The Cytotoxic Necrotizing Factor of *Yersinia pseudotuberculosis* (CNF\textsubscript{Y}) Enhances Inflammation and Yop Delivery during Infection by Activation of Rho GTPases

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**Abstract**

Some isolates of *Yersinia pseudotuberculosis* produce the cytotoxic necrotizing factor (CNF\textsubscript{Y}), but the functional consequences of this toxin for host-pathogen interactions during the infection are unknown. In the present study we show that CNF\textsubscript{Y} has a strong influence on virulence. We demonstrate that the CNF\textsubscript{Y} toxin is thermo-regulated and highly expressed in all colonized lymphatic tissues and organs of orally infected mice. Most strikingly, we found that a cnf\textsubscript{Y} knockout variant of a naturally toxin-expressing *Y. pseudotuberculosis* isolate is strongly impaired in its ability to disseminate into the mesenteric lymph nodes, liver and spleen, and has fully lost its lethality. The CNF\textsubscript{Y} toxin contributes significantly to the induction of acute inflammatory responses and to the formation of necrotic areas in infected tissues. The analysis of the host immune response demonstrated that presence of CNF\textsubscript{Y} leads to a strong reduction of professional phagocytes and natural killer cells in particular in the spleen, whereas loss of the toxin allows efficient tissue infiltration of these immune cells and rapid killing of the pathogen. Addition of purified CNF\textsubscript{Y} triggers formation of actin-rich membrane ruffles and filopodia, which correlates with the activation of the Rho GTPases, RhoA, Rac1 and Cdc42. The analysis of type III effector delivery into epithelial and immune cells in vitro and during the course of the infection further demonstrated that CNF\textsubscript{Y} enhances the Yop translocation process and supports a role for the toxin in the suppression of the antibacterial host response. In summary, we highlight the importance of CNF\textsubscript{Y} for pathogenicity by showing that this toxin modulates inflammatory responses, protects the bacteria from attacks of innate immune effectors and enhances the severity of a *Yersinia* infection.

**Citation:** Schweer J, Kulkarni D, Kochut A, Pezoldt J, Pisano F, et al. (2013) The Cytotoxic Necrotizing Factor of *Yersinia pseudotuberculosis* (CNF\textsubscript{Y}) Enhances Inflammation and Yop Delivery during Infection by Activation of Rho GTPases. PLoS Pathog 9(11): e1003746. doi:10.1371/journal.ppat.1003746

**Editor:** James B. Bliska, Stony Brook University, United States of America

**Received** May 10, 2013; **Accepted** September 20, 2013; **Published** November 7, 2013

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**Funding:** This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB621), the President’s Initiative and Networking Fund of the Helmholtz Association of German Research Centres (HGF) under contract number VH-GS-202, and the Fonds der Chemischen Industrie. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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**Introduction**

Enteropathogenic *Yersinia* species such as *Y. enterocolitica* and *Y. pseudotuberculosis* initially infect the terminal ileum and colonize the Peyer’s patches (PPs) within several hours of infections. Bacteria are subsequently transported to the mesenteric lymph nodes (MLNs) and can also spread systemically to reach liver and spleen via the bloodstream. The infections typically result in enteritis, enterocolitis and mesenteric lymphadenitis where the infected tissues show formation of microabscesses or granuloma-like lesions with central necrosis [1].

Enteropathogenic *yersiniae* have been shown to secrete exotoxins and/or inject effector proteins by specialized secretion machineries to manipulate host cell functions, including cytoskeletal rearrangements, to prevent immune responses and to establish a successful infection. They encode a type III secretion system (T3SS) on a 70 kb virulence-associated plasmid (pYV) that is essential for their defense against the host immune system [2–4]. The *Yersinia* T3SS has been shown to form a syringe-like apparatus with a thin needle-like surface exposed projection [5]. It is used to insert a translocation channel (composed of YopB and YopD) within the host membrane to inject the effector proteins YopE, YopH, YopJ/YopP, YopK/YopQ, YopM, YopO/YpkA, and YopT into the cell’s cytoplasm. Yops target different cell signaling molecules and processes, in particular cytokine production and actin dynamics, often resulting in the inhibition of phagocytosis [6].

YopH is a tyrosine phosphatase that dephosphorylates proteins of the focal adhesion complex [7–10]. The *Yersinia* T3SS is the effector system. The toxins YopE, YopT and YopO/YpkA manipulate the regulation of Rho GTPases, which control the formation of lamellipodia, filopodia and stress fibers [2,11]. YopJ/YopP promotes cell death of macrophages by inactivating the counterregulators of the Toll-like receptor 4-triggered apoptotic pathway, the mitogen-activated protein kinase kinases (MEKs) and the inhibitor of nuclear factor-κB (IKK) β [12–16].
Various toxins and effector proteins of bacterial pathogens have been found to manipulate eukaryotic cell machineries to promote persistence and proliferation within their hosts. Many of these virulence factors target small Rho GTPases, but their role in pathogenesis is often unknown. Here, we addressed the expression and functional consequences of the CNFγ toxin found in some isolates of Y. pseudotuberculosis. We found that CNFγ is a deamidase of RhoA, Rac1 and Cdc42, contributing to increased inflammation and tissue damage. Moreover, CNFγ increases the ability of Yersinia to prevent the attack of the immune system, by enhancing the delivery of antiphagocytic and cytotoxic effectors to professional phagocytes. Our findings provide the first insights into the multi-functional action and severe consequences of the CNFγ toxin on the inflammatory response and disease-associated tissue damage during the natural course of the infection.

YopM forms a complex with RSK and PRK kinase isoforms, traffics to the nucleus, and is important for Yersinia to persist in liver and spleen with a contextual decrease of several proinflammatory cytokines, including IL-1β, IL-12, IL-18, interferon γ, and TNF-α, and depletion of NK cells [17–21]. The effector YopK/YopQ seems to play a role in orchestrating the translocation of effector proteins by modulating the ratio of the pore-forming proteins YopB and YopD. This appears to prevent unintended Yop delivery and neutrophil death, which would enhance the inflammatory response possibly favoring the host [22–24].

Insertion of the YopB/D translocation channel allows Yop delivery while maintaining the host cell membrane intact. The YopB/D complex results in activation of Rho GT-Pases, actin polymerization and pore-formation. However, pore formation is usually prevented by the GT-Pase-downregulating function of YopE and YopT. Yet, expression of constitutively active forms of Rac1 and RhoA leads to a loss of membrane integrity and results in increased pore formation even when YopE and YopT are expressed [25]. In addition, signaling pathways triggered by high affinity-binding of the main Yersinia adhesins YadA and InvA to β1 integrin receptors and YopB/D signaling were shown to induce activity of Rho GT-Pases and actin polymerization which are crucial for efficient translocation of the Yop effectors [26].

Another Yersinia factor shown to activate the small GT-Pase RhoA is the cytotoxic necrotizing factor-Y (CNFγ) [27,28]. CNFγ is prevalent in some Y. pseudotuberculosis isolates, e.g. the widely used Y. pseudotuberculosis strain YPIII. All these strains belong to the serogroup III, but other isolates of this serogroup do not express CNFγ and contain deletions within the corresponding cnfγ gene [27].

On the amino acid level, CNFγ is highly similar (>60%) to the CNF toxins found mainly in E. coli strains isolated from patients and domestic animals with extraintestinal infections (CNF1-3) [29,30]. CNF1 is the best-characterized toxin of this class of bacterial toxins and is transferred to host cells through outer membrane vesicles (OMVs) [31–34]. The CNF1 toxin is a single-chain A-B toxin with an N-terminal delivery domain including subdomains for receptor binding, pore formation and proteolytic cleavage, and a C-terminal deamidase domain [35,36]. Internalization of the toxin into target cells occurs through receptor-mediated endocytosis, which appears to be independent of clathrin and lipid rafts (sphingolipid/cholesterol rich microdomains) [37,38]. After uptake, the 55 kDa C-terminal deamidase domain is autocatalytically cleaved off in the late endosome, and delivered into the cytoplasm in a pH-dependent manner [39].

CNF1 deamidates Glu-61/-63 of RhoA, Rac1 and Cdc42 to Glu-61/-63 resulting in Rho GTPases with a blocked GTP hydrolase activity. Deamidated Rho GTPases induces polymerization of F-actin at focal contacts, increase cell-matrix adhesion, and promote formation of stress fibers, lamellipodia and filopodia, which led to the classification as ‘constitutively active’ [40–43]. Cytoskeletal rearrangements attributed to CNF lead to multinucleated cells due to inhibited cytokinesis with ongoing cell cycle progression [46]. Additionally, CNF1 has been reported to (i) induce phagocytosis in epithelial cells and reduce CR3-mediated phagocytosis in monocytes [47,48], (ii) promote bacterial cell entry [49], (iii) decrease the barrier function of intestinal tight junctions [40,50], (iv) decrease transmigration of polymorphonuclear leukocytes across a TB4 monolayer [51], and (v) induce apoptosis of bladder cells [52].

The overall amino acid sequence of CNFγ of Y. pseudotuberculosis is very similar to CNF1. However, CNFγ is not recognized by neutralizing antibodies against CNF1 [27]. Moreover, CNFγ seems to bind to different cell receptors and preferentially deamidates RhoA (over Rac1 and Cdc42) in cultured epithelial cells [28,38]. Although CNFγ and certain Yop effectors alter the cytoskeleton by affecting the activity of the Rho GT-Pases, little is known about the interplay, cooperation and joint role of these toxins in the pathogenic lifestyle of Y. pseudotuberculosis. Here, we provide evidence that CNFγ is an important virulence factor of Y. pseudotuberculosis YPIII. CNFγ is shown to enhance Yop protein delivery, which is crucial for pathogenicity. Furthermore, the toxin was found to induce inflammatory responses and increase the severity of a Yersinia infection.

**Results**

CNFγ is expressed in all infected tissues and organs throughout the infection.

Since many Y. pseudotuberculosis isolates as well as Y. pestis contain deletions within the cnfγ gene [27], we first tested whether the intact cnfγ toxin gene in the Y. pseudotuberculosis wild-type strain YPIII is expressed and induced under virulence-relevant growth conditions. A cnfγ-lacZ transcriptional fusion was only slightly expressed when Y. pseudotuberculosis was grown at 25°C, but its expression was strongly induced at 37°C and reached its maximum during stationary phase (Fig. S1A). High cnfγ transcription was generally observed in complex media, in particular BHI, whereas only low expression levels were detected in all tested minimal media (Fig. S1B, data not shown). In summary, cnfγ is predominantly expressed at 37°C in a nutrient rich environment, resembling conditions found in the mammalian intestinal tract.

This result prompted us to test expression of the toxin during infection. BALB/c mice were orally infected with 2×10⁶ bacteria of the Y. pseudotuberculosis wild-type strain YPIII expressing a cnfγ-luxCDABE fusion, and the bioluminescent signal was monitored in the mice for six days using an in vivo imaging system. Only very low luciferase activity was measured in the bacterial culture before infection (data not shown) and in the intestinal tract directly after oral ingestion (1 h, Fig. 1). However, a very strong bioluminescent signal of the cnfγ-luxCDABE fusion was detectable during the entire following course of the infection. The most intensive signals were detected two days post infection in the intestine and associated lymphoid tissues (Fig. 1). No light emission was monitored in mice infected with bacteria carrying the promoterless luxCDABE operon in the identical expression system (data not shown).
In order to study cnfY expression in the individual infected tissues, we used a set of established fluorescent fusion vectors for in vivo expression analysis. To do so, Y. pseudotuberculosis YPIII harboring a plasmid-encoded constitutive PhoP-cZsRed2 reporter construct and a compatible PhoP-cZsRed2 reporter construct and a compatible PhoP-cZsRed2 reporter construct and a compatible PhoP-cZsRed2 reporter construct was used to infect BALB/c mice. At indicated time points, mice were anesthesized and bioluminescence was detected with a CCD camera (in vivo imaging system) on the ventral side.

Figure 1. In vivo expression analysis of the cnfY-luxCDABE fusion. 2×10^6 bacteria of Y. pseudotuberculosis YPIII pJNS02 (pconY-luxCDABE) was used to orally infect BALB/c mice. At indicated time points, mice were anesthesized and bioluminescence was detected with a CCD camera (in vivo imaging system) on the ventral side.

In order to study cnfY expression in the individual infected tissues, we used a set of established fluorescent fusion vectors for in vivo expression analysis. To do so, Y. pseudotuberculosis YPIII harboring a plasmid-encoded constitutive PhoP-cZsRed2 reporter construct and a compatible PhoP-cZsRed2 reporter construct was used to infect BALB/c mice. Five days post infection, the small intestine, caecum, colon, PPs, MLNs, spleen and liver were isolated and cryosections were prepared. The bacteria in the tissues were visualized by monitoring dsRed2, and then tested for PhoP-cZsRed2. As shown in Fig. 2, the PhoP-cZsRed2 fusion was expressed in all tested organs. In summary, a temperature shift to 37°C, but most likely no tissue-specific signals are required to induce toxin expression in infected tissues.

CNF_Y is crucial for virulence of Y. pseudotuberculosis YPIII

Absence of a functional toxin gene in other Y. pseudotuberculosis clinical isolates, may suggest that CNF_Y only adds another potential virulence factor to the variety of effector proteins and toxins that are produced by this pathogen. However, high expression of cnfY during the entire course of an infection also indicates that presence of this toxin may enhance the pathogenicity of Y. pseudotuberculosis. To first assess the impact of CNF_Y on pathogenesis, the potential of the Y. pseudotuberculosis wild-type strain YPIII and the isogenic cnfY-deficient strain to cause lethal infections was compared. BALB/c mice were orally infected with 2×10^6 bacteria of the cnfY mutant (YP147) and the wild-type strain (YPIII) harboring the empty vector (pJNS11) or a cnfY-encoding plasmid (pJNS10). Survival and weight of the mice were monitored over two weeks and date of death was recorded (Fig. 3, S2). Mice infected with YPIII showed signs of the infection, e.g. weight loss, piloerection and lethargy, and succumbed to infection between day four and six days. In contrast, none of the mice infected with YPI147 developed severe disease symptoms and all mice were still alive 14 days post infection. Monitoring of body weight demonstrated that despite infection with the cnfY knockout strain YP147 showed a slight reduction in weight, but they recovered quickly and regained weight (Fig. S2). Presence of the cnfY-encoding low-copy number plasmids reverted the avirulent phenotype of the cnfY mutant and reduced the average day of death of the wild-type strain YPIII by one day, most likely due to the overexpression of the toxin. The Y. pseudotuberculosis YPIII isolate, unlike other Y. pseudotuberculosis strains, is unable to replicate in murine macrophages due to a defective allele of phoP [53]. To exclude that CNF_Y influence on virulence is only visible in a phoP-deficient derivative with an overall lower pathogenicity, the inability to grow in macrophages was complemented by an exchange of the allele against the phoP ORF from Y. pseudotuberculosis IP32953. However, when mice were challenged with 2×10^7 CFU of the equivalent phoP strains, 100% of the mice infected with the CNF_Y-positive strain died during the observation period, while 80% of the mice infected with the isogenic cnfY-deficient strain survived and regained weight (Fig. S3).

To gain a deeper insight into the differences in the infection process of CNF_Y-positive and -negative strains, we determined the number of bacteria that colonized the small intestine, caecum, PPs, MLNs, liver and spleen of BALB/c mice at different time points after oral infection with 2×10^6 bacteria (Fig. 4). Comparable amounts of wild-type (YPIII) and the mutant strains (YP147) were recovered from PPs and caecum during infection, and only a very small increase of bacterial counts was observed with the cnfY mutant in the small intestine at day 5–7 post infection (Fig. 4). However, significantly reduced numbers of YP147 were recovered from MLNs and spleen (Fig. 4). The number of cnfY-positive and -negative bacteria in these organs was almost identical up to day three post infection, but the cnfY mutant was eliminated very rapidly later during the infection. At day seven, none or only few mutants were recovered from MLNs and spleen (Fig. 4). The number of YP147 bacteria of the wild-type strain were recovered per gram of both organs. The effect was less pronounced in the liver, but the strongly reduced number of mutant bacteria relative to the wild-type bacteria six and seven days after infection clearly indicated that the presence of CNF_Y is also advantageous for the colonization of the liver (Fig. 4). This demonstrated that loss of CNF_Y, resulting in avirulence of Y. pseudotuberculosis YPIII,
reflected by a fast elimination of the bacteria from MLNs, liver and spleen. Within the first week after infection with wild-type strain YPIII the size of the spleen and liver decreased two-fold, whereby changes of the organ size were first visible at day three post infection (Fig. S4A, B). In contrast, infection with the isogenic cnfY mutant strain YP147 had no effect on the size of the liver and induced a considerable increase of the size of the spleen. In addition, mice infected with wild-type strain YPIII had significantly shorter intestines (30%) at day six and seven post infection than mice infected with the cnfY mutant (Fig. S4C). The shortening of the intestine is a sign of marked intestinal inflammation. This indicated that CNFY not only affects colonization of systemic organs, but has also a strong influence on the host’s inflammatory response against the bacterial infection.

Histopathological examination of the infected host tissues demonstrated marked differences of the overall inflammatory reaction, which was stronger in YPIII-infected animals, especially in the small intestine and spleen compared to YP147-infected mice. In the intestine, inflammation was most prominent in the ileum and caecum in both groups. However, in YPIII-infected mice inflammation was diffuse affecting the entire ileum at day six (Fig. 5A, upper panel, 5B middle panel). In YP147-infected mice inflammation was locally restricted to multifocal lesions characterized by the presence of inflammatory cells from the muscular layer up to the epithelial cells (Fig. 5A, lower panel, 5B right panel). In these areas inflammation led to epithelial cell hyperplasia (increased proliferation) resulting in an increase of the villi length. However, this lesion is only locally restricted and adjacent tissue remains unaltered. In addition, inflammation was more generalized in YPIII- compared to YP147-infected organs. In mice infected with YP147, no bacterial foci (diffuse patches of bacteria) could be detected microscopically in hematoxylin and eosin (H & E) stained sections of the spleen at day six post infection, whereas in the majority of YPIII-infected mice bacterial foci were visible in the histological sections (Fig. 5C). YPIII

Figure 2. Expression of P_cnfY::gfp in the lymphatic tissues and organs. 2×10⁸ bacteria of Y. pseudotuberculosis YPIII pFU228/pJNS03 (P_gapA::dsred, P_cnfY::gfpmut3.1) were used to orally infect BALB/c mice. Three days post infection, mice were sacrificed, and PPs, caecum, MLNs, spleen and liver were isolated. Histological slides were prepared and analyzed by fluorescence microscopy to detect bacteria in the tissues by expression of the reporter protein DsRed2. In parallel cnfY expression in the bacteria was monitored by GFPMut3.1 mediated fluorescence. White bars indicate 20 μm. doi:10.1371/journal.ppat.1003746.g002
infections were accompanied by a more severe inflammation of the spleen, where presence of the bacteria resulted in necrotizing splenitis leading to splenic atrophy with marked depletion of the white pulp. YPIII caused multifocal necrosis in spleen, whereas in YPI47-infected spleens, only mild hyperplasia of the white pulp and increased erythropoiesis were found (Fig. 5C). Taken together, CNFY has a significant influence on the number of microcolonies in the tissues and leads to a more

Figure 3. Influence of cnfY on the survival of BALB/c mice infected with *Y. pseudotuberculosis*. Survival of BALB/c mice (n = 10/strain) were monitored up to 14 days after oral infection with 2×10⁹ cfu of *Y. pseudotuberculosis* YPIII (black line), the cnfY mutant YP147 (red line) harbouring the empty vector pJNS11, and the strains YPIII pJNS10 (cnfY⁺) (green line) and YP147 pJNS10 (cnfY⁺) (blue line).

doi:10.1371/journal.ppat.1003746.g003

Figure 4. Influence of cnfY on the virulence of *Y. pseudotuberculosis*. BALB/c mice were infected intragastrically with an inoculum of 2×10⁸ cfu of *Y. pseudotuberculosis* wild-type YPIII or the cnfY mutant YP147. After 1–7 days of infection, mice were sacrificed and the number of bacteria in homogenized host tissues and organs (PPs, caecum, small intestine, MLNs, spleen, liver) was determined by plating. Data of two independent experiments (5 mice/group) are represented in scatter plots of numbers of CFU per gram as determined by counts of viable bacteria on plates. The statistical significances between the wild-type and the cnfY mutant were determined by the Mann-Whitney test. P-values: *: <0.05; **: <0.01 ***: <0.001.

doi:10.1371/journal.ppat.1003746.g004
severe and widespread inflammation in the small intestine, liver and spleen.

**CNF$_Y$ modulates the innate immune response**

Because of the strong influence of CNF$_Y$ on the colonization of bacteria in MLNs, spleen and liver, it was hypothesized that the toxin might counteract host immune defenses. To test this hypothesis, we infected BALB/c mice with $2 \times 10^8$ bacteria of YPIII and YP147. YPIII: diffuse invasion of inflammatory cells into the lamina propria. YP147: Focal invasion of inflammatory cells into the lamina propria; adjacent tissue was unaffected. Bar represents 200 μm. White boxes indicate magnified areas in the slides below. (B) Magnified section of the ileum of an uninfected mouse (left panel), a mouse infected with YPIII - magnification of the ileum selection shown in A (middle panel), and a mouse infected with YP147 - magnification of the focal invasion of inflammatory cells (right panel), local inflammation is indicated by the circle. Bar represents 50 μm. (C) spleen (6 days post infection); uninfected mouse (left panel). YPIII infected mouse: splenic atrophy and bacterial colony surrounded by focal necrosis. Pictures shown are representatives of multiple fields and samples of 9 mice. Arrow points to the bacterial foci (middle panel). YP147: hyperplasia of the white pulp and activated lymphoid follicle (right panel). W: white pulp indicated by dashed lines, N: necrosis, H: hyperplasia, R: red pulp, M: muscularis mucosa. Bar represents 50 μm.

doi:10.1371/journal.ppat.1003746.g005

Figure 5. Histology of infected organs. Histopathology of H & E stained sections of the ileum and the spleen of mice orally infected with $2 \times 10^8$ bacteria of YPIII and YP147. (A) ileum (6 days post infection); YPIII: diffuse invasion of inflammatory cells into the lamina propria. YP147: Focal invasion of inflammatory cells into the lamina propria; adjacent tissue was unaffected. Bar represents 200 μm. White boxes indicate magnified areas in the slides below. (B) Magnified section of the ileum of an uninfected mouse (left panel), a mouse infected with YPIII - magnification of the ileum selection shown in A (middle panel), and a mouse infected with YP147 - magnification of the focal invasion of inflammatory cells (right panel), local inflammation is indicated by the circle. Bar represents 50 μm. (C) spleen (6 days post infection); uninfected mouse (left panel). YPIII infected mouse: splenic atrophy and bacterial colony surrounded by focal necrosis. Pictures shown are representatives of multiple fields and samples of 9 mice. Arrow points to the bacterial foci (middle panel). YP147: hyperplasia of the white pulp and activated lymphoid follicle (right panel). W: white pulp indicated by dashed lines, N: necrosis, H: hyperplasia, R: red pulp, M: muscularis mucosa. Bar represents 50 μm.

doi:10.1371/journal.ppat.1003746.g005
post infection, when the bacterial load is still similar and the
overall health status of YPIII-infected mice is only slightly and not
severely reduced as at day six. A very pronounced variation of the
immune cell population between the YPIII- and YP147-infected mice was observed (Fig. 6). All types of immune cells were
significantly decreased in the spleen three days after infection with
YPIII when the spleen started to shrink, but the most severe
changes were observed with cells of the innate immune system. In
particular, numbers of macrophages, monocytes and NK cells
were significantly reduced; whereas reduction of neutrophils and
conventional DCs was less pronounced. In contrast, no reduction of
immune cells was detectable in spleens of YP147-infected mice
(Fig. 6). In contrast, a significant higher influx of neutrophils and macrophages/monocytes was observed, which is consistent with
the rapid clearance of mutant bacteria from the spleen upon
triggering of the immune response. To determine whether CNFY
affects the steady-state level of certain cell populations, the
population percentage was also compared and further conﬁrmed
a significant expansion of neutrophils and macrophages/monocytes
in YP147-infected spleens (Fig. S6). These and the histopathological data strongly suggest that the CNFY toxin
reduces inﬂux and/or causes rapid cell death of invading immune
cells in the spleen.

CNFY enhances Yop delivery into macrophages

Our infection experiments clearly demonstrated that absence of
the CNFY toxin renders the bacteria completely avirulent, resulting in the clearance of the bacteria in MLNs, liver and
spleen. A similar attenuation in mouse models of oral infection was observed (i) when the virulence plasmid, encoding the T3SS and the Yop effectors is cured from Y. pseudotuberculosis YPIII, (ii) when multiple yop genes were deleted or (iii) when the regulator LcrF that controls expression of the T3SS/Yops is absent [54,55].
Moreover, a significant influx of neutrophils was observed in the
spleen of mice infected with a yopM mutant strain of Y. pestis, while
the numbers of neutrophils decreased during infection with the
parental strain [20,21]. In addition, YopJ translocation has been shown to promote cell death of professional phagocytes [13,15].
This suggested that the CNFY toxin is important for the efﬁcient injection of the Yop effectors into host cells during the infection
process. In fact, recent work by Mejia et al. [26] demonstrated that
efficient translocation of the Yop effectors requires Rho activation
– a process that has been shown to be stimulated by the CNFY
in vivo [28,38].

To address whether CNFY-mediated activation of Rho
GTPases inﬂuences Yop-translocation into professional phagocytes,
we ﬁrst tested the inﬂuence of recombinant CNFY toxin on
non-activated and PMA-activated macrophages, thus mimicking
its effect on unstimulated and stimulated macrophages during
infection. Intoxiﬁcation of murine macrophages [774A1.1] led to
activation of all three Rho GTPases, RhoA, Cdc42 and Rac1
(Fig. 7A). CNFY further induced a marked increase in cell size with
some giant multinucleated cells (Fig. 7B). These CNFY effects
occurred independently of macrophage stimulation with PMA.
This indicates that CNFY controls actin dynamics in macrophages
through deamidation of Rho GTPases.

Since host actin polymerization by Rho activation plays a role
in Yop translocation by Y. pseudotuberculosis [26] we also tested the
influence of CNFY on Yop delivery. To do so, we generated Y.
pseudotuberculosis strains expressing a YopE-b-lactamase reporter
fusion [56], namely YP173 (YP173-ETEM), YP174 (YP101Δyop-ETEM), and YP217 (YP147Δyop/ETEM), and used these strains to
infect host cells treated with the dye CCF4-AM. CCF4-AM
consists of coumarin and ﬂuorescein conjugated by a lactam ring
and is modiﬁed by cellular esterases, whereby the dye becomes
green ﬂuorescent and is trapped inside the cell. If the b-lactam ring
is cleaved by b-lactamase the dye changes its ﬂuorescence from
green to blue [57,58]. The green to blue conversion allows
identiﬁcation of host cells in which the YopE-b-lactamase fusion
protein has been successfully injected. We ﬁrst used this
an excitation-based system to monitor translocation of the chimeric
protein into HEp-2 cells, and determined the number of green and
blue ﬂuorescent cells by ﬂuorescence microscopy and ﬂow
cytometry. Efﬁcient translocation of YopE-b-lactamase into
epithelial cells was observed upon infection with YP173 (YP111-
ETEM), but not with the secretion-deﬁcient control strain YP174
(YP101ΔyopX-ETEM) (Fig. 8A,B). YopE-b-lactamase translocation by
the yopD-deﬁcient strain YP217 (YP147Δyop/fETEM) was
signiﬁcantly reduced compared to YP173 (YP111-ETEM), whereas
preincubation of the host cells with CNFY increased translocation of
the fusion protein (Fig. 8A, B), indicating that CNFY enhances
effector delivery. Since Y. pseudotuberculosis predominantly injects
the Yops into professional phagocytes in vivo [59], we also tested
CNFY influence on YopE-b-lactamase translocation into murine macrophages, and found that pretreatment with CNFY also boosts
Yop delivery into these phagocytes (Fig. 8C).

Stimulation of Rac1 through YadA and invasin-bound b2-integrons was shown to be essential for Yersinia uptake into epithelial
cells [11], but neither internalization nor activation of Rac1 was
required for Yop translocation by Y. pseudotuberculosis into HeLa cells [26]. This suggested that CNFY-mediated stimulation of Yop
delivery into macrophages might preferentially be caused by
activation of RhoA. To validate this assumption, we pretreated macrophages with the Clostridium botulinum C3 toxin, an ADP-
riboseylating protein that speciﬁcally inhibits RhoA, B and C, or with
inhibitor reduced the percentage of blue macrophages signiﬁcantly,
whereas the Rac inhibitor had no inﬂuence on YopE-b-lactamase
translocation. These ﬁndings indicated that the CNFY toxin
enhances Yop delivery into murine macrophages, and in particular
activation of RhoA seems to play a role in the processes that
stimulate Yop translocation into these professional phagocytes.

It has been reported that translocated effector YopE of Y.
pseudotuberculosis YPIII is a GTPase-activating protein (GAP) for
Rac1 and RhoA and this function appears important to regulate
Yop translocation and modulate host defenses crucial for virulence
[62-65]. This raised the question how YopE and CNFY contribute
to RhoA-GTP and Rac1-GTP levels and Yop translocation. To
address this, we analyzed RhoA and Rac1 activation and Yop
translocation in the presence and absence of YopE in untreated
or CNFY-perturbed murine macrophages. As shown in Fig. 8C, the RhoA/B/C inhibitor reduced the percentage of blue macrophages signiﬁcantly,
whereas the Rac inhibitor had no inﬂuence on YopE-b-lactamase
translocation. These ﬁndings indicated that the CNFY toxin
enhances Yop delivery into murine macrophages, and in particular
activation of RhoA seems to play a role in the processes that
stimulate Yop translocation into these professional phagocytes.

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We next analyzed whether the CNF\textsubscript{Y} toxin affects Yop translocation into host cells in the original tissue environment. MLNs were harvested from uninfected mice and filtered to disrupt the tissue architecture and generate single-cell suspensions. Single cell suspensions were infected with a multiplicity of infection (MOI) of 10, incubated with CCF4-AM, and then analyzed by flow cytometry. As shown in Fig. S8, significantly higher numbers of blue cells with translocated YopE-\textsubscript{b}-lactamase were measured after infection with YPIII, indicating that Yop delivery into host cells can be enhanced by the toxin through activation of Rho GTPases.

**CNF\textsubscript{Y} enhances Yop delivery into phagocytes during infection**

It has been previously reported that \textit{Y. pseudotuberculosis} selectively targets Yops to professional phagocytes in the PPs, MLNs and spleen during the oral route of infection [59]. To analyze whether the CNF\textsubscript{Y} toxin also affects YopE-\textsubscript{b}-lactamase delivery in the course of an infection, we orally infected mice with \textit{2} \texttimes \textit{10}\textsuperscript{9} bacteria \textit{YP173} and the isogenic \textit{cnfY} mutant strain \textit{YP217}. The T3SS-deficient \textit{yscS} mutant, encoding the YopE-\textsubscript{b}-lactamase, and YPIII without the fusion were used as negative controls. At day three post infection mice were sacrificed, the PPs, MLNs, and spleen were harvested, and the translocation of Yops into various immune cell subsets was analyzed by flow cytometry (Fig. S9).

Following infection with the YopE-\textsubscript{b}-lactamase expressing wild-type strain 4.5% of all living cells within PPs were affected by Yop translocation. In contrast, only 1.5% of all living cells in the PPs contained the fusion protein after infection with the \textit{cnfY}-deficient strain (Fig. S10). Yop translocation efficiency was still significantly reduced in tissues infected with the \textit{cnfY}-deficient strain when the percentage of translocated blue cells was normalized to the...
bacterial load of the tissue/organ (Fig. 9). This excludes that lower bacterial numbers account for this effect, but it also assumes that bacteria are infecting different cell types at the same MOI, which is unknown. Yop delivery was also significantly lower in the absence of the CNFY toxin in MLNs and spleen in which the total number of targeted cells was reduced compared to PPs (Fig. 9, S10). We further determined whether CNFY-mediated stimulation of Yop translocation affected specific immune cells more frequently than others. Translocation of YopE-β-lactamase into each immune cell type was compared in MLNs and spleen from mice infected with YP173 (YP173), YP147 ΔcnfY-ETEM (YP217), and YP101 ΔyscS-ETEM (YP174). Cells were labeled with CCF4-AM and analyzed by fluorescence microscopy. Bar: 20 μm (B) HEP-2 cells were untreated or treated with 3 μg/ml (25 nM) purified CNFY toxin for 3 h prior to the infection with Y. pseudotuberculosis strains YP173 (YP173), YP147 ΔcnfY-ETEM (YP217) or YP147 ΔcnfY-ETEM pregrown at 37 °C using a MOI of 10. Cells were labeled with CCF4-AM and percentage of blue HEP-2 cells and macrophages was determined. The graph represents data of two (B) or three (C) independent experiments with 5–6 wells per group. The asterisks indicate the significant difference in the quantity of translocated cells upon infection with the different strains or after toxin treatment based on a Mann-Whitney test with *** (P<0.001).

doi:10.1371/journal.ppat.1003746.g008
Figure 9. Absence of CNF\textsubscript{Y} reduces Yop delivery into neutrophils, macrophages and DCs in PPs, MLNs and spleen during infection. (A) BALB/c mice were orally infected with $2 \times 10^9$ cfu of YP111-ETEM (YP173) and YP147ΔcnfY-ETEM (YP217). YP111 and YP101 ΔyscS-ETEM (YP174) were used as negative controls. Day three post infection the MLNs, liver and spleen were isolated and filtered to generate single-cell suspensions. Cells were labeled with antibodies to the indicated surface markers for macrophages, DCs, neutrophils, NK cells, B and T cells and incubated with CCF4-AM. The percentage of blue cells was analyzed by flow cytometry (see also Fig. S10). Data of 8 mice of which the bacterial burden in the organs has been determined in parallel were normalized to the bacterial loads to determine Yop translocation efficiency. (A) Detection of green and blue cells by flow cytometry in PP, MLNs and spleen of mice infected with YP173 and YP217. The Yop translocation efficiency into all living cells is plotted. (B) The percentage of blue cells of the different analyzed cell types of the MLNs and the spleen were determined and normalized to the bacterial load in the
demonstrate that the CNF\textsubscript{Y} toxin plays a critical role during the infection, facilitating targeting of Yops to host immune cells, in particular professional phagocytes.

**Discussion**

Many bacterial toxins and translocated effector proteins target Rho GTPases, which control crucial eukaryotic signal transduction pathways involved in the organization of the cell cytoskeleton, cell cycle progression, genetic information processing, and host defense processes to promote invasion, survival and replication of pathogens within their hosts [29,67]. In this study we investigated the Rho-activating cytotoxic necrotizing factor CNF\textsubscript{Y} of *Yersinia*. Although much progress has been made unravelling the molecular mechanism of this toxin, the functional consequences for host-pathogen interaction and pathogenesis were largely unknown. Using a murine model for gastrointestinal tract infections we provide evidence that this Rho-activating protein is crucial for virulence of the naturally toxin-expressing *Yersinia*. Previous work demonstrated that CNF\textsubscript{Y} predominantly activates cell cycle progression, genetic information processing, and host-infection pathways involved in the organization of the cell cytoskeleton, Rho GTPases, which control crucial eukaryotic signal transduction pathways involved in the organization of the cell cytoskeleton, cell cycle progression, genetic information processing, and host defense processes to promote invasion, survival and replication of pathogens within their hosts [29,67].

Using a murine model for gastrointestinal tract infections we demonstrate that the CNF\textsubscript{Y} toxin plays a critical role during the infection, facilitating targeting of Yops to host immune cells, in particular professional phagocytes.

### Histological analysis and immune cell composition of the infected tissues suggest that CNF\textsubscript{Y} contributes significantly to the acute characteristics of the inflammatory response and host tissue damage during infection. Histo-pathologic evaluation underlines the finding that CNF\textsubscript{Y} induces apoptosis, as focal necrosis was not seen in YP147-infected animals. Cell death leads to atrophy of the spleen in YPPIII-infected mice. Moreover, a restriction of the inflammation to small foci could be observed in the intestine of YP147-infected animals, whereas the entire ileum was affected by a diffuse inflammation in YPPIII-infected animals, explaining the shortening of the intestine. Hyperplasia of the white pulp seen in YP147-infected mice displays the immune response triggered by the infection. The infection is restricted to small foci in the intestine and is reversible, whereas the infection in YPPIII infected animals is generalized and most probably leads to death by endotoxemia. This inflammatory necrotizing phenotype is reminiscent of earlier studies analyzing the effect of CNF1 of *E. coli* using subcutaneous injections as well as animal models of urinary tract and prostatitis infection [31,68,69].

Infections of the gastrointestinal tract by enteropathogenic *Yersinia* lead to a biphasic inflammatory process in which bacterial adhesion and transmigration through the intestinal epithelial layer triggers an initial antibacterial defense response with little inflammation, e.g. expression of IL-8 by epithelial cells, which is followed by an acute infiltration and activation of neutrophils, cytokine production and tissue necrosis [70]. First recognition of *T. pseudotuberculosis* occurs through contact of the bacterial LPS with TLR4 on naive host macrophages and this leads to proinflammatory cytokine production through activation of MAPK and NF\textsubscript{κ}B. However, translocation of Yop\textsubscript{J} inhibits activation of MAPK and NF\textsubscript{κ}B and induces an apoptotic signaling pathway including activation of initiator caspase-8, and the executioner caspase-3, -7, and -9 [70,71]. Apoptotic macrophages are eliminated and this process also triggers production of anti-inflammatory cytokines such as IL-10 and TGF-\textbeta [72,73]. However, induction of apoptosis is probably not always fully immunologically silent, e.g. phagocytosis of apoptotic cells by other phagocytes, can prime other immune responses such as activation of CD8\textsuperscript{+} T cells [71]. During the course of the infection, the number of activated macrophages increases whereas the number of naive macrophages declines. In activated macrophages *Yersinia* causes cell death by inflammatory pyroptosis. This occurs through activation of a multiprotein complex, called the inflammasome, which forms a platform for the autoprocessing and activation of the cysteine protease caspase-1. Activation of caspase-1 results in the secretion of the inflammatory cytokines such as IL-1\textbeta, IL-1\textbeta, and IL-18, and triggers cell death [66]. LPS, the T3SS and the translocated Yop\textsubscript{J} protein of *Yersinia* were shown to induce caspase-1 activation and pyroptosis [71,74].

Induction of pyroptosis (inflammatory death) in activated macrophages corresponds to later stages of the infection with *T. pseudotuberculosis*, where inflammation and necrosis is evident from histopathology. Based on our results it is very likely that CNF\textsubscript{Y} supports *Yersinia*-induced pyroptosis of activated phagocytes in the spleen during later stages of the infection. CNF\textsubscript{Y} was shown to manipulate the number of immune cells and induce inflammatory responses. The number of macrophages, monocytes and neutrophils decreased strongly (4- to 15-fold) in all lymphoid organs three days post infection. In contrast, infection with the *cnf\textsubscript{Y}* mutant resulted in no reduction, but rather an increase of phagocytes when compared to uninfected control mice, and the overall inflammation of the infected tissues was considerably reduced. Similar to CNF1 of *E. coli* [34], it is possible that CNF\textsubscript{Y} is transported by OMVs, which act as long-range toxin delivery vectors. We further demonstrate that CNF\textsubscript{Y} enhances Yop delivery into phagocytes during infection. This strongly suggests that increased translocation of Yop\textsubscript{J} could stimulate cell death in the spleen. Consistent with this assumption, Yop\textsubscript{J} and CNF\textsubscript{Y} promote systemic dissemination following oral infection. Work by Monack et al. [75] showed that a *yop\textsubscript{J}* mutant is deficient for spread from the PPs to other lymphoid tissues (MLNs, spleen), similar to the *cnf\textsubscript{Y}* mutant investigated in this study. Moreover, wild-type *Yersinia* induce apoptosis of macrophages from infected spleens [75], implying that mainly Yop\textsubscript{J} is used to eliminate immune cells in the spleen to dampen the immune response against *Yersinia* during infection. Yop\textsubscript{J} was also shown to subvert the NOD2/RICK/TAK1 pathway, activate caspase-1 and induce IL-1\textbeta secretion within PPs, which is associated with increased barrier permeability [76]. This suggests that CNF\textsubscript{Y} action also enhances Yop\textsubscript{J}-dependent intestinal barrier disruption and promotes the dissemination of *Yersinia* by exploiting the mucosal inflammatory response. In addition, CNF\textsubscript{Y} seems to contribute to depletion of NK cells in the spleen. It was observed that *T. pseudotuberculosis* had a significant global decrease in NK cell numbers [21], indicating that NK cell depletion is enhanced by CNF\textsubscript{Y}-mediated activation of YopM translocation.

Here, we observed that Rhoa, Racs1 and Cdc42 are activated in CNF\textsubscript{Y}-intoxicated macrophages, which is reflected by the high content of actin cables/stress fibres, the formation of lamellipodia and filopodia, pronounced cell spreading and inhibited cytokinesis. Previous work demonstrated that CNF\textsubscript{Y} predominantly activates Rhoa in epithelial cells [27,28]. However, a very recently published study also reported CNF\textsubscript{Y}-mediated activation of...
Rac1 and Cdc42 in HeLa cells [77]. Use of different toxin concentrations, incubation times and cell types (human epithelial cells versus murine macrophages) in which the CNF toxins may display a different selectivity and different efficiencies of cell toxicities are likely to account for these variations. In fact, CNFv-mediated Rho GTPase activation pattern varies during intoxication whereby RhoA activation is generally more pronounced than Rac1 and Cdc42 2–3 h after toxin addition [J. Schweer, unpublished results; [77]]. This suggests that at very early time points and/or under low toxin concentrations predominantly RhoA might be activated. A previous report demonstrates that *Y. pseudotuberculosis* selectively modulates RhoA activity (e.g. by signals triggered by the YopB/D translocus and/or from engagement of β₂-integrin receptors) to induce cellular changes that control TSS pore formation and effector translocation [26]. Here, we strengthen this observation, as CNFv-mediated stimulation of Yop delivery of *Y. pseudotuberculosis* was sensitive to the Rho inhibitor C3-transferase of *C. botulinum*, but insensitive to Rac1 inhibition by TcdBF toxin of *C. difficile*. In contrast, new experiments addressing the influence of CNFv on Yop translocation of *Y. enterocolitica* demonstrated that CNFv also stimulates effector delivery by this pathogen, although this process seemed entirely dependent on Rac and not on Rho GTPases [77]. Different YadA/InvA-promoted signalling events, differences in Yop protein abundance (e.g. RhoA-inactivating YopT is absent in YPIII) and differences in the regulation of Yop delivery by Rho GTPases between the different species may be responsible for this discrepancy.

Some effector proteins, in particular YopE, were shown to inhibit Yop delivery by inactivation of RhoA and Rac1 most likely as part of an intra-cellular control mechanism which measures and adapts the amount of protein translocated by *Yersinia* during infection. This is reflected by elevated levels of Yop effector translocation into epithelial cells by yopE-deficient strains [11,63–65,78]. Our analysis demonstrated that absence of YopE caused no or only a small increase in Rac1/RhoA activation and Yop translocation during infection of murine macrophages with *Y. pseudotuberculosis* YPIII with or without treatment with CNFv. This indicates that intracellular YopE is not able to counteract CNFv in these phagocytes. Recently published work showing that none of the Rho inhibiting effectors (YopE, YopT and YopO) could reduce the effect of CNFv on Yop translocation by *Y. enterocolitica* into human epithelial cells supports this observation [77]. However, we cannot exclude the possibility that other conditions (e.g. conditions which enhance (i) deamidation and subsequent ubiquitin-dependent degradation of the modified Rho GTPases or (ii) YopE translocation and activity) allow counterregulation.

Very recently, it has also been reported that NOD1, a pattern recognition receptor that senses cytosolic microbial products similar to NOD2, monitors the activation state of all three Rho GTPases. Activation of Rho GTPases triggered the NOD1 signalling cascade with consequent RIP2-mediated induction of NF-κB-dependent inflammatory responses [79]. NOD1 activation was triggered by activation of Rac1 and Cdc42 by the *Salmonella* effector SopE. In line with this, all three Rho GTPases, Rac1, RhoA and Cdc42 were previously shown to activate the NF-κB pathway [80] and particular Rac1 has been reported to contribute to NF-κB activation by CNF1 of *E. coli* by clustering the NF-κB inhibitor IkBa and components of the IkBζ E3-ubiquitin ligase into membrane ruffles [79].

Based on our current knowledge we envision a model in which the CNFv toxin exerts its function in a multi-step process (Fig. 10). The first step corresponds to the uptake of the CNFv toxin by infiltrating innate immune cells (e.g. neutrophils, macrophages, DCs) in the early phase of the infection process. This triggers activation of the Rho GTPases, in particular RhoA, in the phagocytes. Induced actin polymerization resulting from Rho GTPase activation enhances Yop delivery into host cells to counteract innate and adaptive immune responses. As a consequence, invading immune cells are inhibited and undergo apoptosis leading to uncontrolled proliferation of the pathogens. Higher CNFv toxin concentrations by replicating pathogens potentiate activation of RhoA, Rac1 and Cdc42 which triggers inflammatory responses e.g. via the NOD1-RIP2 signalling cascade. In addition, interaction of *Yersinia* with increased numbers of activated macrophages causes cell death by inflammatory pyroptosis leading to strong inflammation and necrosis of the organs during later phases of the infection process. In summary, our data identify CNFv as an important Rho GTPase-activating toxin which is instrumental for *Yersinia* to amplify crucial virulence factor functions which determines the success of the infection and the severity of the associated disease.

### Material and Methods

#### Ethics statement

All animal work was performed in strict accordance with the German Recommendations of the Society for Laboratory Animal Science (GV-SOLAS) and the European Health Recommendations of the Federation of Laboratory Animal Science Associations (FELASA). The animal protocol was approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittel sicherheit: animal licensing committee permission no. 33.9-42502-04-055/09. Animals were handled with appropriate care and welfare, and all efforts were made to minimize suffering.

#### Bacterial strains, cell culture, media and growth conditions

The strains used in this study are listed in Table 1. Overnight cultures of *E. coli* were routinely grown at 37°C, *Yersinia* strains were grown at 25°C or 37°C in LB (Luria-Bertani) broth. The antibiotics used for bacterial selection were as follows: carbenicillin 100 μg/ml, chloramphenicol 30 μg/ml, kanamycin 50 μg/ml, and gentamicin 50 μg/ml. For infection experiments, bacteria were grown at 25°C or 37°C, washed and diluted in PBS prior to infection. For invasion assays and mouse infections, bacteria were grown to stationary phase, washed and resuspended in PBS. For the *in vitro* Yop delivery assay, bacteria were grown in LB medium at 37°C.

Human HEp-2 cells were cultured in RPMI 1640 media with an alternative to L-glutamine with increased stability (Invitrogen) supplemented with 7.5% newborn calf serum (Sigma Aldrich). Murine J774A.1 macrophages were cultured in the same medium supplemented with 5% fetal calf serum (PAA). All cell lines were cultivated at 37°C in the presence of 5% CO₂.

#### DNA manipulations and construction of plasmids

All DNA manipulations, PCR, restriction digestions, ligations and transformations were performed using standard techniques as described previously [81,82]. Plasmids used in this study are listed in Table 1. Plasmid pJNS01 (cgrTaux) was constructed by amplification of cgrT (yph_2615) from genomic DNA of *Y. pseudotuberculosis* YPIII with primers I1795/I1795 and integrated into the XhoI/NheI sites of pET28a (Novagen).

For construction of the luxCDABE-gfpmut3.1 and lacZ reporter gene fusions encoded on plasmids pJNS2-4, the promoter region of cgrT (primer pair II996/II998) was amplified and ligated into the BamHI/SalI sites of pFU54, pFU58 and pFU68.
Plasmid pJNS10, containing the cnfY promotor region with the cnfY gene, was constructed by the insertion of a PCR fragment amplified with primers II896 and IV16 from chromosomal DNA of Y. pseudotuberculosis YPIII into the BamHI/NotI sites of pFU234. All clones were confirmed by sequencing and restriction.

Construction of mutant strains

For generation of the mutant strain YP147, a cnfY::KanR mutation encoded on plasmid pJNS05 was constructed. To do so, the kanamycin resistance gene was amplified using the kan primers (I661/I662) and plasmid pACYC177 as template. Next, Y. pseudotuberculosis YPIII genomic DNA was used as a template to amplify 500-bp regions flanking the target gene cnfY. The upstream fragment was amplified with the primer pair III710/715 of which the reverse primer contained 20 nt at the 5'-end which are homologous to the start of the kanamycin resistance gene. The downstream fragment was amplified with primer pair III712/III714 of which the forward primer contained additional 20 nt at the 3'-end which are homologous to the end of the kanamycin resistance gene. A PCR reaction was performed with the forward primer and the reverse primer using the upstream and downstream PCR products of the target gene and the kanamycin gene fragment as templates and cloned into pAKH3. The resulting plasmid pJNS05 was integrated into the cnfY locus of YPIII via conjugation as described [83]. Chromosomal integration of the fragments was selected by plating on LB supplemented with kanamycin. Excision of the plasmid including the defective cnfY allele of YPIII was obtained by plating of the strain on 10% sucrose and generated strain YP147 was analyzed by PCR and DNA sequencing. For the construction of strain YP56 (DphoPQ), a phoPQ::KanR PCR fragment was generated. For this purpose, the kanamycin resistance cassette was created using the primers 360/361 and pACYC177 as template. The primes contain homology regions (20 nt) to the upstream or downstream region of the phoPQ gene. A fragment including sequences of the phoPQ upstream region was generated by PCR using the primers 538/539, a fragment including sequences of the phoPQ downstream region was amplified using the primers 540/541. Primer 539 and 540 contain 20 nt of the kanamycin resistance cassette. A PCR fragment consisting of these three fragments was amplified using the primers 538/541. The product was transformed into Y. pseudotuberculosis YPIII pKOBEG-sacB and a phoPQ::KanR mutant (YP56) was generated and selected for as described [84]. For the construction of YP149, the phoPQ gene of strain IP32953 was amplified by PCR with primer III926 and III927, creating SacI and XhoI restriction sites. The fragment was cloned into plasmid pDM4, generating vector pVP1. Integration of the plasmid was obtained through conjugation of strain S17pir pVP1 with the YP56 as described [83]. Excision of the plasmid including the defective phoPQ allele was obtained by plating of the strain on 10% sucrose. PCR and DNA sequencing proved presence of the intact phoPQ allele. To generate strain YP188 the cnfY gene was destroyed as described.

Figure 10. Model of CNFγ toxin function. Adhesin-mediated interaction of Y. pseudotuberculosis with macrophages induces activation of RhoA which induces T3SS-promoted Yop delivery into the phagocyte. Yop translocation leads to blockage of phagocytosis and YopJ-mediated apoptotic cell death. In CNFγ-producing Y. pseudotuberculosis, prior or parallel to the interaction of the bacteria with phagocytes, the CNFγ toxin is secreted via outer membrane vesicles. Most likely CNFγ-containing vesicles are internalized through receptor-mediated endocytosis, transferred via early endosomes (EE) to acidic compartments (late endosomes, LE) where the toxin is translocated across the bilayer into the cytosol by a low pH-dependent mechanism. Liberation of CNFγ leads to activation of small Rhô GTPases, in particular RhôA, and this amplifies Yop delivery and cell death. Rhô GTPase activation was shown to promote pore formation of the T3SS, which appear to activate caspase-1 [26,74]. Furthermore, enhanced cell death can lead to an accelerated proinflammatory immune response known to raise the level of activated macrophages in which Yersinia triggers activation of caspase-1 [66]. Induction of caspase-1 leads to secretion of inflammatory cytokines and triggers cell death by inflammatory pyroptosis. In parallel, uncontrolled proliferation of the bacteria will lead to higher toxin concentrations, which will further enhance activation of Rac1, Cdc42 and RhôA and potentiate inflammatory pyroptosis.

doi:10.1371/journal.ppat.1003746.g010
| Strains, Plasmids | Description | Source and reference |
|------------------|-------------|---------------------|
| **Bacterial strains** | | |
| E. coli K-12 | | |
| DH101 | F− endA1 recA1 galE16 rpsL ΔlacZΔM15 araD139 Δara,leuUV57697 mcrA [Δmrr-hsdRMS-mcrBC] λ− | Invitrogen |
| BL21 λDE3 | F− ompT gal dcm lon hsdB(rpo−m5) gal λDE3 | [86] |
| CC118 λpir | F− Δara-leu7697 ΔlacZΔM15 ΔphoA20 araD139 galE galK thi rpsE rpoB arfE recA1 | [87] |
| S17-1 λpir | Ty* Sm*, recA, thi, pro, hisD, M* RP4.2-Tc:MuKm Tn7 | [88] |
| **Y. pseudotuberculosis** | | |
| YP111 | pBl1, wild-type | [89] |
| YP56 | YP111 ΔphoPQ, KanR | this study |
| YP101 | YP111 ΔyscS, KanR | Rebekka Steinmann |
| YP147 | pBl1, ΔcnfY, KanR | this study |
| YP149 | YP111 phoPQ+p22353 | this study |
| YP173 | YP111 ETEM, amino acids 1 to 100 of YopE+TEM1 | this study |
| YP174 | YP111 ΔyscS ETEM, amino acids 1 to 100 of YopE+TEM1 | this study |
| YP188 | YP149 ΔcnfY, KanR | this study |
| YP217 | YP147 ETEM, amino acids 1 to 100 of YopE+TEM1 | this study |
| YP275 | pBl1, ΔyopE, KanR | this study |
| IP32953 | wild-type, pYV | [90] |
| **Plasmids** | | |
| pACYC177 | cloning vector, p15, ApR, KanR | [91] |
| pAKH3 | pGP704, sacB+, AmpR | [83] |
| pDM4 | R6K derivative, sacB+, CmR | [92] |
| pET28a | T7 promoter based expression vector, KanR | Novagen |
| pFU54 | promoterless luxCDBE, pSC101* ori, AmpR | [93] |
| pFU58 | promoterless gfpmut3.1, pSC101* ori, AmpR | [93] |
| pFU68 | promoterless lacZ, pSC101* ori, AmpR | [93] |
| pFU166 | gapA-luxCDBE, colE1 ori, AmpR | [93] |
| pFU228 | gapA-dsRed2, colE1 ori, CmR | [93] |
| pFU234 | dip*, pSC101* ori, KanR | [94] |
| pKOBEG-sacB | recombination vector, sacB+, CmR | [95] |
| pSR47s-E-TEM2 | YopE-TEM1, KanR | [56] |
| pJSN01 | pET28a, cnfY, KanR | this study |
| pJSN02 | pcnfY-luxCDBE, pSC101* ori, AmpR | this study |
| pJSN03 | pcnfY-gfpmut3.1, pSC101* ori, AmpR | this study |
| pJSN04 | pcnfY-lacZ, pSC101* ori, AmpR | this study |
| pJSN05 | pAKH3, cnfY, KanR, sacB+, AmpR | this study |
| pJSN09 | pFU234, AmpR | this study |
| pJSN10 | pJSN09, cnfY, pSC101* ori, AmpR | this study |
| pJSN11 | pJSN09, pSC101* ori, AmpR | this study |
| pJSN13 | pAKH3, yopE-KanR, sacB+, AmpR | this study |
| pVP1 | pDM4, phoPQ+p22353 | this study |
| **Primers** | | |
| 360 | 5’-GGTTATTTGAACTTTTCTGTTG-3’ | |
| 361 | 5’-CCAGTTGACACCAAATGAAA-3’ | |
above for YP147 (ΔopT). Strains YP173, YP174 and YP217 were constructed by chromosomal integration of the YopE-β-lactamase (ETEM) fusion plasmid pSR47s-E-TEM1 into the yopE locus. Integration was obtained through conjugation of E. coli K-12 strain S17Δpir pSR47s-E-TEM1 with the Y. pseudotuberculosis strains YPIII, YP101 (ΔyseS) and YP147 (ΔopT) as described [83]. For generation of the mutant strain YP275, a yopE::KanR mutation encoded on plasmid pJNS01 was constructed as described above for YP147. Primer V553/V554 and V555/V556 were used to amplify 500-bp regions flanking the target gene for YP147. Primer V553/V554 and V555/V556 were used to amplify 500-bp regions flanking the target gene for YP147. Primer V553/V554 and V555/V556 were used to amplify 500-bp regions flanking the target gene for YP147. Primer V553/V554 and V555/V556 were used to amplify 500-bp regions flanking the target gene for YP147.

β-galactosidase assays

β-galactosidase activity was determined of three independent cultures of bacteria harboring the yopE-lacZ fusion as described [83]. The activities were calculated as follows: [β-galactosidase activity OD_{420} • 6.75 OD_{600}^{-1} • Δt (min)^{-1} • vol (ml)^{-1}.

Purification of CNF\textsubscript{Y}-His\textsubscript{6}

For overexpression of CNF\textsubscript{Y} E. coli strain BL21ΔDE3 was transformed with the cnfY expression plasmid pJNS01 and grown at 37°C in LB medium to an OD\textsubscript{600} of 0.6. Subsequently, P\textsubscript{cnfY}-driven expression was induced upon addition of 250 μM IPTG and grown at 17°C overnight. CNF\textsubscript{Y}-His\textsubscript{6}, production was tested by western blot analysis using an antibody directed against the His-tag (Qiagen). For purification of CNF\textsubscript{Y}, cells were harvested, resuspended in 50 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 8.0, 300 mM NaCl, 10 mM imidazole and lysed with a French press (120,000 psi). The soluble CNF\textsubscript{Y}-His\textsubscript{6} extract was separated from insoluble cell material by centrifugation at 25,000 g. The CNF\textsubscript{Y}-His\textsubscript{6} protein was then purified by affinity chromatography on Ni-NTA agarose (Qiagen). The column was washed with three column volumes of 50 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 8.0, 300 mM NaCl, 20 mM imidazole and eluted with 50 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 8.0, 300 mM NaCl containing 250 mM imidazole.

Visualization of the actin cytoskeleton

In order to study the influence of the recombinant CNF\textsubscript{Y} protein on actin cytoskeleton rearrangements, 1 × 10^6 J774A.1 cells were incubated with purified CNF\textsubscript{Y} toxin (10 nM) or PBS for 24 h. Subsequently, cells were fixed with 4% paraformaldehyde (in PBS) for 10 min at room temperature, washed with PBS and permealized with 0.1% Triton X-100 in PBS for 5 min. The actin cytoskeleton was stained with Phalloidin-FITC (0.5 μg/ml PBS; Invitrogen) for 15 min at room temperature. Cells were washed in PBS, and the nuclei were stained with DAPI (1 μg/ml in TBST) for 5 min at room temperature. Cells were visualized using a fluorescence microscope (Axiovert II with Axiocam HR, Zeiss, Germany) and the AxioVision program (Zeiss, Germany).

Rho GTPase activation assay

Activation of RhoA was tested using the Rho activation assay kit 17-294 (Millipore, Billerica, MA, USA) and activation of Rac1 and Cdc42 was determined with the Rho/Rac/Cdc42 Activation Assay Combo Kit (Cell Biolabs, San Diego, CA, USA). Approximately 1 × 10^6 cells of the macrophage cell line J774A.1 were starved for at least 20 h in RPMI 1640 without FCS, and incubated with PBS or 25 nM (3 μg/ml) recombinant CNF\textsubscript{Y} for 2–3 h. To test the influence of YopE on Rac1 and RhoA activation, macrophages were subsequently infected for 20 min with wild-type strain YPIII or the isogenic yopE mutant YP275.
with an MOI of 100. Cells were lysed and activation of small Rho GTPases was tested and visualized by western blotting according to the manufacturer’s protocol.

**Yop delivery assay**

The Yop delivery assay was performed as described previously [85]. 5×10⁶ (for fluorescence microscopy) or 1×10⁶ (for flow cytometry) HEp-2 or J774A.1 cells were incubated with recombinant CNF₇ (25 nM/3 µg/ml), exoenzyme C3 transferase from *C. botulinum* (CTD4, Cytoskeleton. Inc) (0.5 µg/ml, 1 µg/ml), *C. difficile* toxin Tcβlf (85 ng/ml, 250 ng/ml) [60,61], or the same amount of PBS before the cells were infected with bacteria with a MOI of 10. After 1 h cells were washed and dried with CCF4-AM and according to the manufacturer’s protocol using the LiveBLAzer-FRET B/G Loading Kit from Life Technologies. Yop translocation was visualized by a fluorescence microscope (Axiovert II with AxioVision HR, Zeiss, Germany) or detected with an LSR Fortessa cell analyzer (Zeiss, Germany) using the AxioVision program (Zeiss, Germany) or detected with an LSR Fortessa cell analyzer (BD Bioscience). Acquired data of flow cytometry were then analyzed with FlowJo software (Treestar).

To compare Yop translocation of YPIII and YP275 (ΔyopE), both strains were pregrown at 37°C and added with an MOI of 100 to approximately 1×10⁶ cells of murine macrophages incubated with PBS or 25 nM recombinant CNF₇ for 3 h. One hour post infection, cells were washed with PBS, resuspended in SDS sample buffer and separated on 12% SDS polyacrylamide gels. Proteins were blotted onto a membrane and intracellular localization was visualized by a fluorescence microscope (Axiovert II with AxioVision HR, Zeiss, Germany) or detected with an LSR Fortessa cell analyzer (BD Bioscience). Acquired data of flow cytometry were then analyzed with FlowJo software (Treestar). For the *in vitro* analysis of Yop delivery into primary cells, mesenteric lymph nodes from uninfected 6- to 8-week-old BALB/c mice were removed. To generate single-cell suspensions the cells were rinsed with sterile PBS and incubated with 100 µg/ml gentamicin in order to kill bacteria on the luminal surface. After 30 min, gentamicin was removed by washing with PBS. Subsequently, all organs were weighted and homogenized in PBS at 30,000 rpm for 30 sec using a Polytron PT 2100 homogenizer (Kinematica, Switzerland). To determine the bacterial load of the organs serial dilutions of the homogenates were plated on LB plates with and without antibiotics. The colony forming units (cfu) were counted and are given as cfu per g organ/tissue.

**Yop delivery assay during mouse infection**

BALB/c mice were infected intragastrically with 2×10⁶ bacteria of strain YPIII-ETEM (YP173) and the isogenic *cnfY* mutant YP147-ETEM (YP217), wild-type YPIII and YP101-ETEM (YP174). Infection was allowed to proceed for three days. Subsequently, the infected lymphatic tissues (PPs, MLNs, and spleen) were isolated and single cell suspensions were generated in PBS by pressing the cells through a cell strainer (70 µm, Falcon). To eliminate erythrocytes, spleen cells were incubated for 3 min in lysis buffer (7.8 mM NH₄Cl, 10 mM KHCO₃, 100 µM EDTA). All cells were resuspended in PBS containing 0.2% BSA and total cell number was determined using an Accuri C6 flow cytometer (BD Bioscience). For flow cytometry analysis 1×10⁶ cells were transferred per tube and FcγR was blocked using CD16/CD32 (Becton Dickinson). Anti-mouse CD16/CD32 antibody for 15 min at 4°C. Immune cells were first stained for 15 min at 4°C using a biotin–conjugated antibody against CD19. Subsequently, other cellular surface marker for innate immune cells or T cell panel were conjugated antibody against CD19. Subsequently, other cellular surface marker for innate immune cells or T cell panel were conjugated. Cells were then stained for 15 min at 4°C in FACS buffer (PBS+0.2% BSA) using the following antibodies: SA-PerCP-Cy5.5, CD11c-APC, CD11c-PE-Cy7, Gr1-A750, CD3-PE, CD4-APC-Cy7, CD3-PE-Cy7, NKp46-PE and CD25-APC. Samples were washed twice in FACS buffer and labeled with 1 µg/ml CCF4-AM using the LiveBLAzer-FRET B/G Loading Kit (Life Technologies) for 1 hour at 20°C in the presence of 1.5 mM probenecid (Sigma) and 50 µg/ml gentamicin. Cell subsets were defined as following: B cells (CD19⁺ CD3⁻), T cells (CD19⁻ CD3⁺), NK cells (CD19⁻ CD3⁻ NKp46⁺), neutrophils (CD19⁻ CD3⁻ CD49b⁺ Ly6G⁺ CD11b⁺), macrophages/monocytes (CD19⁻ CD3⁻ CD49b⁻ Ly6G⁺ CD11b⁻), and DCs (CD19⁻ CD3⁻ CD49b⁻ Ly6G⁻ B220⁻ F4/80⁺ CD11c⁺). Cells were analyzed in a LSR Fortessa cell analyzer (BD Bioscience). Acquired data were analyzed with FlowJo software (Treestar). Cells from tissues that were not treated with CCF4-AM and/or antibodies were used as negative controls.

**CNF₇ influence in the host immune response**

To characterize the host immune response induced upon infection with the wild-type strain YPIII or the isogenic *cnfY* mutant strain YP147, mice were orally infected with approximately 2×10⁶ bacteria of *Y. pseudotuberculosis* strains YPIII or YP147 (Δ*cnfY*). Three and six days after infection, PPs, MLNs and spleen were isolated. Single cell suspensions were obtained by mechanical disruption of the organs through a cell strainer. To eliminate erythrocytes, spleen cells were also incubated for 3 min in erythrolysis buffer (7.8 mM NH₄Cl, 10 mM KHCO₃, 100 µM EDTA). All cells were resuspended in FACS buffer and total cell number was determined using an Accuri C6 flow cytometer (BD Bioscience). Amounts of 1–2×10⁶ CFU of the homogenates were plated on LB (Kinematica, Switzerland). To determine the bacterial load of the organs serial dilutions of the homogenates were plated on LB plates with and without antibiotics. The colony forming units (cfu) were counted and are given as cfu per g organ/tissue.
Cellular surface markers for either lymphoid or myeloid panel were stained for 15 min at 4°C in PBS-BSA (0.2%) using the following antibodies: CD3-APC, CD4-PerCP-Cy5.5, CD8- 

In vitro expression of CNFY

YPIII harboring a PfoN::gfpmut3.1 fusion (pJS03) and a 
PfoN::dRed2 expression construct (pFU226) were grown in LB medium at 25°C overnight. Mice were infected orally with 2×10^8 bacteria. After five days mice were sacrificed by CO_2 asphyxiation. For sections, the small intestine, colon, caecum, MLNs, spleen and liver were frozen in Tissue-Tek OCT freezing medium (Sakura Finetek) on dry ice. Sections of 8–10 μm were made using a Zeiss cryostat Hryax C50 and mounted on SuperFrost Plus slides (Thermo Scientific). Air-dried sections were fixed for 10 min in ice-cold acetone and washed twice with PBS. For visualization of the nuclei in the fixed tissue, samples were stained with 49,6-diamidino-2-phenylindole (DAPI, Sigma) for 3–5 min, air-dried in ice-cold acetone and washed twice with PBS. For visualization of the bacteria were visualized by a fluorescence microscope (Axiovert II with Axioscan HR, Zeiss, Germany) using the AxioVision program (Zeiss, Germany).

To detect the cnfY gene expression during the infection process Y. pseudotuberculosis wild-type strain YPIII harboring the PfoN::lacZ fusion vector pJS02 or the empty vector pFU54 were grown in LB medium at 25°C overnight. Mice were infected orally with 2×10^8 bacteria. After five days mice were sacrificed by CO_2 asphyxiation. Biotin-conjugated antibodies were incubated with streptavidin for 15 min at 4°C. After staining cells were fixed with the Foxp3 staining buffer set from eBioscience. Incubated with streptavidin for 15 min at 4°C and the strains YPIII pJNS10 (cnfY') (green line) and YPI147 pJNS10 (cnfY') (blue line).

In vivo expression of CNFY

YPIII harboring a PfoN::gfpmut3.1 fusion (pJS03) and a 

Figure S2 Influence of cnfY on the bodyweight of BALB/c mice infected with Y. pseudotuberculosis. Weight of the spleen (A) and the liver (B) of BALB/c mice (n = 10/strain) were monitored up to 14 days after oral infection with 2×10^8 cfu of Y. pseudotuberculosis YPIII (black), the cnfY mutant YP147 (red,). Length of the intestine of BALB/c mice infected with Y. pseudotuberculosis YPIII (black), the cnfY mutant YP147 (red,). Stars indicate results of the organs infected with YP147 that differed significantly from those infected with YPIII with * (P<0.05), ** (P<0.01) and *** (P<0.001).

Gating strategy of splenocytes from YP147 

Figure S5 Gating strategy for the analysis of immune cells recruited to the spleen after infection with Y. pseudotuberculosis YPIII or YP147. Exemplary gating strategy of splenocytes from YP147 (ΔcnfY)-infected mice at day three post infection. (A) T cells = CD19^-CD3^+, B cells = CD19^-CD3^-, natural killer (NK) cells = CD19^-CD3^-NKp46^+. (B) Neutrophils = CD49b^-CD11b^- Ly-6G^-CD11b^, dendritic cells (DCs) = CD49b^-CD11b^- Ly-6G^-CD11c^+ macrophages/monocytes = CD49b^-CD11b^- Ly-6G^-CD11c^-CD11b^.

Figure S6 Analysis of immune cells recruited to the spleen after infection with Y. pseudotuberculosis YPIII or YP147. About 2×10^8 bacteria (YP11, YP147) were used to infect BALB/c mice. Three days after infection, mice were sacrificed, the spleens were isolated, homogenized and the cell suspensions were used for flow cytometric analysis. Values on the x-axis indicate the number of cells isolated from spleen with wild-type strain YP11 or the cnfY mutant strain YP147. CD11b^-Ly6G^- macrophages/monocytes; CD11b^-Ly6G^- neutrophils; CD11c^-DCs; NKp46^+ NK cells, CD3^+, T cells, CD19^- B cells. The data show the median from at least two different experiments each done with groups of 4-6 mice. The asterisk indicates that there was a significant difference in the number of the indicated cells type in the whole organ based on a Mann-Whitney test. Stars indicate results that differed significantly from those of YPIII with * (P<0.05), ** (P<0.01) and *** (P<0.001).

Figure S7 Effect of YopE on CNFY-induced Rho GTPase activation in macrophages. Murine macrophages were either incubated for 3 h with 25 nM purified CNFY or left untreated. After incubation, cells were infected with Y. pseudotuberculosis YPIII
(wild-type) or the yopE mutant YP275 grown at 37°C with a MOI of 100 from a culture grown at 37°C. Cells were lysed and aliquots of the cell lysates were taken for westernblot analysis. (A) Remaining lysates were incubated with beads, coupled with GTPase-binding domains and signaling molecules interacting with GTP-bound RhoA or Rac1/Cdc42. The total and activated amounts of the RhoA and Rac1 GTPases in the lysates were analyzed with specific antibodies. (B) Intracellular Yops were visualized using an antiserum directed against all secreted Yops (α-Yop). The size of the molecular marker (kDa) is given on the left. Strain YP101 (∆cndα) was used as negative control to rule out permeabilization of the membrane in the detergent solubility assay.

Figure S8 Absence of CNFY reduces Yop delivery into primary cells of MLNs. Single cell suspension of MLNs of six 8-week-old BALB/c mice were prepared and infected with YP147-ETEM (YP173) and YP147 ΔyscS-ETEM (YP217) at an MOI of 10 for 1 h. YP111 and YP174 was used as negative controls. Two independent experiments were performed each done with groups of three mice. The percentage of blue cells of the suspensions was plotted and the median is presented. The asterisks indicate that percentage of blue cells differed significantly in the organ based on a Mann-Whitney test. Stars indicate results of YP147 that differed significantly from those of YPHII with ** (P<0.01).

Figure S9 Gating strategies for the analysis of CNFY on Yop delivery. Exemplary gating strategies of MLNs cells of YP147-ETEM infected mice at day three post infection. (A) Cells were subjected to CCF4-AM treatment. Alive cells are “green”, translocated cells are “blue”. (A) T cells = CD19^+ CD3^+ , B cells = CD19^+ CD3^+ , Neutrophils = CD19^- CD3^- Ly-6G^+ CD11b^- , dendritic cells (DCs) = CD19^- CD3^- CD11c^+ , Macrophages/Monocytes = CD14^+ CD19^- CD3^- Ly-6G^+ CD11b^- . (B) Natural killer (NK) cells = CD19^- CD3^- NKp46^- .

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