Original Paper

Increased Susceptibility to *Yersinia enterocolitica* Infection of *Tff2* Deficient Mice

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Key Words

Cytoprotective peptides • Trefoil factor family • Gastrointestinal tract • *Yersinia* infections

Abstract

TFF2 is one of the members of the trefoil factor family, known for its role in protection of gastrointestinal epithelia upon injury; however, recent studies suggest that TFF2 could also play an important role in the immune system. In the present study *Tff2* deficient and wild type mice were infected by *Y. enterocolitica* which resulted in a lethal outcome in all *Tff2* deficient mice, but not in WT animals. *Yersinia* invaded Peyer's patches more efficiently as shown by high bacterial titers in the KO mice while wild type mice displayed lower titers and a visible bacterial accumulation in the intestine. Bacterial accumulation in Peyer's patches of *Tff2* deficient mice was accompanied by increased recruitment of macrophages. While an increased level of MAC-1 positive cells was observed in the spleens of both *Tff2* deficient and WT mice at third day post infection, bacterial dissemination to liver, lung and kidneys was observed only in *Tff2* knock-out mice. Analysis of the cellular composition of spleen did not reveal any substantial alteration to WT animals, suggesting possible dysregulation of hemopoietic cells involved in immune response to *Y. enterocolitica*. These new data indicate that *Tff2* plays an important role in immune response by protecting the organism from consequences of infection and that *Tff2* knock-out mice react adversely to bacterial infections, in this case specifically to *Y. enterocolitica*.

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Introduction

The trefoil factor family (TFF), comprises of three polypeptides, TFF1, TFF2 and TFF3 (7–12 kDa) secreted to mucosal surfaces by mucus producing cells, most prominently in the gastrointestinal (GI) tract [1–3]. TFFs play an important role in the process of restitution after mucosal injury and protect the integrity of the epithelial barrier [4–6]. In addition, treatment with exogenous TFF peptides demonstrated both protective and healing functions in stomach and colon [7, 8]. Their expression is rapidly and coordinately up-regulated at the margins of mucosal injury [9, 10], in gastric ulceration [11] and inflammatory bowel disease [12, 13]. In addition, they participate in the mucosal immune response by stimulating leukocyte migration [14]. Furthermore, TFF2 deficiency has been shown to exacerbate inflammatory response upon gastric injury and cause higher susceptibility to *H. felis* induced gastritis. *In-vitro* experiments including lymphoblastic Jurkat cells revealed a modulatory role of TFF2 in Ca^{2+} and AKT signaling through the CXCR4 receptor [15].

Trefoil family factor 2 (TFF2), also known as spasmodic peptide, is a low-molecular-weight protein that is up-regulated in gastric tissues during inflammatory conditions. Studies of gene activity in *Tff2* deficient mice by microarrays demonstrated differential expression patterns of multiple genes involved in innate immunity [15]. Furthermore, TFF2 and TFF3 were found to be expressed in organized lymphoid tissues, like spleen, lymph nodes and bone marrow [16]. Study by Kurt-Jones et al. showed that the inflammatory and proliferative responses of macrophages and lymphocytes are dysregulated in *Tff2* knockout mice. *Tff2* deficient cells are hyperresponsive to interleukin 1 beta stimulation but respond normally to lipopolysaccharide, suggesting a specific role for TFF2 in interleukin 1 receptor but not Toll-like receptor 4 signalling via their Toll-interleukin 1 resistance domains [15].

Y. enterocolitica causes acute or chronic enteric disorders such as enteritis, enterocolitis, and mesenteric lymphadenitis, which are normally self-limited [17, 18]. *Y. enterocolitica* has evolved efficient mechanisms to overcome the innate immune system, thus the acquired immunity including CD4 helper and CD8 cytotoxic T cells, particularly interferon- γ -producing CD4 T helper 1 (Th1) cells in cooperation with activated macrophages play a crucial role in host defence against *Y. enterocolitica* [19, 20].

The precise role of trefoil peptides in the immune responses against *Y. enterocolitica* is yet to be elucidated, therefore, in the present study we investigated the onset of intestinal inflammation in *Tff2* deficient mice and their wild type controls by infecting with *Y. enterocolitica* and subsequent followed-up of the course of infection.

Materials and Methods

Animals

Tff2 knock-out (129/Sv) [16] and wild type (129/Sv) female mice (Charles River, Germany) 4–6 weeks old were used for three independent types of experiments. Animals were kept in a specific pathogen-free (SPF) facility of the University Clinic of Tuebingen with conditions of 12 h dark/light cycle, and temperature of 22°C. Food and water were accessible *ad libitum*. Commercially gained animals were acclimatized for a period of 7 days before the experiments. To avoid unnecessary suffering, all efforts were made to minimize the number of animals used (total n=26). Care and use of the animals and the experimental protocol were reviewed and approved by the regional board for scientific animal experiments in Tuebingen.

Bacterial strain and growth conditions

We used *Y. enterocolitica* WA-314 serotype O:8 which is a clinical isolate of serotype O:8 harboring virulence plasmid pYVO8 [21, 22]. *Y. enterocolitica* WA-314 serotype O:8 were grown overnight in Luria-Bertani (LB) medium under aerobic conditions at 27°C. For the infection experiments, bacteria in the exponential growth phase were used. 1:25 dilution of the bacterial culture was incubated for additional 3 hours at 37°C, then washed once with phosphate-buffered saline (PBS; Invitrogen, Karlsruhe, Germany) and the optical density at 600nm was determined.

Assessment of survival efficiency

Animals from both groups (*Tff2* knock-out and wild type, each n=7 animals, the same age and gender) were starved for four to six hours prior to oral infection. Pre- and post-infection genotyping of *Tff2* knock-out and wild type mice was performed via polymerase chain reaction (PCR) assays on genomic DNA isolated from ear and tail clips respectively. Mice were orally infected with 3×10^9 *Y. enterocolitica* WA 314 0:8. All animals were observed daily, and mortality was scored for 15 days. Data were statistically analyzed by logrank test using GraphPad software.

Quantification of *Y. enterocolitica* in the infected Peyer's patches

To investigate bacterial burden in the targeted tissues i.e. ileal Peyer's patches, *Tff2* knock-out and wild type mice (each group n=6) were orally infected and sacrificed by CO₂ asphyxiation. The entire small intestine was removed and extensively washed with cold PBS to remove bacteria associated with the mucosal surface of Peyer's patches. Then, the Peyer's patches of each mouse were carefully excised and pooled. The number of bacteria was determined by homogenizing the Peyer's patches in PBS and plating serial dilutions of the homogenates onto Mueller-Hinton agar at 27°C for 48 hours. T-test was performed with calculated p-value using GraphPad prism software.

Histopathology

To monitor histopathological effects, wild type and *Tff2* knock-out were sacrificed on 3rd day after infection with 3×10^9 *Y. enterocolitica* WA-314 serotype 0:8. Infected animals were perfused with PBS and later on with 4% paraformaldehyde. Various organs (stomach, duodenum, jejunum, ileum, caecum, colon, Peyer's patches, spleen, liver, kidney and lung) were collected to monitor histopathological changes due to infection. Isolated tissues were kept in 4% paraformaldehyde over night and embedded in paraffin. After cutting, tissue slides were preceded for hematoxylin and eosin (H & E) staining. Slides were investigated in a blind fashion in a separate laboratory using established MIRAX Viewer software.

Immunohistochemistry of Mac3

Paraffin-embedded tissue sections from Peyer's patches and ileum of infected *Tff2* knock-out as well as wild type mice were stained with rat anti-Mac3 antibody (Zytomed). Dewaxed 5µm thick tissue sections were incubated with a rat anti-Mac3 antibody (dilution, 1:400) for 60 minutes, followed by a peroxidase-conjugated goat-anti-rat secondary antibody (Vector) for 45 minutes (dilution, 1:100). Incubation of ABC-PO (Elite-Kit, Vector) was done for 30 minutes followed by using DAB (3,3-diaminobenzidine) (Dako DAB + Chromogen) as chromogene to detect positive cells. Mayers Haemalaun was used to counterstain the tissues.

Immunofluorescence staining

Liver, kidney, lung, spleen and ileum of *Y. enterocolitica* infected mice were embedded in Tissue-Tek and shock frozen in liquid nitrogen. Different antibodies were used, as follows: PE-anti-CR1.2 (clone 7G6, BD Biosciences), anti-CD4 (clone H129.19, BD Biosciences), anti-MAC-1 (clone M1/70 from ATCC). FITC-anti-MARCO (IBL-12) and anti-sialoadhesin (IBL-13) mAbs were used for the identification of marginal zone macrophage subpopulations [23]. Marginal zone B cells and follicular B cells were detected using anti-IgD (clone 11-26c.2a, BD Biosciences) and FITC-anti-IgM (clone B7.6 produced in-house). For detecting red pulp macrophages and granulocytes F4/80 and anti-Gr-1 (clone RB6-8C5) mAb as hybridoma supernatants obtained from ATCC were used. Biotinylated peanut agglutinin (Sigma-Aldrich, Budapest) was used for identification of germinal centres in the spleen. Rat hybridoma cell line secreting anti-B220 mAb (clone RA3-6B2) was obtained from the American Type Culture Collection. For multicolour analysis, anti-B220 mAb was conjugated with Alexa 647 (Invitrogen, Csertex Ltd, Budapest); anti-IgM and anti-MARCO were conjugated with FITC (Sigma-Aldrich Hungary, Budapest) according to standard procedures. PE-goat-anti-rat IgG (BD Pharmingen), FITC-mouse anti-rat kappa chain IgG (clone OX-12, produced in-house) and Streptavidin-Alexa Fluor 350 (Invitrogen, Budapest) were used as secondary reagents. Single indirect labelling was performed using anti-MAC-1, anti-F4/80 or anti-Gr-1 followed by FITC-conjugated OX-12. For multiple immunofluorescence first unlabeled rat mAb (anti-IgD or anti-sialoadhesin) was detected using PE-conjugated anti-rat IgG followed by saturation step with 20-fold diluted normal rat serum. Subsequently labelled rat mAb (FITC-anti-MARCO or FITC-anti-IgM) were applied. After mounting, the sections were

Fig. 1. Survival curve of *Tff2*^{-/-} and wild type mice upon infection with 3×10^9 *Y. enterocolitica* (n= 7 each) (p=0.0003).

Fig. 2. Bacterial load in Peyer's patches of *Tff2*^{-/-} and wild type mice with calculated p-value (p=0.0156).

viewed under an Olympus BX61 fluorescent microscope. Presence of *Y. enterocolitica* was detected by anti-*Yersinia* WA-vital as a primary and Cy2-goat anti-rabbit-IgG as a secondary antibody.

Immunization with human red blood cells (hRBC)

Human blood was collected into tubes containing EDTA, centrifuged and the supernatant discarded. Following, cells were tenfold diluted in PBS and 100 μ L/mouse was applied intraperitoneally. Seven days later mice were sacrificed and their spleens were processed for immunofluorescent staining. For the detection of germinal centre reaction PNA lectin was used.

Results

Anatomical differences in the expression of *Tff*-family members in the absence of *Tff*

The cluster of the three *TFF* genes is organized (direction of transcription) in the order of *TFF1* $\rightarrow$ *TFF2* $\rightarrow$ *TFF3* in humans as well as in mice. Our deletion construct affected solely sequences within *Tff2*. Due to their close genomic positioning, we also tested for expression of *Tff1* and *Tff3* in our mouse model in different tissues. While the level of expression of *Tff1* remained unchanged (*Tff2* wild type vs *Tff2* knock-out), deleting *Tff2* sequences resulted in up-regulation of the downstream *Tff3* gene by factor 41 (in Brunner's glands) or factor 1.9 (in duodenum) as judged by expression microarrays [16].

Shorter life span of *Tff2*^{-/-} mice after *Y. enterocolitica* infection and morphologic changes following infection

To monitor the effect of impaired *Tff2* under bacterial load, we challenged *Tff2* knock-out as well as wild type mice by infecting them orally with *Y. enterocolitica*, a species exerting severe virulent effects. Bacterial infection caused 100% mortality in *Tff2* knock-out mice within 7 days while 80% of the wild type animals survived (Fig. 1). This dramatic effect reflects diminished defence when murine *Tff2* is deleted.

Post infection of *Tff2* knock-out animals showed that blood vessels in the small intestine were injured or bloody and lymph nodes were swollen as compared to wild type animals. Stomach was swollen due to gas, and caecum was bloody in some *Tff2* knock-out mice (data not shown).

Higher bacterial burden in the ileal Peyer's patches of *Tff2*^{-/-} mice and altered distribution of lamina propria macrophages following *Y. enterocolitica* infection associated with *Tff2*-deficiency

Yersinia species are normally taken up by M cells, specialized epithelial cells which

Fig. 3. Macrophage staining in the ileum (Mac1 – top row, Mac3 – row below, respectively). Similarly, Mac3 staining is shown in the Peyer's patches of infected *Tff2*^{-/-} and wild type mice. The arrows point at Mac+ positive cells.

Fig. 4. Post-infection staining (bright) of *Yersinia* in different tissues (liver, kidney and lung) of *Tff2* knock-out as well as wild type mice.

occur within the follicle-associated epithelium of the Peyer's patches. M cells expose $\beta 1$ -integrins at their apical surface, which may bind to the *Yersinia* invasin protein [24]. To quantify the penetration of the epithelial barrier by *Y. enterocolitica* in animals lacking *Tff2* and the bacterial invasion of the Peyer's patches, we determined the bacterial titer, which demonstrated a higher bacterial load ($p=0.0156$) as compared to wild type (Fig. 2). On the other hand, this mechanism enables *Yersinia* to invade the host tissue, and most likely subsequently disseminate by lymphatics and blood, but on the other hand, in our knock out model, *Yersinia* crossed mucosal barriers and/or the innate immune response initiated in the Peyer's patches more vigorously resulting in tissue dissemination.

Fig. 5. T and B cell compartmentalization in the spleen of *Tff2* deficient and wild type mice (A), and the presence of different type of phagocytic cells including macrophages (F4/80+) and polymorphonuclears (Gr-1+). Antibodies against CR1.2 were used to identify the follicular dendritic cells. Marginal zone B cells are defined by high level of IgM expression with low to negligible levels of IgD. Marginal zone macrophages were identified by their MARCO (green) and metallophilic macrophages by sialoadhesin (red) expression, respectively.

Fig. 6. Distribution of MAC-1 positive cells in the spleen of pre- and post-infection *Tff2* deficient and wild type mice (A). Germinal centre reaction was identified by PNA lectin staining upon immunization with human red blood cells (B).

In addition to adhering to M cells, *Yersinia* may also enter the mucosal barrier through the site where efficient immune responses are initiated in the GI tract. To compare the distribution of intestinal pathogens, we investigated their gut regions in more details. In the wild type, the majority of bacteria was limited to epithelium of the ileum and did not spread to the Peyer's patches in any significant numbers. Here we found more numerous activated macrophages within the ileum than in the *Tff2* knock-out strain indicating a more efficient defence in the wild type animals. In addition it was observed that there is an impaired migration of macrophages to the intestinal villi (Fig. 3) in *Tff2* knock-out mice. In contrast, the reduced amount of macrophages in *Tff2* knock-out mice in intestinal lamina propria

allowed *Y. enterocolitica* to cross the epithelial barrier of the ileum more vigorously and to spread to other target tissues, such as Peyer's patches, more efficiently. As a consequence, more activated macrophages were noted in Peyer's patches of *Tff2* knock-out than in wild type animals (Fig. 3).

Bacterial dissemination, splenic architecture and the germinal centre formation

In addition to causing intestinal inflammation, *Y. enterocolitica* may also colonize other organs. To investigate the degree of bacterial dissemination in *Tff2*-deficiency, immunofluorescence staining of *Y. enterocolitica* was performed. Figure 4 shows that *Y. enterocolitica* spread to liver, lung and kidneys of *Tff2*-KO, but not in wild type mice. Observed dissemination of *Yersinia* in *Tff2*-KO is consistent with lethal infection seen in these mice. These data suggest that *Tff2*-KO mice can not mount immune responses as effectively in their intestines as wild-type mice, thus the capacity of mutant mice to limit bacterial dissemination to other organs is substantially impaired.

Hematogenic spreading of pathogens is controlled by the spleen. Enhanced colonization of extraintestinal organs suggests a reduced bacterial filter capacity by this organ. To investigate the effect of *Tff2*-deficiency on the lymphoid organization of spleen, we used immunohistological analysis. Investigation of the cellular composition of spleen in the *Tff2* deficient mice pre- and post-infection did not reveal any significant alteration from normal wild type animals. All constituent cells, including subpopulations of B and T cells, macrophages and polymorphonuclear leukocytes, were present at expected locations (Fig. 5). Thus both follicular B cells and follicular dendritic cells as well as marginal zone B cells were readily detectable, together with properly structured marginal zone macrophage compartments. In addition, MAC-1 positive macrophages were also increased at third day following infection, associated with detectable bacterial dissemination to the spleen (Fig. 6). To test whether the absence of *Tff2* interferes with the spleen's ability to mount T-dependent immune response, *Tff2* deficient and wild type mice were immunized by hRBC to induce germinal centre reaction. We found that both groups of mice were similarly able to mount a humoral immune response, as proven by the occurrence of PNA-positive germinal centres on the 8th day.

Expression of mucosal Tff2 deficiency relevant immunomodulatory genes

When comparing wild type to *Tff2* knock-out animals, numerous genes displayed modulated expression, as reported previously [16]. Assessing expression levels upon *Y. enterocolitica* infection of genes relevant to mucosal immune response (*Rbp2*, *Gusb*, *Timp1*, *Lyz1*, *Psmb5*, *Bag2*, *Tap1*, *Tff3*, *Mmp7*, *Ctsc*, *Crip1*) using quantitative real time PCR indicated that these genes were expressed at comparable levels resulting in expressional ratio (wild type vs. *Tff2* knock-out) close to 1 (data not shown).

Discussion

Three pathogenic species of the genus *Yersinia* (*Y. pestis*, *Y. enterocolitica*, *Y. pseudotuberculosis*) cause serious human diseases [25], with *Y. pestis* being the etiological agents for the often fatal plague, and the two remaining species being enteropathogens that also cause infections of the gastrointestinal tract. Infections caused by *Y. enterocolitica* are normally self-limited. All species of genus *Yersinia* share common tropism for lymphoid tissues and a common capacity to resist non-specific immune response, particularly phagocytosis and killing by macrophages and PMN [26]. Thus the clearance of *Yersinia* is promoted primarily by adaptive immune responses, including activation and proliferation of CD4 and CD8 T cells at the site of infection [19, 20].

In addition to its production in the mucosa of gastrointestinal and respiratory tract, *Tff2* is also highly expressed in specialised lymphoid organs like lymph nodes, spleen and bone marrow, whereas *Tff2* deficiency has been shown to modulate several genes involved in the

immune system [27]. Supporting the role of TFF2 in mucosal defence, *in-vivo* studies on *Tff2* deficient mice have shown higher susceptibility to infection and inflammation [15].

Although extensive research efforts on the *Tff2* deficient mice have identified several alterations in the genes involved in the immune system, as well as in lymphocytes' dysregulation *in vitro*, the *in vivo* relevance of these findings remains elusive. In addition, information on the structure of the lymphoid organs of *Tff2* deficient mice is scarce. Therefore *Tff2* knock-out mice were challenged with *Y. enterocolitica* to investigate the effect of TFF2 deficiency on the course of infection. Survival rate, histopathology, bacterial load in the Peyer's patches, *Yersinia* dissemination, expression of the genes previously found to be diversely expressed in *Tff2* KO mice, and cellular composition of the spleen were determined and compared between wild type and *Tff2* KO mice.

The infection by *Y. enterocolitica* resulted in a lethal outcome in all *Tff2* deficient mice, but not in WT animals. *Yersinia* invaded Peyer's patches more efficiently as shown by high bacterial titers in the KO mice while wild type mice displayed lower titers and a reduced bacterial accumulation in the intestine. While an increased level of MAC-1 positive cells was observed in the spleens of both *Tff2* deficient and WT mice at third day post infection, bacterial dissemination to liver, lung and kidneys was observed only in *Tff2* knock-out mice.

The pathogenesis of *Yersinia* infection as well as the mechanism of defence have been well described previously [28, 29]. Usually, the host's defence strategy involves phagocytosis of bacteria by macrophages, while in the case of *Yersinia*, blocking of phagocytes leads to increased bacterial growth, tissue dissemination and destruction. Further bacterial growth is hindered by recruitment of macrophages and T cells which involves various cytokines and integrins [30-33]. These cells produce cytokines such as IFN- γ and IL-2, but not IL-4 or IL-10, indicating the importance of Th1 polarized immune response in yersiniosis [20, 24, 34]. In addition, study of Matteoli et al. confirmed the importance of INF- γ and IL-6 by using mice deficient for INF- γ and IL-6, respectively [35]. During the course of infection with *Y. enterocolitica* splenic neutrophils (MAC-1+, Gr-1+) undergo a 10-fold increase in absolute number by day one post infection, whereas an increase in the number of macrophages (MAC-1+/F4/80+) appears with delay and reaches 3-fold of the increase of neutrophils after 72 h [36]. However, we have identified a significant increase in MAC-1 positive cells in the spleen of both wild type and *Tff2* deficient mice at 3rd day post infection. In addition, there was no alteration in the presence and positioning of other constituting cell types of the spleen when compared to the wild type controls, suggesting dysregulation of the effector mechanisms involved in defence against *Yersinia*.

Microarray studies in gastric mucosa of *Tff2*-deficient animals have identified altered expression levels (mainly up-regulation) of genes linked to immunity, such as defensins and MHC class I-associated genes [27]. An inspection of the promoter region of the stomach trefoils TFF1 and TFF2 showed the presence of consensus motifs for a number of transcription factors including AP-1, NF- κ B, STAT 6, C/EBP β , PPARE and USF, commonly utilized by both pro- and anti-inflammatory cytokines and growth factors to regulate target gene transcription [37].

In a recent study TFF2^{-/-} macrophages were hyperresponsive to interleukin 1 beta stimulation but showed normal responses to lipopolysaccharide, suggesting a specific role for TFF2 in interleukin 1 receptor but not Toll-like receptor 4 signaling via their Toll-interleukin 1 resistance domains. These results suggest a possible role of TFF2 in negative regulation of IL-1R signalling [15]. On the other hand, IL-6, one of the cytokines induced by IL-1 signalling, has an important protective role during *Yersinia enterocolitica* infection [38]. Thus, increased susceptibility to *Y. enterocolitica* observed in our study adds more evidence to dysregulated inflammatory and proliferative responses seen *in vitro*.

In summary Tff2 plays an important role in the defence system of an organism and *Tff2* knock-out mice are severely affected by bacterial infections, in this case specifically to *Y. enterocolitica*.

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