PAGE, transferred to a membrane and visualized and filtered to generate a single-cell suspension. Cells were incubated with CCF2-AM and labelled with antibodies to the indicated cell surface marker(s). Fluorescence intensity was analysed by flow cytometry. (A–C) The percentage of blue^high^ green^-^ cells (white bars) and the percentage of blue^low^ 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 four times and the bars are the average ± SEM from all the experiments.

Fig. S5. Neutrophils, macrophages and dendritic cells are also preferentially translocated with HTEM in the blue^-^/green^-^ during Yptb infection. Mice were ovariographically infected with 2 ¥ 10^5 cfu of WT-HEM. Day 5 post infection tissues were harvested and filtered to generate a single-cell suspension. Cells were incubated with CCF2-AM and labelled with antibodies to the indicated cell surface marker(s). Fluorescence intensity was analysed by flow cytometry. (A–C) The percentage of blue^high^ green^-^ cells (white bars) and the percentage of blue^low^ 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 four times and the bars are the average ± SEM from all the experiments.

Fig. S6. Western blot of Invasin and YadA in WT, Δinv, ΔyadA and ΔinvΔyadA. Bacteria were grown at 26°C for 2 h and then grown at 37°C for 2 h in 2×YT. 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).

Fig. S7. Translocation into splenocytes after infection at moi 40:1 with WT-HEM, Δinv-HEM, ΔyadA-HEM and ΔinvΔyadA-HEM. 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.

**Fig. S8.** YadA is a key virulent factor for Yptb survival in the PP. Eight-week-old female BALB/c mice were infected orally with 2 \times 10^9 cfu with WT-IP2666 or ΔyadA and sacrificed at 6 h, 24 h and 48 h 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 labelled 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.

Table S1. % Blue cells in infected tissues.
Table S2. *Yersinia* strains used in this study.
Table S3. Primers used in this study.

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