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Critical Roles of TIPE2 Protein in Murine Experimental Colitis

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Both commensal bacteria and infiltrating inflammatory cells play essential roles in the pathogenesis of inflammatory bowel disease. The molecular mechanisms whereby these pathogenic factors are regulated during the disease are not fully understood. We report in this article that a member of the TNF-α–induced protein 8 (TNFAIP8) family called TIPE2 (TNFAIP8-like 2) plays a crucial role in regulating commensal bacteria dissemination and inflammatory cell function in experimental colitis induced by dextran sodium sulfate (DSS). Following DSS treatment, TIPE2-deficient mice, or chimeric mice that are deficient in TIPE2 only in their hematopoietic cells, lost less body weight and survived longer than wild-type controls. Consistent with this clinical observation, TIPE2-deficient mice exhibited significantly less severe colitis and colonic damage. This was associated with a marked reduction in the colonic expression of inflammatory cytokines, such as TNF-α, IL-6, and IL-12. Importantly, the ameliorated DSS-induced colitis in TIPE2−/− mice also was associated with reduced local dissemination of commensal bacteria and a weaker systemic inflammatory response. Combined with our previous report that TIPE2 is a negative regulator of antibacterial immunity, these results indicate that TIPE2 promotes colitis by inhibiting mucosal immunity to commensal bacteria. The Journal of Immunology, 2014, 193: 000–000.

Human inflammatory bowel diseases (IBDs), represented primarily by ulcerative colitis and Crohn’s disease, cause considerable morbidity and significantly increase the risk for cancer in the colon and rectum (1). Ulcerative colitis exhibits a characteristic profile of chronic, relapsing and remitting inflammation involving the distal colon and rectum, and it is generally considered an immune-mediated disorder resulting from abnormal interactions between colonic microflora and mucosal immune cells (2). The relapsing and remitting course of the disease varies greatly among patients, with ~15% developing an “acute severe” form some time in their lives (3). Although the precise etiology of ulcerative colitis is not well understood, it is widely accepted that both genetic and environmental factors are involved (4). Several animal models of experimental colitis have been developed to help investigate the molecular and cellular mechanisms of the disease. Of these, the dextran sodium sulfate (DSS)-induced experimental colitis model is one of the best studied. It is initiated by DSS-induced damage to the intestinal epithelial cells but is dependent on the presence of both commensal microflora and myeloid (but not lymphoid) cells (5–7). Similar to human IBD, DSS-induced colitis is limited to the colonic mucosa and is characterized by diarrhea, bloody feces, weight loss, colonic ulceration, and a histopathological picture of inflammation, consisting mainly of infiltrating macrophages and granulocytes (5, 6).

TIPE2, TNF-α–induced protein 8 (TNFAIP8) like-2, is a member of the TNFAIP8 family (8) and is preferentially expressed in hematopoietic cells (8–10). TIPE2 serves as a negative regulator of immunity that maintains immune homeostasis (8, 11). Abnormal expression of TIPE2 has been found in patients with systemic lupus erythematosus, hepatitis B, diabetic nephropathy, and childhood asthma (12–15). TIPE2 is also implicated in the development of atherosclerosis and experimental stroke (16–18). TIPE2 may control innate immunity to bacteria and dsRNA viruses by targeting the Rac GTPases (19, 20). However, the role of TIPE2 in IBDs has not been reported. To address this issue, we studied DSS-induced colitis in TIPE2-deficient mice. We report in this article that TIPE2 plays an important role in the development of acute colitis by promoting dissemination of commensal bacteria in the colon.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 (B6) and CD45.1+ B6 mice were purchased from The Jackson Laboratory. B6 mice that carry a TIPE2 gene–null mutation were generated by backcrossing TIPE2−/− 129 mice to B6 mice for 12 generations, as described previously (8, 19). All mice used were male and 8–12 wk old and were maintained under pathogen-free conditions in the University of Pennsylvania Animal Care Facilities. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Induction and evaluation of DSS-induced colitis

Experimental colitis was induced by adding DSS (m.w. = 36,000–50,000; MP Biomedicals, Solon, OH) to the drinking water to a final concentration of 4% (w/v). Subsequently, mice were switched to regular drinking water
until the end of the experiment. Mice were examined daily to determine their clinical Disease Activity Index (DAI), which was based on the degree of body weight loss, stool consistency, and fecal blood (ranging from 0 to 12), as described previously (21). Briefly, DAI was scored as follows: weight loss (no change = 0; <5% = 1; 6–10% = 2; 11–20% = 3; >20% = 4), stool (normal = 0; soft, well-formed = 1; soft without pellets = 2; diarrhea = 4), and blood (no blood = 0; visible blood in rectum = 1; gross bleeding in rectum = 2; visible blood on fur = 4). For histological analysis, the distal colon and cecum specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections were stained with H&E, and pathological scores, ranging from 0 to 6 (combining inflammatory cell infiltration score and tissue damage score), were determined as follows (22): inflammatory cell infiltration in the lamina propria (occasional inflammatory cells = 0; increased inflammatory cells = 1; confluence of inflammatory cells extending to the submucosa = 2; transmural extension = 3) and tissue damage (no mucosal damage = 0; lymphoepithelial lesions = 1; surface mucosal erosion = 2; extensive mucosal damage and extension into deeper structures of the bowel wall = 3).

### Real-time quantitative PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Two micrograms of total RNA were reverse transcribed using SuperScript II transcriptase (Invitrogen). Real-time quantitative PCR was performed in an Applied Biosystems 7500 System with Power SYBR Green PCR Master Mix (Applied Biosystems). Quantitect Primers for mouse GAPDH, TIPE1, TIPE2, and TIPE3 were purchased from QIAGEN. TIPE primer sequences used were as follows: forward, 5'-AGCGATTCAACTTCCGGGGAACAG-3' and reverse, 5'-GTCGATACGGTGTGCGGATG-3'. Each sample was run in triplicate. The relative changes in gene expression were calculated using the 2-ΔΔCt method and compared using the log-rank test. Two-tailed Student t-test was used for all other comparisons. A p < 0.05 was considered statistically significant. All statistical analyses were performed with Prism 5.0 for Windows (GraphPad, San Diego, CA).

### Blood cell counts

Before euthanizing the mice, the entire colon was removed under aseptic conditions. The terminal 3-cm segment of distal colon was washed, weighed, homogenized, and serially diluted. Different dilutions of the suspensions were plated in triplicate on brain heart infusion agar and blood agar (BD Biosciences) plates and incubated at 37°C for 24 h to quantify the bacterial colonies.

### Assessment of intestinal permeability

Intestinal barrier permeability in vivo was measured using an FITC-labeled dextran method, as previously described (25). In brief, WT and TIPE2−/− mice were deprived of water and food overnight and were administered permeability tracer FITC-dextran (molecular mass 4 kDa; Sigma-Aldrich) at 400 mg/kg body weight by oral gavage. Blood was collected 4 h later by retro-orbital bleeding. Fluorescence intensity of the serum was measured using a fluorescent spectrophotometer with an excitation wavelength of 490 nm and an emission wavelength of 530 nm. FITC-dextran concentrations were calculated using standard curves generated by a serial dilution of FITC-dextran.

### Results

#### Expression of TIPE family members in the murine colon

Unlike TIPE2−/− 129 mice that develop systemic inflammation early in their lives, young TIPE2−/− mice of the B6 background are relatively healthy, with a normal gastrointestinal tract (Supplemental Fig. 1). TIPE2 mRNA was readily detected in the colon tissue homogenates from WT, but not TIPE2−/−, mice (Fig. 1A). In contrast, similar levels of TNF-α, IL-6, and IL-12 were measured in eBioscience and BD Biosciences Pharmingen. Quantitative ELISA was performed according to the manufacturer’s instructions.

#### Bone marrow chimeric mice

Bone marrow cells were harvested by flushing the femurs and tibias from donor WT and TIPE2−/− mice to express either CD45.1 or CD45.2, as previously described (18, 24). Recipient mice were sublethally irradiated twice, at a dose of 4.5 Gy, 3 h apart. Following the second irradiation, bone marrow cells (10 million cells/mouse) were transferred i.v. into WT and TIPE2−/− mice via the tail vein. Four chimeric groups were generated: WT → WT (WT cells expressing CD45.2 into WT mice expressing CD45.1), TIPE2−/− → WT (TIPE2−/− cells expressing CD45.2 into WT mice expressing CD45.1), WT → TIPE2−/− (WT cells expressing CD45.1 into TIPE2−/− mice expressing CD45.2), and TIPE2−/− → TIPE2−/− (TIPE2−/− cells expressing CD45.2 into TIPE2−/− mice expressing CD45.2). For the first 2 wk after bone marrow transfer, recipient mice received antibiotics in their drinking water, followed by a 5-kd in engraftment in 10% FBS. Six weeks after the transplantation, the degree of bone marrow reconstitution was determined by staining peripheral blood leukocytes with PE-conjugated anti-CD45.1 and PerCP-Cy5.5-conjugated anti-CD45.2 (BD Biosciences). As we reported, in the chimeric mice so generated, >90% of the hematopoietic cells were derived from donor bone marrow (18, 24).

#### Bacterial culture

After euthanizing the mice, the entire colon was removed under aseptic conditions. The terminal 3-cm segment of distal colon was washed, weighed, homogenized, and serially diluted. Different dilutions of the suspensions were plated in triplicate on brain heart infusion agar and blood agar (BD Biosciences) plates and incubated at 37°C for 24 h to quantify the bacterial colonies.

#### Ashman’s death test

Four groups of chimeric mice were generated: WT → WT (WT cells expressing CD45.2 into WT mice expressing CD45.1), TIPE2−/− → WT (TIPE2−/− cells expressing CD45.2 into WT mice expressing CD45.1), WT → TIPE2−/− (WT cells expressing CD45.1 into TIPE2−/− mice expressing CD45.2), and TIPE2−/− → TIPE2−/− (TIPE2−/− cells expressing CD45.2 into TIPE2−/− mice expressing CD45.2). For the first 2 wk after bone marrow transfer, recipient mice received antibiotics in their drinking water, followed by a 5-kd engraftment in 10% FBS. Six weeks after the transplantation, the degree of bone marrow reconstitution was determined by staining peripheral blood leukocytes with PE-conjugated anti-CD45.1 and PerCP-Cy5.5-conjugated anti-CD45.2 (BD Biosciences). As we reported, in the chimeric mice so generated, >90% of the hematopoietic cells were derived from donor bone marrow (18, 24).

#### Statistical analysis

Quantitative data are presented as mean ± SEM of two or three experiments. The survival curves were plotted according to the Kaplan–Meier method and compared using the log-rank test. Two-tailed Student t-test was used for all other comparisons. p < 0.05 was considered statistically significant. All statistical analyses were performed with Prism 5.0 for Windows (GraphPad, San Diego, CA).

#### Bone cell counts

Before euthanizing the DSS-treated mice, blood was collected, and whole-blood cell counts were determined using a Drew Hemavet 950F (Drew Scientific, Oxford, U.K.).
Consistent with the mortality data, TIPE2−/− mice lost considerably less weight than did the WT controls, commencing on day 5. The body weight difference increased gradually until the end of the study (Fig. 2B). No weight loss was observed in mice drinking regular water (Fig. 2B). Consistent with these results, the clinical manifestation of the disease, as reflected by DAI, was significantly less severe for TIPE2−/− mice compared with WT mice (Fig. 2C). Differences in colon weight and colon length also were apparent between the two groups. Without DSS treatment, TIPE2−/− mice had colon weights and colon lengths similar to those of WT mice. After DSS treatment, the colon weight of TIPE2−/− mice was 23% more than that of WT mice (Fig. 2D), whereas the colon length of TIPE2−/− mice was 27% longer than that of WT mice (Fig. 2E, Fig. 2F, right panel).

Histological examination also was performed to validate the clinical data. DSS treatment induced significant histopathological changes in the colons of WT mice that were characterized by massive inflammatory infiltrates and disruption of mucosal structures (Fig. 2F, right panel), consistent with previous reports (29). However, TIPE2−/− mice displayed less severe injury compared with WT mice (Fig. 2F, right panel). The histopathological score of TIPE2−/− mice was significantly lower than that of WT mice (2.6 ± 0.4 versus 4.6 ± 0.6, respectively) (Fig. 2G). Taken together, these results demonstrate that TIPE2−/− mice may be significantly less susceptible to DSS-induced colitis.

Reduced inflammatory cytokine expression and inflammatory cell infiltration in TIPE2−/− colon

DSS-induced colitis is an inflammatory disease mediated by many proinflammatory cytokines (30). To determine the effect of TIPE2 deficiency on the production of proinflammatory cytokines at the site of DSS-induced inflammation, we examined the expression levels of several cytokines by ELISA. We found that proinflammatory cytokines, such as TNF-α, IL-6, and IL-12, were markedly increased in WT mice after DSS treatment. However, TIPE2−/− mice produced significantly less of these cytokines relative to WT mice (Fig. 3A–C). In contrast, TIPE2−/− mice produced comparable amounts of IL-1β, IL-4, and IFN-γ to WT mice (data not shown).

To characterize the inflammatory cells involved, leukocytes isolated from lamina propria of the colon were analyzed by flow cytometry. Various inflammatory cell subsets were found in WT and TIPE2−/− colons after DSS feeding. In comparison with WT colon, the TIPE2−/− colon had markedly reduced neutrophils (8.89 ± 0.55 versus 2.92 ± 0.32, p < 0.001) (×104/g colon), macrophages (12.63 ± 0.63 versus 6.92 ± 0.69, p < 0.001) (×104/g colon), and dendritic cells (24.04 ± 3.65 versus 11.97 ± 0.27, p < 0.05) (×104/g colon) (Fig. 3D–F). Importantly, a significant proportion of the leukocytic infiltrate in both WT and TIPE2−/− mice consisted of Ly6G–CD11b+ macrophages (Fig. 3D–F).

TIPE2 deficiency in hematopoietic cells ameliorates colitis

Because both colonic epithelial and immune cells play important roles in the development of colitis, we next determined the cell populations that are critical for a TIPE2-mediated effect. We detected no significant difference in colonic epithelial cell apoptosis, proliferation, or permeability between WT and TIPE2−/− mice administered 4% DSS for 3 d (Y. Lou, H. Sun, and Y. Chen, unpublished observations), suggesting that TIPE2 may not have a primary effect on colonic epithelium in DSS-induced colitis.
To determine the potential roles of hematopoietic cells, we generated four groups of bone marrow chimeras. Six weeks after bone marrow transplantation, we found that >90% of the blood leukocytes were of donor origin, confirming successful engraftment (Supplemental Fig. 4). Following DSS treatment, knockout (KO) → KO chimeric mice developed the least severe colitis (Fig. 4A, 4B). Transplant of KO bone marrow to WT recipient mice (KO → WT) markedly reduced body weight loss (Fig. 4A), improved DAI (Fig. 4B), preserved the colon length (Fig. 4C), and reduced the histopathological score (Fig. 4D, 4E). These results indicate that TIPE2-deficient hematopoietic cells are responsible for the reduced colonic inflammation in TIPE2-KO mice.

Reduced local dissemination of commensal microflora and systemic inflammation in TIPE2−/− mice

It is well established that commensal microflora in the lumen of the colon play an essential role in the development of human IBD and murine experimental colitis (31–33). Our previous study showed that TIPE2-KO macrophages and neutrophils had enhanced phagocytic and bactericidal activities, and TIPE2-KO mice were resistant to Listeria monocytogenes and Staphylococcus aureus. 

FIGURE 3. Reduced inflammatory cytokine production and inflammatory cell infiltration in TIPE2−/− colon. WT (n = 5) and TIPE2−/− (n = 5) mice were administered 4% DSS or water ad libitum for 5 d, followed by recovery on regular drinking water for 2 d. Colonic lysates from control and DSS-fed WT and TIPE2−/− mice were analyzed by ELISA for the amounts of TNF-α (A), IL-6 (B), and IL-12 (C). Data are mean ± SEM cytokine/mg of total colon protein. (D) Flow cytometric profiles of neutrophils (Ly6G+CD11b+), macrophages (Ly6G−CD11b+), and dendritic cells (CD11c+) isolated from the colonic lamina propria of WT mice (upper panels) and TIPE2−/− mice (lower panels). Numbers indicate the frequencies of cells in each subset. Total numbers of neutrophils (Ly6G+CD11b+) (E), macrophages (Ly6G−CD11b+) (F), and dendritic cells (CD11c+) (G) per milligram of colon, as determined by flow cytometry. Data are mean ± SEM of one representative experiment. The experiments were repeated three times with similar results. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 4. TIPE2 deficiency in hematopoietic cells reduces colonic inflammation. Bone marrow chimeric mice (n = 5) were administered 4% DSS for 5 d, followed by recovery on regular drinking water for 2 d. (A) Body weight loss was measured daily. (B) DAI was scored daily. (C) Colon lengths at the end of the experiment (day 7). (D) Colonic histopathologic scores of mice treated with DSS. (E) Representative images of H&E-stained colonic sections of chimeric mice on day 7 (original magnification ×100). Data are mean ± SEM of one representative experiment of three. *p < 0.05, **p < 0.01.
infections (19). Because we observed a reduced inflammatory response in DSS-induced colitis in TIPE2−/− mice, we asked whether TIPE2 deficiency affected commensal microflora dissemination in mice. As shown in Fig. 5A and 5B, significantly less bacteria were detected in the colon of TIPE2−/− mice relative to DSS-treated WT mice. We also found a significant decrease in plasma FITC fluorescence in TIPE2−/− mice after DSS-induced injury compared with WT mice (Fig. 5C), indicating less colon tissue disruption and less permeability (Fig. 2F). It is known that systemic dissemination of bacteria and bacterial components leads to increased peripheral blood leukocyte counts, as well as triggers an exuberant cytokine inflammatory response. In agreement with the reduced bacterial dissemination in TIPE2−/− mice, the numbers of total peripheral blood leukocytes and monocytes were markedly reduced in TIPE2−/− mice relative to DSS-treated WT mice (Fig. 5E). Although there appeared to be a trend toward decreased neutrophils and lymphocytes in the KO mice, the differences were not statistically significant (Fig. 5D). Furthermore, serum concentrations of the proinflammatory cytokine IL-6 also were lower in TIPE2−/− mice compared with WT mice (Fig. 5D). Taken together, these results indicate that the reduced DSS-induced morbidity and mortality in the absence of TIPE2 may be caused by enhanced immunity to commensal bacteria.

To interrogate gut microbial diversity, we performed quantitative real-time PCR amplification of 16S rRNA gene sequences. The total DNA in stool was extracted, and real-time PCR was conducted using specific 16S rRNA primers for the following major groups: E. rectale–C. coccoides, Bacteroides spp., and Enterobacteriaceae. In addition, the total bacterial (eubacteria) numbers were determined using standard curves constructed with reference bacteria specific for each group. We found no significant differences in the numbers of total bacteria or the three representative bacterial groups between naive WT and TIPE2−/− mice at 6–8 wk of age (Fig. 6), indicating that bacterial composition was not affected by TIPE2 deficiency.

Discussion

The results reported in this article indicate that TIPE2 is an important regulator of DSS-induced colitis. TIPE2−/− mice are resistant to DSS-induced colitis, and enhanced bacterial clearance after DSS treatment may be responsible. TIPE2 function has been studied in several diseases, including autoimmune diseases, bacterial infection, chronic inflammatory diseases, and cancer (12, 18, 19, 34). TIPE2 is constitutively expressed at high levels in immune cells, especially in macrophages and neutrophils (8, 19, 20), which play crucial roles in acute ulcerative colitis. To our knowledge, little is known about the function of TIPE2 in intestinal disorders. Acute ulcerative colitis induced by DSS is characterized by massive infiltration of inflammatory cells, such as macrophages, neutrophils, and CD4+ T cells, within the colonic walls, which destroy epithelium and shorten the colon length (35). These infiltrated inflammatory cells are major producers of inflammatory mediators, such as TNF-α, IL-6, IL-12, IFN-γ, and IL-1β, that contribute to pathogenesis (6). We found that TIPE2−/− mice produced markedly less TNF-α, IL-6, and IL-12, but not IFN-γ or IL-4, relative to WT mice (36).

Our previous studies showed that TIPE2-deficient macrophages produced significantly more IL-6 and IL-12 upon stimulation with LPS, and TIPE2-deficient mice were more susceptible to LPS-induced septic shock (8). We also found that TIPE2-deficient mice were hypersensitive to polyinosinic-polycytidylic acid lethalitity (20). In addition, TIPE2 deficiency exacerbates cerebral ischemia/reperfusion injury (16) and atherosclerosis in Ldlr−/− mice (18). Based on these findings, we expected TIPE2−/− mice to be more susceptible to colitis development than WT mice. On the contrary, TIPE2 deficiency rendered mice resistant to DSS-induced colitis. Adoptive transfer of TIPE2-deficient bone marrow cells was capable of rescuing colonic injury phenotype in WT mice, suggesting that TIPE2 expression in hematopoietic cells may play an important role in the development of colitis. Our previous studies also showed that TIPE2 serves as a negative regulator of phagocytosis and oxidative burst during infection. TIPE2-deficient mice exhibited resistance to bacterial challenge, and TIPE2-deficient macrophages and neutrophils exhibited enhanced bacterial clearance (19). Indeed, significantly fewer bacteria were found in the colon of TIPE2−/− mice relative to DSS-treated WT mice. The number of leukocytes and serum concentrations of proinflammatory cytokine IL-6 were markedly reduced in TIPE2−/− mice, consistent with a reduced bacterial dissemination in these mice. Thus, in colitis, enhanced bacterial clearance in TIPE2−/− mice likely ameliorated the disease.
The authors have no financial conflicts of interest.

References
28. Ghia, J. E., F. Galeazzi, D. C. Ford, C. M. Hogaboam, B. A. Vallance, and S. Collins. 2008. Role of M-CSF-dependent macrophages in colitis is driven by...


Fig. S1. Young $TIPE2^{-/-}$ B6 mice display normal colonic morphology. Representative images of HE-stained colonic cross-sections (A) and longitudinal sections (B) of unmanipulated six-week-old WT and $TIPE2^{-/-}$ B6 mice. Original magnifications for A, X50; original magnifications for B, X100. Data are representative of two independent experiments (n=3).
Fig. S2. *TIPE2*−/− mice have normal water consumption. WT and *TIPE2*−/− mice (n=5) were fed with 4% DSS for 5 days. Water consumption was monitored daily. Data shown are means + SEM of water consumption per day per mice, pooled from four independent experiments.
Fig S3. TIPE2 deficiency prolongs the lives of mice with DSS-induced colitis. WT and TIPE2<sup>−/−</sup> (n=5) mice were fed with 5% DSS in the drinking water for 5 days and then switched to regular drinking water. The difference in survival between the two groups is statistically significant as determined by Kaplan-Meier analysis (P<0.05).
Fig. S4. Confirmation of reconstitution of bone marrow chimeric mice. Representative flow cytometric profiles of peripheral blood leukocytes of the chimeric mice stained with anti-CD45.1-PE (for WT cells) and anti-CD45.2-Percp-Cy5.5 (for TIFE2<sup>−/−</sup> cells).