Yersinia enterocolitica YopH-deficient Strain Activates Neutrophil Recruitment to Peyer's patches Promoting Clearance of the Virulent Strain

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Running title: YopH-deficient Ye Activates Neutrophil Recruitment

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Abstract: 228 words

Text: 7511 words

ABSTRACT

Yersinia enterocolitica evade the immune response by injecting *Yersinia* outer proteins (Yops) into the cytosol of host cells. YopH is a tyrosine phosphatase critical for *Yersinia* virulence. However, the mucosal immune mechanisms subverted by YopH during *in vivo* orogastric infection with *Y. enterocolitica* remain elusive. The results of this study revealed neutrophil recruitment to Peyer's patches (PP) after infection with YopH-deficient mutant strain (*Y. enterocolitica* Δ*yopH*). While the *Y. enterocolitica* wild-type (WT) strain in PP induced the major neutrophil chemoattract CXCL1 mRNA and protein levels, infection with the *Y. enterocolitica* Δ*yopH* mutant strain exhibited a higher expression of the CXCL1 receptor, CXCR2, in blood neutrophils leading to efficient neutrophil recruitment to the PP. In contrast, migration of neutrophils into PP was impaired upon infection with *Y. enterocolitica* WT strain. *In vitro* infection of blood neutrophils revealed the involvement of YopH in CXCR2 expression. Depletion of neutrophils during *Y. enterocolitica* Δ*yopH* infection raised bacterial load in PP. Moreover, the clearance of *Y. enterocolitica* WT strain was improved when an equal mixture of *Y. enterocolitica* WT and *Y. enterocolitica* Δ*yopH* was used in infecting the mice. This study indicates that *Y. enterocolitica* prevents early neutrophil recruitment in the intestine and that the effector protein YopH plays an important role in the immune evasion mechanism. The findings highlight the potential use of *Y. enterocolitica* YopH-deficient strain as an oral vaccine carrier.

Key words: *Yersinia enterocolitica,* YopH, mucosa, innate immune response, neutrophils, CXCR2

INTRODUCTION

The genus *Yersinia* includes three human pathogenic species: *Yersinia pseudotuberculosis, Y. pestis,* and *Y. enterocolitica. Y. pestis* cause plague, and *Y. pseudotuberculosis* and *Y. enterocolitica* cause gastroenteritis (1). *Y. enterocolitica* infections are characterized by fever, abdominal pain, and diarrhea resulting in gastroenteritis and lymphadenitis, which are commonly self-limiting in humans (2). The bacteria are usually ingested through contaminated food or water and travel to the terminal ileum, where they attach to and invade through the M cells of Peyer's patches (PP). *Y. enterocolitica* then survive and undergo extracellular replication in the PP, and may eventually disseminate to deeper tissues of the mesenteric lymph nodes, liver, spleen, and lung (3).

For survival in host tissues, pathogenic *Yersinia* species have a plasmid-encoded Type 3 Secretion System (T3SS) that translocates virulence proteins, the so-called *Yersinia* outer proteins (Yops), into the cytosol of target cells, suppressing the host immune response and enabling extracellular replication of the bacteria in lymphatic tissue (4). Yops include YopH, which is a tyrosine phosphatase critical for virulence of *Yersinia* (5-7) and targets tyrosine kinases and their adapters in a variety of cell types. The substrates of YopH thus far identified include the following: the adapters p130Cas and paxilin in epithelial cells (8, 9); the adapters ADAP, SKAP-HOM and the tyrosine kinase FAK in macrophages (10, 11); and the tyrosine kinase LcK and ZAP-70, and the adapters SLP-76 and LAT in T cells (12, 13). Rolán et al have recently demonstrated that PRAM-1/SKAP-HOM and SLP-76 are molecular targets of YopH in polymorphonuclear neutrophils (PMN) during animal infection (14). As a result, YopH

promotes inhibition of phagocytosis (15), blocks specific bactericidal function of PMN (16), inhibits cytokine production by T-cells and T-cell proliferation, and prevents the expression of the co-stimulatory receptor CD86 on B-cells (17, 18). In addition, YopH inhibits the phosphatidylinositol 3 kinase (PI3K)/Akt signaling pathway in macrophages, preventing the expression of the chemokine monocyte chemoattractant protein-1 (MCP-1, CCL2), an important chemotactic factor for macrophages (17). Different reports have shown that the lack of YopH reduces *Y. enterocolitica* virulence in mice, underlining the relevance of YopH for the full virulence of *Y. enterocolitica* (5-7, 19, 20). However, the immune mechanisms involved in the control of YopH-deficient *Y. enterocolitica* strain have yet to be fully clarified.

In previous studies we demonstrated that the *Y. enterocolitica* mutant strain (Δ*sycH*), which is unable to secrete the virulence protein YopH, is reduced in virulence, colonizes PP (7) and induces mucosal and systemic *Yersinia*-specific IgA levels (21). In the present study, we show that a YopH-deletion mutant (*Y. enterocolitica* Δ*yopH*) is avirulent after orogastric infection. Interestingly, while mRNA and protein levels of CXCL1, the major neutrophil chemoattract, associated with higher bacterial load of the *Y. enterocolitica* wild-type (WT) strain in PP, circulating blood neutrophils expressed higher level of the CXCL1 receptor, CXCR2, in *Y. enterocolitica* Δ*yopH* -infected mice. Indeed, we observed higher CXCR2 expression after *in vitro* infection of blood neutrophils with the mutant strain. Moreover, the elimination of *Y. enterocolitica* Δ*yopH* was impaired in neutrophil-depleted mice, supporting the contribution of recruited neutrophils in the intestinal defense against *Y. enterocolitica*. Finally, the clearance of virulent *Y. enterocolitica* WT was improved when mice were co-infected

with *Y. enterocolitica* WT - *Y. enterocolitica* Δ*yopH*. The data shed new light on the role of YopH in the context of animal infection, and support the notion of a protective response induced by the *Y. enterocolitica* Δ*yopH* mutant when mice are co-infected with the virulent *Y. enterocolitica* strain. These findings highlight the potential of this attenuated strain as an oral vaccine vector.

MATERIALS AND METHODS

Mice

C57BL/6 wild-type (WT) mice were purchased from the Animal Facilities of the National University of La Plata (La Plata, Argentina). Breeding colonies were established at the Animal Facility of the National University of San Luis (San Luis, Argentina). Mice were kept in a positive-pressure cabinet (EHRET, Emmendingen, Germany) and provided with sterile food and water *ad libitum*. Six- to eight-week-old mice were used for the experiments. All animal procedures were performed according to the rules and standards for the use of laboratory animals of the National Institute of Health, USA. Animal experiments were approved by the Institutional Committee of Care and Use of Animals (CICUA) of the Faculty of Chemistry, Biochemistry and Pharmacy, at the National University of San Luis (San Luis, Argentina) (Protocol number B-163/13).

Bacterial strains and infection

The following strains were used in this study: *Y. enterocolitica* WA-314 wild-type (pYV+, serotype O:8; clinical isolate; WA-314 pYVO8^{+}; Nal^{r}; *Y. enterocolitica* WT) (22), and *Y. enterocolitica* WA-314 deficient in YopH (pYV^{+}, WA-C pYV *yopH* Δ17-

455 Nal^r Kan^r; *Y. enterocolitica* Δ*yopH*) (23). Bacteria were cultured and prepared as previously described (7). For infection, mice were first starved for 3 h before and after orogastric infection with $5x10^8$ yersiniae in 0.2 ml phosphate-buffered saline (PBS) using a gastric tube. Control mice received PBS. In co-infection experiments, *Y. enterocolitica* Δ*yopH* were administered in combination with *Y. enterocolitica* WT or with *Y. enterocolitica* WT GFP (24), with an equal dose of $2.5x10^8$ or $1x10^{10}$CFU, respectively. The number of inoculated bacteria was controlled by plating of serial dilutions of the inoculated suspension on Trypticase Soy Agar (TSA) and counting the CFU number after incubation at 27°C for 48 h. To determine the bacterial burden after infection, spleen, PP and feces were obtained and homogenates were prepared in isotonic saline solution or in a cold extraction buffer (50 mM EDTA, 30 mg/ml Soybean trypsin inhibitor, 1% bovine serum albumin in PBS) for feces. Serial dilutions were plated on Igarsan-Mac Conkey agar plates for PP and feces, or on TSA plates for spleen samples. Plates were incubated for 48 h at 27°C and CFU numbers were determined. In co-infection experiments, the *Y. enterocolitica* WT clearance was calculated as the differential CFU number between plates with and without kanamycin, since *Y. enterocolitica* Δ*yopH* is kanamycin resistant. The limit of CFU detection is $\log_{10}$ of 25 CFU = 1.4 (7).

Cell preparation and flow cytometry

PP were finely cut and digested for 10 min at 37 ºC in Hank´s balanced salt solution containing collagenase (0.5mg/ml; type IV; Sigma-Aldrich) and DNAse I (15 µg/ml, Roche). Flow-cytometric staining was conducted as previously described (25). Cells

were first incubated with anti-mouse CD16/32 (Fc block) for 15 min at 4ºC and then stained with anti-F4/80-FITC (clone CI:A3-1), anti-CD11b-APC (clone M1/70), anti-CD11b PerCp-Cy5.5 (clone M1/70), anti-CD11c-APC (clone HL3) and anti-Ly6G-PE (clone 1A8) for 30 min at 4ºC. Anti-CD16/32 and anti-CD11b were from BD Biosciences (San Jose, CA, USA), anti-Ly6G and anti-F4/80 were from Biolegend (San Diego, CA, USA). CXCR2 staining was performed with anti-CXCR2-PE (clone 242216) (R&D, Minneapolis, MN, USA) generously provided by Dr. Cristina Pistoresi (Córdoba, Argentina) or with anti-CXCR2-Alexa Fluor 647 (clone SA045E1) from Biolegend with their appropriate matched isotype control antibodies. To exclude dead cells, 7-aminoactinomycin D (7-AAD; Sigma-Aldrich, St. Louis, MO, USA) was used. Neutrophils cells were identified as $Ly6G^+$ $CD11b^+$, and macrophages as $F4/80^+$ $CD11b^+$ cells. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR). A total of 1-2x10^6events were acquired.

Determination of Chemokines by bead array or ELISA

The PP were homogenized in PBS containing 0.5% bovine serum albumin, 0.4 M NaCl, 1 mM EDTA, 0.05% Tween 20 and 1% protease inhibitor cocktail (Sigma-Aldrich), and centrifuged at 10,000 g for 10 min [adapted from (26)]. CXCL1 was quantified in the homogenates using the CBA Assay (BD Biosciences), and the level of CXCL2 was quantified using the specific ELISA kit (Peprotech, Mexico DF) following manufacturer's instructions. These antibodies were generously provided by Dr. Eva Acosta (Córdoba, Argentina). The chemokine levels were normalized to protein

concentration, which was determined by Qubit fluorometric quantification (Invitrogen, San Diego, CA, USA).

RT-PCR and quantitative PCR analysis.

The total RNA from the PP of the vehicle-treated and infected mice was isolated using the TRIzol reagent (Invitrogen), according to the instructions of the manufacturer. Each total RNA sample was treated with the RQ1 RNase-free DNase according to the manufacturer's instructions (Promega, Madison, WI, USA). First-strand cDNA was synthesized using ProtoScript M-MuLV First Strand cDNA Synthesis Kit according to the manufacturer's instructions (New England Biolab, Ipswich, MA, USA). Quantitative PCR analysis was performed using an ABI PRISM 7500 instrument (Applied Biosystems, Pleasanton, CA, USA) with SBR Green PCR master mix (Applied Biosystems). The following primers were used for PCR amplification: for mouse *β-actin* cDNA, sense, 5'-CGTTGACATCCGTAAAGACCT-3', and antisense, 5'-CTTGATCTTCATGGTGCTAGGAG-3'; for mouse *cxcl1,* sense, 5'-TCCAGC ACTCCAGACTCC-3', and antisense, 5'-TGACAGCGCAGCTCATTG-3'. Forty cycles of PCR amplification were performed in duplicate for each primer set. The fold change in the quantity of gene transcripts was measured and compared to the β-actin gene using the comparative $2^{-\Delta\Delta Ct}$ method.

Histological evaluation

The histological examination of PP was carried out after routine fixation and paraffin embedding. Five-micrometer-thick sections were cut, stained with hematoxylin-eosin

and examined under a light microscope. Photographs were taken using an Olympus BX40 light microscope equipped with Sony SSC-DC5OA camera.

Immunofluorescence studies

The PP were embedded in the Tissue-Tek OCT compound (Sakura, Zoeterwoude, The Netherlands) and frozen at -80ºC, and 7 µm cryostat sections were prepared. Tissue sections were fixed for 10 min at room temperature with 4% paraformaldehyde, washed three times with PBS and then incubated with 50 mM ammonium chloride. The tissue sections were washed twice with PBS and permeabilized with 0.1% Triton X-100. After incubation with anti-mouse CD16/32 (BD Biosciences) and a biotin blocking kit (Vector Laboratories, Burlingame, USA), the sections were washed three times and incubated 30 min at room temperature with a biotin anti-Gr1 antibody (generously provided by Dr. Cristina Pistoresi, Córdoba, Argentina, 100 µg/ml in PBS-10% FBS), washed and then incubated with streptavidin-AlexaFluor 594 (1µg /ml in PBS-10% FBS) for 30 min at room temperature. The slides were mounted in Mowiol (Carl Roth, Karlsruhe, Germany). Labeled cells were visualized with an Axiovert 40 CFL fluorescence microscope (Zeiss, Esslingen, Germany).

Expression of CXCR2 after *in vitro* infection

Y. enterocolitica WT and *Y. enterocolitica* Δ*yopH* were grown overnight at 27°C in Trypticase Soy Broth (TSB) supplemented with 20 mM magnesium chloride and 20 mM sodium oxalate. A 1:20 dilution of the overnight bacterial culture was incubated for

additional 3 h at 37°C. The bacteria were washed once with saline and the optical density at 600 nm was determined.

Whole blood was collected from uninfected mice and erythrocytes were removed by treatment with a lysis buffer containing 0.15 M NH_4Cl, 10 mM K_2CO_3 and 0.1 mM EDTA, followed by centrifugation. The remaining leucocytes containing 11% of neutrophils were suspended in RPMI 1640 (Invitrogen) medium supplemented with 10% FCS (Sigma), 2 mM glutamine (Invitrogen), 50 mM 2-mercaptoethanol (Sigma), and 1 mM sodium pyruvate (Invitrogen) without antibiotics, and infected with *Y. enterocolitica* WT or *Y. enterocolitica* Δ*yopH* at multiplicity of infection (moi) 50:1. The bacteria were sedimented onto the cells at 400 g for 5 min. After 30 min of infection, the cells were washed with gentamicin (100 µg/ml; Sigma) diluted in saline. The CXCR2 expression in $Ly6G^+CD11b^+$ cells was analyzed by flow cytometry.

Neutrophil depletion and bacterial counting.

To deplete neutrophils, mice were injected intraperitoneally with 100 µg of the monoclonal anti-Gr1antibody (clone RB6-8C5, generously provided by Dr. Cristina Pistoresi, Córdoba, Argentina) diluted in 100 µl of sterile saline 1 day before, and on days 2 and 3 after *Y. enterocolitica* Δ*yopH* infection. Control mice received the same dosage of saline. Neutrophil depletion was measured by counting the $Ly6G^+CD11b^+$ cells in peripheral blood using flow cytometric analysis. To further monitor the effect of neutrophil depletion on *Y. enterocolitica* Δ*yopH* infection, the CFU number was determined in PP and spleen of depleted and control mice 3 days after infection, four hours after the last anti-Gr1antibody dose.

Statistical analysis

Multiple comparisons were tested using one-way ANOVA, followed by Bonferroni's post test. For a comparison of data of two groups, Student's *t*-test was used. Statistical analysis of survival was performed by using the Log-rank test. Results with $P<0.05$ were considered statistically significant. Data were analyzed using GraphPad Prism 5.0 Software (GraphPad Software, La Jolla, CA, USA).

RESULTS

Deletion of YopH decreases *Y. enterocolitica* virulence in mice

To analyze how YopH deletion affects *Y. enterocolitica* infection, C57BL/6 mice were orogastrically infected with the *Y. enterocolitica* WT strain or *Y. enterocolitica* Δ*yopH* mutant strain ($5x10^8$ CFU/mice). While 45% of the mice infected with the *Y. enterocolitica* WT strain died, all the mice infected with the *Y. enterocolitica* Δ*yopH* mutant strain survived the infection (Fig. 1A), demonstrating that YopH is critical for *Y. enterocolitica* virulence after oral infection. When the intestinal elimination of these strains was examined in feces at different days after infection, we found significant higher intestinal clearance of this mutant strain than *Y. enterocolitica* WT strain on days 3, 7, 14 and 21 ($P<0.001$ at days 3, 7 and 14; $P<0.01$ at day 21) (Fig. 1B and Table 1). We previously reported that *Y. enterocolitica* WT colonization of gut tissues continues at high levels (log CFU/mg feces of 4-5) for at least 5 weeks (21). Therefore, we monitored for how long *Y. enterocolitica* Δ*yopH* was shed from the intestinal tract, and found that this mutant strain had been completely eliminated from the intestine by day

42 after infection (Fig. S1). Next, the bacterial load in PP and in the spleen was analyzed in both groups of mice on days 3, 5, 7 and 21 after infection. The bacterial burden was significantly higher in the PP of mice infected with the *Y. enterocolitica* WT throughout the period of infection, and on day 7 in the spleen, compared to mice infected with *Y. enterocolitica* Δ*yopH* mutant strain (Fig. 1C and 1D). The bacterial load in PP decreased significantly after *Y. enterocolitica* Δ*yopH* infection on days 3, 5 and 7 ($P<0.05$ on day 5; and $P<0.001$ on days 3 and 7 compared with *Y. enterocolitica* WT) to undetectable CFU on day 21 ($P<0.05$). Moreover, after infection with *Y. enterocolitica* WT, we observed that bacterial load improved from day 5 to day 7 in the mice that survived (Fig. 1C and D).

Neutrophil recruitment is increased in *Y. enterocolitica* Δ*yopH*-infected mice

Since *Y. enterocolitica* Δ*yopH* was eliminated soon after infection, and phagocytes of the innate immune system contribute to an early antibacterial defense (27), we analyzed the recruitment of neutrophils in PP 3 days post-infection (dpi) with *Y. enterocolitica* WT or *Y. enterocolitica* Δ*yopH* mutant strain. Notably, although the bacterial burden was lower after the infection with *Y. enterocolitica* Δ*yopH* mutant strain, a significantly higher frequency and absolute number of $CD11b^{+}Ly6G^{+}$ neutrophils was detected in PP of *Y. enterocolitica* Δ*yopH*-infected mice compared to *Y. enterocolitica* WT-infected mice ($P<0.001$) (Fig. 2). Moreover, we did not detect differences in CD11b expression in $Ly6G^{+}$ cells in PP of mice infected with *Y. enterocolitica* Δ*yopH* or *Y. enterocolitica* WT (Fig. S2). Therefore, our results reveal that the activation state of neutrophils is not different after *Y. enterocolitica* Δ*yopH* infection. These findings indicate that the mutant

strain *Y. enterocolitica* Δ*yopH* induces early recruitment of neutrophils to the PP and point to the involvement of YopH in neutrophil migration to the infection site.

YopH is not associated with CXCL1 mRNA and protein expression

The increase of neutrophils in PP induced by *Y. enterocolitica* Δ*yopH* suggested that YopH may be involved in the early suppression of chemoattractants in this organ. Because the chemokines CXCL1 and CXCL2 are of central importance for neutrophil recruitment, we explored the expression of these chemokines. Although we did not detect the induction of CXCL2 protein in PP within infected mice (the levels were lower than the limit of detection, 0.1pg/ml), both CXCL1 protein and mRNA expressions were higher in PP of *Y. enterocolitica* WT compared to *Y. enterocolitica* Δ*yopH*-infected mice (Fig. 3A and B). These results correlate with the bacterial load in PP as a stimulus for chemokine expression (Fig. 1C). Previous studies have also demonstrated that CXC chemokine secretion is triggered through recognition of bacterial peptidoglycan or LPS causing migration of neutrophils to the infection site (27-30). Since resident macrophages and newly recruited monocyte-derived macrophages are the main source of CXCL1 (31), we analyzed the number of macrophages in PP of infected mice. Accordingly, we detected increased macrophage ($F4/80^{+}CD11b^{+}$) influx in PP 3 dpi with *Y. enterocolitica* WT compared to infection with *Y. enterocolitica* Δ*yopH* mutant strain (Fig. 4). However, these results did not explain the massive neutrophil recruitment in PP after *Y. enterocolitica* Δ*yopH* infection. Therefore, since in response to infection, neutrophils are rapidly mobilized from the bone marrow, resulting in a rise in circulating cell numbers and followed by rapid trafficking of neutrophils into the

infected tissue (32), we compared the neutrophil number in blood of mice infected with both strains. Neutrophil numbers in the blood of *Y. enterocolitica* WT-infected mice were significantly higher compared to PBS-treated and *Y. enterocolitica* Δ*yopH*-infected mice (Fig. 5A). However, the levels of neutrophils infiltrating into the PP (calculated as the absolute number of PP neutrophils to blood neutrophils in each mouse) were augmented after *Y. enterocolitica* Δ*yopH* infection (Fig. 5B). Furthermore, histological studies analyzing migration of neutrophils into PP revealed that in non-infected mice the neutrophils were predominantly located in the blood vessels, while low neutrophil numbers were in the tissue at 3 dpi with the *Y. enterocolitica* WT strain (Fig. 5C and D). In contrast, increased neutrophil numbers were observed in the tissue of *Y. enterocolitica* Δ*yopH*-infected mice (Fig. 5C and D).

YopH prevents CXCR2 expression in blood circulating neutrophils

We then analyzed the CXC receptor 2 (CXCR2) expression on blood neutrophils of infected mice since CXC chemokines induce migration of neutrophils to the site of infection predominantly through the signaling of CXCR2 (33). As depicted in Figure 6, higher CXCR2 expression was detected on blood neutrophils from *Y. enterocolitica* Δ*yopH*-infected mice compared to blood neutrophils from *Y. enterocolitica* WT-infected mice. However, no changes in CXCR2 expression were detected in PP upon infection with both strains (Fig. 6). Moreover, CXCR2 levels were higher on blood neutrophils following *in vitro* infection with *Y. enterocolitica* Δ*yopH* strain compared to infection with *Y. enterocolitica* WT (Fig. 7). Together, these data suggest that neutrophil

infiltration into PP upon *Y. enterocolitica* Δ*yopH* infection is presumably due to increased CXCR2 expression on circulating neutrophils in the blood.

Neutrophils play a critical role in *Y. enterocolitica* Δ*yopH* elimination

To study the function of neutrophils in the elimination of *Y. enterocolitica* Δ*yopH*, we depleted neutrophils in the mice with the monoclonal antibody RB6-8C5 prior to and during *Y. enterocolitica* Δ*yopH* infection. The analysis of peripheral blood of the mice showed that neutrophil depletion was achieved in more than 95% in mice treated with RB6-8C5 (Fig. S3). On day 3 after infection, a significant increase in bacterial load (more than 100 fold) was detected in PP of neutrophil-depleted mice (Fig. 8A). Remarkably, in contrast with control mice, high bacterial levels were detected in the spleen of mice treated with RB6-8C5 (Fig. 8B). These data indicate that neutrophils play a significant role in controlling *Y. enterocolitica* Δ*yopH* in PP and consequently in limiting the systemic spread of infection.

Promotion of *Y. enterocolitica* WT clearance during co-infection with *Y. enterocolitica* Δ*yopH*

Neutrophils are important targets of Yop translocation (34); however, enhanced local recruitment of neutrophils improved bacterial clearance upon *Y. enterocolitica* infection (25). Therefore, we evaluated the clearance of *Y. enterocolitica* WT in mice infected with an equal mixture of *Y. enterocolitica* Δ*yopH* (2.5 x10^8 of each strain). The colonies were dissected by culture on kanamycin agar due to *Y. enterocolitica* Δ*yopH* mutant strain resistance to kanamycin (23). Co-infection resulted in a complete elimination of

344 the *Y. enterocolitica* WT strain. This contrasted with the high bacterial load after
345 infection with *Y. enterocolitica* WT alone in both PP and in feces 3 dpi (Fig. 9A and B).
346 Accordingly, immunofluorescence analyses of the PP revealed reduced numbers of
347 GFP^+ *Y. enterocolitica* colonies upon co-infection with GFP-expressing *Y.*
348 *enterocolitica* WT- *Y. enterocolitica yopH* compared with GFP-expressing *Y.*
349 *enterocolitica* WT strain alone (Fig. 9C). Moreover, augmented neutrophil recruitment
350 was detected after co-infection (Fig. 9C). Together, the data indicate that *Y.*
351 *enterocolitica* Δ*yopH* improve rapid intestinal elimination of the *Y. enterocolitica* WT
352 strain.
353
354 **DISCUSSION**
355 Infections are recognized by the innate immune system, eliciting in this system an
356 immediate defense, which works to promote long-lasting adaptive immunity (35). The
357 ability of professional phagocytes to ingest and kill microorganisms is central to innate
358 immunity and host defense. One strategy of bacterial pathogens is to evade
359 phagocytosis during early immune response (36). *Y. enterocolitica* prevent phagocytosis
360 by host cells and proliferate extracellularly in lymphatic tissue (15, 37). YopH plays a
361 critical function in this process promoting intestinal colonization and persistence of *Y.*
362 *pseudotuberculosis* (6).
363 We previously demonstrated that *Y. enterocolitica* Δ*sycH*, a functional YopH
364 mutant, colonizes the PP without causing systemic infection, indicating that yersiniae
365 lacking the YopH function can be eliminated by mechanisms that do not require an IL-
366 12-, IL-18-, and TNFRp55-dependent immune defense (7). Further studies

demonstrated that IFN-γ and IL-6 are not necessarily required for clearance of *Y. enterocolitica* Δ*yopH* (38). These results raised intriguing questions, namely, what early immune response controls *Y. enterocolitica* Δ*yopH* after oral infection. In this study, we also investigated whether this immune response would protect mice against the infection with the fully virulent *Y. enterocolitica* strain.

Our results clearly demonstrate the attenuation of the *Y. enterocolitica* Δ*yopH* strain after oral infection. The survival rate of mice increased and bacterial colonization in PP decreased when we compared with the *Y. enterocolitica* WT strain. Moreover, the bacterial dissemination to the spleen was strongly limited in the YopH-deficient mutant strain. These results are consistent with previous studies performed with *Y. enterocolitica* Δ*sycH* mutant strain (7, 21). Notably, infection with the *Y. enterocolitica* WT strain revealed a biphasic bacterial load in PP, indicating that a fully virulent *Y. enterocolitica* strain coping with the host immune response during early stages of infection would provoke a second increase of the bacterial load in PP and dissemination to the spleen. Moreover, recent studies in *Y. pestis*-infected mice have demonstrated a biphasic nature to the progression of the disease (28). In contrast, an early immune response induced by *Y. enterocolitica* Δ*yopH* infection was able to control this infection without a biphasic course of infection.

Neutrophils are fundamental cells in the primary innate immune defense and they have been shown to be important for controlling systemic *Yersinia* infection (27). *Yersinia* species target neutrophils through the transfer of Yops (34, 39). YopH has been identified as one of the major contributors to the antiphagocytic capacity of *Yersinia* on neutrophils, and their target has been defined in *in vitro* assays and during *in vivo*

systemic and oral murine infections (8-12, 14-16). Upon infection by invading microorganisms, neutrophils migrate to the infected tissue through circulation (40). Notably, we found dramatic increases of neutrophils in PP of mice infected with the YopH-deficient mutant strain. Consistent with this result, other authors have recently reported rapid neutrophil infiltration in the lungs of mice infected with an avirulent *Y. pestis* strain, but they observed no significant changes in the levels of neutrophils in the lungs after infection with a fully virulent *Y. pestis* (28). Moreover, we observed that depletion of neutrophils impaired *Y. enterocolitica* Δ*yopH* elimination in PP. Accordingly, Westermark *et al* used neutrophil-depleted mice to demonstrate that the virulence-attenuated *yopH* mutant of *Y. pseudotuberculosis* was clearly more virulent in the absence of these cells (41).

Chemokines CXCL1 and CXCL2 are potent chemoattractants for neutrophils, and increased serum levels of these chemokines are a hallmark of infection and inflammation in peripheral tissues (42). Our results are in line with previous studies that detected high CXCL1 levels in the sera of systemically infected mice with *Y. enterocolitica* WT, which correlated with bacterial burden (43). The process of neutrophil recruitment within individual organs is dictated by inciting infection and the response of organ-specific tissue-resident cells (44). Moreover, resident macrophages are an important source of neutrophil-attracting chemokines in bacterial infections (31, 45). Increased recruitment of phagocytes to PP was observed in an oral *Salmonella* mouse infection model (46). Furthermore, purified monocytes from *Salmonella*-infected mice preferentially produced chemokines and neutrophils from infected mice migrated towards these chemokines (47). Accordingly, a recent study using a mouse model of

413 urinary tract infection with the uropathogenic *Escherichia coli* nicely showed that
414 tissue-resident and recruited macrophages work together to create an effective
415 neutrophilic response to infection by producing CXCL1 (48). Moreover, splenic
416 $CD11b^+$ cells, which include macrophages, were shown, in contrast to the *yopH* mutant,
417 to have increased CXCL-1 mRNA levels after systemic infection with *Y. enterocolitica*
418 WT (38). In line with this, we also observed augmented numbers of macrophages in PP
419 and increased CXCL-1 mRNA and protein levels after oral infection with *Y.*
420 *enterocolitica* WT. Thus, our findings suggest that PP resident cells sense bacterial
421 infection, leading to CXCL-1 production in relation to the bacterial load.

422 Although high CXCL1 levels were detected in PP after infection with *Y.*
423 *enterocolitica* WT, neutrophil recruitment was lower compared with infection with the
424 YopH-deficient mutant strain. Our findings showed that infection with *Y. enterocolitica*
425 WT decreased CXCR2 expression in blood neutrophils compared with non-infected and
426 *Y. enterocolitica* Δ*yopH*-infected mice. Therefore, we assume that neutrophils rapidly
427 mobilize from blood to PP after *Y. enterocolitica* Δ*yopH* infection, and that the YopH
428 virulence factor in the *Y. enterocolitica* WT strain targets CXCR2 expression on
429 circulating neutrophils, affecting neutrophil influx into PP. Moreover, in other
430 infections, including *Y. pestis*, the absence of CXCR2 resulted in increased colonization
431 and decreased neutrophil recruitment to the infected site (29, 49-51). Additionally,
432 consistent with the study reporting that phagocytosing neutrophils down-regulate the
433 expression of chemokine receptors (52), we detected that CXCR2 expression decreased
434 when neutrophils reach PP. Correspondingly, other authors found that neutrophils from
435 the PP of *Salmonella*-infected mice down-regulated CXCR2 expression (46). In

summary, our findings suggest that the YopH-dependent blocking of neutrophil recruitment into PP is a key event for *Y. enterocolitica* to evade the immune response at the intestinal mucosa. We are currently directing our studies to define the molecular targets of YopH involved in the down regulation of CXCR2 on neutrophils during *Yersinia* infection.

We hypothesized that YopH impair neutrophil influx into PP to enhance survival of *Y. enterocolitica*. Notably, we observed that the *Y. enterocolitica* WT was eliminated when co-infection with *Y. enterocolitica* Δ*yopH* was performed. Moreover, by combining the findings that: a) *Y. enterocolitica* selectively delivers Yops to neutrophils (53); b) these cells are preferentially injected with YopH (34); c) Yop injection depends on bacterial adhesion on neutrophils (54), which leads to phagocytic uptake of pathogens (55); and d) Yop translocation increases when more neutrophils are present in PP (34), we can speculate that the the *Y. enterocolitica* WT strain is preferentially phagocyted by neutrophils after co-infection.

In conclusion, in this study we reported differential neutrophil recruitment upon oral *Y. enterocolitica* WT or *Y. enterocolitica* Δ*yopH* infection. In addition, we argue that YopH of *Y. enterocolitica* may modulate neutrophil chemotaxis into the infection site. The mutant *Y. enterocolitica* Δ*yopH* contributed to the complete elimination of *Y. enterocolitica* WT. Therefore, our findings emphasize the importance of the early immune response in mucosa during intestinal infections and the potential use of the *Y. enterocolitica* Δ*yopH* strain as an oral vaccine carrier.

ACKNOWLEDGMENTS

The authors thank Fabian Mohamed, Angelina Bernardi and Maria Elena Arce (National Universitiy of San Luis) for their collaboration in the histological and immunofluorescence studies.

FUNDING INFORMATION

This work was supported by grants from the National Agency for Promotion of Science and Technology (PICT-2008-763; PICT-2011-0732), the National University of San Luis (PROICO-2-1114), the Alexander von Humboldt Foundation, and the CONICET-DFG cooperation Project. RJE, VPF, CVG and MSDG are members of the Scientific Career of the National Council of Scientific and Technical Investigations (CONICET); MND, JES, and MBJ are fellows from the CONICET.

The authors have no conflict of interest to declare.

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652 **TABLE 1. Comparative kinetic of elimination of *Y. enterocolitica* Δ*yopH* vs *Y.***
653 ***enterocolitica* WT.**

	Days	*Y. enterocolitica* WT (% elimination)[#]	*Y. enterocolitica* Δ*yopH* (% elimination)[#]	Elimination of Ye Δ*yopH* relative to Ye WT strain
PP	3	40.1 ± 1.7	64.2 ± 7.9*	1.6
	7	45.5 ± 5.5	79.9 ± 5**	1.8
	21	63.3 ± 5.5	100***	1.6
Feces	3	44.4± 2.3	60.3 ± 2.1***	1.4
	7	48.8 ± 2.8	57± 2.2**	1.2
	21	55.6 ± 0.3	69.3 ± 1.9**	1.3

654 *P<0.05, ** P<0.01,*** P<0.001; [#]% elimination: (% bacterial load at final versus initial time
655 point)
656

FIGURE LEGENDS

Figure 1. Survival curves and bacterial load following oral infection of mice with *Y. enterocolitica* strains. A) Survival curves of C57BL/6 mice infected with $5x10^8$ CFU *Y. enterocolitica* (Ye) wild-type (WT) strain or with a similar dose of the mutant that lacks YopH (Ye Δ*yopH*). Results shown are the summary results of 3 experiments. n =12 mice per group, Log-rank test was used (**P<0.01). Kinetics of bacterial clearance in feces (B), and the bacterial load in Peyer´s patches (PP) (C), and spleen (D) of mice infected with Ye WT or with Ye Δ*yopH* at the indicated days post infection (dpi). Data in B-D are shown as mean and SEM from the summary results of 2 experiments. n=3-5 mice per day for each group of mice (*P<0.05, **P<0.01, ***P<0.001).

Figure 2. Neutrophil infiltration in PP following *Y. enterocolitica* WT or *Y. enterocolitica* Δ*yopH* infection. C57BL/6 mice were infected orally with $5x10^8$ CFU of *Y. enterocolitica* (Ye) WT strain or Ye Δ*yopH* mutant strain. Control mice received PBS. Cells were collected from Peyer´s patches (PP), stained for the neutrophil markers Ly6G and CD11b, and subjected to flow cytometry analysis. A) Representative dot plot showing analysis of neutrophils in PP from control (PBS), Ye WT and Ye Δ*yopH* infected mice. The numbers in the plots indicate the percentages of labeled cells. Percentage (B) and absolute neutrophil number (C) in the PP of mice at day 3 after infection with Ye WT or Ye Δ*yopH* strains are presented. The data in B and C are the summary results of 3 experiments. Each symbol represents an individual mouse; horizontal lines indicate the mean. (***P<0.001),

679 **Figure 3. CXCL1 expression in PP.** C57BL/6 mice were infected with $5x10^8$ CFU *Y.*
680 *enterocolitica* (Ye) WT or with the same dose of the avirulent Ye Δ*yopH*. At 3 days
681 post-infection (dpi), concentration of CXCL1 protein in homogenates of uninfected
682 (PBS) and infected Peyer´s patches (PP) was quantified by flow cytometry using CBA
683 assay (A). Moreover, RNA was purified from the PP and subjected to qPCR analysis of
684 CXCL1 gene expression (B). The data are the summary results of 2 experiments. Each
685 symbol represents an individual mouse; horizontal lines indicate the mean (*P<0.05,
686 ***P<0.001).

687

688 **Figure 4. Macrophage infiltration in PP following *Y. enterocolitica* WT or *Y.**
689 ***enterocolitica* Δ*yopH* infection.** Macrophages in Peyer´s patches (PP) of infected mice
690 (day 3 after infection) were stained with F4/80 and CD11b and subjected to flow
691 cytometry analysis. A) Representative dot plot showing analysis of macrophages in PP
692 from control (PBS), Ye WT and Ye Δ*yopH* infected mice. The numbers in the plots
693 indicate the percentages of labeled cells in representative mice. Percentage (B) and
694 absolute macrophages number (C) in the PP of *Y. enterocolitica* (Ye) WT and the Ye
695 Δ*yopH* infected mice are presented. The data are the summary results of 2 experiments.
696 Each symbol represents an individual mouse; horizontal lines indicate the mean
697 (*P<0.05, **P<0.01).

698

699 **Figure 5. Neutrophil homing from blood to Peyer´s patches.** A) The graphic shows
700 the absolute neutrophil number in the blood of mice at day 3 after infection with Ye WT
701 or Ye Δ*yopH* strains. B) Relation of absolute number of neutrophils in Peyer´s patches
702 (PP) to absolute number of neutrophils in blood of each infected mouse at 3 days post-
703 infection (dpi) are presented. C) Histological analysis of migration of neutrophils.
704 Sections of PP from mice that received PBS (control) and from infected mice 3 dpi were
705 stained with hematoxylin-eosin. Photographs show blood-neutrophils (arrows) and
706 neutrophils in PP (arrowhead); (magnification is indicated in the figure). In Ye WT
707 infection, the insert shows an amplification of two neutrophils in blood vessels (v). In
708 Ye Δ*yopH* infection, the insert shows in augment an extravasing neutrophil.
709 Photographs are representative of one out of 4 mice per group of 2 independent
710 experiments. D) Quantification of multiple independent images (n=5 per group)**.** The
711 data in A and B are the summary results of 2 experiments. Each symbol represents an
712 individual mouse in A and B, or image in D; horizontal lines indicate the mean
713 (*P<0.05, ***P<0.001).

714

715 **Figure 6. CXCR2 expression in blood circulation and Peyer's patches neutrophil.**
716 Expression of cell surface CXCR2 on circulating neutrophils isolated from the
717 peripheral blood, and in neutrophils of Peyer's patches (PP) 3 days post infection (dpi)
718 with *Y. enterocolitica* (Ye) WT or Ye Δ*yopH* ($5x10^8$CFU). A) Representative overlaid
719 flow cytometry histogram analysis showing CXCR2 expression on neutrophil
720 ($CD11b^+F4/80^-CD11c^-$) gate compared with PBS mice. Isotype control (grey peak). B)
721 The average CXCR2 mean fluorescence intensity (MFI) levels are indicated in the

graphics. The data are the summary results of 2 experiments. Each symbol represents an individual mouse; horizontal lines indicate the mean (*P<0.05). ns: not significant.

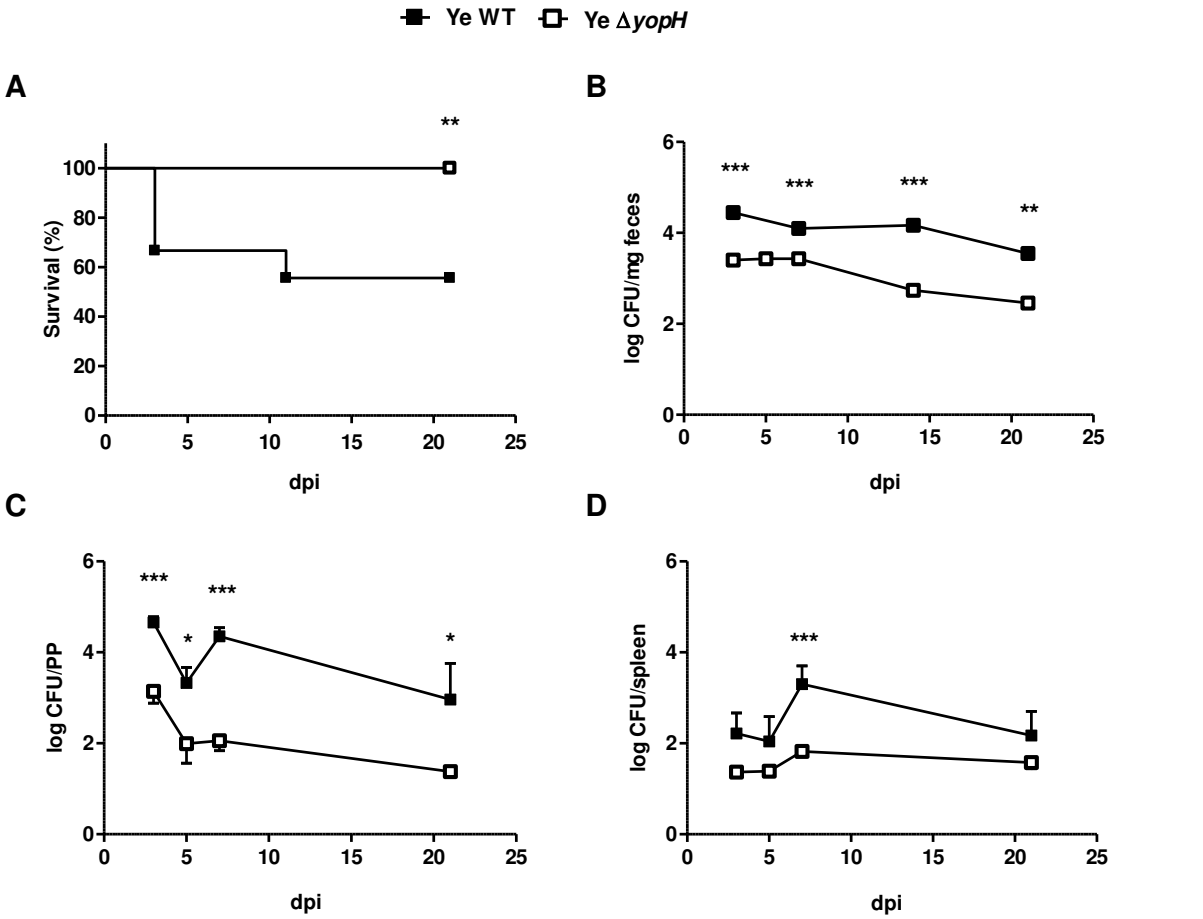
Figure 7. Expression of CXCR2 after *in vitro* infection.

Blood neutrophils were *in vitro* infected with *Y. enterocolitica* WT or *Y. enterocolitica* Δ*yopH* at multiplicity of infection (moi) 50:1 for 30 min. The cells were washed and the CXCR2 expression in $Ly6G^{+}CD11b^{+}$ cells was analyzed by flow cytometry. A) Representative overlaid flow cytometry histogram analysis showing CXCR2 expression on neutrophil ($Ly6G^{+}CD11b^{+}$) gate compared with uninfected cells (medium). Isotype control (grey peak). B) The average CXCR2 mean fluorescence intensity (MFI) levels are indicated in the graphics. The data are the summary results of 2 experiments. Each symbol represents cells from an individual mouse; horizontal lines indicate the mean. (*P<0.05). ns: not significant.

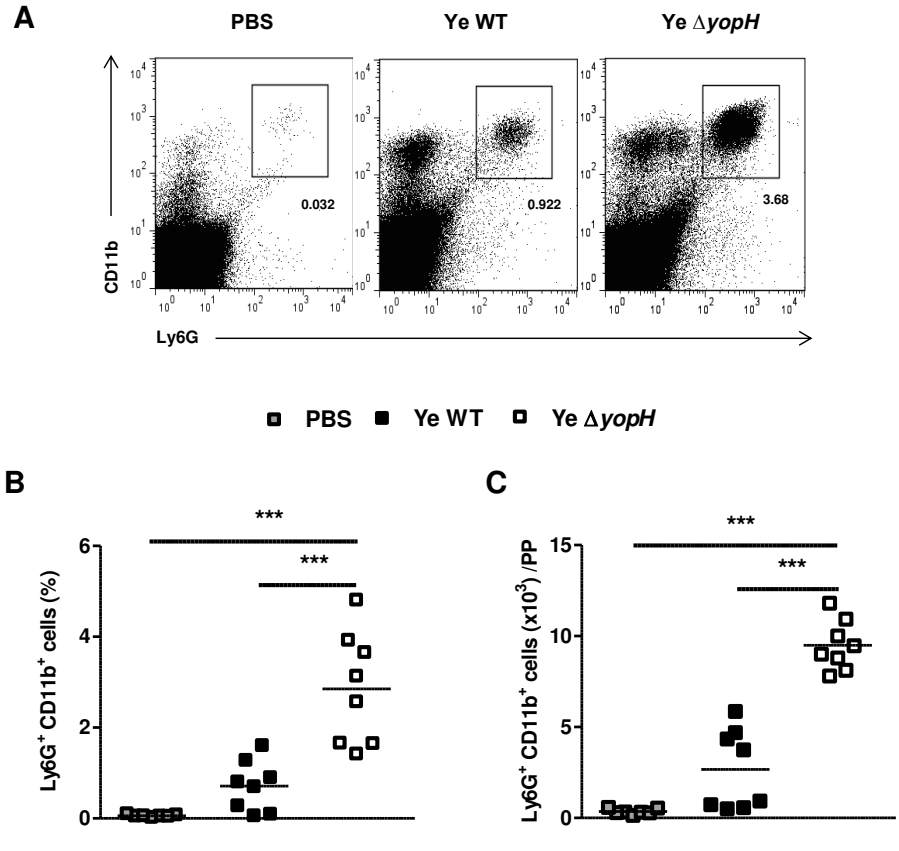
Figure 8. Impact of neutrophil depletion on the outcome of *Y. enterocolitica* (Ye) Δ*yopH* infection. Neutrophil-depleted mice were injected intraperitoneally with 100 µg of the monoclonal anti-Gr1antibody (clone RB6-8C5) 1 day before, and on days 2 and 3 after intragastric *Y. enterocolitica* (Ye) Δ*yopH* infection. Control mice received the same dosage of saline. Bacterial load (CFU) in the Peyer´s patches (PP) (A) and spleen (B) of neutrophil-depleted mice (Ye Δ*yopH* + RB6-8C5) and control mice (Ye Δ*yopH*) were assessed at day 3 after infection with Ye Δ*yopH*. Each symbol represents an individual mouse; horizontal lines indicate the mean. *** P<0.001.

744

745 **Figure 9. *Y. enterocolitica* Δ*yopH* promotes *Y. enterocolitica* WT clearance during**
746 **co-infection.** C57BL/6 mice were infected with $5x10^8$ CFU *Y. enterocolitica* (Ye) WT
747 or with an equal mixture of Ye WT and Ye Δ*yopH* ($2.5x10^8$ of each strain). At day 3
748 after infection, bacterial load of Ye WT was determined in Peyer´s patches (PP) (A) and
749 feces (B). C) Immunofluorescence analysis of abscess at day 3 after Ye WT-GFP
750 infection or Ye WT-GFP:Ye Δ*yopH* co-infection. Neutrophil infiltration was detected
751 with Ly6G-PE antibody (Red). Photographs are representative of one out of 4 mice per
752 group of 2 independent experiments. The data in A and B are the summary results of 2
753 experiments. Each symbol represents an individual mouse; horizontal lines indicate the
754 mean (***P<0.001).



Dave et al, Figure 1



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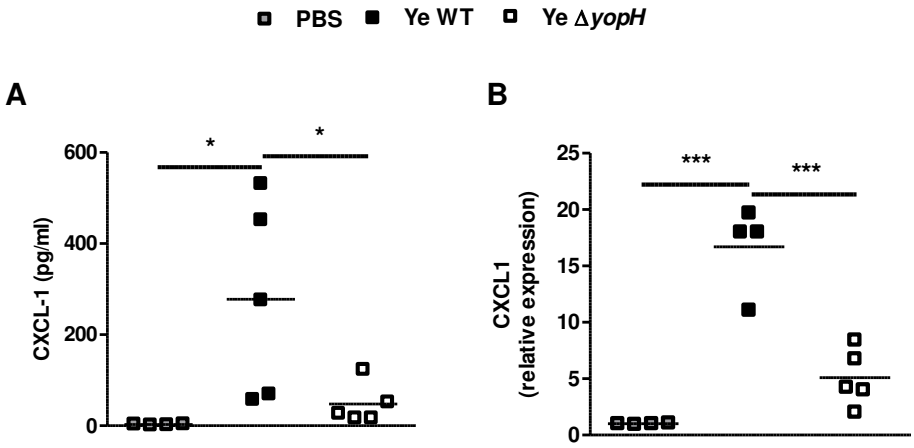

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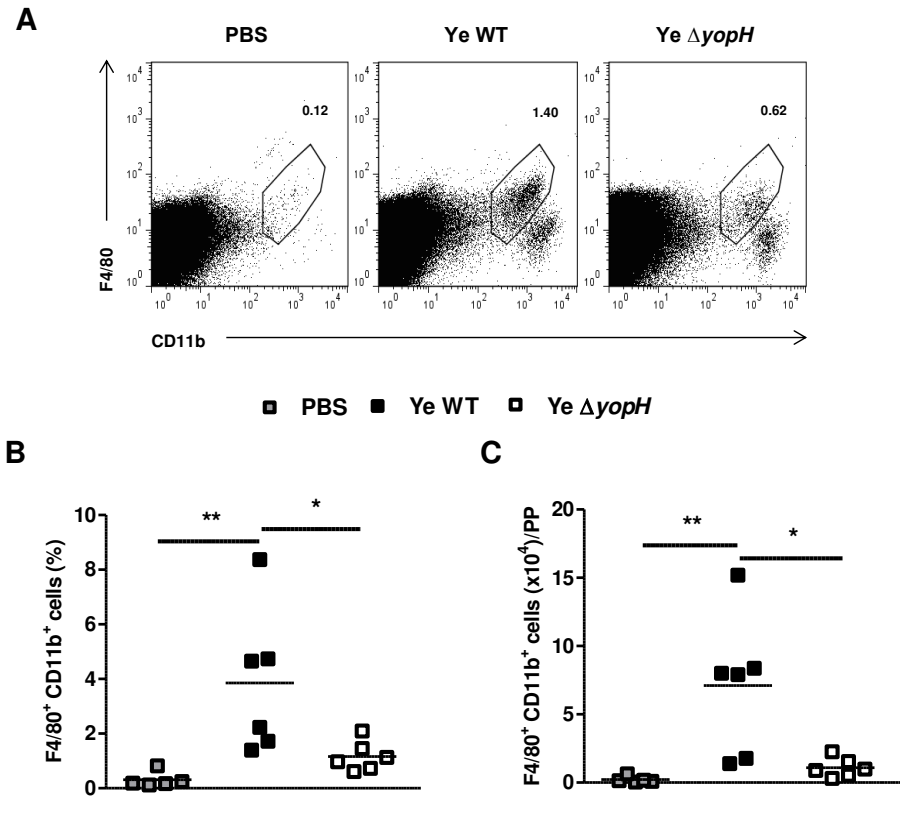

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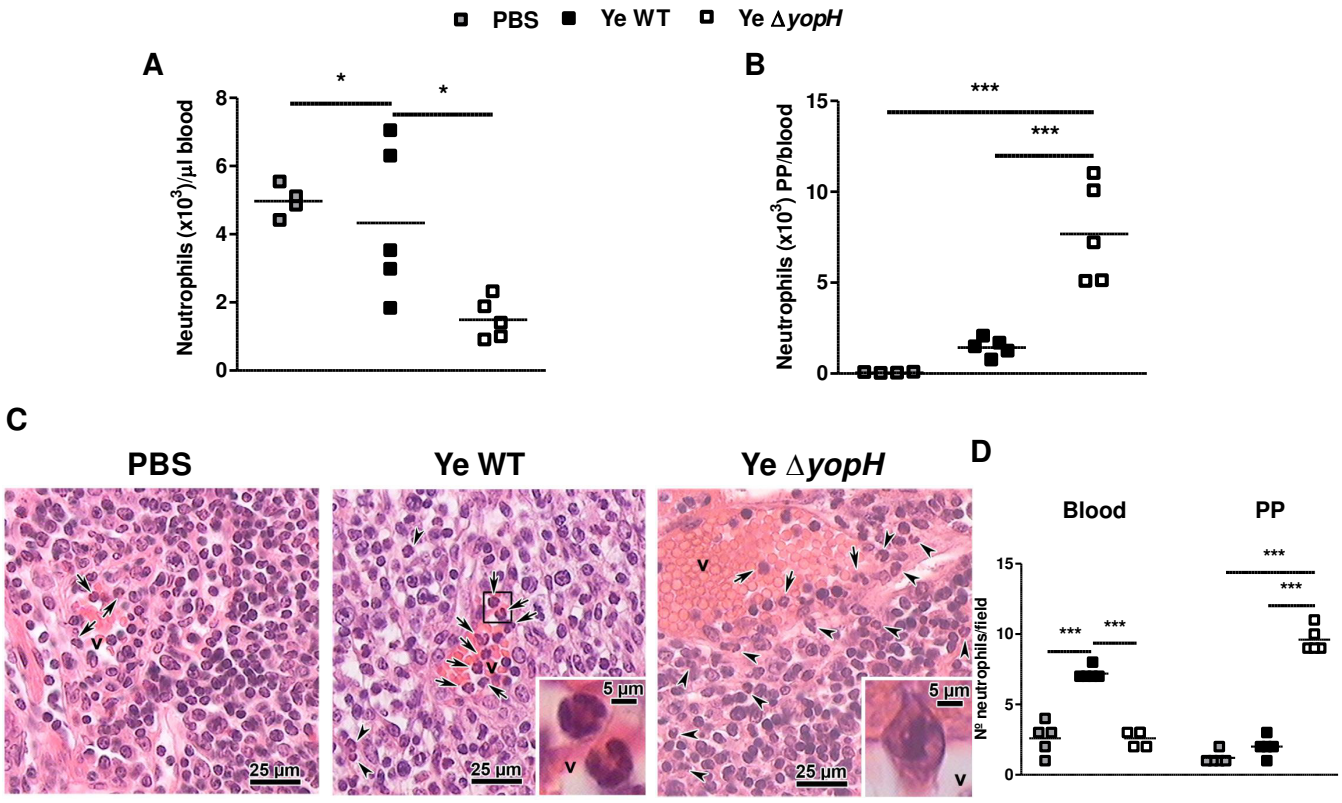

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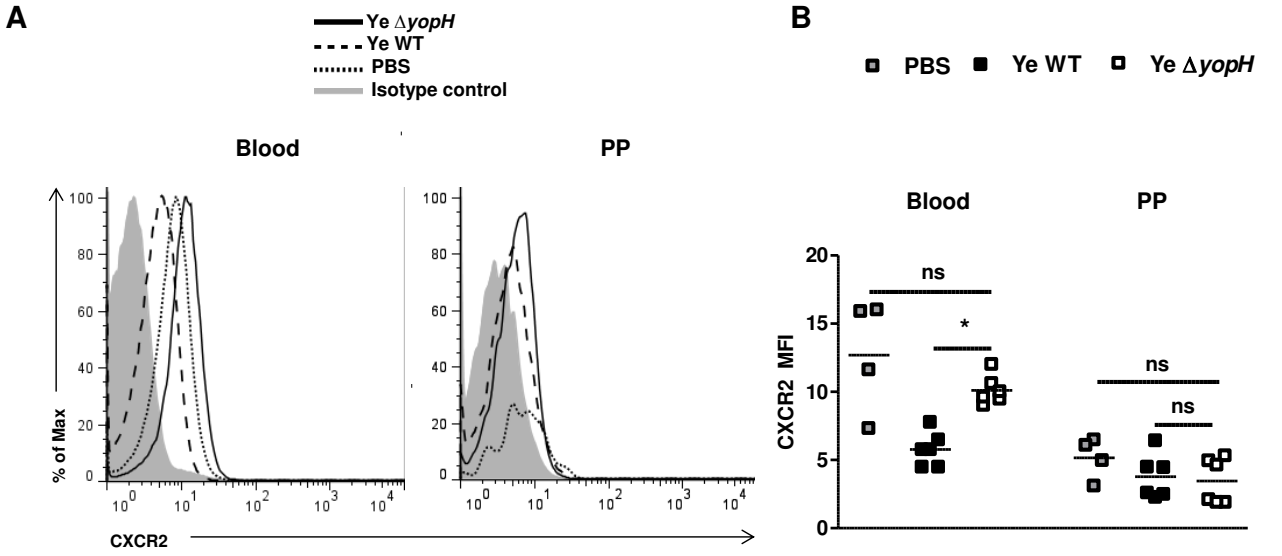

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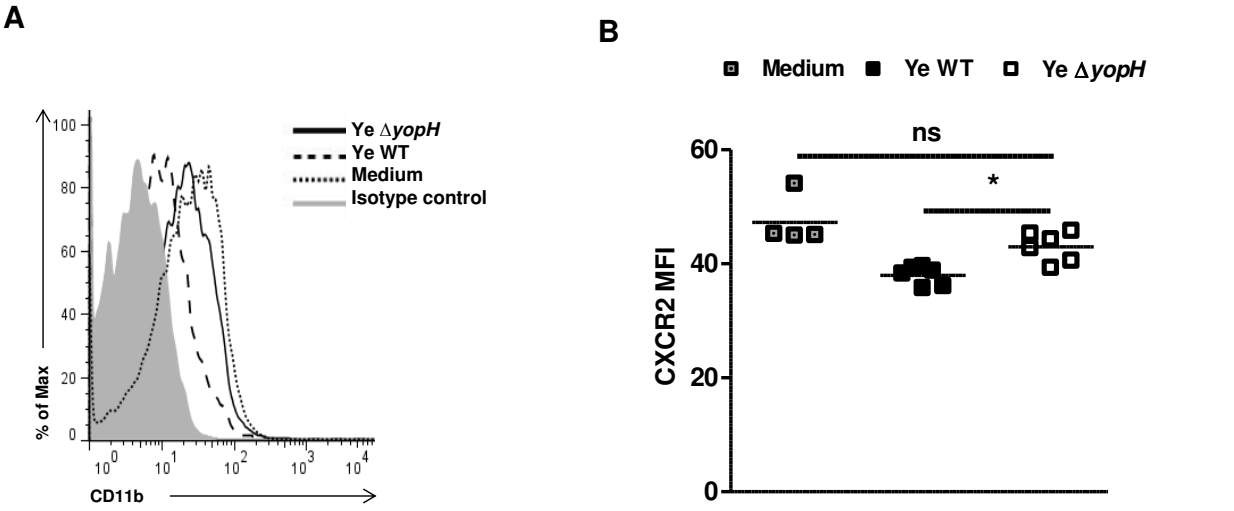

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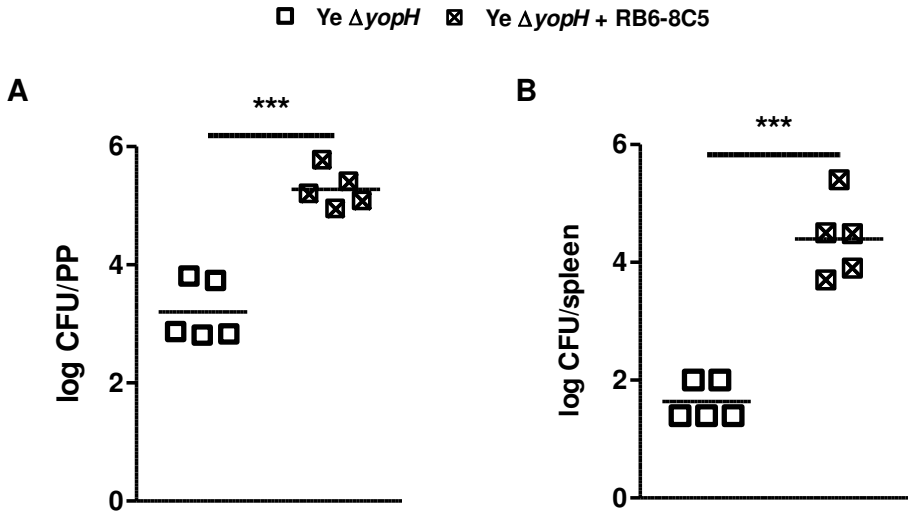

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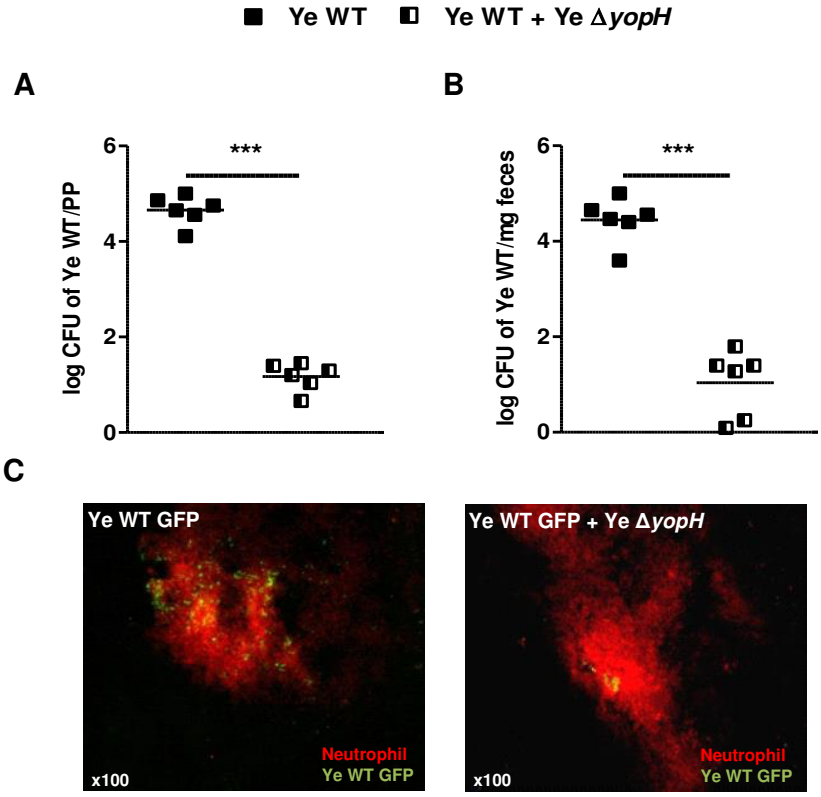

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