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TFF PEPTIDES PLAY A ROLE IN THE IMMUNE RESPONSE FOLLOWING ORAL INFECTION OF MICE WITH *TOXOPLASMA GONDII*

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The peptide trefoil factor family 3 (TFF3) is a major constituent of the intestinal mucus, playing an important role in the repair of epithelial surfaces. To further understand the role of TFF3 in the protection of intestinal epithelium, we tested the influence of TFF3 in a murine *Toxoplasma gondii*-induced ileitis model. Surprisingly, $TFF3^{KO}$ mice showed a reduced immune response in the ileum when compared to wild-type animals. Interleukin-12 and interferon-γ expression levels as well as the number of $CD4^+$ lymphocytes were reduced in the infected $TFF3^{KO}$ mice. These effects were in line with the trend of elevated parasite levels in the ileum. Moreover, TFF1 expression was upregulated in the spleen of infected mice. These initial results indicate that TFF3 is involved in the immune pathology of *T. gondii* infection-induced intestinal inflammation. Thus far, the mechanisms of how TFF3 influences the immune response are not fully understood. Further studies should identify if TFF3 affects mucus sensing of dendritic cells and how TFF3 is involved in regulating the immune response as an intrinsic secretory peptide of immune cells.

Keywords: TFF3, TFF1, TFF peptide, trefoil factor, *Toxoplasma gondii*, intestinal inflammation, FCGBP

Introduction

The peptide TFF3 (formerly intestinal trefoil factor, P1.B) is a member of the trefoil factor family (TFF) and is a typical secretory product of mucous epithelia (reviews: [1–3]). The predominant expression occurs in intestinal goblet cells together with the mucin Muc2 [4, 5]. However, TFF3 and Muc2 expression are not coordinately regulated [6]. From cDNA cloning, the size of the mature peptide was predicted to be 59 amino acid residues including seven cysteine residues [5]. Surprisingly, mature intestinal TFF3 exists predominantly as a disulfide-linked heteromer with the mucus-associated IgG Fc binding protein (FCGBP, M_r: >300.000); about 15–20% of TFF3 occurs in a monomeric form and only very little as a homo-dimer [7]. Other mucous epithelia with exocrine TFF3 synthesis are the salivary glands [8], the esophagus [9], the gastric cardia and antrum [9, 10], the Vater's ampulla [11], the respiratory tract [12], the uterus [13], the vagina [14], the urinary tract [15], the conjunctiva [16], and the efferent tear ducts [17]. In addition, TFF3 is also secreted by the endocrine pancreas [18], the central nervous system [19–21], and by certain cell subsets of the immune system [20, 22].

TFF3 is considered to play a key role in intestinal protection and repair mechanisms *in vivo* after damages by various noxes (compilation: [3]). For example, the intraperitoneal application of recombinant TFF3 significantly reduced the inflammatory index in a trinitrobenzene sulfonic acid (TNBS)-induced murine colitis model which was paralleled by downregulated toll-like receptor 4 (TLR4) and nuclear factor κB (NF-κB) expression [23]. Interestingly, in two rat colitis models, only the direct luminal application of recombinant TFF3 homo-dimer by a catheter was protective [24], whereas systemically administered TFF3 aggravated the colitis scores, particularly the TFF3 monomer [24]. Furthermore, the ectopic expression of TFF3 in the jejunum of transgenic mice resulted in a reduced sensitivity to indomethacin [25] as well as active delivery of TFF3 by genetically modified *Lactococcus lactis* protected against dextran sulfate sodium (DSS)-induced

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acute colitis [26]. In contrast, TFF3-deficient ($TFF3^{KO}$) mice were markedly more sensitive in a DSS colitis model (50% of the animals died in contrast to 5% of the wild-type animals) [27]. $TFF3^{KO}$ mice were also more susceptible to chemotherapy- and radiation-induced mucositis, and oral recombinant TFF3 was able to reduce the severity of the mucositis [28].

In vitro, a relatively weak motogenic effect (compilation: [3]) due to chemotaxis [29] as well as both pro- and anti-apoptotic effects have been reported for TFF3 [30, 31]. Thus far, all attempts have failed to characterize specific TFF3 receptors. Chemokine receptors might be candidates [32]. However, recombinant dimeric TFF3 has been reported to bind $DMBT1^{gp340}$ in a Ca^{2+} dependent manner [33]. Interestingly, TFF3 – similar to TFF1 [34] and TFF2 [35, 36] – has also been reported to act as a lectin [34]. This would enable TFF3 to bind to a plethora of transmembrane glycoproteins modulating different biological effects.

TFF3 is typically upregulated in chronically inflamed epithelia, particularly in the ulcer-associated cell lineage (UACL) [2, 5, 37]. The TFF3 regulatory regions are complex (review: [3]); a hallmark being the hypoxia-inducible factor-1 (HIF-1) [38]. Of note, strong immediate induction of TFF3 expression has been observed after TLR2 activation, whereas the ulcerative colitis-associated TLR2-R753Q variant is functionally deficient in the ability to induce TFF3 synthesis [39]. Furthermore, TFF3 was also induced in the injured rat jejunum after treatment with indomethacin possibly due to increased nitric oxide (NO) synthesis and stabilization of hypoxia-inducible factor-1 (HIF-1) [40]. Generally, inflammatory bowel disease (IBD) is characterized by increased TLR4/NF-κB signaling which triggers the upregulation of pro-inflammatory cytokines and contributes to the development of ulceration. In a recent study, the application of recombinant TFF3 promoted a protective effect against colitis and was followed by downregulated TNF levels in the colon. Thus, TFF3 inhibition of TLR4/NF-κB expression is a potential therapeutic in treatment of IBD [23].

TFF3–FCGBP and MUC2 are the major components of intestinal mucus, and they both colocalize in the gut [7, 41, 42]. When TFFs and mucin were combined, they were more effective in protecting the barrier function of epithelial cells [43]. Only within the last years did it become evident that intestinal mucus is not just a static barrier but directly communicates with the gut microbiota and immune cells of the gut. The mucus layer, which overlays the epithelium, forms an important barrier covering the entire gastrointestinal tract [44, 45]. This shield serves as a barrier to protect the host tissue from pathogens [46]. Recent studies indicate that mucus secretion by intestinal goblet cells involves crosstalk with the gut microbiota and food components via the process of autophagy [47, 48]. Both autophagy and endosome formation are required to trigger secretory granule accumulation and efficient mucus secretion via the generation of reactive oxygen species (ROS) [49]. Additionally, mucus also influences the function of antigen presenting cells by educating them to develop tolerance towards food and commensal antigens. Dendritic cells (DCs) are exposed to the mucus, and MUC2 in particular suppresses the responses of DCs to microbe-derived signals and promotes their capacity to stimulate the production of regulatory T (T_{reg}) cells [50, 51]. Thus, it is not too surprising that aberrant mucus production is often associated with inflammatory responses and has profound consequences on tissue homeostasis [51] because mucus is part of a complex feedback loop regulating both invasion of microbes as well as oral tolerance.

In a further attempt to investigate the potential protective role of TFF3 for the intestinal epithelium, we tested the influence of TFF3 on the intestinal inflammation caused by *Toxoplasma gondii* in a low-dose model [52]. The acute phase of infection followed by oral infection of mice with the parasite is associated with intestinal inflammation, and the high dose infection is a well-established model for acute ileitis [53]. Both acute and chronic stages of the infection are controlled by the pro-inflammatory cytokine interferon (IFN)-γ. Interleukin (IL)-12, mainly produced by DCs, macrophages, and polymorphonuclear neutrophils (PMNs), further drives the production of IFN-γ [54–57]. As a consequence, inflammatory monocytes are recruited to the ileum in a Ccr2- and Ccl2-dependent manner and act as a first line of defense expressing antimicrobial activities during *T. gondii* infection [52, 55, 58].

TFF2, another member of the trefoil factor family, has been previously reported to antagonize IL-12 release by macrophages and DCs after *T. gondii* infection [59]. Thus, TFF2 deficiency was associated with elevated IL-12 production and increased T-cell recruitment in naive mice. Infected $TFF2^{KO}$ mice displayed lower parasite numbers and reduced gut immunopathology [59]. In contrast, TFF2 positively regulates type 2 immunity and IL-33 production, e.g., after infection with the hookworm parasite *Nippostrongylus brasiliensis* [60].

Materials and methods

Murine oral T. gondii *infection model*

$TFF3^{KO}$ mice [27] were originally obtained from Prof. D.K. Podolsky (Harvard Medical School). These animals were then backcrossed to 129/Sv and C57BL/6 mice leading to a mixed background. As described previously, TFF3 homozygous sister lines were established (now crossings for more than ten generations) representing a TFF3-deficient genotype ($TFF3^{KO}$) and a wild-type (WT, $TFF3^{+/+}$) line, respectively [61–63].

Animal care and experimental procedures were performed according to legal regulations, and *T. gondii* infection experiments were approved by the state authorities (Landesverwaltungsamt Sachsen–Anhalt, Halle). The animals were kept in standard cages under specific-pathogen free (spf) conditions at the animal facility of the Medical Faculty, maintained on laboratory food and tap water

ad libitum in a regular 12 h dark–light cycle with a temperature of 22 °C.

To obtain *T. gondii* cysts, NMRI mice (Harlan-Winkelmann, Borchen, Germany) were orally infected with ten cysts of a type II strain (ME49) 5–6 months previously, and the tissue cysts in the brain homogenates were counted as described previously [57]. Experimental mice (age: 2–3 months) were orally infected with a brain inoculum equivalent to three cysts per mouse. Seven days post-infection, animals were anesthetized and transcardially perfused with 50 ml 0.9% NaCl, and tissue samples were collected for reverse transcription-polymerase chain reaction (RT-PCR) analysis and histological studies, respectively.

DNA and RNA extraction, PCR analysis

For genotyping the animals, genomic DNA was isolated from tail clippings taken at weaning and purified with Invisorb® spin tissue mini kit (1032100 300, STRATEC Molecular GmbH, Berlin, Germany) following the manufacturer's instruction. One percent of the DNA was used for PCR analysis (primer pairs: TFF3 MB1871/98, neomycin resistance gene/Neo MB1920/1921).

Total RNA/DNA of tissues was isolated and purified using TRIzol® reagent (Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer's protocol. One microgram DNA isolated from the ileum was used for PCR analysis to test infection with *T. gondii* (primer pair MB2342/2343). Alternatively, RNA was isolated with the Isolate RNA mini Kit (BIO-52073, Bioline GmbH, Luckenwalde, Germany).

Prior to reverse transcription, RNA preparations were digested with RNAse-free DNAse I (Thermo Fisher Scientific, Fermentas Walldorf, Germany) as described previously [64]. The concentration and purity of the RNA were estimated with a Nanodrop ND-1000 spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). First

Table 1. Oligonucleotides used for (RT)-PCR analysis and calculated size of the products. Actb, β-actin; Il-12a, Il-12p35 transcript variant 2, Iba1/Aif1, ionized calcium binding adapter molecule 1

Genes	Accession No.	Primer No.	Primer Pairs	Nucleotide Positions	Tm (°C)	Size (bp)	Intron Spanning
Actb	NM_007393.4	MB1912 MB1913	CCCTCACGCCATCCTGCGTC ACGCAGCTCAGTAACAGTCCGC	622-641 1259-1238	60	638	yes
Actb	NM_007393.4	MB2166 MB2167	AGTACCCCATTGAACATGGC GTAAAACGCAGCTCAGTAACAG	312-331 1264-1243	60	953	yes
Iba1/Aif1	NM_019467.2	MB1727 MB1728	GGATTTGCAGGGAGGAAAAG GCCACTGGACACCTCTCTAA	329-348 602-583	60	274	yes
Ifnγ	NM_008337.3	MB2054 MB2055	TCCTCCTGCGGCCTAGCTCTG TGGCGCTGGACCTGTGGGTT	83-103 494-475	60	412	yes
Il-1β	NM_008361.3	MB2038 MB2039	GTGGCTGTGGAGAAGCTGTGGC CAGGGTGGGTGTGCCGTCTT	270-291 659-640	60	390	yes
Il-10	NM_010548.2	MB2154 MB2155	CTGCTCTTACTGACTGGCAT GGAGTCGGTTAGCAGTATG	95-114 274-255	60	180	yes
Il-12a	NM_008351.3	MB2133 MB2134	CACAGTCCTGGGAAAGTCCTG TAGCCAGGCAACTCTCGTTC	9-29 410-391	60	402	yes
TFF1	NM_009362.2	MD7 MD8	AAACATGTATCATGGCCC GAATTCGAGGACTAAAAGTCTG	128-145 450-429	57	323	yes
TFF2	NM_009363.3	MD5 MD6	TTCCACCCACTTCCAAAC AATGCTGTGTCTAGCCACTG	242-259 551-532	57	310	yes
TFF3	NM_011575.2	MB1847 MB1848	TCTGGCTAATGCTGTTGGTG TCAGATCAGCCTTGTGTTGG	52-71 443-424	60	392	yes
Tnfα	NM_013693.3	MB2052 MB2053	GCAGCCAACCAGGCAGGTTCT ACGTAGTCGGGGCAGCCTTGT	94-114 622-602	60	529	yes
Actb/promoter	NC_000071.6	MB1783 MB1784	GATGCTGACCCTCATCCACT ATGAAGAGTTTTGGCGATGG	142907148-129 142906954-973	60	195	no
Asl	NC_000071.6		TCTTCGTTAGCTGGCAACTCACCT ATGACCCAGCAGCTAAGCAGATCA	130022680-657 130022575-598	60	106	
Neo	AM235741.1	MB1920 MB1921	TGCTCTGATGCCGCCGTGTT GCACGAGGAAGCGGTCAGCC	1073-1092 1709-1690	60	637	
TFF3	AJ271004.1	MB1871 MB98	CTGTCACATCGGAGCAGTGT TGACCCTGTGTCATCACCCT	4943-4962 5233-5214	60	291	e2-i2
***T. gondii* B1**	KC607827.1	MB2342 MB2343	TCCCCTCTGCTGGCGAAAAGT AGCGTTCGTGGTCAACTATCGATTG	113-133 210-186	60	98	

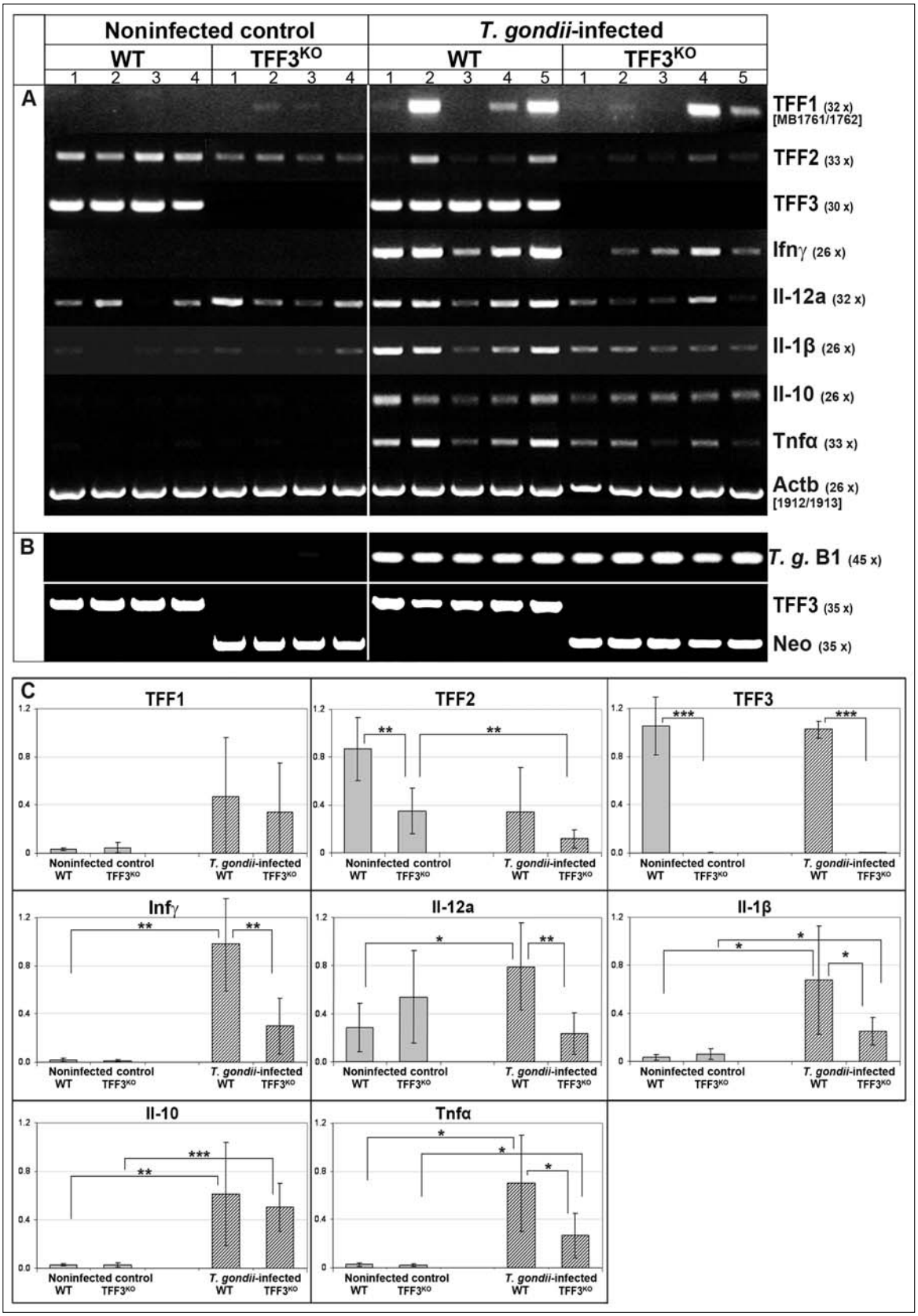

Fig. 1. (RT)-PCR analysis of the ileum. (A) TFF1, TFF2, TFF3, interferon-γ (Ifnγ), interleukin-12a (Il-12a), Il-1β, Il-10, and tumor necrosis factor α (Tnfα) expression were monitored in the ileum of noninfected control animals (4 WT and 4 $TFF3^{KO}$ mice, respectively) and orally *T. gondii*-infected animals (5 WT and 5 $TFF3^{KO}$ mice, respectively). The number of amplification cycles is given in parentheses. (B) Results of the *T. gondii* infection test (PCR analysis of DNA from the ileum for the *T. gondii* gene B1) and the genotyping (PCR analysis of genomic DNA from tail clippings for TFF3 and the neomycin resistance gene/Neo). (C) Results of the semiquantitative RT-PCR analyses (relative gene expression levels normalized against β-actin). Significant differences are indicated by asterisks

strand complementary DNA (cDNA) synthesis was performed with 1.0 μg RNA primed with oligo$(dT)_{18}$ using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific, Fermentas Walldorf, Germany). The relative expression level of selected genes was monitored by RT-PCR analysis including semiquantitative evaluation as described previously [64]. Generally, the highest intensity for each gene was set to 100, and then the relative intensities were normalized against the corresponding relative β-actin intensities. As a control for the integrity of the cDNA preparations, β-actin transcripts were amplified in parallel reactions. The cDNA was also checked for contaminating chromosomal DNA by amplification of a promoter sequence from the β-actin gene (primer pair MB1783/1784). The specific primer pairs used in this study are listed in *Table 1* or have been published previously [65].

Toxoplasma real-time PCR

Semiquantitative real-time PCR analyses were performed to determine parasite loads in brains as described previously [66]. FastStart Essential DNA Green Master (Roche, Grenzach-Wyhlen, Germany) was used with 90 ng genomic DNA in a reaction volume of 20 μL. Triplicate reactions were developed in a LightCycler 480 Instrument II (Roche, Grenzach-Wyhlen, Germany). After an initial activation step (95 °C for 10 min), 45 amplification cycles were run, comprising of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and elongation at 72 °C for 15 s. The primers manufactured by Tib MolBiol (Berlin, Germany) were used at a final concentration of 0.3 μM. The specific primer pairs are listed in *Table 1* (*T. gondii* B1 gene, murine argininosuccinate lyase/Asl as a reference). For normalization against Asl, target–reference ratios were calculated with the LightCycler 480 Software release 1.5.0 (Roche, Grenzach-Wyhlen, Germany). The resulting data were further normalized against a control group, i.e., the ileum of WT animals.

Histological studies

Tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin, and 5 μm sections were stained with hematoxylin and eosin (H&E) as described previously [52]. Immunohistochemistry was performed using a rabbit monoclonal anti-CD4 antibody (1:50; Epitomics, Burlingame/CA, USA) and a Benchmark XT automated slide staining system (Ventana, Tucson, AZ, USA) according to the manufacturer's recommendations.

Microscopic findings were evaluated using a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) with Olympus DP26 digital camera and CellSens Entry software (Olympus, Tokyo, Japan). The height of the mucosa was measured in triplicate, and a mean height value was calculated for each specimen. The number of $CD4^+$ lymphocytes in the mucosa was counted in three different high power fields (HPF), and a mean value of $CD4^+$ lymphocytes per HPF was calculated.

Statistical analysis

Student's *t*-test was performed using the Excel 2003 software package (Microsoft, USA). The error bars in *Figs 1–4* represent ±SEM. Significant differences between the mean values of the different experimental groups are indicated by asterisks ($P \leq 0.05$: significant, *; $P \leq 0.01$: highly significant, **; $P \leq 0.001$: extremely highly significant, ***).

Results

Altered immune response in the ileum of TFF3^{KO} and WT mice upon T. gondii *infection*

Clearly, the expression profile of the noninfected control ileum (4 WT and 4 $TFF3^{KO}$ mice, respectively) is altered when compared to the *T. gondii*-infected samples (5 WT and 5 $TFF3^{KO}$ mice, respectively) concerning eight selected genes as well as β-actin as a control *(Fig. 1)*. *Figure 1c* shows a semiquantitative analysis of these eight genes. Furthermore, the infection with *T. gondii* was controlled by amplifying the B1 gene *(Fig. 1b)*. WT and $TFF3^{KO}$ mice were affirmed by amplifying the TFF3 or the neomycin resistance gene, respectively *(Fig. 1b)*.

As anticipated, *T. gondii* infection is followed by the elevated expression level of several inflammatory markers *(Fig. 1a* and *1c)*. Notably, the expression of Ifnγ, IL-12, IL-1β, and Tnfα genes is significantly diminished in *T. gondii*-infected $TFF3^{KO}$ animals when compared with *T. gondii*-infected WT animals *(Fig. 1c)*.

Of note, TFF2 expression is downregulated in the ileum of noninfected $TFF3^{KO}$ mice when compared with the noninfected WT mice *(Fig. 1c)*. A reduced TFF2 expression trend can also be observed upon *T. gondii* infection *(Fig. 1c)*. In contrast, TFF1 expression of WT or $TFF3^{KO}$ mice is not altered *(Fig. 1c)*. Furthermore, there is a tendency to increased TFF1 expression in *T. gondii*-infected mice (this is not significant because of relatively large individual variations; *Fig. 1c*).

Based on the observation that the differently expressed inflammatory genes differ significantly in the ileum of WT and $TFF3^{KO}$ mice after oral *T. gondii* infection, the ileum of these mice was subject to a brief pathological review *(Fig. 2)*. In the mucosa of *T. gondii*-infected WT mice, typical inflammatory changes including increased cellularity and necrosis with a loss of villi were observed *(Fig. 2a)*. Furthermore, the height of the mucosa *(Fig. 2b)* and the number of CD4-positive lymphocytes per HPF were measured *(Fig. 2a)*. In particular, the height of the mucosa of *T. gondii*-infected animals was significantly shorter in the WT mice when compared with the $TFF3^{KO}$ mice, whereas

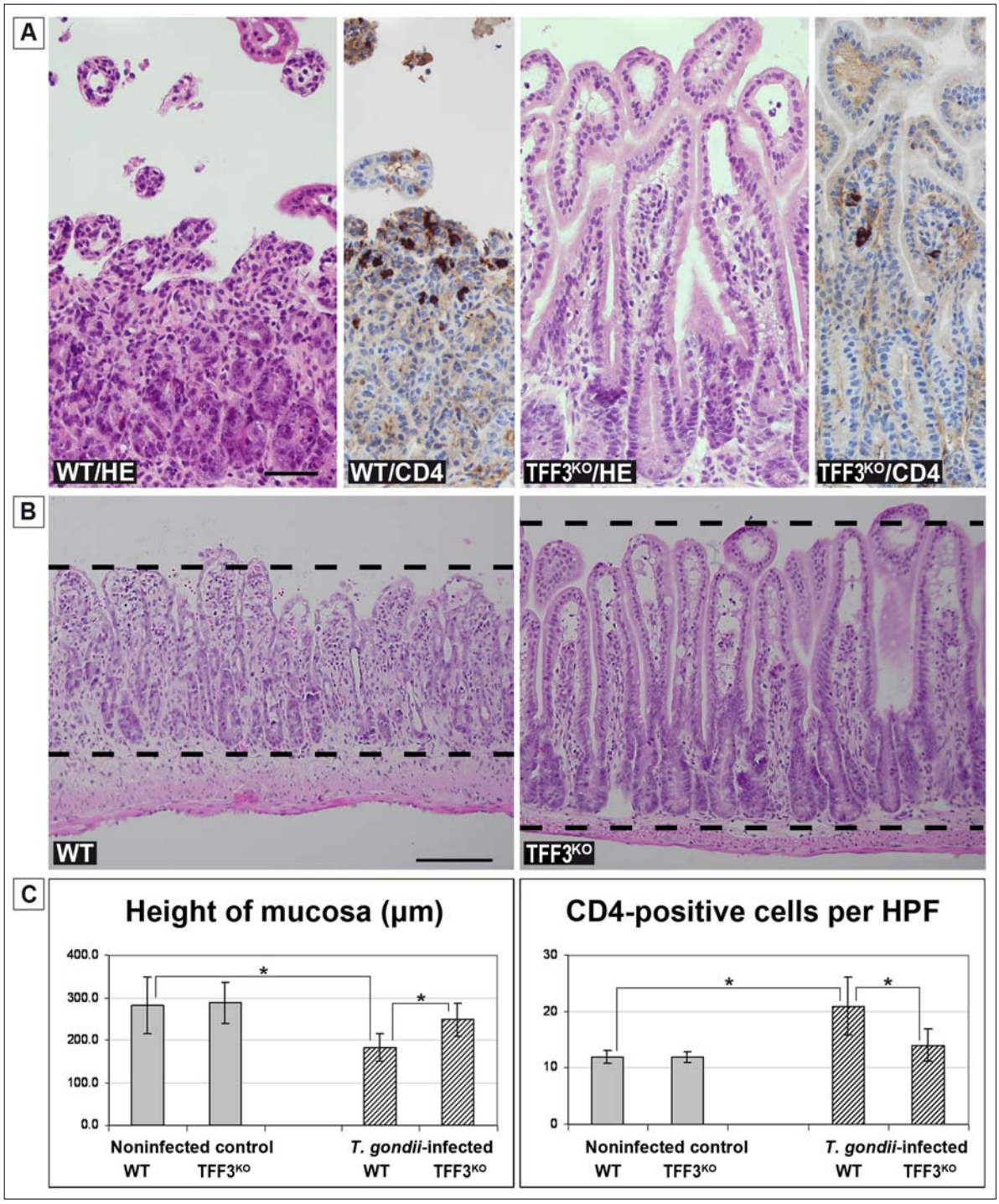

Fig. 2. Histological analysis of the murine ileum. (A) Representative hematoxylin and eosin (H&E) stained sections and CD4 immunohistochemistry, respectively, of the ileum of *T. gondii*-infected animals (WT and TFF3^{KO} mouse, respectively). Scale bar: 50 µm. (B) Representative H&E stained sections of the ileum of *T. gondii*-infected animals (WT and TFF3^{KO} mouse, respectively). The dashed lines indicate the height of the mucosa. Scale bar: 50 µm. (C) Height of the mucosa (left) and the number of CD4^{+} lymphocytes per high power field (HPF; right) of the different animal groups (the infected animals investigated here are the same as in *Fig. 1*, whereas the noninfected control animals originate from a different series as those in *Fig. 1*). The significance of the differences between the different groups is indicated by asterisks

the number of CD4-positive lymphocytes was significantly increased in the WT mice *(Fig. 2c)*.

Taken together, WT mice – in comparison to TFF3^{KO} mice – showed a significantly increased inflammatory response (RT-PCR analysis) along with microscopic inflammatory changes including necrosis, a reduced height of the mucosa, and an increased number of CD4^{+} lymphocytes in the ileum after *T. gondii* infection. Thus, TFF3^{KO} mice seem to be partially protected from low-dose oral infection with *T. gondii*.

Gene expression profiling in the spleen and brain after oral T. gondii *infection*

In order to get a rough estimate on how far oral *T. gondii* infection affected other organs, expression profiling of the spleen and brain was performed concerning inflammatory genes (Ifnγ and Tnfα), the ionized calcium binding adapter molecule 1 (Iba1/Aif1) as an established marker for activated microglial cells, and the TFF genes *(Fig. 3)*.

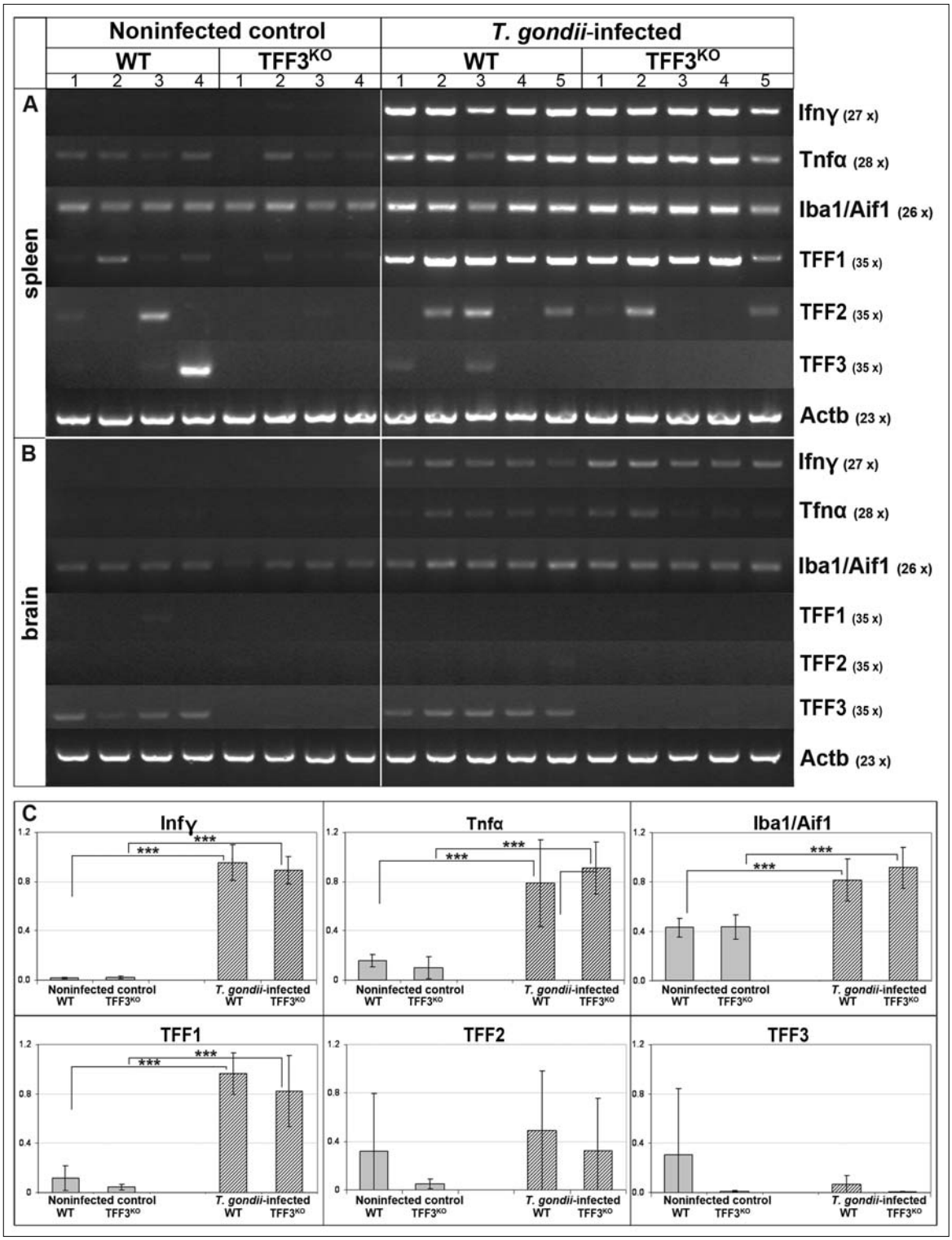

Fig. 3. RT-PCR analysis of spleen and brain. Interferon-γ (Ifnγ), tumor necrosis factor α (Tnfα), ionized calcium binding adapter molecule 1 (Iba1/Aif1), and TFF1 expression were monitored in the spleen (A) as well as the brain (B) of noninfected control animals and orally *T. gondii*-infected animals (same animals as in *Fig. 1*). The number of amplification cycles is given in parentheses. The integrity of the cDNAs was tested by monitoring the transcript level of β-actin. (C) Results of the semiquantitative RT-PCR analyses of the spleen (relative gene expression levels normalized against β-actin). Significant differences are indicated by asterisks

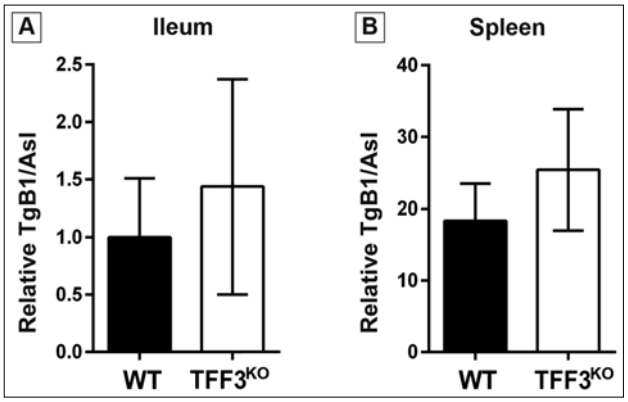

Fig. 4. Semiquantitative real-time PCR analysis of the *T. gondii* burden in the ileum and spleen. *T. gondii* B1 (TgB1) load was measured in the ileum (A) as well as in the spleen (B) of orally *T. gondii*-infected animals. Relative parasite DNA levels were normalized against murine argininosuccinate lyase (Asl)

In the spleen *(Fig. 3a)*, expression of Ifnγ and Tnfα was significantly upregulated in the *T. gondii*-infected animals *(Fig. 3c)*. No difference was detectable between WT and TFF3^{KO} mice. Of note, TFF1 was significantly upregulated in the *T. gondii*-infected animals *(Fig. 3c)*.

Furthermore, also the *T. gondii* burden was estimated in the infected animals by semiquantitative real time PCR *(Fig. 4)*. In both the spleen and the ileum, the *T. gondii* B1 levels were slightly elevated in the TFF3^{KO} mice when compared to the WT animals. However, these results were not significant.

In the brain, Ifnγ and Tnfα expression was slightly induced in *T. gondii*-infected mice, whereas Iba1 expression was hardly changed at this early time point *(Fig. 3b)*. In contrast to spleen, TFF1 expression was hardly detectable in the brain *(Fig. 3b)*.

Discussion

The mechanisms regulating the mucosal barrier and inflammatory response in the intestine are complex and incompletely understood thus far. In particular, the involvement of TFF peptides is still enigmatic. Based on the fact that TFF3–Fcgbp is a major component of the intestinal mucus [7], one may expect that TFF3^{KO} mice, which are markedly more sensitive in a DSS colitis model [27], are also more susceptible to *T. gondii* infection because of the defective mucus barrier. Thus, it is surprising that TFF3^{KO} mice appear to be partially protected from *T. gondii* infection-induced inflammation and disparate to the report that TFF2^{KO} mice show reduced immunopathology after oral low-dose *T. gondii* infection [59]. In the latter study, TFF2 was shown to negatively regulate IL-12 levels, and thus, TFF2^{KO} mice had an increased baseline intestinal inflammation, which is protective. In this report, infected TFF2^{KO} mice displayed lower parasite rates compared to WT controls. In contrast, in the current experiments presented in *Fig. 4*, slightly elevated *T. gondii* levels were detected in the ileum and the spleen of TFF3^{KO} mice. The reduced proinflammatory cytokine response in the TFF3^{KO} mice could explain the tendency of the elevated parasite numbers in these animals, because insufficient immune cells and response are available for parasite control.

As shown in *Fig. 1c*, in the *T. gondii*-infected animals, the IL-12a expression is significantly reduced in the TFF3^{KO} animals when compared with the WT indicating a diminished inflammatory response in TFF3^{KO} mice. This is in line with the reduced expression of most other inflammatory marker genes *(Fig. 1c)*, which is further supported by the histological examination *(Fig. 2)*. This reduced inflammatory response could be a consequence of the increased IL-12a expression level tendency in the noninfected TFF3^{KO}, compared to WT animals *(Fig. 1c)*. Elevated IL-12 levels may protect TFF3^{KO} mice from initial *T. gondii* infection. Of note, the TFF2 expression is significantly lower in the noninfected TFF3^{KO} mice, compared to those in WT animals *(Fig. 1c)*. Thus, the situation in the TFF3^{KO} mice shows some similarity with that of the TFF2^{KO} mice, both having diminished TFF2 expression levels in comparison to WT animals.

Thus far, it is not known how TFF3 influences the immune response following *T. gondii* infection. Considering that TFF3 is a major mucus constituent, the recently described mucus function to educate DCs in developing tolerance against common gut content and to influence T_{reg} cell production [50, 51] could be altered in the absence of TFF3. Also, Fcgbp could possibly be involved in this process. To confirm this theory, further experiments are needed to evaluate DC and T_{reg} dynamics and function. Another possible hypothesis is that TFF3 is directly involved in regulating the immune response as an intrinsic secretory product of immune cells. This assumption is based on the report that TFF3 (and TFF2) is synthesized in lymphoid tissues, such as the spleen (see also *Fig. 3*), thymus, lymph nodes, or bone marrow and it stimulates migration of monocytes [22]. TFF3 is predominately synthesized in the mesenterial lymph nodes (Stürmer, Znalesniak, and Hoffmann, unpublished results). TFF3 is also synthesized by activated microglial cells *in vitro,* which represent the primary immune cells of the central nervous system [20]. For TFF2, a direct function in regulating the immune response has already been demonstrated [67, 68].

In agreement with a previous report [22], TFF3 and TFF2 are also expressed in the spleen of both noninfected and *T. gondii*-infected animals *(Fig. 3)*. There are relatively large individual variations, and TFF2 expression levels might be elevated in the *T. gondii*-infected animals.

In contrast, TFF1 expression is clearly increased in the *T. gondii*-infected animals with no difference between the WT and the TFF3^{KO} mice. This is the first report that, in the spleen, TFF1 expression is specifically induced during an inflammatory response. Currently, the consequences are not understood and are subject to detailed analysis. Based on results on epithelial cells [64], TFF1 can be expected to act as a motogen/chemokine in the immune system. Of note, a comparable induction of TFF1 expression has been observed in the brain of a murine *Toxoplasma* encephalitis

model following intraperitoneal infection with *T. gondii* [63]. Thus, TFF1 seems to be generally involved in inflammatory responses. However, TFF1 expression was not induced in the brain 7 days after oral *T. gondii* infection *(Fig. 3)* because, at that early time point, the brain is virtually unaffected.

These first outcomes suggest that TFF3 is engaged in the immune pathology of intestinal inflammation followed by *T. gondii* infection. However, further experiments should endorse and scrutinize how TFF3 is involved in the pathological processes, immune cell recruitment and function in the *T. gondii* infection-induced inflammation model. The potential targets of TFF peptides in the immune system are still not identified, though interesting relations with chemokine receptors such as CXCR4 were previously described [32]. Importantly, recent developments point toward the potential role of TFF peptides in binding to a plethora of transmembrane glycoproteins due to their different lectin activities modulating different signal transduction processes, particularly in the immune system [45].

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