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Mononuclear phagocytes contribute to intestinal invasion and dissemination of *Yersinia enterocolitica*

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ABSTRACT

Enteropathogenic *Yersinia enterocolitica* (Ye) enters the host via contaminated food. After colonisation of the small intestine Ye invades the Peyer's patches (PPs) via M cells and disseminates to the mesenteric lymph nodes (MLNs), spleen and liver. Whether Ye uses other invasion routes and which pathogenicity factors are required remains elusive. Oral infection of lymphotoxin-β-receptor deficient mice lacking PPs and MLNs with Ye revealed similar bacterial load in the spleen 1 h post infection as wild-type mice, demonstrating a PP-independent dissemination route for Ye. Immunohistological analysis of the small intestine revealed Ye in close contact with mononuclear phagocytes (MPs), specifically CX_3CR1^+ monocyte-derived cells (MCs) as well as $CD103^+$ dendritic cells (DCs). This finding was confirmed by flow cytometry and imaging flow cytometry analysis of lamina propria (LP) leukocytes showing $CD103^+$ DCs and MCs with intracellular Ye. Uptake of Ye by LP $CD103^+$ DCs and MCs was dependent on the pathogenicity factor invasin, whereas the adhesin YadA was dispensable as demonstrated by Ye deletion mutants. Furthermore, Ye were found exclusively associated with $CD103^+$ DCs in the MLNs from wild-type mice, but not from $CCR7^{-/-}$ mice, demonstrating a CCR7 dependent transport of Ye by $CD103^+$ DCs from LP to the MLNs. In contrast, dissemination of Ye to the spleen was dependent on MCs as significantly less Ye could be recovered from the spleen of $CX_3CR1^{GFP/GFP}$ mice compared to wild-type mice. Altogether, MCs and $CD103^+$ DCs contribute to immediate invasion and dissemination of Ye. This together with data from other bacteria suggests MPs as general pathogenic entry site in the intestine.

1. Introduction

Yersinia enterocolitica (Ye) are Gram-negative predominantly extracellular located bacteria that cause food borne acute or chronic gastrointestinal and systemic diseases (Cover and Aber, 1989). Ye pathogenicity factors are encoded on the bacterial chromosome (Revell and Miller, 2001) and the 70 kb virulence plasmid pYV (Cornelis et al., 1998). Adhesion to the host cell is mediated by YadA, encoded on the pYV plasmid, which binds to extracellular matrix proteins like cellular fibronectin, collagen and laminin (Flügel et al., 1994; Roggenkamp et al., 1995; Schulze-Koops et al., 1993). Inactivation of the *yadA* gene in Ye impairs invasion of the mouse intestine (Kapperud et al., 1987). Ye was shown to invade through M cells of the PPs via binding of the outer membrane protein invasin to β1 integrins on the luminal surface of M cells which leads to the translocation of Ye across the M cells into the underlying tissue and eventual dissemination to the lymph nodes, spleen, lung, and liver (Autenrieth et al., 1996; Clark et al., 1998; Pepe and Miller, 1993; Schulte et al., 2000). 3 h post orogastric infection Ye were observed within venules (Autenrieth and Firsching, 1996) suggesting that dissemination occurs not only via the lymph, but also via the blood.

Abbreviations: Ye, *Yersinia enterocolitica*; PPs, Peyer's patches; MLNs, mesenteric lymph nodes; MCs, monocyte-derived cells; DCs, dendritic cells; LP, lamina propria; MPs, mononuclear phagocytes; LTβR, lymphotoxin-β-receptor; GFP, green-fluorescent protein; RFP, red-fluorescent protein; CFU, colony-forming unit.

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Using mice lacking PP, it was shown that villous M cells exist in the small intestine, which serve as a gateway for the sampling of *Yersinia pseudotuberculosis*, *Salmonella*, and *Escherichia coli* (Jang et al., 2004). Moreover, *Yersinia* species were found in the spleen starting* one day post infection in mice lacking PP, indicating an PP-independent dissemination route (Barnes et al., 2006; Handley et al., 2005). It was suggested that *Y. pseudotuberculosis* needs to replicate in the intestinal lumen to efficiently colonize the liver and spleen (Barnes et al., 2006). However, the exact mechanism for Ye lamina propria (LP) invasion and dissemination independently of PP remains unknown.

The induction of active immunity and maintenance of intestinal homeostasis is mediated by intestinal mononuclear phagocytes (MPs), comprising dendritic cells (DCs) and macrophages. Specialized MP subsets fulfill distinct yet complementary functions. Intestinal CX_3CR1^+ monocyte-derived cells (here referred to as MCs and characterized as $CD11c^+MHCII^+CD103^-CD11b^+CX_3CR1^{hi/+}$) reside in the LP and act as innate effector cells destroying bacteria, secreting cytokines, and maintaining intestinal homeostasis (Cerovic et al., 2014). The main functions of LP $CD103^+$ DCs (characterized as $CD11c^{hi}MHCII^+CD103^+CD11b^+$) are the migration from the intestine to the mesenteric lymph nodes (MLNs) and the initiation of adaptive immune responses by priming naıve T cells (Bogunovic et al., 2009; Coombes et al., 2007; Guilliams et al., 2014; Johansson-Lindbom et al., 2005; Rescigno, 2009; Schulz et al., 2009; Varol et al., 2009).

MPs sample antigens and microorganisms via different routes. M cells are located in the villous epithelium and transport antigens directly to MPs in the LP (Jang et al., 2004). Goblet cells are also able to deliver soluble antigens to underlying $CD103^+$ DCs (McDole et al., 2012). Moreover, LP MCs are able to open tight junctions between enterocytes in a CX_3CR1-dependent manner and extend their dendrites in the intestinal lumen to directly sample luminal antigens in the steady state and after TLR-stimulation of *Salmonella typhimurium* infection (Chieppa et al., 2006; Niess et al., 2005; Rescigno et al., 2001b; Vallon-Eberhard et al., 2006; Varol et al., 2009). Nevertheless, $CD103^+$ DCs are also capable to directly sample luminal Salmonella via transepithelial dendrite formation (Farache et al., 2013).

The transport of sampled antigens to the MLNs by the different MPs and subsequent stimulation of naïve T cells is still a matter of debate. It was longtime accepted that $CD103^+$ DCs, unlike CX_3CR1^+ MCs, migrate to MLNs and stimulate naïve T cells (Milling et al., 2010; Ng et al., 2010). Supporting this notion, other DC subsets are also qualified to form transepithelial dendrites to sample bacteria or bacterial antigens are passed from CX_3CR1^+ MCs to a migrating population to transport them to the lymph nodes (Ng et al., 2010). In contrast, it was recently shown that CX_3CR1^+ MCs migrate to the MLNs and transport at least non-invasive bacteria to the MLNs (Cerovic et al., 2012; Diehl et al., 2013).

Thus, at present, multiple mechanisms have been shown being involved in the initial LP invasion as well as the dissemination of Ye. However, the actual contributions of the various mechanisms remain unclear. Here, we addressed the role of (i) intestinal MPs and of (ii) bacterial virulence factors for Ye invasion into the LP of the small intestine and subsequent dissemination.

2. Materials and methods

2.1. Mice

Animal experiments were performed in strict accordance with the German regulations of the Society for Laboratory Animal Science (*GV-SOLAS*) and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The protocol was approved by the Regierungspräsidium Tübingen (Anzeigen 01.12.10, 09.10.07; Permit Numbers: H2/05, IZ1/11). All efforts were made to minimize suffering.

Female C57BL/6JolaHsd mice were purchased from Janvier (St Berthevin Cedex, France). $LT\beta R^{-/-}$ mice (Fütterer et al., 1998), $CX_3CR1^{GFP/GFP}$ and $CX_3CR1^{GFP/+}$ mice (Jung et al., 2000), $CCR7^{-/-}$ mice (Forster et al., 1999) with a genetic C57BL/6 background were bred under specific pathogen-free conditions in the animal facilities of the University of Tübingen or the Helmholtz Center for Infection Research, Braunschweig. Mice used for experiments were between 6 and 12 weeks of age and were provided food and water ad libitum.

2.2. Bacterial strains and culture conditions

In this study the virulent wild-type strain *Y. enterocolitica* WA-314 pYV^+ (WAP) serotype O:8 clinical isolate (Heesemann et al., 1983), the invasin-deficient mutant strain of WAP (Inv^-) (Ruckdeschel et al., 1996), the YadA-deficient mutant of WAP ($YadA^-$) (Roggenkamp et al., 1995) and the WA-314 strain expressing GFP or RFP (Oellerich et al., 2007) were used. To generate the GFP-expressing YadA-deficient WAP ($YadA^-$-GFP) or invasin-deficient WAP (Inv^--GFP) mutant strains of WAP, the GFP plasmid was isolated from the WAP (GFP) by alkaline lysis, purified over a column (Peqlab) and was electroporated into WAP ($YadA^-$) and WAP (Inv^-). Positive colonies were screened by selection with chloramphenicol.

The YadA-deficient, Inv-deficient double mutant was generated from WA-314 pYV^+YadA^- by allelic exchange as described previously (Kaniga et al., 1994). A Yersinia-compatible suicide plasmid was generated by removing PstI endonuclease restriction sites from pSB890 (Kaniga et al., 1994) by site directed mutagenesis. Suicide plasmids for Inv deletion were constructed by Gibson assembly according to standard protocols (Gibson et al., 2009). Primers and plasmids for site-directed mutagenesis and allelic exchange are listed in Table S1.

The strains of Ye were grown overnight at 27 °C in Luria-Bertani broth supplemented with nalidixic acid (10 μg/ml). For the WAP (GFP^+) and WAP (RFP^+) expressing mutant strains chloramphenicol (25 μg/ml) was additionally supplemented, for WAP ($YadA^-$) and WAP (Inv^-) kanamycin (50 μg/ml) was added. A 1:20 dilution of the overnight *Y. enterocolitica* culture was incubated for an additional 2–3 h at 27 °C. The bacteria were washed once with phosphate-buffered saline (PBS) and the optical density at 600 nm (OD600) was determined to calculate bacterial numbers.

2.3. Intragastric infection

Mice were infected intragastrically with the indicated amount of Ye in 400 μl PBS, control mice were injected with 400 μl PBS. Mice were subjected to fasting at least 3 h prior and after infection. The administered infection dose was determined by plating serial dilutions onto Müller-Hinton agar plates. Mice were sacrificed at the indicated time points after infection by CO_2 asphyxiation, and small intestines, PPs, MLNs and spleens were aseptically removed.

2.4. Loop infection

Mice were anesthetized intraperitoneally with a mixture of fentanyl (0.05 mg/kg bodyweight), midazolam (5 mg/kg bodyweight) and medetomidin (0.05 mg/kg bodyweight) for the entire duration of the experiment. Stretches of the small intestine of nearly two centimeters long were ligated at both extremities with surgical thread. 5×10^9 bacteria were resuspended in 50 μl and injected into the loop. After 15 min intestines were removed and pieces with loop infection and without treatment (internal control) were collected. The tissue sections were either fixed overnight in 1%