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TNFAIP8 Deficiency Exacerbates Acute Graft Versus Host Disease in a Murine Model of Allogeneic Hematopoietic Cell Transplantation

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Abstract

Background.—Gastrointestinal acute graft-versus-host disease (GVHD) occurring after allogeneic hematopoietic cell transplant is an allo-reactive T cell and inflammatory cytokine driven organ injury with epithelial apoptosis as 1 of its hallmark findings and is associated with significant mortality. Tumor necrosis factor (TNF)-alpha-induced protein 8 (TNFAIP8 or TIPE) acts as a negative mediator of apoptosis via inhibition of caspase-3 activation, promotes cell proliferation and *Tipe*$^{-/-}$ deficiency is associated with increased inflammation.

Methods.—To evaluate the role of TIPE in acute GVHD, naive C57BL/6 and *Tipe*$^{-/-}$ C57BL/6 mice were conditioned with 1000 cGy single dose total body irradiation, followed by transplantation of 10 million bone marrow cells and 20 million splenocytes from either syngeneic C57BL/6 or allogeneic BALB/c donors.

Results.—Allo TIPE-deficient mice developed exacerbated gut GVHD compared with allo controls and had significantly decreased survival (6 wk overall survival: 85% versus 37%; $P < 0.05$), higher clinical GVHD scores, more profound weight loss, increased serum proinflammatory cytokines (interleukin-17A, TNF, interleukin-6, and interferon-γ). T-cell infiltration into the ileum was increased; epithelial proliferation was decreased along with significantly higher levels of chemokines KC and monokine induced by gamma interferon. Using bone marrow chimeric experiments, TIPE was found to have a role in both hematopoietic and nonhematopoietic cells.

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Conclusions.—Absence of TIPE results in excessive inflammation and tissue injury after allo-HCT, supporting that TIPE confers immune homeostasis and has tissue-protective function during the development of gut GVHD and may be a potential future target to prevent or treat this complication after allogeneic HCT.

INTRODUCTION

Allogeneic hematopoietic cell transplantation (allo-HCT) is increasingly utilized in the treatment of hematologic malignant and nonmalignant diseases. Currently, there are >25 000 transplants carried out annually worldwide. Allo-HCT involves the transfer of donor hematopoietic stem cells and donor immune cells into the patient. One of the principal therapeutic benefits of this procedure is known as the graft-versus-leukemia effect, in which allo-reactive T-cell responses can exert antileukemic effects. The activity of these allo-reactive cells can result in better disease control and reduced risk of relapse, but also allows for interactions between the donor immune system and the patient tissue, resulting in graft-versus-host disease (GVHD).[1] This disease is the largest barrier to increased therapeutic use of allo-HCT and is associated with significant mortality and morbidity.[2]

Acute GVHD accounts for 15% to 30% of deaths that occur following allo-HCT carried out to treat malignant diseases and is a major cause of morbidity in up to 50%–70% of transplant recipients.[2] The pathophysiology of acute GVHD (aGVHD) can be outlined in the following 3 sequential phases: (1) conditioning-related tissue injury and inflammatory cytokine storm leading to the activation of antigen-presenting cells, (2) donor T-cell activation, and (3) the effector cell phase. The result is a combined injury to the patient's tissue by antigen-specific alloreactive T cells and by inflammatory cytokines, such as tumor necrosis factor (TNF), with the primary organs affected being the intestine, liver, skin, and the hematopoietic system. The initiation of these events involves injury to the gut,[3] propagating donor T-cell activation and a cascade of inflammatory events through translocation of pathogen-associated molecular pattern molecules and damage-associated molecular pattern molecules.[4]

TNF-alpha-induced protein 8 (TNFAIP8 or TIPE) family are recently identified proteins, which are important for maintaining immune homeostasis.[5] The mammalian TNFAIP8 family consists of the following 4 members: TNFAIP8 (TIPE), the first identified member of this family; TNFAIP8L1; TIPE1; TIPE2; and TIPE3, which share high degrees of sequence homology. TIPE is the founding member of its family that was cloned and studied.[6-8] TIPE is a newly identified regulator of cancer and infection; however, its precise roles and mechanisms of actions remain unrevealed. TIPE is induced by TNF-α and NF-κB[7,9,10] TIPE acts as a negative mediator of TNF-mediated apoptosis via inhibition of caspase-3 activation.[7,9] By contrast, TIPE may promote glucocorticoid-induced apoptosis of normal thymocytes in culture.[11] TIPE can play different roles in apoptosis depending on the environmental stimuli factors. Overexpression of TIPE in tumor cell lines also enhances cell proliferation and migration.[6] TIPE deficiency may induce increased cell death in the colon by inhibiting the PI3K-AKT signaling.[12] Sun et al[12] previously showed that *Tipe*$^{-/-}$ mice are hypersensitive to dextran sulfate sodium-induced colitis and develop severe colitis along with enhanced inflammatory responses. *Tipe*$^{-/-}$ mice with experimental colitis show higher

levels of interleuken (IL)-17A and IL-6 in serum and higher levels of IL-17A and IL-6, IL-1β in the colon. *Tipe*$^{-/-}$ mice with experimental colitis also show increased bacterial invasion of the colonic tissue and decreased epithelial cell survival and proliferation, primarily due to TIPE deficiency in nonhematopoietic cells (NHC).

Given the importance of cytokines like TNF, IL-6, and IL-17 and of epithelial injury in the pathophysiology of aGVHD, we hypothesized that TIPE deficiency in allogeneic HCT recipients will result in worsened GVHD.

MATERIALS AND METHODS

Mice

Female BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory and *Tipe*$^{-/-}$ C57BL/6 were kindly provided by Youhai H. Chen (Philadelphia, PA).[12]

Transplant Model

Nine to 12 weeks old C57BL/6 and *Tipe*$^{-/-}$ C57BL/6 mice were conditioned with total body irradiation using a Cesium source irradiator (University of Kentucky), at a total dose of 1000cGy. Animals then received 10 million bone marrow (BM) cells with 20 million splenocytes from either syngeneic C57BL/6 or allogeneic BALB/c donors via tail vein injection. Mice were housed in sterilized micro isolator cages and received autoclaved chow and water. All animals were handled and used as per the University of Kentucky Institutional Animal Care and Use Committee guidelines on approved protocol 2015-2022.

Generation of BM Chimera Mice

To further delineate the impact of *Tipe*$^{-/-}$ NHC and *Tipe*$^{-/-}$ hematopoietic (HC) cells in GVHD pathophysiology, we generated BM chimera mice for TIPE deficiency in hematopoietic cells or in NHCs. Four groups of chimera mice were established by lethal irradiation with 1000cGy following the infusion of T-cell–depleted BM cells: (1) C57/BL6 wild type (WT) mice received C57BL/6 WT cells, (2) C57BL/6 WT were transplanted with BM from *Tipe*$^{-/-}$ C57BL/6 animals to replace wt hematopoiesis with *Tipe*$^{-/-}$ hematopoiesis, (3) *Tipe*$^{-/-}$ C57BL/6 received cells from C57BL/6 WT animals to have TIPE deficiency only present in the NHCs, and (4) *Tipe*$^{-/-}$ C57BL/6 received *Tipe*$^{-/-}$ C57BL/6 cells. Groups 1 and 4 were included to allow for age and treatment adjusted controls, in which both hematopoietic cells and NHCs either were WT or *Tipe*$^{-/-}$, respectively. Three months later, BM chimeras were used as recipients and were reirradiated at a total dose of 1000 cGy on day 0, followed by hematopoietic cell transplantation from allogeneic donors, then analyzed at day +42.

Assessment of Clinical GVHD

Recipient mice were monitored daily for survival, and clinical aGVHD was assessed weekly. Mice were scored for clinically by assessment of 5 parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity. Individual mice from coded cages received a score of 0 to 2 for each criteria, which was used as an index of severity and progression of disease.[13]

Genotyping of *Tipe*$^{-/-}$ Mice

Genomic DNA was extracted from tail snips using the DNA extraction kit (Gene jet genomic DNA purification kit, Thermo Scientific) as per the manufacturer protocol. A common WT forward primer 1 (5′-CCAAAGGCTCAACATGCTCT-3′) was paired with a reverse primer 3 (5′-CCAATAAACCCTCTTGCAGTTGC-3′) against the sequence on gene trap vector to produce a 190 bp polymerase chain reaction (PCR) fragment from *Tipe*$^{-/-}$ allele, and a WT reverse primer 2 (5′-CCCATGTGTGCAAGTGAAAA-3′) to generate a 334-bp PCR fragment from the WT allele.[12] PCR reactions were performed on PCR icycler (Bio-Rad) using PCR Master Mix (Thermo Scientific, Rockford, IL).

Flow Cytometric Analysis

Spleen was collected from the animals in all groups, and splenocytes were phenotyped for T cells (CD3) and their subtypes (CD4 and CD8). Cells were washed with phosphate-buffered saline containing 4% FBS and incubated with FcR-block anti-mouse CD16/CD32 (Clone:93, eBioscience, San Diego, CA) for 15 minutes at 4°C. Following a washing step, cells were incubated in a predetermined optimized concentration of antibodies for 15 minutes at 4°C while shaking. The following antibody clones were used: CD3-FITC (17A2) (Biolegend, San Diego, CA). Cells were analyzed on BD LSR II (BD Biosciences). Flow cytometry data were analyzed using FlowJo_V10 software (Tree Star, Ashland, OR).

Serum Cytokine Analysis

The levels of serum cytokines were determined using the Cytometric Bead Array Mouse Cytokine kit (BD Biosciences). Mouse cytokine-specific bead sets and standards were implemented as per the manufacturer's instructions. The fluorescence produced by the beads was measured using BD LSR II and analyzed with FCAP Array software as per manufacturer instructions.

Histopathology and Immunohistochemistry

Upon necropsy liver, lung, ileum, and colon tissues were preserved and embedded in paraffin for histological examination. Tissues were then sectioned and stained with hematoxylin and eosin. Histological examination and GVHD damage assessment were carried out by pathologists in a completely blinded fashion.[13-15]

For immunohistochemistry, sections were deparaffinized in xylene and dehydrated in Ethanol series (100% and 95%) and subjected to antigen retrieval in a pressure cooker using Target Retrieval Solution (Dako, Carpinteria, CA) after rehydration in phosphate buffer saline. After retrieval, sections were incubated with respective primary antibodies against Ki-67 (Dako M7249), cleaved caspase-3 (Invitrogen-700182), or CD3e (CST-99940), followed by incubation with rabbit-derived HRP labeled polymer (Dako EnVision + System-HRP Labelled Polymer). The sections -were developed using Dab buffer (Dako, Carpinteria, CA). Pictures were captured using Nikon eclipse 55i microscope analyzed through ImageJ software.

Tissue Cytokine and Chemokine Analysis

At time of analysis, tissues were retrieved and snap-frozen. Supernatant of homogenized tissue was obtained as described before.[16] Total protein concentration in the supernatant was determined using bicinchoninic acid assay (Thermo Scientific, Rockford, IL) to allow for cytokine and chemokine concentration normalization. Cytokine analysis was performed on tissue homogenates using enzyme-linked immunosorbent assay for IL-6, IL-17A, interferon (IFN)-γ, and TNF (BD Biosciences, San Diego, CA). The tissue homogenates and serum samples were subjected for chemokine quantitation using LEGENDplex mouse proinflammatory chemokine panel (13-plex) assay kit which analyzes CCL5, CCL11, CCL17, KC/chemokine (C-X-C motif) ligand 1 (CXCL1), MIG (monokine induced by gamma interferon)/CXCL9, CXCL10, CXCL13, CXCL5, CCL22, CCL2, CCL3, and CCL4.

Statistical Analysis

Experimental data are expressed as means ± SD. Differences between groups were analyzed either by the 2-tailed *t* test or 1-way ANOVA, followed by multiple comparisons. A $P \leq 0.05$ was considered statistically significant. Survival data were analyzed using the Log-rank test. All data were prepared using GraphPad Prism software (GraphPad, San Diego, CA).

RESULTS

Tipe$^{-/-}$ Allogeneic Recipients Show Exacerbated Intestinal GVHD After Allogeneic Transplant

Tipe$^{-/-}$ C57BL/6 and C57BL/6 WT animals were transplanted as described in Material and Methods. At day +42 posttransplant all syngeneic recipients survived but only 37% survived in *Tipe*$^{-/-}$ allogeneic recipient group compared to 85% in allogeneic controls ($P < 0.05$) (Figure 1A). This increased mortality was associated with significantly higher clinical GVHD scores in *Tipe*$^{-/-}$ allogeneic recipients from day +28 onwards compared to allogeneic WT recipients (Figure 1B). Similarly, significantly higher weight loss was observed for *Tipe*$^{-/-}$ allogeneic recipients from day +14 onwards compared with allogeneic WT recipients (Figure 1C).

Intestinal Injury in *Tipe*$^{-/-}$ Mice Following Allogeneic Transplant Is Progressive

The observed progressive weight loss in allogeneic *Tipe*$^{-/-}$ recipients was associated with significantly increased gut histopathology scores compared to allogeneic controls on day 42 (Figure 1E) but not on day 7 (Figure 1D). At both time points, gut pathology was increased when compared to syngeneic controls. No differences in pathology of lung and liver were seen at either time point between allogeneic groups (data not shown). Increased gut injury was associated with increased TNF ($P < 0.05$), IL-6 ($P < 0.01$), IL-17A ($P < 0.01$), and IFN-γ ($P < 0.01$) serum levels in *Tipe*$^{-/-}$ allogeneic recipients compared to allogeneic control (Figure 2E-H), though no differences were observed at day +7 (Figure 2A-D).

TIPE Deficiency Affects Regeneration and Healing Process in Small Intestine After Allo-HCT

To explore cell proliferation and apoptosis in the gut, we analyzed the expression of proliferation marker (Ki-67) and apoptosis marker (active caspase-3). Immunohistochemical analysis of Ki-67 expression in the gut revealed a significant decrease in ileum of allogeneic *Tipe*$^{-/-}$ recipients when compared to allogeneic controls at day +7 (Figure 3A) and day +42 (Figure 3C and D), however, no differences were seen in the colon (Figure 3B, E, F) at either time point. Although not significant, a trend for increased active caspase-3 expression was found in the ileum (Figure 3G and H; $P = 0.0632$) and colon ($P = 0.0686$) (Figure 3I and J) of *Tipe*$^{-/-}$ allogeneic recipients when compared to allogeneic controls at day +42. No differences were seen for active caspase-3 expression in ileum and colon at day +7 (data not shown).

T-Cell Infiltration Into the Small Bowel of *Tipe*$^{-/-}$ Recipients Is Increased After Allo-HCT

Histopathologic analysis of T-cell infiltration in ileum and colon by immunohistochemistry for CD3 revealed increased infiltration in ileum of *Tipe*$^{-/-}$ allogeneic recipients at day +7 (Figure 4A; $P < 0.05$) and day +42 (Figure 4C and D; $P < 0.05$) when compared to allogeneic controls. In colon samples, no significant difference was observed either at day +7 (Figure 4B) or day +42 (Figure 4E and F). Analysis of splenic T cell absolute counts showed no differences compared to allogeneic controls either at day +7 (Figure 4G) or at day+42 (Figure 4H).

Tipe$^{-/-}$ Allogeneic Recipients Show Enhanced Inflammatory Responses in Tissue Compared to Allogeneic Recipients

We analyzed protein levels of proinflammatory chemokines in ileum and colon tissue homogenates at day +42 as described in materials and methods. We observed increased level of KC/CXCL1 and MIG/CXCL9 in ileum samples of *Tipe*$^{-/-}$ allogeneic recipients (Figure 5A [$P < 0.01$] and Figure 5B [$P < 0.05$]), respectively. No differences for other chemokine were observed. KC/CXCL1 and MIG/CXCL9 significantly correlated with GVHD scores and pathology score (Figure 5C-F). No difference for these proinflammatory chemokines was observed in colon samples of *Tipe*$^{-/-}$ allogeneic recipients (data not shown). Analysis of tissue cytokines by enzyme-linked immunosorbent assay showed a nonsignificant trend for IFN-γ and IL-17A in ileum and IFN-γ in colon of *Tipe*$^{-/-}$ allogeneic recipients compared to allogeneic recipients at day +42 tissue homogenates (data not shown).

In addition to this, we analyzed the infiltration of neutrophils in the hematoxylin and eosin-stained ileum and colon sections by counting neutrophils in 10 high power fields. We observed no difference in the neutrophil count in both ileum and colon sections of *Tipe*$^{-/-}$ allogeneic recipients compared to allogeneic controls at day +42 (Figure 5G and H).

TIPE Deficiency in NHCs, as Well as HCs, Is Responsible for Exacerbated GVHD in TIPE-Deficient Mice

Analysis of retransplanted chimeric mice at day +42 confirmed our observation of exacerbated clinical GVHD in *Tipe*$^{-/-}$ allogeneic recipients (*Tipe*$^{-/-}$ → *Tipe*$^{-/-}$) chimera mice, in which both hematopoietic and NHCs were deficient of TIPE, when compared to

WT allogeneic controls (*Tipe*$^{+/+}$ → *Tipe*$^{+/+}$) chimera, leading to decreased survival of 50% versus 87.5% ($P < 0.05$), respectively (Figure 6A). Allo chimera recipients in which only the hematopoietic cells (*Tipe*$^{-/-}$ → *Tipe*$^{+/+}$] or the NHCs (*Tipe*$^{+/+}$ → *Tipe*$^{-/-}$), were TIPE deficient, showed an intermediate clinical course, with survival of 75% versus 73%, respectively (Figure 6A).

Mortality in allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{-/-}$) chimera was associated with significantly higher clinical GVHD scores compared with WT allogeneic controls (*Tipe*$^{+/+}$ → *Tipe*$^{+/+}$) chimera from day +28 onwards ($P < 0.001$) (Figure 6B) and higher weight loss at +35 (Figure 6C, $P < 0.01$), respectively. This observation is consistent with the increased gut pathology in allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{-/-}$) chimera compared with allo controls at day +42 ($P < 0.05$) (Figure 6D).

Allogeneic chimera recipients (*Tipe*$^{-/-}$ → *Tipe*$^{+/+}$) and (*Tipe*$^{+/+}$ → *Tipe*$^{-/-}$) showed no differences for clinical GVHD scores and weight loss in comparison to WT allogeneic controls (*Tipe*$^{+/+}$ → *Tipe*$^{+/+}$) chimera (Figure 6B and C). Gut pathology scores were significantly higher in (*Tipe*$^{-/-}$ → *Tipe*$^{+/+}$) chimera, and no significant difference was seen in (*Tipe*$^{+/+}$ → *Tipe*$^{-/-}$) recipients when compared to allogeneic controls (Figure 6D).

We further analyzed the expression of proliferation (Ki-67), apoptosis (active caspase-3) and T-cells infiltration markers in ileum and colon sections of these retransplanted mice. Allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{-/-}$) chimera showed significantly reduced Ki-67 expression (Figure 6E) and increased cleaved caspase-3 expression and T-cell infiltration (Figure 6F and G) in ileum compared to allogeneic controls. Colon tissues of allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{-/-}$) chimera mice showed significantly increased T-cells infiltration compared to allogeneic controls (Figure 6J). However, no difference was observed for Ki-67 and caspase-3 expression in the colon between allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{-/-}$) and allogeneic controls (Figure 6H and I).

Comparison of allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{+/+}$) chimera with allogeneic controls revealed no differences for Ki-67 in either ileum or colon samples (Figure 6E and H). Allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{+/+}$) chimera mice showed significantly higher caspase-3 expression in ileum but not in colon in comparison to allogeneic controls (Figure 6F and I). A significant increase in T-cell infiltration in colon was observed alongside a nonsignificant trend ($P = 0.06$) in ileum of allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{+/+}$) chimera when compared to allogeneic controls (Figure 6G and J).

Allogeneic (*Tipe*$^{+/+}$ → *Tipe*$^{-/-}$) chimera showed a significant decrease in Ki-67 expression in ileum when compared with allogeneic controls (Figure 6E). No differences were seen for T-cell infiltration in ileum but a significant increase in caspase-3 expression was observed in allogeneic (*Tipe*$^{+/+}$ → *Tipe*$^{-/-}$) chimera compared with allogeneic controls (Figure 6F and G). Colon samples of allogeneic (*Tipe*$^{+/+}$ → *Tipe*$^{-/-}$) chimera showed significant increase in T-cell infiltration (Figure 6J), and no differences for Ki-67 and caspase-3 expression were seen when compared with allogeneic controls (Figure 6H and I).

DISCUSSION

In the current study, we showed that *Tipe*$^{-/-}$ mice have significantly exacerbated intestinal GVHD, greater body weight loss, increased mortality, and higher clinical GVHD scores, indicating a key role for TIPE in protection against gastrointestinal GVHD. While tissue damage and pathology were not significant at the early time point (d +7), we observed worsened gut clinical GVHD and increased gut pathology at the later time point (d +42).

TIPE has been reported as an antiapoptotic molecule in tumor cells,[6] but knocking down its expression by RNAi protects thymocytes from glucocorticoid-mediated apoptosis.[11] Thus, TIPE may play divergent roles in apoptosis depending on the environmental cues and cell type. Overexpression in tumor cell lines also enhances tumor proliferation and migration.[6]

We observed a significant decrease of Ki-67 expression in the ileum and a trend for increased intestinal epithelial cell apoptosis in *Tipe*$^{-/-}$ allogeneic recipients compared to allogeneic controls. These observations point to TIPE protein playing an important role in healing and inflammatory conditions. The epithelial barrier integrity in the gastrointestinal system is crucial for protecting against environmental insults, including toxins and microbes.[17] Increased epithelial cell death and decreased proliferation are associated with GVHD.[18-22] The loss of epithelial integrity caused by increased epithelial cell death and reduced proliferation in *Tipe*$^{-/-}$ mice can lead to increased dissemination of commensal bacteria and enhanced leukocyte infiltration and inflammatory responses. Increased serum endotoxin level due to damaged gut epithelium after transplant affect the gastrointestinal tract healing and regeneration leading to worsened GVHD at later stages.[13,23,24] Sun et al[12] also showed that TIPE deficiency affects epithelial cell death and proliferation in a colitis mouse model. The increased mortality in *Tipe*$^{-/-}$ mice could be explained by increased cell death and decreased proliferation of colonic epithelial cells during GVHD.

Furthermore, increased T-cell infiltration seen in ileum of TIPE-deficient mice could be responsible for inducing epithelial cell apoptosis and thus affecting epithelial cell regeneration. T cells are well known to induce apoptosis in small intestine.[25] This further explains how TIPE deficiency may play an important role in exacerbating gut GVHD. It is, therefore, possible that controlled overexpression of TIPE might be an innovative therapeutic approach for GVHD development.

The increased level of proinflammatory cytokines seen in serum of *Tipe*$^{-/-}$ allogeneic recipients at later time point of GVHD supports that TIPE deficiency contributes to gut GVHD. TNF-α plays an important role during establishment of GVHD both through direct effects leading to apoptosis on GVHD target tissues, and indirectly by inducing activation and proliferation of T cells, the main cellular effector of GVHD.[26] IL-6 has been identified as a critical inflammatory cytokine that alters the balance between the effector and regulatory arms of the immune system and drives a proinflammatory phenotype that is a defining characteristic of GVHD.[27]

The pathogenic role of inflammatory cytokines and stimulation of mature donor T cells is well known in acute GVHD. MIG/CXCL9 is a CXC chemokine, produced by IFN-γ stimulated monocytes, macrophages, and endothelial cells and is involved in the

pathogenesis of aGVHD.[28] CXCR3, the chemokine receptor for ligands CXCL9, 10, and 11, is highly expressed on effector T cells and plays an important role in T-cell trafficking and function. In a well-defined experimental BMT model where acute GVHD is mediated by donor $CD8^+$ T cells, it was shown that $CXCR3^{-/-}$ donor T cells had diminished ability to infiltrate the small bowel and were associated with reduced gastrointestinal tract as well as liver damage and overall mortality.[29] Therefore, the increased levels of MIG/CXCL9 in the gut of *Tipe*$^{-/-}$ allogeneic recipients support the concept of chemokine-mediated T-cell recruitment into the tissue and tissue injury propagation.

Increased KC/CXCL1 expression in gut and the correlation with disease severity in *Tipe*$^{-/-}$ allogeneic recipients further supports that TIPE deficiency in allogeneic recipients exacerbates GVHD. Neutrophils are known to be involved in the pathogenesis of GVHD in both mice and humans, and it is known that neutrophil depletion reduces GVHD-related mortality. Neutrophils can further amplify tissue damage during an allogeneic immune response by promoting T-cell activation due to their ability to cleave chemokines and produce reactive oxygen species.[30,31]

Tipe$^{-/-}$ C57BL/6 mice have been shown not to differ in innate immune cell function compared with C57BL/6. Porturas et al[32] have shown that neutrophil chemotaxis remained similar between cells of C57BL/6 mice and *Tipe*$^{-/-}$ C57BL/6 mice. Contrary to our expectation and despite increased tissue KC/CXCL1 expression, no difference in neutrophil infiltration was observed. In our study, this may be explained by crosstalk between CXCL9 and neutrophils, as it has been previously shown that the COOH-terminal CXCL9 domain can bind glycosaminoglycans (GAGs) and may compete with several cytokines for GAG binding thereby inhibiting neutrophil migration to inflammatory sites. Thus, no difference in neutrophil number within the intestinal tract, observed in our study, could reflect the balance between prochemotactic CXCL1 mediated and indirectly mediated antichemotactic effects.[33-36]

It is previously reported that phagocytic capacity of BM-derived macrophages from C57BL/6 and *Tipe*$^{-/-}$ C57BL/6 mice does not differ. BM-derived macrophages did not show a difference in the production of TNF-α and IL-6 upon stimulation with peptidoglycans in vitro.[32] We observed that TNF production from LPS-induced peritoneal macrophages of WT and KO mice did not differ, which supports the previous observation (data not shown). To determine whether TIPE deficiency primarily affected the hematopoietic or nonhematopoieic systems, in GVHD pathophysiology, we generated chimeric mice and retransplanted after 3 months with donor cells from allogeneic donors as detailed in material methods. The retransplanted mice were analyzed at day +42, and this analysis further validated our observation of aggravated GVHD seen in *Tipe*$^{-/-}$ allogeneic recipients compared with allogeneic controls. Allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{-/-}$) chimera showed significantly higher clinical GVHD scores and reduced survival, which is associated with higher pathology scores in gut reflecting more aggressive GVHD compared to allogeneic controls. However, allogeneic (*Tipe*$^{-/-}$ → *Tipe*$^{+/+}$) chimera and allogeneic (*Tipe*$^{+/+}$ → *Tipe*$^{-/-}$) chimera showed somewhat similar parameters for increased GVHD but remained higher than allogeneic recipients, although similar or lower than *Tipe*$^{-/-}$ allogeneic recipients. This observation indicates that TIPE deficiency in both NHCs and hematopoietic cells contributes

to worsening gut GVHD. Based on these data, it is possible that overexpression of TIPE in a controlled manner may play a protective or therapeutic role in GVHD pathophysiology.

TNFAIP8 (TIPE) deficiency in allogeneic recipients resulted in increased mortality after transplant, which was associated with significant increase in proinflammatory cytokines expression, decreased proliferation and increased apoptosis of intestinal epithelial cells at later post-allo-HCT time point (d +42), suggesting not only exaggerated inflammatory damage but also decreased epithelial regeneration leading to increased gut GVHD. The negative impact on GVHD and transplant outcome seems to depend on lack of TIPE signaling in both nonhematopoietic and hematopoietic cells. Our study is the first to show a protective role for TNFAIP8/TIPE, a member of the TNF receptor family, in GVHD pathophysiology, which may be targeted to mitigate the role of the gastrointestinal tract as a critical site for acute GVHD initiation.

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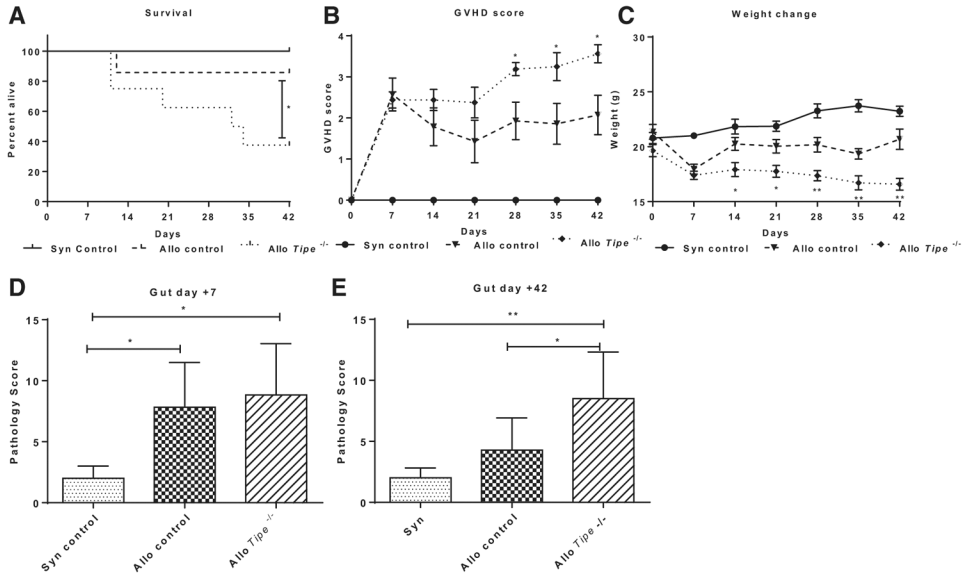

FIGURE 1.

Tipe$^{-/-}$ allogeneic recipients show exacerbated intestinal graft-vs-host disease (GVHD) after allogeneic transplant. A, Survival at day +42 after hematopoietic cell transplant (HCT). Animals underwent transplantation as described in Materials and Methods. B, Clinical GVHD score at day +42 after HCT as described in Materials and Methods. C, Weight graph of the transplanted mice; asterisks in B and C represent the statistical comparison between allo control and allo *Tipe*$^{-/-}$ group. D, Pathology of the gut by hematoxylin and eosin (H&E) staining at day +7 after HCT. E, Pathology of the gut by H&E staining at day +42 after HCT using scoring systems as described in Materials and Methods. Sample size at day +7; syngeneic control: n = 3; allogeneic HCT (allo-HCT) control: n = 6; allo *Tipe*$^{-/-}$: n = 6. Sample size at day +42; syngeneic control: n = 3; allo-HCT control: n = 7; allo *Tipe*$^{-/-}$: n = 8. Data shown are from 1 experiment. These observations were independently confirmed in the chimera experiment. $*P < 0.05$.

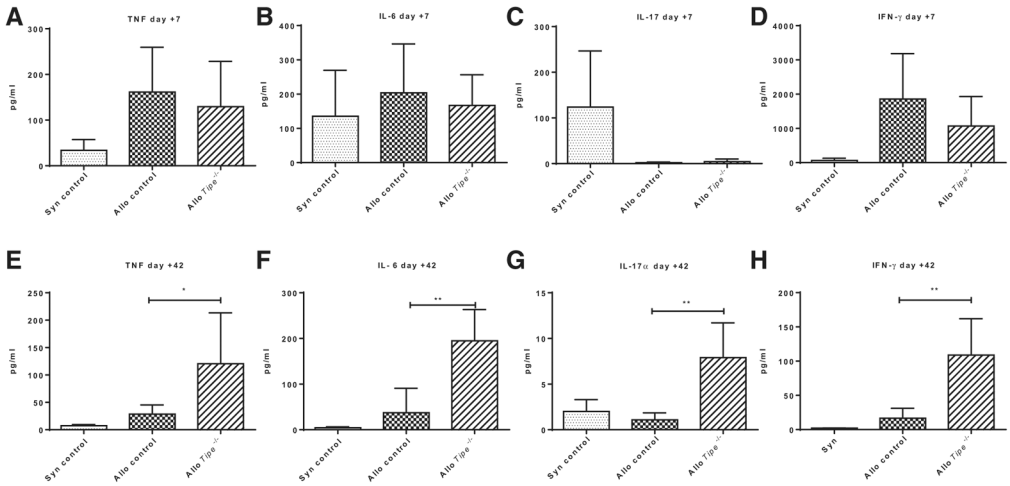

FIGURE 2.

Tipe$^{-/-}$ allogeneic recipients show increased serum proinflammatory cytokines at day 42 post allogeneic transplant. A–D, Cytokine levels in serum at day +7 posthematopoietic cell transplant (HCT) by Cytometric Bead Array Mouse Cytokine kit. E–H, Cytokine levels in serum at day +42 post-HCT by Cytometric Bead Array Mouse Cytokine kit (sample size at d +7; syngeneic control: n = 3; allo-HCT control: n = 6; allo *Tipe*$^{-/-}$: n = 6). Sample size at day +42; syngeneic control: n = 3; allo-HCT control: n = 6; allo *Tipe*$^{-/-}$: n = 3. *$P < 0.05$; **$P < 0.01$.

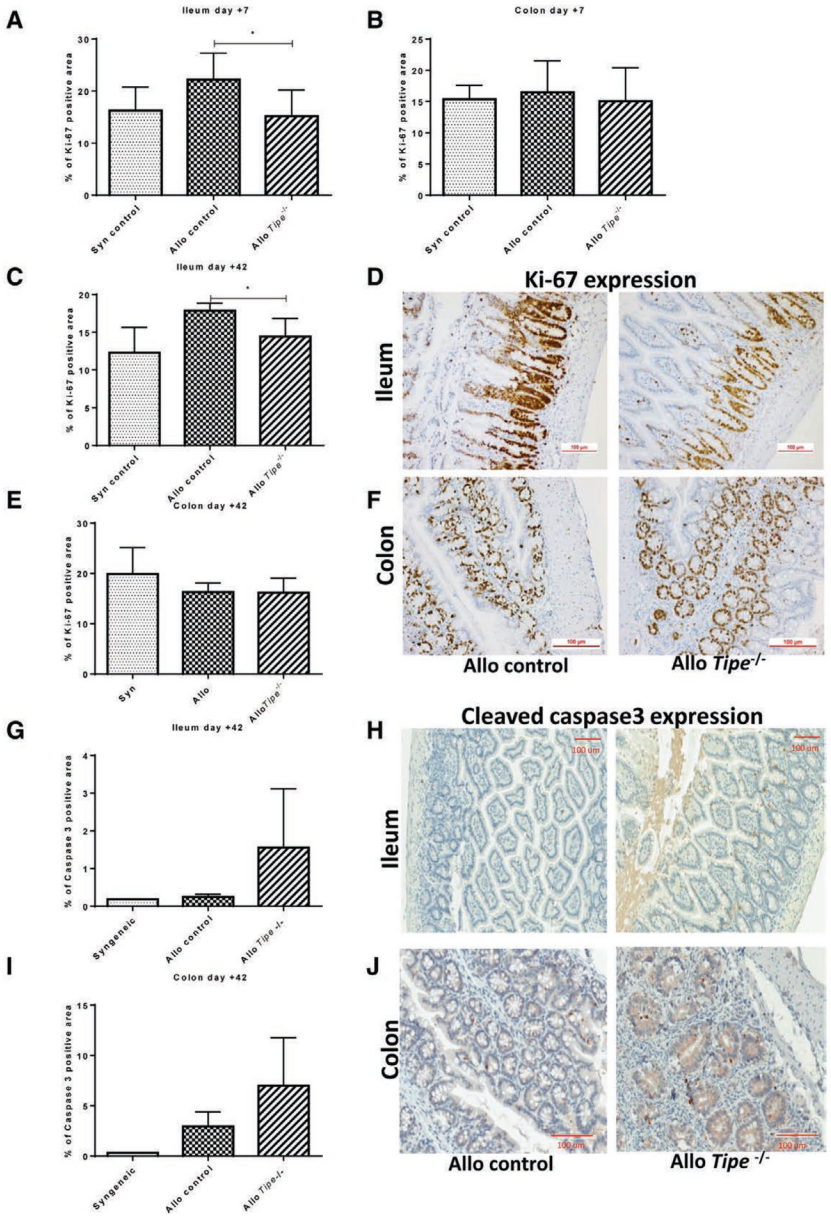

FIGURE 3.

Tumor necrosis factor-alpha-induced protein 8 (TIPE) deficiency affects regeneration and healing processes in small intestine after allogeneic hematopoietic cell transplant (allo-HCT). A and B, Expression of Ki-67 in ileum and colon sections by immunohistochemistry (IHC) at day +7. C and E, Expression of Ki-67 by IHC in ileum and colon at day +42 post-HCT. D and F, Representative pictures of Ki-67 stained ileum and colon sections of allogeneic control and allo *Tipe*$^{-/-}$ at day +42 after HCT. G and I, Expression of cleaved caspase-3 by IHC in ileum and colon at day +42 post-HCT. H and J, Representative pictures of cleaved caspase-3 stained ileum and colon sections of allogeneic control and allo *Tipe*$^{-/-}$ at day +42 after HCT. Sample size at day +7; syngeneic control: n = 3; allo-HCT control: n = 6; allo *Tipe*$^{-/-}$: n = 6. Sample size at day +42; syngeneic control: n = 3; allo-HCT control: n = 7; allo *Tipe*$^{-/-}$: n = 8. **P* < 0.05; ***P* < 0.005.

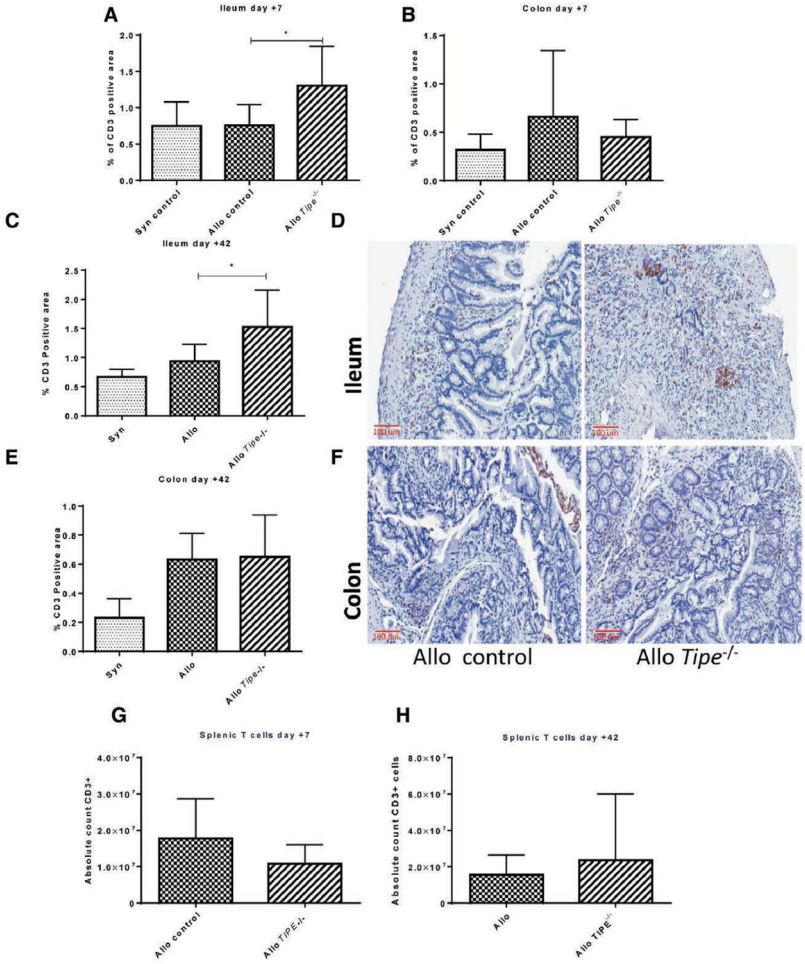

FIGURE 4.

T-cell infiltration in gut of *Tipe*$^{-/-}$ allogeneic recipients exacerbates graft-vs-host disease (GVHD) development. A and B, T-cell infiltration by CD3 staining through immunohistochemistry (IHC) in ileum and colon tissue section at day +7 posthematopoietic cell transplant (HCT). C and E, T-cell infiltration shown by CD3 staining through IHC in ileum and colon tissue section at day +42 post-HCT. D and F, Representative pictures of CD3 stained ileum and colon sections of allogeneic control and allo *Tipe*$^{-/-}$ at day +42 after HCT. G–H, Absolute count of splenic T cells ($CD3^{+}$), by flow cytometry at day +7 and day +42 after HCT. $*P < 0.05$.

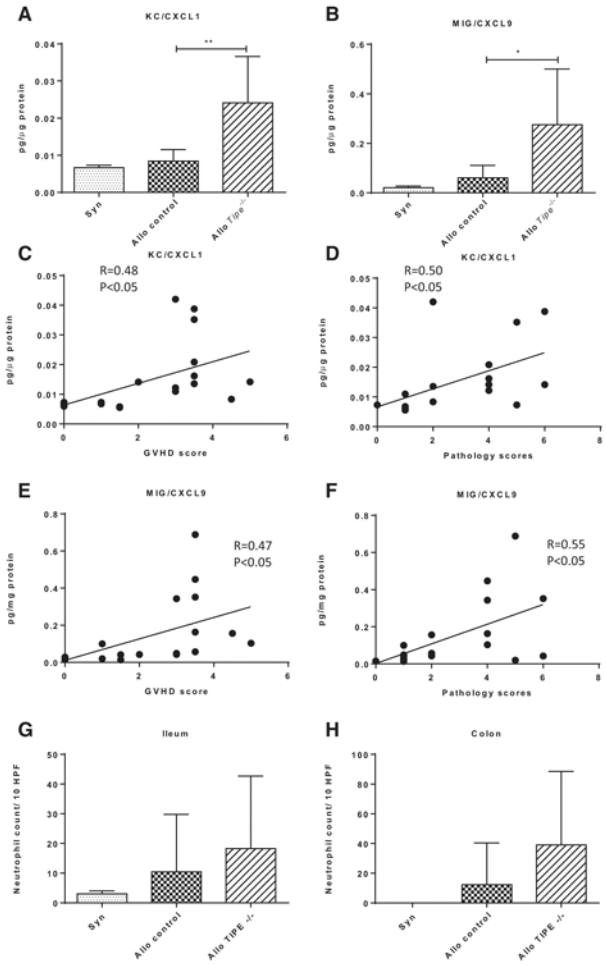

FIGURE 5.

Tipe$^{-/-}$ allogeneic recipients show enhanced inflammatory responses in tissue compared to allogeneic recipients. A and B, Chemokine expression for KC/chemokine (C-X-C motif) ligand 1 (CXCL1) and monokine induced by gamma interferon (MIG)/CXCL9 in ileum tissue homogenate measured by LEGENDplex mouse proinflammatory chemokine panel assay kit as described in material and methods. C–D, Correlation of KC/CXCL1 with graft-vs-host disease (GVHD) scores and pathology scores, respectively. E and F, Correlation graphs for MIG/CXCL9 with GVHD scores, pathology scores respectively. G–H, Total neutrophil count per 10 high power field in ileum and colon after day +42. R represents the Pearson coefficient. *$P < 0.05$; **$P < 0.008$.

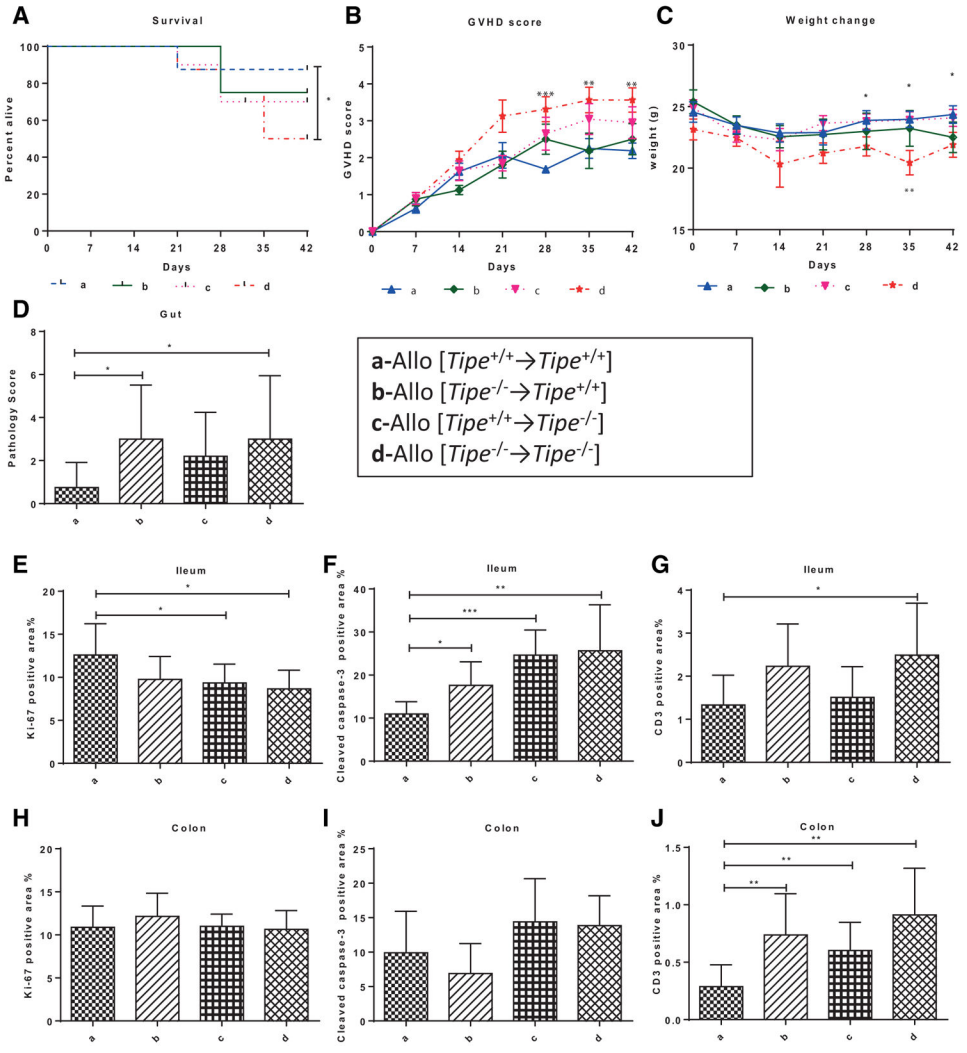

FIGURE 6.

Tumor necrosis factor-alpha-induced protein 8 (TIPE) deficiency in nonhematopoietic cells, as well as hematopoietic cells, is responsible for the exacerbated graft-vs-host disease (GVHD) in TIPE-deficient mice. Chimeric mice were generated by transplanting C57BL/6 wild type (WT) → C57BL/6 WT (n = 8); *Tipe*$^{-/-}$ C57BL/6→C57BL/6 WT (n = 8); C57BL/6 WT → *Tipe*$^{-/-}$ C57BL/6 (n = 10); *Tipe*$^{-/-}$ C57BL/6→*Tipe*$^{-/-}$ C57BL/6 (n = 8)mice. Three months after reconstitution, bone marrow (BM) chimeras were used as recipients and transplant was performed as described in materials and methods. Analysis of retransplanted chimeric mice at day +42 confirmed our observation of exacerbated clinical GVHD in *Tipe*$^{-/-}$ allogeneic recipients. A, Survival analysis (B) GVHD scores at day +42 after hematopoietic cell transplant (HCT) as described in Materials and Methods. C, Weight graph of the transplanted mice. Asterisks in B and C represent the statistical comparison between allo control and allo *Tipe*$^{-/-}$ C57BL_/6→*Tipe*$^{-/-}$ C57BL/6 Chimera group. D, Pathology scores of gut by H&E staining at day +42 after HCT using scoring systems as described in Materials and Methods. E–G, Expression of Ki-67 and cleaved Caspase-3 and T-cell infiltration in ileum tissue sections of transplanted mice at day +42 post-HCT. H–J,

Expression of Ki-67 and cleaved Caspase-3 and T-cell infiltration in colon tissue sections of transplanted mice at day +42 post-HCT. *$P < 0.05$; **$P < 0.009$; ****$P < 0.0001$.