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Trefoil factor 2 negatively regulates Type 1 immunity against *Toxoplasma gondii*

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Abstract

Interleukin 12 (IL-12)-mediated Type 1 inflammation confers host-protection against the parasitic protozoan *Toxoplasma gondii*. However, production of interferon gamma (IFN-γ), another Type 1 inflammatory cytokine, also drives lethality from excessive injury to the intestinal epithelium. As mechanisms that restore epithelial barrier function following infection remain poorly understood, this study investigated the role of Trefoil factor 2 (TFF2), a well-established regulator of mucosal tissue repair. Paradoxically, TFF2 antagonized IL-12 release from DC's and macrophages, which protected TFF2 deficient mice ($TFF2^{-/-}$) from *T. gondii* pathogenesis. Dysregulated intestinal homeostasis in naïve $TFF2^{-/-}$ mice correlated with increased IL-12/23p40 levels and enhanced T cell recruitment at baseline. Infected $TFF2^{-/-}$ mice displayed low rates of parasite replication and reduced gut immunopathology, whereas WT mice experienced disseminated infection and lethal ileitis. p38 MAPK activation and IL-12p70 production was more robust from $TFF2^{-/-}$ $CD8^+$ DC compared to WT $CD8^+$ DC and treatment of WT DC with rTFF2 suppressed TLR-induced IL-12/23p40 production. Neutralization of IFN-γ and IL-12 in $TFF2^{-/-}$ animals abrogated resistance shown by enhanced parasite replication and infection-induced morbidity. Hence, TFF2 regulated intestinal barrier function and Type 1 cytokine release from myeloid phagocytes, which dictated the outcome of oral *T. gondii* infection in mice.

Keywords

macrophage; epithelial cells; injury; inflammation

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Introduction

Toxoplasma gondii is an obligate intracellular protozoan that, upon peroral infection, rapidly crosses the gastrointestinal epithelium and disseminates through lymphatic, hepatic and nervous tissues (1, 2). Host-mediated control of parasite replication is dependent upon the production of IL-12 and IFN-γ from myeloid and lymphoid lineages, respectively (3–6). Oral inoculation of C57BL/6 mice with *T. gondii* tissue cysts causes severe inflammatory bowel disease, characterized by weight loss, massive granulocytic inflammation, excessive production of T_H1 associated cytokines, epithelial invasion of enteric microbes, and mortality within 9–15 days (1, 7). TLR activation and excessive inflammatory cytokine release are considered to drive the epithelial cell injury that results from *T. gondii* infection in C57BL/6 mice (8), but the pathogenesis of oral toxoplasmosis remains poorly understood.

Trefoil factor 2 (TFF2) is one of three "trefoil-motif" containing proteins (TFF1-3) that promotes restitution, the rapid and directed movement of epithelia to cover exposed areas of basement membrane tissue following mucosal insult (9, 10). The predominant sources of TFF2 are stromal cells (epithelia, endothelia, fibroblasts), but TFF2 mRNA transcripts are also expressed by tissue macrophages (10, 11). Although TFF2 and TFF3 can both down-regulate gastric and colonic inflammation (11–13), the non-redundant mechanisms of regulation of intestinal homeostasis or pathogen-specific immunity by TFF2 are currently unclear.

This report demonstrates that TFF2 functions as a regulator of intestinal homeostasis that suppresses *T. gondii*-driven Type 1 inflammation. TFF2 suppresses p38 MAPK activation and IL-12p70 release from $CD8^+$DC and limits IL-12/23p40 production from macrophages. Oral inoculation of $TFF2^{-/-}$ mice with *T. gondii* results in the rapid clearance of parasites preventing the development of infection-induced immunopathology. These data extend the importance of TFF2 from mucosal barrier function to a previously unrecognized role in the suppression of the IL-12/IFN-γ axis that drives host immunity against parasitic protozoa.

Methods

Mice and *T. gondii* infection model

Six to ten week-old, sex-matched WT or $TFF2^{-/-}$ C57BL/6 mice bred in-house were used for all studies. For oral *T. gondii* (ME49 strain) infections, brain cyst homogenates were obtained from chronically infected mice and cyst suspensions were prepared at the concentrations indicated. Mice were infected by oral gavage with 15–50 cysts using a 21 gauge ball-tipped feeding needle. Weight was monitored daily. Moribund mice (>20% weight-loss) were sacrificed according to the Institutional Animal Care and Use Committee at the Cincinnati Children's Hospital Medical Center.

Histological staining and immunohistochemistry

Toxoplasma antigen-specific immunohistochemistry on paraffin embedded tissue was performed with anti-*T. gondii* primary antibody (US Biologicals) as previously described (14). For immunofluoresence, paraffin-embedded tissue sections were immersed in 4% Donkey Serum (Millipore) for 2 hours at room temperature to prevent non-specific binding of primary antibodies. Rabbit anti-CD3 (Dako), 1:100, and 5ug/ml of rat anti-mouse F4/80 or anti-CD11b (eBioscience) were applied to tissue sections overnight, washed and incubated with 1% BSA incubated with Donkey anti-Rat-594 and Donkey anti-Rabbit 488 (Invitrogen) for 2 hours for detection of primary antibody. DAPI-Fluormount (SouthernBiotech) was used for nuclear staining.

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QRT-PCR

Total RNA was purified from BMDM or DC cultures using Trizol reagent according to manufacturer's instructions (Invitrogen). cDNA was prepared using the Taqman cDNA synthesis kit (Roche). Gene expression was measured using the Lightcycler 480 and data were normalized to beta-actin. Biopsies of small intestine (duodenum, jejunum and ileum) were pooled, weighed and DNA was extracted using the DNeasy blood and tissue extraction kit (Qiagen). Primers used for amplification of *Toxoplasma gondii* B1 gene: Forward: 5′-CTGGCAAATACAGGTGAAATG-3'; and Reverses Reverse: 5′-GTGTACTGCGAAAATGAATCC-3′ as described previously (15). PCR reactions were setup in a final volume of 20μl, using 5μl of total tissue DNA, 1μl of 20μM forward and reverse primer, and 2× of SYBR Green I master mix (Roche). RT-PCR analysis was performed on a Light Cycler 480 System (Roche). Relative quantification was performed using standard curve analysis of purified parasite DNA from a defined number of parasites and expressed as the number of parasites per mg of tissue.

Flow cytometry

Mesenteric lymph node cells were washed in FACS buffer (HBSS, 1% FCS and 0.2% sodium azide) and incubated with anti-FcγRII/RIII mAb (2.4G2). Lamina propria cells were isolated as previously described (16). Single cell suspensions were stimulated with phorbol myristic acid (PMA)/ionomycin/Golgi-stop (BD Pharmingen) and stained with APC-F4/80 (clone BM8), FITC-anti-CD11b (clone M1/70) and intra-cellularly stained with PE-anti-IL-12/23p40 (R&D systems). For T cell staining, MLN were stimulated with anti-CD3 (1mg/ml) 16h with Golgi-Plug added during the last 4h, followed by anti-TCR-β, anti-CD4, anti-CD8, and anti-IFN-γ, mAb's (eBioscience). Intracellular staining with mAb specific for p38 or p42/44 MAPK (Cell Signaling) was performed according to manufacturer's protocol. Acquisition was performed with a BD FACS Calibur and analyzed with Flojo 7.5.5 software.

Bone marrow derived macrophages (BMDM) and splenic dendritic cells

BMDM were grown from the mononuclear fraction of bone marrow cultures for 6 days in M-CSF generated by CMG cell line as previously described (16). Spleen-derived CD11c cells were obtained after Collagenase D digestion, followed by CD11c-MACS beads magnetic separation (cell purity >85% as determined by flow cytometry). Soluble tachyzoite antigen (STAg) was generated from RH-strain tachyzoites grown from HS27 fibroblasts. CpG was purchased from a commercial vendor (Coley pharmaceutical, oligodoxyucleotide 1826).

Antibody neutralization

Rat-anti-mouse IL-12/IL-23p40 (C17.8), rat-anti-mouse IFN-γ (XMG1.2) or rat Ig control antibody (GL113) were purified from culture supernatant using thiophilic agarose chromatography, dialyzed against PBS and sterile filtered through a 0.2μm filter.

Evaluation of intestinal permeability

1 cm segments of mucosa were mounted in U2500 Dual Channel Ussing chambers that exposed 0.30 cm^2 of tissue to 10 ml of Krebs buffer. Agar-salt bridges and electrodes were used to measure the potential difference. Following a 15 min equilibrium period, Basal short-circuit current (Isc), tissue resistance (TER), and permeability to FITC-dextran (2.2 mg/ml, molecular mass 4.4 kDa; Sigma-Aldrich) was determined as previously described (17).

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Statistical analysis

Statistical significance was assessed by either two-tailed Student's t test (two groups) or analysis of variance (ANOVA) for multiple groups with a post-hoc test to determine significance using Prism Graph Pad 4.0 software.

Results

Trefoil factor 2 is necessary to maintain mucosal barrier function within the small intestine and limits baseline production of IL-12/23 p40 from myeloid phagocytes

Treatment of rodents with recombinant hTFF2 ameliorates mucosal injury and TFF2 deficiency in mice exacerbates Helicobacter infection-induced mucosal inflammation (9, 12). However, it was unclear whether TFF2 regulated mucosal barrier function under homeostatic conditions. To address this issue, jejunal tissue segments from naïve WT and $TFF2^{-/-}$ mice were mounted on Ussing chambers to evaluate both trans-epithelial resistance (TER) (Ω cm^2) (Fig. 1A) and paracellular permeability (basolateral to apical flux of FITC-dextran) (Fig. 1B). Strikingly, jejunum tissue from $TFF2^{-/-}$ mice had significantly less TER and significantly more permeability to FITC-dextran than WT tissues (Fig. 1A-B). This indicated that TFF2 served a non-redundant role in the mucosal barrier function within the proximal small intestine of mice.

Given this finding, we postulated that the TFF2 deficiency might have led to a dysregulation of immune cell composition or a baseline increase in intestinal inflammation. Evaluation of jejunal biopsies via H&E staining revealed that $TFF2^{-/-}$ mice had a moderate accumulation of leukocytes within the lamina propria, but did not possess signs of overt immunopathology (Fig. 1C). Immunofluorescence staining for $F4/80^+$ and $CD3^+$ cell populations was used to indicate the relative abundance of myeloid phagocytes and T lymphocytes, respectively. Results show that $TFF2^{-/-}$ mice had a greater accumulation of both cell populations in the lamina propria, as compared to WT mice (Fig. 1D).

To determine whether cytokine dysregulation accompanied these cell compositional changes, 3-cm segments of jejunum were digested with Liberase™ and evaluated for intracellular IL-12/23p40 levels within the lamina propria macrophage population ($CD11b^+$/$F4/80^+$). Strikingly, the $CD11b^+$/$F4/80^+$ cell population from $TFF2^{-/-}$ mice expressed 4-fold greater levels of IL-12/23p40 than WT (Fig. 1E). IL-12p40 mRNA levels within the jejunum were also higher in $TFF2^{-/-}$ tissues than WT, but there were no differences in the baseline expression for IFN-γ or IL-17A as determined by QRT-PCR(data not shown). Taken together, these results indicated that TFF2 served an essential role in the baseline regulation of small intestinal barrier function and inflammatory cell composition/function within the small intestine at baseline.

$TFF2^{-/-}$ mice control early parasite dissemination without developing intestinal immunopathology following oral infection with *T. gondii* tissue cysts

As there were marked perturbations in $TFF2^{-/-}$ mice at baseline, we sought to determine whether these abnormalities would alter the course of disease caused by an oral infection with *T. gondii*. WT and $TFF2^{-/-}$ mice were orally inoculated with Me49 tissue cysts at varying inoculum doses and monitored for changes in weight as an indicator of disease progression. Oral inoculation with 50 cysts (high dose) caused rapid weight loss in both WT and $TFF2^{-/-}$ strains (Fig. 2A), accompanied by marked infection-induced immunopathology within the liver and intestine (Supplemental Fig. 1). However, infection with 25 cysts (medium dose) or 15 cysts (data not shown) did not cause cachexia or mortality in $TFF2^{-/-}$ mice, whereas WT animals lost >20% of their original weight and experienced 30–40% mortality by 8–9 days post-inoculation (Fig. 2B). $TFF2^{-/-}$ mice inoculated with 25 tissue

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cysts did not develop splenomegaly (Fig. 2C) or intestinal inflammation (Fig. 2D), whereas infected WT mice developed severe transmural ileitis characterized by extensive granulocytic infiltration (Fig. 2C-D). Congruent with reduced intestinal inflammation in $TFF2^{-/-}$ mice, the expression levels of IL-10, a key immunosuppressive cytokine, were significantly higher in the ileal tissue of $TFF2^{-/-}$ mice compared to WT at day 7 post-inoculation (Fig. 2E).

Furthermore, the marked differences between strains in the susceptibility to infection-induced ileitis prompted experiments to test whether reduced immunopathology in $TFF2^{-/-}$ mice correlated with parasite burden. Tachyzoite-specific PCR revealed that parasite numbers in the intestine of $TFF2^{-/-}$ mice inoculated with 25 cysts were significantly reduced compared to WT at day 3 and day 5 post-infection (Fig. 3A). Tachyzoite-specific immunohistochemistry verified these findings, as there was robust staining for parasite-antigen within the lamina propria of WT mice but very few parasites within $TFF2^{-/-}$ intestinal tissues at day 5 post-infection (Fig. 3B-C). Taken together, these data demonstrate that at low inoculum doses, TFF2 deficiency protects the host from infection-induced immunopathology, which is associated with early control of parasite replication and increased IL-10 expression within the intestine.

Cell-intrinsic expression of TFF2 in macrophages and dendritic cells negatively regulates MAPK activation and IL-12 production

Although epithelial cells are considered as the major source of TFF2, monocyte/macrophage lineage cells also express TFF2 mRNA. Thus, it was possible that enhanced host resistance to *T. gondii* infection in $TFF2^{-/-}$ mice involved dysregulation of myeloid phagocyte function(s). In order to test whether TFF2 served a direct role in the regulation of pro-inflammatory and anti-inflammatory cytokine release from DC and macrophages, experiments were conducted with TLR-activating microbial antigens. Splenic DC from naïve WT and $TFF2^{-/-}$ mice were isolated by magnetic bead sorting (>85% $CD11c^{+}$), exposed to soluble tachyzoite antigen (STAg), and evaluated for IL-12/23p40 mRNA transcript levels by QRT-PCR. Strikingly, $TFF2^{-/-}$ DC up-regulated IL-12/23p40 mRNA transcripts more rapidly than WT DC at 4 hrs, although there were no differences between strains by 16 hrs (Fig. 4A). Increased IL-12/23 p40 message levels were accompanied by higher amounts of IL-12p70 produced from $TFF2^{-/-}$ DC cultures as compared to WT DC following exposure to STAg or CpG (Fig. 4B). Profillin, a selective TLR11 agonist, also induced greater amounts of IL-12p70 in $TFF2^{-/-}$ DC cultures than WT (Fig. 4C). CpG, a TLR 9 agonist, induced IL-10 production that was significantly higher in $TFF2^{-/-}$ DC cultures as compared to WT (Fig. 4D). Collectively this demonstrated that $TFF2^{-/-}$ DC's were hyper-responsive to diverse TLR-activating antigens resulting in pro-inflammatory and anti-inflammatory cytokine production.

Due to the rapid accumulation of IL-12/23p40 transcripts and elevated production of IL-12p70 within $TFF2^{-/-}$ DC, we then asked whether the proximal signaling molecules responsible for IL-12 production were also differentially regulated by TFF2. Given the essential role for MAPK activation in IL-12 release from APC (18), the kinetics of p38 MAPK phosphorylation were determined in both $CD8^{+}CD11c^{+}$ and $CD8^{-}CD11c^{+}$ populations exposed to STAg. $CD8^{+}$ $CD11c^{+}$ DC from $TFF2^{-/-}$ mice generated more robust phosphorylation of p38, as compared to WT (Fig. 4E and Supplemental Fig. 2). However, p38 activation was no different between strains within the $CD8^{-}CD11c^{+}$ sub-population (Fig. 4F), a minor contributor to IL-12 production (19, 20). No detectable differences were found between strains with regard to p42/44 (ERK) activation in either DC subset (data not shown).

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In addition, we sought to determine whether TFF2 could function as a direct suppressor of microbial antigen-induced IL-12 production. Thus, the reverse experiments were performed to determine whether IL-12 production could be inhibited by prior exposure to rTFF2. Splenic DC cultures isolated from naïve WT and $TFF2^{-/-}$ mice were either left untreated or treated with rTFF2 (40ng/ml) followed by stimulation with STAg (10μg/ml). IL-12/23p40 levels were measured 16hrs later. rTFF2 pre-treatment markedly reduced STAG induced IL-12/23p40 production from DC cultures (Fig. 4G) and suppressed *E. coli* lipopolysaccharide (LPS)-induced IL-12/23p40 expression and cytokine release from BMDM (Fig. 4H). Combined, these data indicate that cell-intrinsic TFF2 expression as well as exogenous rTFF2 pretreatment antagonizes microbial antigen-induced pro-inflammatory cytokine production from myeloid APC's.

Host immunity in TFF2-deficient mice is dependent upon the IL-12/IFN-γ axis

IL-12-driven Type 1 inflammation has a central role in driving host protection against *T. gondii* in mice. Thus, we addressed whether enhanced resistance afforded by TFF2 deficiency resulted in a preferential expansion of IFN-γ producing effector lymphocytes (21). Intracellular IFN-γ content within $TCR\beta^+CD4^+$ and $TCR\beta^+CD8^+$ populations was determined 24 hr following CD3 stimulation (1μg/ml) of mesenteric lymph node cells at day 5 (data not shown) and day 7 following oral inoculation with 25 Me49 tissue cysts. Consistent with our prediction, $TFF2^{-/-}$ animals generated a 5-fold greater percentage of $IFN\text{-}\gamma^+$ T_H1 cells (Fig. 5A) and 3-fold greater percentage of $IFN\text{-}\gamma^+$ CD8 effectors (Fig. 5B) than infected WT animals. Importantly, the baseline production of IFN-γ from these T cell subsets were no different between strains, which lends further support to the hypothesis that $TFF2^{-/-}$ APC preferentially induced the expansion of IFN-γ-producing lymphocytes following *T. gondii* infection.

To determine whether the lack of TFF2 indeed protected mice against *T. gondii* infection through accelerated IL-12/IFN-γ production during acute infection, we tested whether neutralization of these cytokines prior to infection would abrogate protective immunity. Naïve WT and $TFF2^{-/-}$ mice were given a single dose of α-IL-12p40 (1mg) and α-IFN-γ (1mg) neutralizing mAb's one day prior to oral inoculation with 25 *T. gondii* tissue cysts. Results show that $TFF2^{-/-}$ mice treated with α-IL-12p40/α-IFN-γ mAb's lost >20% of their original body weight by 9 days post-inoculation, whereas $TFF2^{-/-}$ mice treated with isotype control mAb did not develop *T. gondii*-induced cachexia (Fig. 5C). As expected, WT mice treated with α-IL-12p40/α-IFN-γ mAb's also developed worse disease than isotype control treated animals (22).

Lastly, we predicted that the enhanced disease severity in $TFF2^{-/-}$ mice correlated with an inability to control parasite dissemination. The gut-draining mesenteric lymph nodes (mLN) were probed with specific mAb for *T. gondii* antigens at day 7 post-inoculation in both strains. While $TFF2^{-/-}$ mice given control IgG harbored few parasites, WT mLN contained many tachyzoites (Fig. 5D-E). In contrast, administration of α-IL-12p40/α-IFN-γ mAb's to $TFF2^{-/-}$ mice caused a dramatic increase of parasite numbers in the mesenteric lymph node, similar to WT mAb-treated animals (Fig. 5F-G). Similar results were observed following immunostaining of splenic tissues (data not shown). These data support our hypothesis that the canonical Type 1 cytokines IL-12 and IFN-γ protected $TFF2^{-/-}$ mice against susceptibility to *T. gondii* infection-induced lethality and immunopathology.

Discussion

The mechanisms that regulate mucosal barrier function, tissue repair and inflammation within the gastrointestinal tract are incompletely understood. Herein, oral inoculation of mice with the human parasite *T. gondii* was used to demonstrate a novel role for TFF2

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(known to promote epithelial restitution) in the negative regulation of infection-induced IL-12 production and Type 1 immunity. TFF2 deficiency increased baseline intestinal inflammation and augmented Type 1 cytokine production from $CD8^+$ DC, $CD4^+$ and $CD8^+$ T lymphocytes. This provided a selective advantage for the $TFF2^{-/-}$ strain in the control of early parasite replication, such that infection-induced immunopathology was averted. Interestingly, TFF2 functioned through cell-intrinsic and extrinsic mechanisms to suppress TLR-driven IL-12 production from macrophages and DC suggesting that homeostatic TFF2 release within the small intestine has multiple functions that maintain mucosal barrier integrity and down modulate myeloid APC activation and cytokine production in response to microbial antigens.

The pro-inflammatory cytokine IL-12 drives host immunity against a variety of microbial, fungal, and viral pathogens (5, 21, 23) including rodents infected with *T. gondii* (24). Unlike human toxoplasmosis, oral inoculation of C57BL/6 strains with *T. gondii* tissue cysts causes rapid and severe weight loss (cachexia), intestinal immunopathology, and host mortality within 7–12 days (25). Lethal ileitis caused by oral *T. gondii* infection bears striking similarity to murine and human inflammatory bowel disease, as both are due to excessive IL-12 and IL-23-driven inflammatory responses aggravated by gut microbes (26, 27). One hypothesis for the similarities in phenotypes of IBD and murine toxoplasmosis is that microbial flora enter the intestinal tissue through areas of damaged epithelium (28).

Naïve $TFF2^{-/-}$ mice had higher baseline levels of IL-12/23 p40 in the intestine than WT, which was concurrent with impaired TER and increased paracellular permeability. These parameters are a central feature of IBD in mice and humans and can be used to indicate the degree of injury to the mucosal barrier within the intestine (29–31). Defective barrier function at baseline in $TFF2^{-/-}$ mice was unexpected, as TFF family members have functionally redundant roles in mucosal epithelial cell repair (32, 33). While the explanation(s) for this defective barrier function remains undefined, one possibility is that $TFF2^{-/-}$ mice have defective expression/function of gap-junction proteins due to the role of TFF2 in the regulation of cell adhesion molecule expression (34). Alternatively, TFF2 deficiency could result in excess intestinal epithelial cell death because several TFF family members block apoptosis (35, 36). Irrespective of the exact mechanism, our evidence for decreased barrier function in naive TFF2 mice may partially explain why $TFF2^{-/-}$ mice have increased susceptibility to chemical and infection-induced models of gastritis and colitis (11).

This work demonstrates that TFF2 suppressed IL-12 production in splenic DC and macrophages through extrinsic and intrinsic mechanisms, which demonstrates a previously unrecognized role for TFF2 in the regulation of Type 1 inflammation. $CD8^+$ $CD11c^+$ DC, a major source of *T. gondii* infection-induced IL-12p70, produced more IL-12 when isolated from $TFF2^{-/-}$ mice compared to WT. This production was suppressed when WT DC were exposed to exogenous rTFF2. IL-12/23p40 production from intestinal lamina propria macrophages was highly elevated in $TFF2^{-/-}$ mice as compared to WT. This classically activated macrophage phenotype is consistent with reports showing that $TFF2^{-/-}$ mice are hyper-responsive to IL-1β stimulation and that $TFF2^{-/-}$ macrophages show enhanced NF-kβ activation compared to WT macrophages (13). In addition, $TFF2^{-/-}$ monocytes release greater amount of reactive nitrogen intermediates and inflammatory cytokines than WT monocytes (11, 13). As there were no obvious differences between WT and $TFF2^{-/-}$ macrophages or DC in the absence of microbial antigen stimulation, we favor a hypothesis that TFF2 limits antigen-induced activation of myeloid APC.

Our data may suggest that systemic release of TFF2 following tissue injury could antagonize cell-mediated immunity. Indeed, dysregulation of this protein has been documented in a

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variety of disease contexts. Elevated TFF2 levels are associated with several different types of cancer (35, 37–39) and may predict the stage of tumor progression (40). Furthermore, administration of rTFF2 accelerates the rate of tissue repair in models of burn injury (41), ocular damage (10), gastritis and colitis (12, 42). In these disease contexts, TFF2 driven suppression of IL-12 production could presumably have a beneficial role in tissue regeneration and the restoration of homeostasis. Taken together, our data show two biological roles for TFF2, which are not mutually exclusive: *i*) a negative regulator of IL-12 production from myeloid APC and *ii*) positive regulator of homeostatic mucosal barrier function. Thus, it is likely that TFF2$^{-/-}$ mice may also develop enhanced systemic inflammation in a variety of disease contexts.

Demonstration that TFF2$^{-/-}$ mice were resistant to *T. gondii*-induced ileitis was unexpected as infection-induced ileitis is largely the result of pro-inflammatory cytokine production (26, 43). However, TFF2 deficiency only protected mice against a low dose of parasites and succumbed to multi-organ inflammation and pathology when inoculated with 50 *T. gondii* tissue cysts instead of 25 or 15 cysts. Therefore, the selective advantage of TFF2 deficiency is only evident up to a certain threshold of parasite inoculum. Once this is exceeded, the baseline inflammation within TFF2$^{-/-}$ mice is no longer sufficient to limit parasite replication and dissemination throughout the host. This is supported by our demonstration that neutralization of IL-12/IFN-γ prior to infection resulted in uncontrolled tachyzoite replication and cachexia in TFF2$^{-/-}$ mice.

On the other hand, anti-inflammatory cytokines such as interleukin 10 have been demonstrated to serve an essential role in limiting excessive IL-12/IFN-γ associated immunopathology following *T. gondii* infection in rodents. Enhanced IL-10 production from infected TFF2$^{-/-}$ mice and TFF2$^{-/-}$ DC could have contributed to suppression of excess immunopathology. However, rTFF2-treatment of DC or macrophages did not induce IL-10 or TGF-β mRNA expression (data not shown). Thus, while the anti-inflammatory effects of TFF2 are unlikely to be IL-10-dependent, the enhanced IL-10 in TFF2$^{-/-}$ mice may have served an important biological role in the context of *T. gondii* infection. The mechanism(s) responsible for TFF2's immunosuppressive effects are currently under investigation.

The role of cross-talk between epithelial cells and leukocytes in the regulation of mucosal and systemic immunity has become increasingly apparent. Our data show that TFF2, a major component of mucus, serves an essential role in the negative regulation of IL-12/23p40 and IL-12p70 production from macrophages and DC as well as enforcement of homeostatic mucosal barrier integrity. Taken together, our work is consistent with the emerging concept that epithelial cell repair proteins have direct immunomodulatory functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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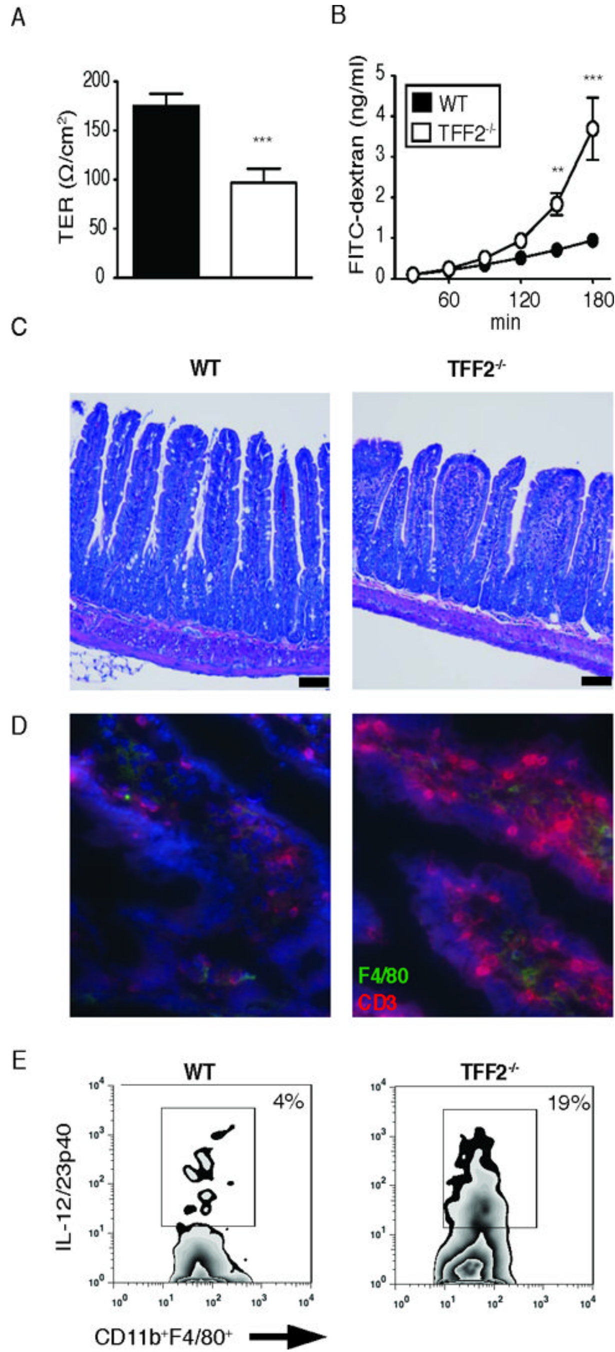

Figure 1. TFF2 deficiency impairs mucosal barrier function and increases the baseline frequency of IL-12$^+$ macrophages in the small intestine

Muscle-free segments of jejunum isolated from naïve WT and TFF2$^{-/-}$ mice were evaluated for (A) trans-epithelial resistance (TER) and (B) permeability to FITC-dextran (4.4 kDa). Data show mean ± SEM from 6 mice/group. (C) Representative images of H&E-stained jejunum from naïve WT (left panel) and TFF2$^{-/-}$ (right panel) mice. Scale bar = 20mm at 200×. (D) Immunofluorescence staining for F4/80 (green) and CD3 (magenta) in paraffin-embedded sections of naïve jejunum from WT (left panel) and TFF2$^{-/-}$ (right panel) mice (E) Percentage of IL-12/23p40$^+$ events within the FSC^{hi} SSC ^{low} CD11b$^{+/}$F4/80$^+$ gate from the lamina propria mononuclear cell fraction of naïve WT and TFF2$^{-/-}$ mice. Pooled

samples from 2–3 mice are shown. Data are representative of 3 independent experiments. (**=p<0.01 and***=p<0.001)

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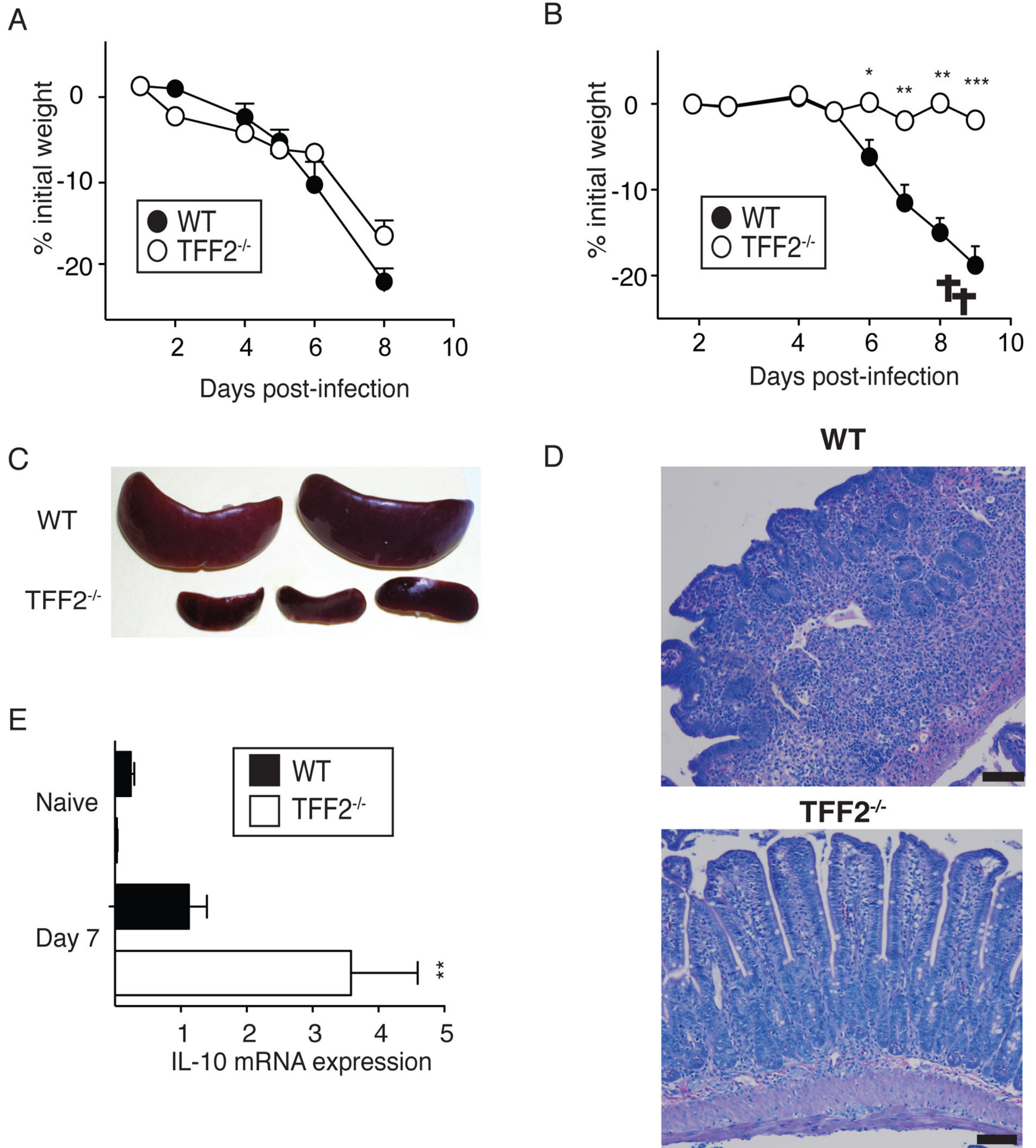

Figure 2. Low dose oral infection with *Toxoplasma gondii* in TFF2$^{-/-}$ mice does not lead to cachexia, parasite dissemination, or intestinal immunopathology

(A) WT and TFF2$^{-/-}$ mice were orally inoculated with 50 Me49 brain cysts or (B) 25 cysts and monitored for weight change. Data show mean ± SE from 4–6 mice/group. Experiment was repeated 4 times. Death indicated by cross. (**=p<0.01) (C) Representative photographs of spleens from orally infected WT (top panel) and TFF2$^{-/-}$ mice (bottom panel) at 8 days post-inoculation. (D) H&E stained small intestine from WT (top panel) and TFF2$^{-/-}$ (bottom panel) mice at 8 days post-inoculation following oral inoculation from experiment described in “B”. (E) IL-10 mRNA transcript levels within the jejunum from

naïve or *T. gondii* infected mice at 7 days post-infection with 25 Me49 cysts. Data show mean ± SE from 4–6 mice/group. Experiment performed twice with similar results.

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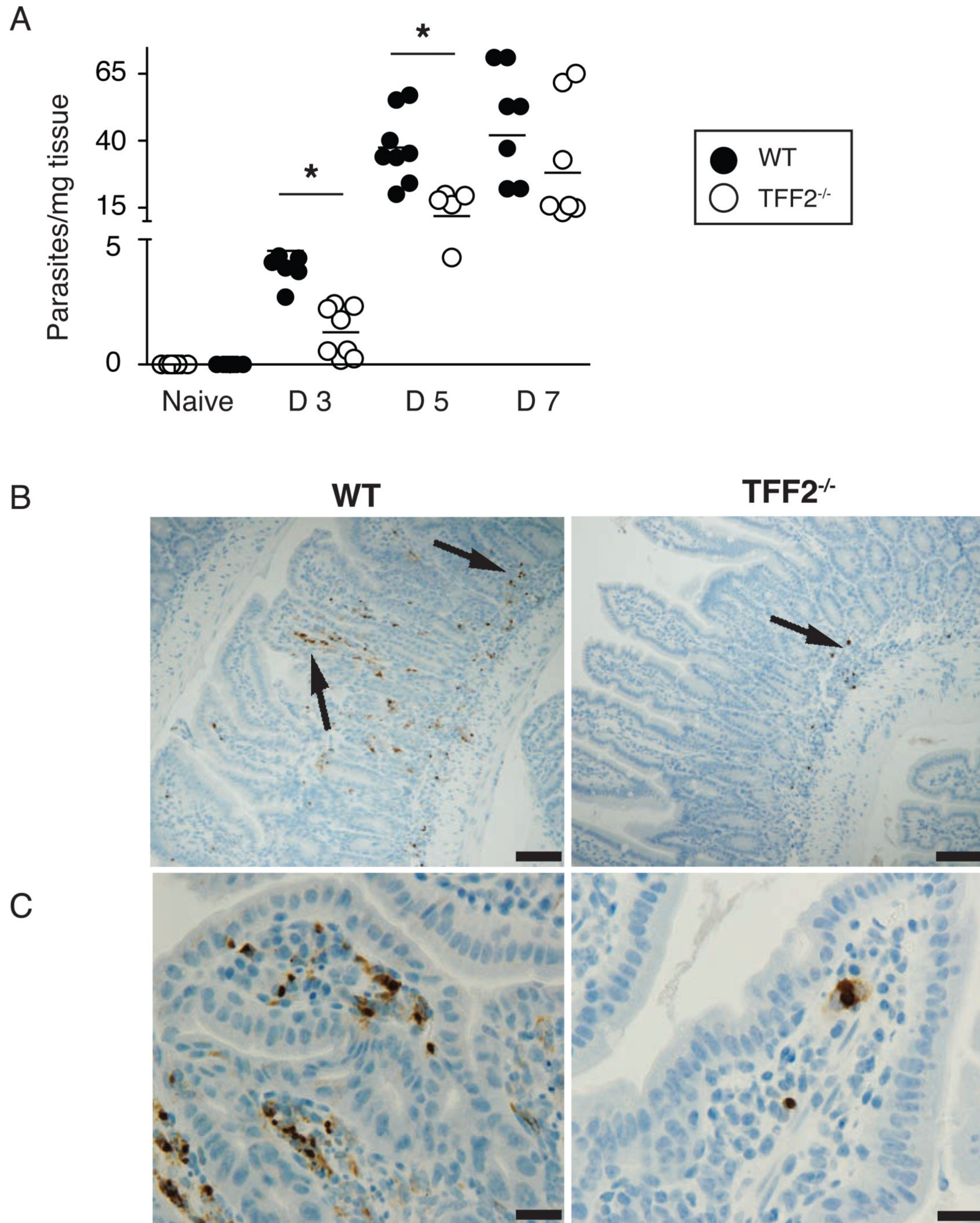

Figure 3. TFF2 deficiency results in early control of *T. gondii* replication following ingestion

(A) *T. gondii* B1-gene specific PCR was performed on small intestine from mice orally inoculated with 25 Me49 brain cysts. Experiment performed 3 times with similar results. (*=p<0.05). *T. gondii* tachyzoite-specific immunohistochemistry was used to detect parasites (dark brown precipitate) within the small intestine of WT and TFF2$^{-/-}$ mice at 5 days post-infection from experiment described in “A”. (B) 100×, (C) 400×. Arrows indicate parasite-infected cells.

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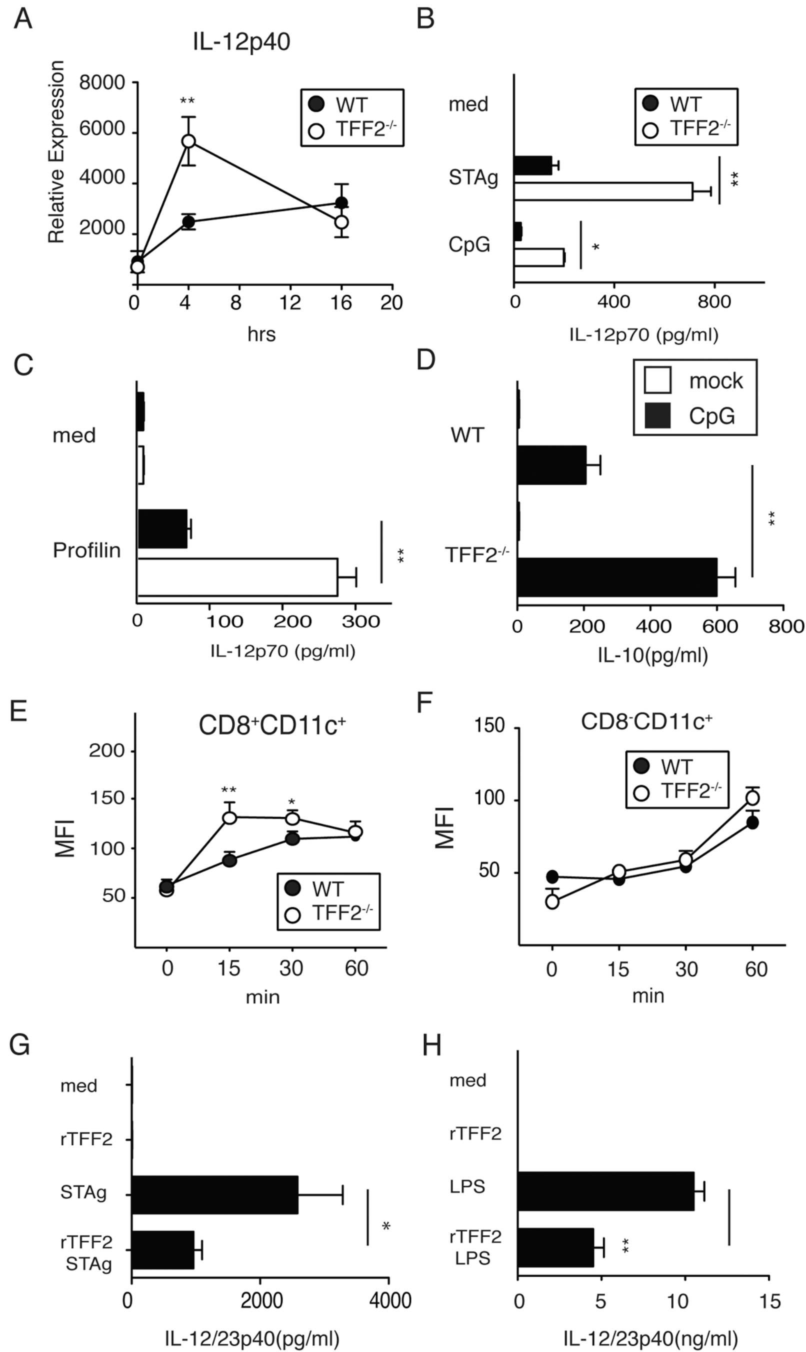

Figure 4. TFF2 negatively regulates p38 MAPK activation and IL-12 production in splenic DC and bone marrow derived macrophages

(A) Splenic DC from naïve WT and TFF2$^{-/-}$ mice were stimulated with STAg (10μg/ml) and monitored for IL-12p40 mRNA transcripts. Data show mean ± SE from 6 mice/group. Experiment was performed twice. (B) IL-12p70 levels produced from WT and TFF2$^{-/-}$ splenic DC left untreated (med), stimulated with STAg (10 μg/ml) or CpG (1 μg/ml) for 48hrs. Data show mean ± SE from 6 mice/group. Experiment performed three times. (C) Splenic DC from naïve WT and TFF2$^{-/-}$ mice were stimulated with rProfilin or (D) CpG for 24 hrs and measured for IL12p70 and IL-10 levels, respectively. Data show mean ± SE from 6 mice/group. (E) Phospho-p38 levels within splenic CD11c^{+}CD8^{+} and (F) CD11c^{+}CD8^{-}

DC populations from naïve WT and TFF2$^{-/-}$ mice that were exposed to STAg (10 μg/ml). Mean ± SE from 8 mice/group. Experiment performed twice. (G) IL-12p40 protein levels produced from WT splenic DC or (H) bone marrow derived macrophages (BMDM) that were either: untreated (med), exposed to rTFF2 (40 ng/ml), LPS (100 ng/ml), or pre-exposed to rTFF2 (16hr) prior to stimuli for 24hrs. Experiments were performed three times. (*=$p<0.05$ and **=$p<0.01$)

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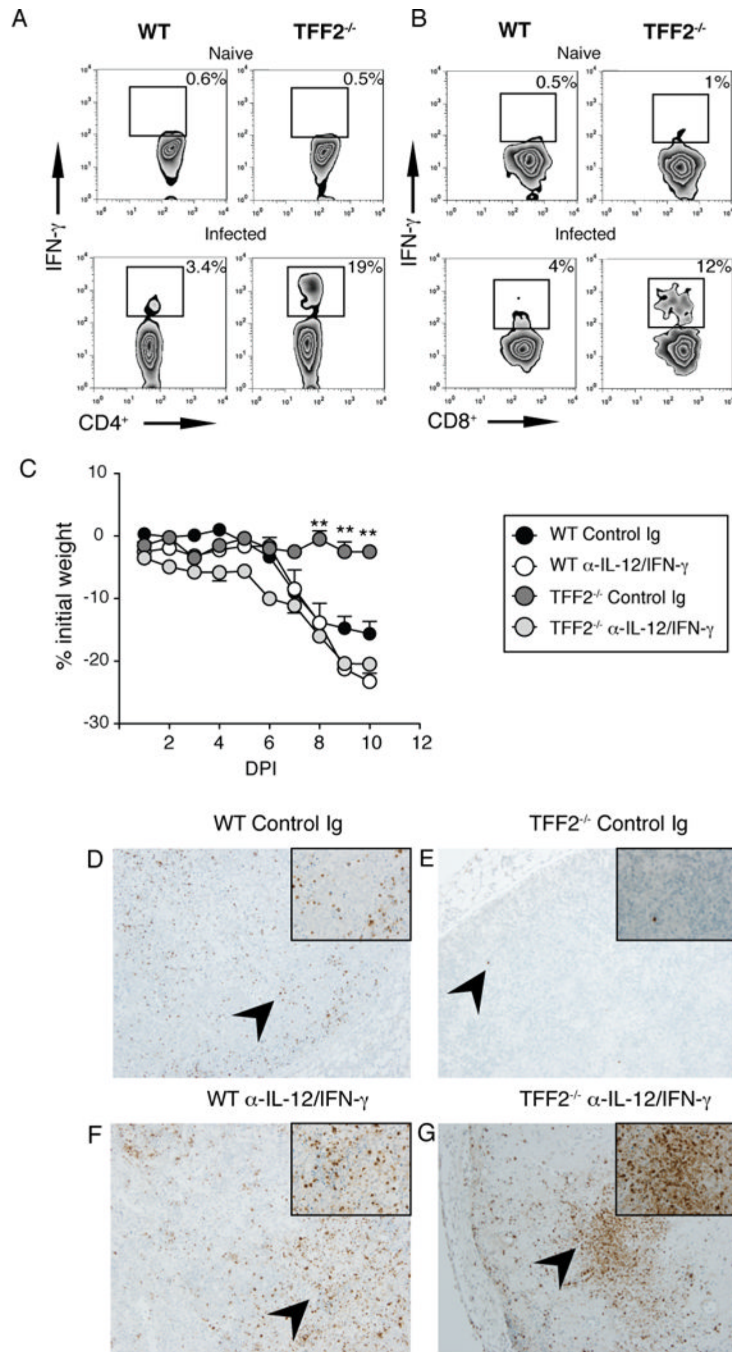

Figure 5. Lack of TFF2 increases IFN-γ effector cell expansion and limits tachyzoite dissemination to lymph nodes

(A) Intracellular IFN-γ staining within $CD4^+$ and (B) $CD8^+$ T lymphocytes within the mesenteric lymph nodes of WT and $TFF2^{-/-}$ mice that were either naïve or 7 days post-oral inoculation with 25 Me49 brain cysts. Representative plots from 8 individual mice analyzed/ group. (C) WT and $TFF2^{-/-}$ mice were administered a combination of anti-IL-12p40/anti-IFN-γ mAb (1 mg each) or isotype control mAb (2 mg) on day -1 followed by oral inoculation with 25 Me49 brain cysts on day 0 and monitored for weight change. Data show mean ± SE shown from 4–6 mice/group. Experiment was performed 3 times. (D) Mesenteric lymph node sections at day 8 post-inoculation from experiment described in “C” in WT

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control mAb treated, (E) TFF2$^{-/-}$ control mAb treated, (F) WT αIL-12/αIFN-γ mAb treated and (G) TFF2$^{-/-}$ αIL-12/αIFN-γ mAb treated groups stained with tachyzoite-specific IgG anti-sera. Magnification 100×. Arrowhead indicates the area within inset panel (400×). Representative images are shown. **=$p<0.01$