Inhibition of 5-lipoxygenase alleviates graft-versus-host disease

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Leukotriene B_4 (LTB_4), a proinflammatory mediator produced by the enzyme 5-lipoxygenase (5-LO), is associated with the development of many inflammatory diseases. In this study, we evaluated the participation of the 5-LO/LTB_4 axis in graft-versus-host disease (GVHD) pathogenesis by transplanting 5-LO–deficient leukocytes and investigated the effect of pharmacologic 5-LO inhibition by zileuton and LTB_4 inhibition by CP-105,696. Mice that received allogeneic transplant showed an increase in nuclear 5-LO expression in splenocytes, indicating enzyme activation after GVHD. Mice receiving 5-LO–deficient cell transplant or zileuton treatment had prolonged survival, reduced GVHD clinical scores, reduced intestinal and liver injury, and decreased levels of serum and hepatic LTB_4. These results were associated with inhibition of leukocyte recruitment and decreased production of cytokines and chemokines. Treatment with CP-105,696 achieved similar effects. The chimerism or the beneficial graft-versus-leukemia response remained unaffected. Our data provide evidence that the 5-LO/LTB_4 axis orchestrates GVHD development and suggest it could be a target for the development of novel therapeutic strategies for GVHD treatment.

INTRODUCTION

Graft-versus-host disease (GVHD) is a systemic inflammatory syndrome that occurs after allogeneic BM transplantation. The interaction of donor T cells with APCs is followed by an inflammatory storm targeting the skin, liver, and intestine (Goker et al., 2001; Ball et al., 2008; Ferrara et al., 2009; Robb and Hill, 2012; Teshima et al., 2016). Clinical and experimental evidence suggests that the gastrointestinal tract is the major organ involved in GVHD pathophysiology and that it participates in the amplification of systemic disease and mortality (Hill and Ferrara, 2000; Ferrara et al., 2009; Pasquini et al., 2010; Ramadan and Paczesny, 2015). Current GVHD prophylaxis and treatment are only partially effective, with an increased risk of infections, disease relapse, and long-term adverse effects. Despite intense efforts, there have been no major advances in effective approaches to prevent and control GVHD (Holtan and MacMillan, 2016; Teshima et al., 2016).

The 5-lipoxygenase (5-LO) pathway is associated with several inflammatory diseases, including cerebral ischemia (Silva et al., 2015), atherosclerosis (Ketelhuth et al., 2015), colitis (Zingarelli et al., 1993; Breganó et al., 2014), and pancreatic cancer (Zhou et al., 2015). The 5-LO pathway is necessary for leukotriene production, including leukotriene B_4 (LTB_4), which is an eicosanoid lipid mediator derived from phospholipase-released arachidonic acid. After cellular stimulation, 5-LO is relocated to the nuclear membrane and activated by the integral nuclear-membrane protein known as 5-LO–activating protein. This enzyme first generates 5-hydroperoxyeicosatetraenoic acid and then the unstable intermediate LTA_4. LTA_4 is metabolized to LTB_4 by LTA_4 hydrolase (Funk, 2001; Luster and Tager, 2004). LTB_4 has an important role in the inflammatory response, promoting leukocyte chemotaxis, degranulation, and endothelial cell adhesion (Ford-Hutchinson et al., 1980). LTB_4 is produced predominantly by inflammatory cells, including mast cells, neutrophils, eosinophils, basophils, monocytes/macrophages, B cells, DCs, and T cells (Funk, 2001). Specifically, LTB_4 has been associated with the development of several diseases, including gout (Amaral et al., 2012) and gut ischemia and reperfusion (Souza et al., 2002). A previous study (Takatsuka et al., 2000) also showed an association between LTB_4 and intestinal injury in human GVHD. The higher serum LTB_4 levels observed in the preconditioning phase were closely related to the severity of intestinal GVHD and to increased levels of IL-2 and IFN-γ in the initial phase of the disease and IFN-γ and TNF in the later phase (Takatsuka et al., 2000). However, the relevance of the 5-LO pathway in GVHD pathogenesis remains poorly understood.

In this context, we investigated the potential role of the 5-LO pathway in GVHD pathophysiology and tested the hy-

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Abbreviations used: 5-LO, 5-lipoxygenase; GVHD, graft-versus-host disease; GVL, graft-versus-leukemia; NAG, *N*-acetyl glucosaminidase.

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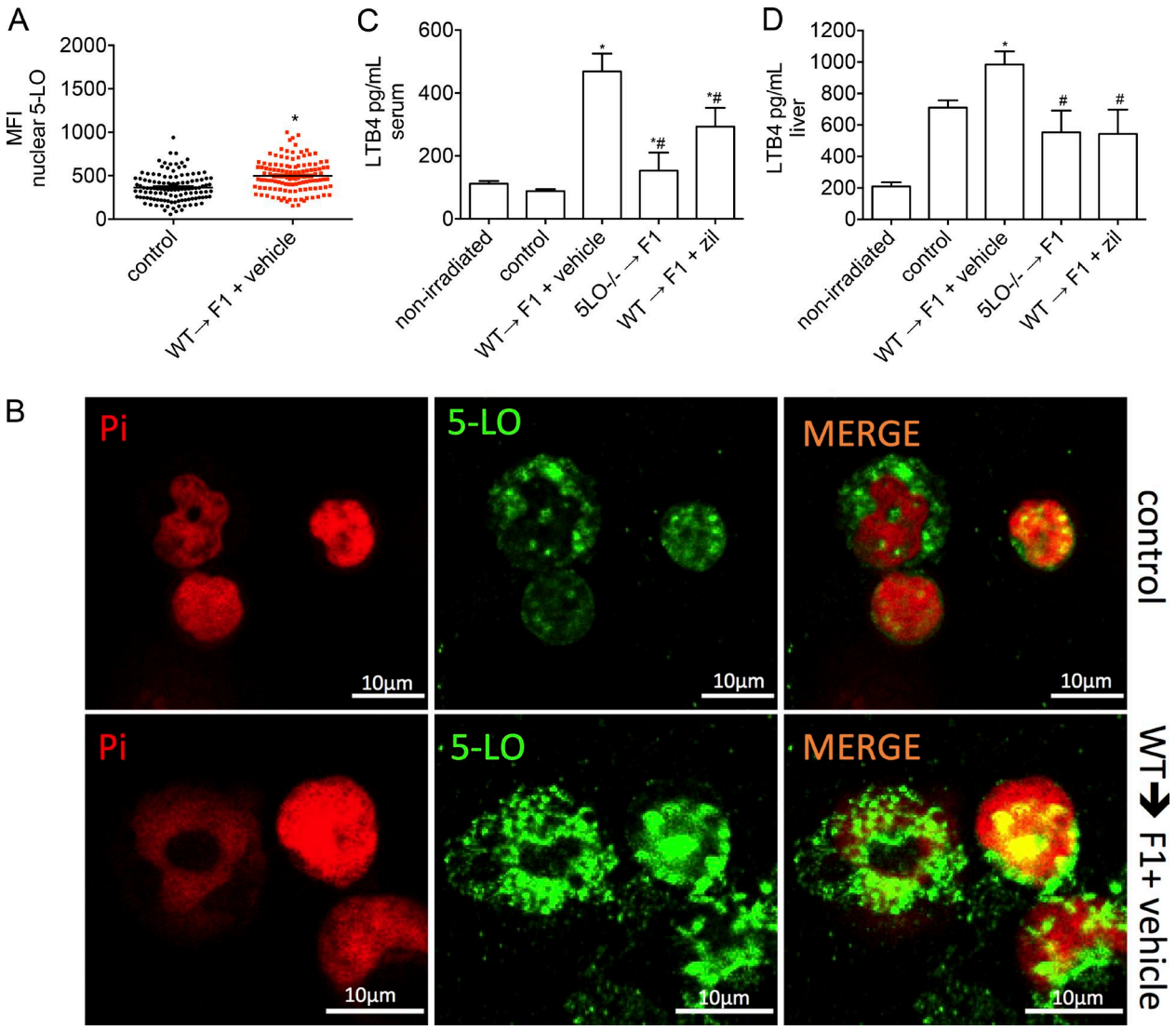

Figure 1. **GVHD increases nuclear 5-LO expression and serum and hepatic LTB_4 production.** GVHD was induced by the adoptive transfer of 10^7 BM cells + 3 × 10^7 splenocytes from WT SV129 (WT → F1 + vehicle group; WT → F1 + zil group) or 5-$LO^{-/-}$ SV129 (5-$LO^{-/-}$ → F1 group) mice donors to B6D2F1 mice. Mice that received syngeneic (B6D2F1) BM cells and splenocytes did not develop any disease and were considered the control group. To pharmacologic 5-LO inhibition, recipient mice were treated with zileuton by gavage (30 mg/kg, 12 h/12 h) on day 0 after transplant until the onset of GVHD clinical signs. 6 d after transplant, the mice were killed, and the splenocytes were isolated and evaluated by confocal microscopy. 200 cells/mice were analyzed to mean fluorescence intensity (MFI) nuclear 5-LO (A and B). Bars, 10 µm. The results are presented as means ± SEM (n = 3). In the onset of mortality, the mice were killed and the serum (C) and liver (D) were sampled for LTB_4 level dosage. The results are representative of two independent experiments and are presented as means ± SEM (n = 5); * and #, $P < 0.05$ when compared with the control and WT → F1 + vehicle group, respectively, using unpaired Student's t test (A) and ANOVA with a multiple-comparison test (C and D).

pothesis that zileuton, a 5-LO inhibitor, could be used as a therapy for experimental GVHD.

RESULTS

Impaired function of 5-LO is associated with reduced mortality, control of body weight loss, and improvement of GVHD clinical signs

We first assessed the expression of nuclear 5-LO in splenocytes of mice subjected to GVHD. Nuclear 5-LO expression was increased in mice that received allogenic transplants, suggesting 5-LO activation after GVHD development (Fig. 1, A and B). Importantly, on day 3 after transplant, there was an increase in LTB_4 in the serum of mice subjected to GVHD. LTB_4 was not detected in WT mice transplanted with 5-LO–deficient leukocytes (control, 88 ± 6; WT → F1 + vehicle, 125 ± 12; 5-$LO^{-/-}$ → F1, 0 ± 0). Moreover, in serum (Fig. 1 C) and liver (Fig. 1 D), on day 14 after transplant (at the onset of mortality), there

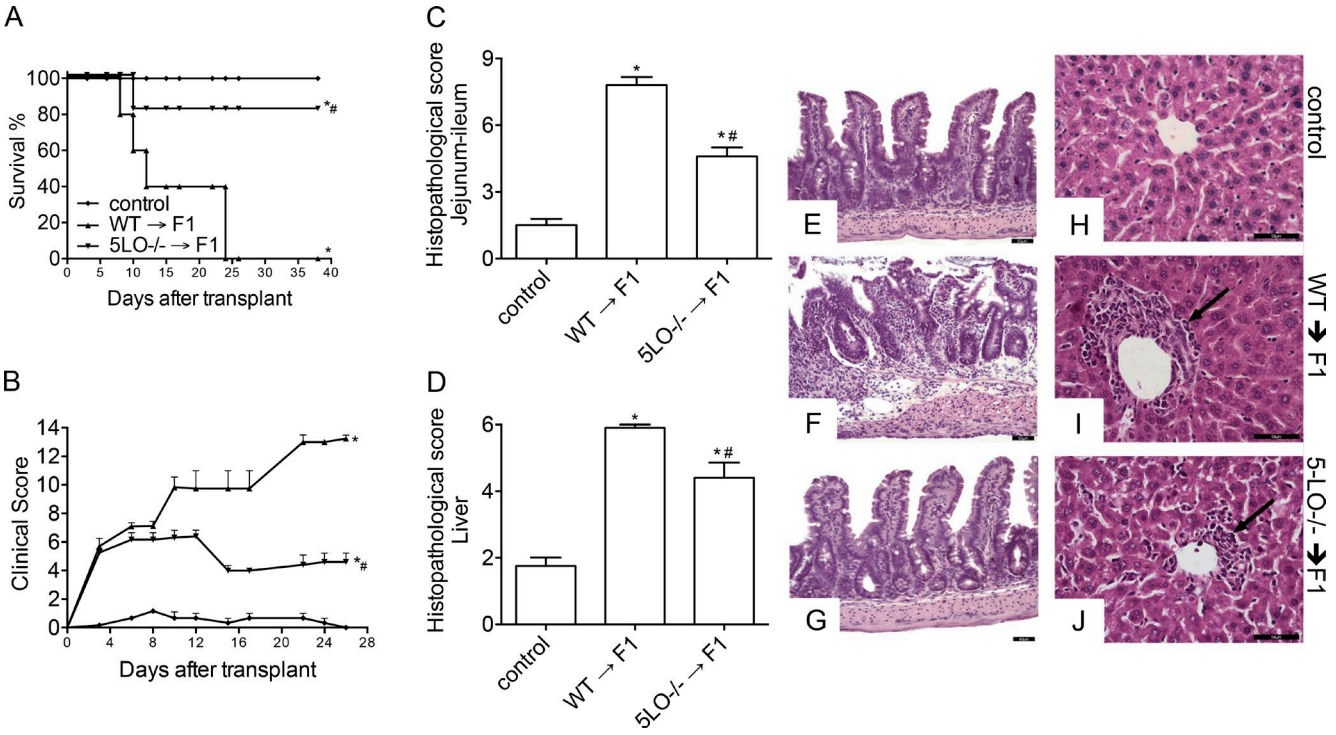

Figure 2. **5-LO–deficient leukocyte transplants are associated with reduced mortality, improved GVHD clinical signs, and fewer target organs injuries in mice subjected to GVHD.** GVHD was induced by the adoptive transfer of 10^7 BM cells + 3×10^7 splenocytes from WT SV129 (WT → F1 group) or 5-LO$^{-/-}$ SV129 (5-LO$^{-/-}$ → F1 group) mice donors to B6D2F1 mice. Mice that received syngeneic (B6D2F1) BM cells and splenocytes did not develop any disease and were considered the control group. After the GVHD induction, the mice were evaluated every 2 d for survival (A) and clinical scoring (B). The results are shown as means ± SEM, and the numbers of animals were as follows: control group (◆), $n = 6$; WT → F1 group (▲), $n = 6$; and 5-LO$^{-/-}$ → F1 group (▼), $n = 6$. At the onset of mortality, mice were killed, and the jejunum–ileum (C and E–G) and liver (D and H–J) tissues were sampled for histopathologic analysis. (E–G) Histologic aspects of hematoxylin and eosin (H&E)–stained small-intestine sections from control, WT → F1, and 5-LO$^{-/-}$ → F1 groups, respectively. Bars, 50 µm. (H–J) Histologic aspects of H&E-stained liver sections from control, WT → F1, and 5-LO$^{-/-}$ → F1 groups, respectively. Black arrows in I and J indicate inflammatory infiltrate around the central lobular vein. Bars, 20 µm. The results are representative of three independent experiments and are presented as means ± SEM ($n = 6$); * and #, $P < 0.05$ when compared with the control and WT → F1 group, respectively, using the log-rank test (A) and ANOVA with a multiple-comparison test (B–D).

was a decrease of LTB_4 levels in mice transplanted with 5-LO–deficient leukocytes. In the jejunum–ileum, there were no differences among any of the groups at the onset mortality (control, 816 ± 58; WT → F1 + vehicle, 802 ± 67; 5-LO$^{-/-}$ → F1, 608 ± 88).

Next, we evaluated whether 5-LO–deficient leukocyte transplant could prevent GVHD-associated morbidity and mortality. The control group did not develop the disease and showed 100% survival rate at the end of the experiment. Mice that received leukocytes isolated from WT mice developed acute GVHD, which was confirmed by 100% lethality 24 d after transplantation (Fig. 2 A) and high clinical scores (Fig. 2 B). In contrast, at 24 d, 5-LO–deficient leukocyte-transplanted mice showed effective protection against the disease, resulting in 83% survival (Fig. 2 A) and significant reduction of clinical disease (Fig. 2 B). Actually, all mice that received 5-LO–deficient leukocytes alive on day 24 were still alive on day 38 after transplant, when they were euthanized (Fig. 2 A).

Transplant of cells deficient in 5-LO ameliorates intestinal and hepatic injury in mice subjected to GVHD

To confirm the hypothesis that 5-LO is involved in development of GVHD, we analyzed the architecture of the small intestine and liver. Mice subjected to GVHD displayed severe injury in those organs at the onset of mortality. Histologic analysis of the intestine showed partial loss of organ architecture, increased cellularity, edema, and congestion. Severe degenerative changes, ulcerations of the mucosa, and areas of focal necrosis in the muscular and serous layers were also observed at that time (Fig. 2, C and F). The transplant of donor cells deficient in 5-LO was associated with decreased overall GVHD pathological score (Fig. 2, C and G). In the liver, histopathology analysis demonstrated an increase in the inflammatory infiltration, mainly around the central lobular vein in mice subjected to GVHD (Fig 2, D and I). In contrast, the livers of mice transplanted with cells deficient in 5-LO exhibited normal parenchymal tissue with lower inflammatory infiltration (Fig. 2, D and J).

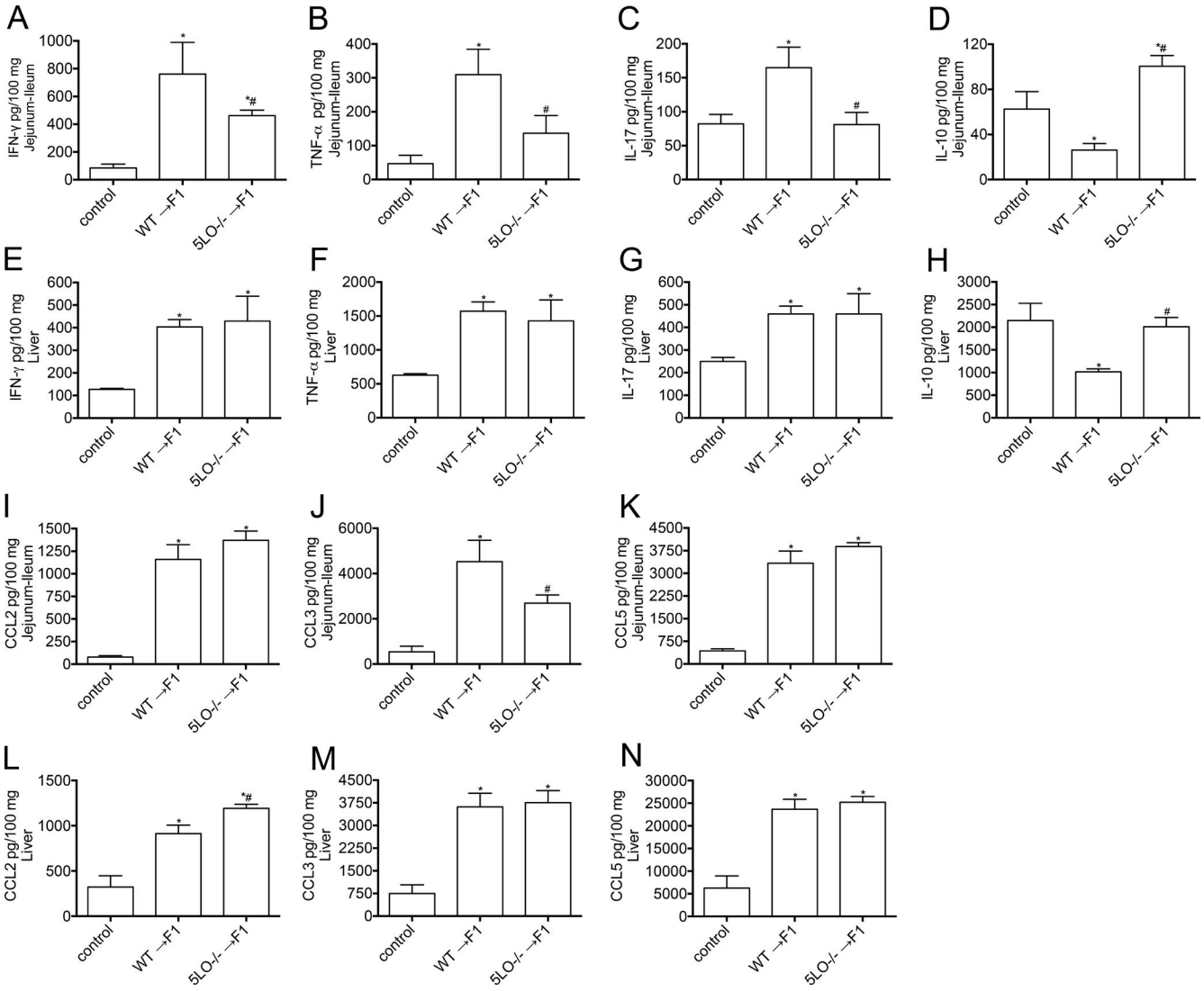

Figure 3. **Transplant of 5-LO–deficient leukocytes reduces the concentration of certain pro-inflammatory cytokines and chemokines related to GVHD in the jejunum–ileum.** GVHD was induced by the adoptive transfer of 10^7 BM cells + 3×10^7 splenocytes from WT SV129 (WT → F1 group) or 5-LO$^{-/-}$ SV129 (5-LO$^{-/-}$ → F1 group) mice donors to B6D2F1 mice. Mice that received syngeneic (B6D2F1) BM cells and splenocytes did not develop any disease and were considered the control group. At the onset of mortality, the mice were killed, and the concentrations of IFN-γ, TNF, IL-17, IL-10, CCL2, CCL3, and CCL5 in the intestinal and hepatic homogenates were evaluated by ELISA for intestinal (A–D) and hepatic (E–H) cytokines, and intestinal (I–K) and hepatic (L–N) chemokines. The results are representative of two independent experiments and are presented as means ± SEM ($n = 5$); * and #, $P < 0.05$ when compared with the control and WT → F1 group, respectively, using ANOVA with a multiple-comparison test.

Transplant of 5-LO–deficient leukocytes reduces the concentration of certain proinflammatory cytokines and chemokines related to GVHD in the jejunum–ileum

We found increased levels of the proinflammatory cytokines IFN-γ, TNF, and IL-17 (Fig. 3, A–C) and reduced levels of the anti-inflammatory cytokine IL-10 (Fig. 3 D) in the intestines of mice that received WT leukocytes. In contrast, mice that received 5-LO–deficient leukocytes showed reduced levels of those proinflammatory cytokines (Fig. 3, A–C) and increased levels of IL-10 (Fig. 3 D). In liver, there was no difference in the levels of those proinflammatory cytokines in the 5-LO–deficient, leukocyte-transplanted mice and WT donor, leukocyte-transplanted mice (Fig. 3, E–G). However, the IL-10 level in the 5-LO–deficient, leukocyte-transplanted mice remained similar to that in the control group (Fig. 3 H).

There was no difference in the intestinal levels of the chemokines CCL2 and CCL5 in the mice that received WT leukocytes or 5-LO–deficient leukocytes; the levels in both groups of mice increased compared with the levels in the control group (Fig. 3, I–K). Nevertheless, the CCL3 level was lower in the 5-LO–deficient, leukocyte-transplanted group

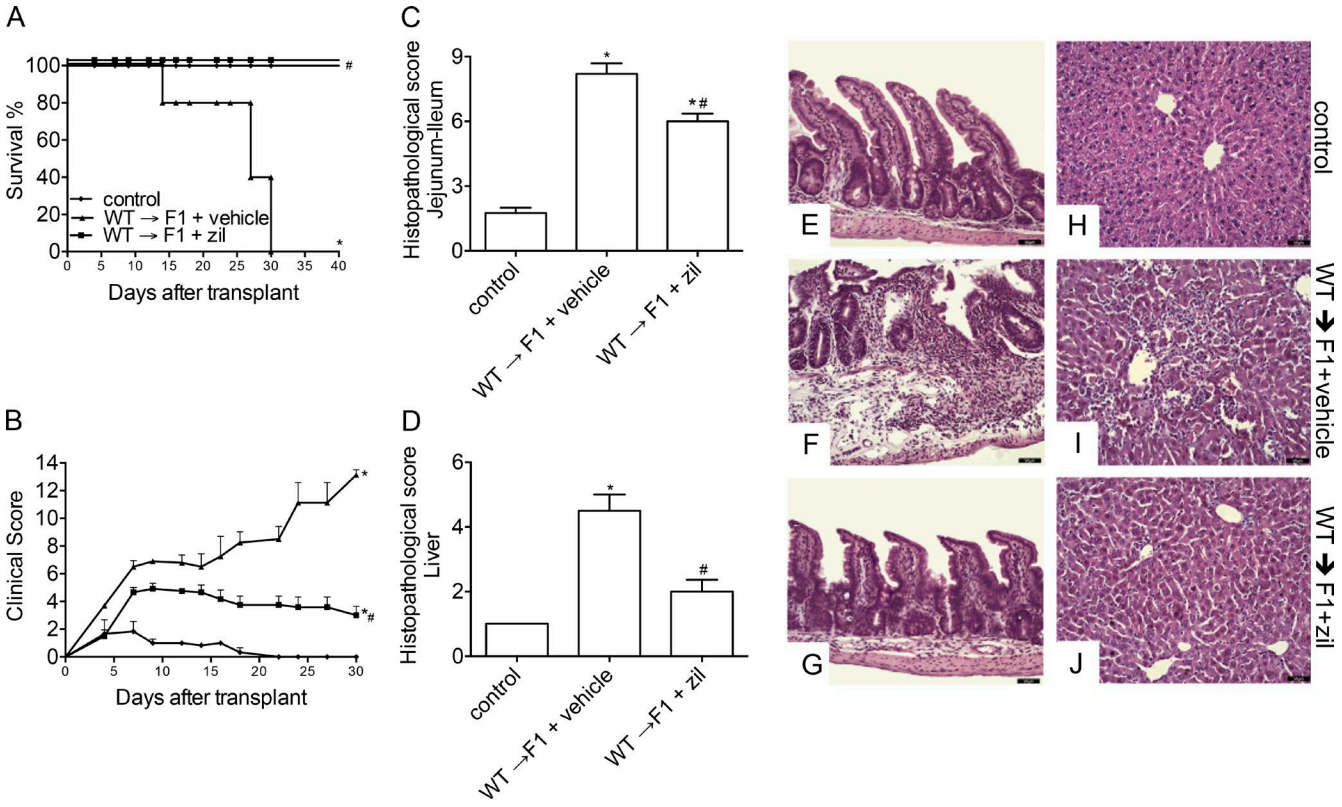

Figure 4. **Zileuton treatment is associated with reduced mortality, improvement in GVHD clinical signs, and reduced intestinal and hepatic injuries in mice subjected to GVHD.** GVHD was induced by the adoptive transfer of 10^7 BM cells + 3 × 10^7 splenocytes from C57BL/6 mice donors to B6D2F1 mice. Mice that received syngeneic (B6D2F1) BM cells and splenocytes did not develop disease and were considered the control group. To pharmacologic 5-LO inhibition, recipient mice were treated with zileuton by gavage (30 mg/kg, 12 h/12 h) on day 0 after transplant until the onset of GVHD clinical signs. After the GVHD induction, the mice were evaluated every 2 d for survival (A) and clinical scoring (B). The results are shown as means ± SEM, and the numbers of animals were as it follows: control group (◆), $n = 6$; WT → F1 + vehicle group (▲), $n = 6$; and WT → F1 + zil (■), $n = 6$. At the onset of mortality, the mice were killed, and the jejunum–ileum (C and E–G) and liver (D and H–J) tissues were sampled for histopathologic analysis. (E–G) Histologic aspects of hematoxylin and eosin (H&E)–stained small-intestine sections from control, WT → F1 + vehicle, and WT → F1 + zil groups, respectively. Bars, 50 µm. (H–J) Histological aspects of H&E-stained liver sections from control, WT → F1 + vehicle, and WT → F1 + zil group, respectively. Bars, 20 µm. The results are representative of three independent experiments and are presented as means ± SEM ($n = 6$); * and #, $P < 0.05$ when compared with the control and WT → F1 + vehicle groups, respectively, using the log-rank test (A) and ANOVA with a multiple-comparison test (B–D).

than it was in the group of mice that received WT leukocytes (Fig. 3 J). In liver, the CCL2 level was higher in the 5-LO–deficient, leukocyte-transplanted mice compared with control and WT, leukocyte-transplanted mice (Fig. 3 L). The CCL3 and CCL5 levels were higher in both the 5-LO–deficient, leukocyte-transplanted mice and WT, leukocyte-transplanted mice compared with the control group (Fig. 3, M and N).

Pharmacologic 5-LO inhibition by zileuton improves survival and protects GVHD target organs

Next, we evaluated the survival and clinical signs of GVHD after treatment with zileuton, an orally active inhibitor of 5-LO used clinically for the treatment of asthma. Mice were treated with zileuton until onset of clinical signs. Mice that received syngeneic leukocytes did not develop disease and showed 100% survival to the end of the experiment. Mice subjected to GVHD developed the disease, which was confirmed by 100% lethality by d 30 after transplantation (Fig. 4 A) and high clinical scores (Fig. 4 B). In contrast, mice subjected to GVHD and treated with zileuton showed effective protection from GVHD, resulting in 100% survival (Fig. 4 A) and reduced clinical signs of the disease (Fig. 4 B), as compared with untreated mice. All mice treated with zileuton actually survived until day 40 after transplantation, when they were euthanized (Fig. 4 A). These results show that treatment with zileuton until onset of clinical disease is sufficient to attain full protection in the model.

To confirm the beneficial effects of zileuton treatment in GVHD, we tested this treatment in mice that had been subjected to a different model of GVHD. To that end, leukocytes isolated from BALB/c mice were transplanted into C57BL/6 mice, as described in the Materials and methods. Zileuton treatment was also protective in this model (Fig. 5).

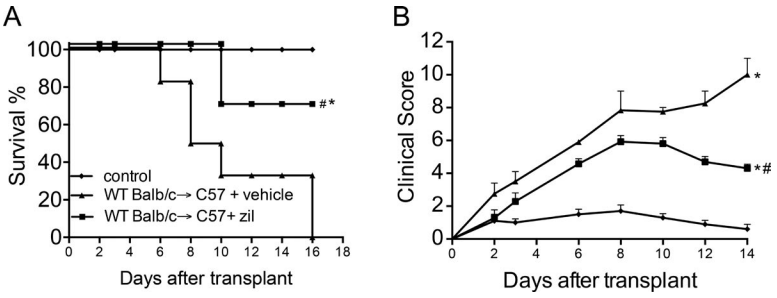

Figure 5. **Zileuton treatment is associated with reduced mortality and improved GVHD clinical signs after transplant of leukocytes isolated from BALB/c to C57BL/6 mice.** GVHD was induced by the adoptive transfer of 10^7 BM cells + 3×10^7 splenocytes from BALB/c mice donors to C57BL/6 mice. Mice that received syngeneic (C57BL/6) BM cells and splenocytes did not develop disease and were considered the control group. To pharmacologic 5-LO inhibition, recipient mice were treated with zileuton by gavage (30 mg/kg, 12 h/12 h) at day 0 after transplant until the onset if the GVHD clinical signs. After GVHD induction, the mice were evaluated every 2 d for survival (A) and clinical scoring (B). The results are representative of two independent experiments and are presented as means ± SEM. The numbers of animals were as follows: control group (◆), $n = 6$; WT BALB/c → C57 + vehicle group (▲), $n = 6$; and WT BALB/c → C57 + zil (■), $n = 7$. * and #, $P < 0.05$ when compared with the control and WT → F1 + vehicle groups, respectively, using the log-rank test (A) and ANOVA with a multiple-comparison test (B).

Next, we evaluated serum, intestinal, and hepatic levels of LTB_4 in mice treated with zileuton. At the onset of mortality (at day 14 after transplant), the level of LTB_4 in the jejunum–ileum in this group was similar to that in the other groups analyzed (control, 816 ± 58; WT → F1 + vehicle, 802 ± 67; 5-$LO^{-/-}$ → F1, 608 ± 88; WT → F1 + zileuton, 813 ± 36). However, we observed decreased LTB_4 levels in serum (Fig. 1 C) and liver (Fig. 1 D) of mice treated with zileuton at that time.

We also examined whether zileuton treatment could affect the histopathologic alterations seen in target organs of animals subjected to GVHD. Treatment with zileuton reduced intestinal and hepatic injuries, resulting in the preservation of small-intestine architecture, reduced inflammatory infiltration into the lamina propria, and modest accumulation of inflammatory cells in the muscle and serous layer (Fig. 4, C and G). In the liver, modest infiltration in the area surrounding the central lobular vein was observed in the zileuton-treated mice (Fig. 4, D and J).

Zileuton-treated mice also showed reduced levels of IFN-γ, TNF, IL-17, and IL-12 in the jejunum–ileum (Fig. 6, A–D). Moreover, there was a reduction in the levels of IFN-γ (Fig. 6 E) and IL-17 (Fig. 6 G) in the livers of zileuton-treated mice. There was no difference in IL-10 (not depicted) or IL-12 level (Fig. 6 H) in treated and untreated mice, and the level of TNF was similar in all groups (Fig. 6 F).

The levels of all intestinal chemokines evaluated were lower in the zileuton-treated mice compared with the vehicle-treated mice (Fig. 6, I–K). The hepatic levels of CCL2 and CCL3 were also reduced after zileuton treatment, compared with vehicle alone (Fig. 6, L and M). No differences were observed in the hepatic CCL5 level in the zileuton-treated mice and vehicle-treated mice (Fig. 6 N). Altogether, these results are consistent with our other results, which showed reduced target-organ injury when we transplanted 5-LO–deficient leukocytes.

5-LO is involved in the production of both LTB_4 and cysteinil-leukotrienes (cys-leukotrienes; Funk, 2001). To further explore the specific involvement of each type of leukotriene in the pathogenesis of GVHD, mice were subjected to GVHD and treated with CP-105,696, a potent and selective antagonist receptor of LBT_4, or with montelukast, a cys-leukotriene receptor antagonist. The treatment with CP 105,696 improved GVHD clinical signs (Fig. S1 A). CP-105,696–treated mice also showed reduced intestinal and hepatic injury (Fig. S1, B and C) and reduced levels of TNF, IFN-γ, IL-17, CCL2, and CCL5 in the jejunum–ileum (Fig. S1, L–P). In contrast, mice subjected to GVHD and treated with montelukast had a clinical score similar to that of untreated mice (Fig. S1 A). Although the histopathologic score of the intestine and the liver was reduced (Fig. S1, B and C), the concentration of cytokines and chemokines in the jejunum–ileum of montelukast-treated mice was similar to that of vehicle-treated mice (Fig. S1, L–P).

Because leukotrienes may cause significant lung dysfunction (Cingi et al., 2015; Qian et al., 2015; Sekioka et al., 2015; Ee et al., 2016; Shigematsu et al., 2016) and the lung is a target organ of GVHD (Ferrara, 2009; Haddad, 2013; Xu et al., 2013; Kambham et al., 2014), we evaluated the effect of treatment with zileuton, CP-105,696, and montelukast in the pulmonary injury that follows GVHD. We observed that there was significant pulmonary injury at the onset of mortality caused by the induction of GVHD (Fig. S2). Treatment with either zileuton or CP-105,696 decreased pulmonary injury, as observed by the lower histopathological score in these animals. Lung injury was not ameliorated by montelukast treatment (Fig. S2). Therefore, whereas treatment with the LTB_4 antagonist seemed to have protective effects in most parameters observed, treatment with montelukast had little protective effective. Overall, these data suggest there is a preferential role for LTB_4, rather than cys-leukotrienes, in driving GVHD in mice.

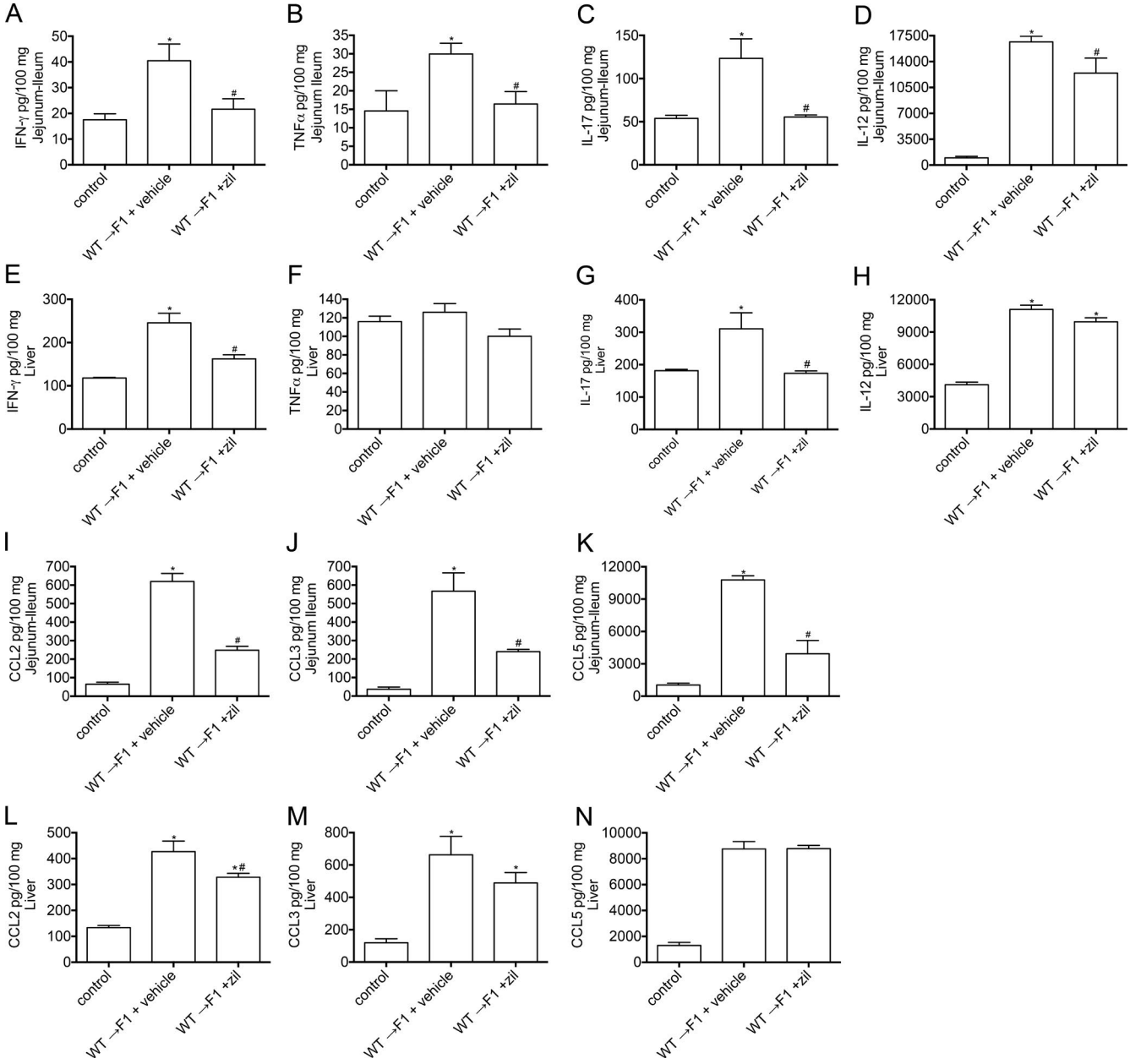

Figure 6. **Zileuton treatment reduces the concentration of proinflammatory cytokines and chemokines in GVHD target organs.** GVHD was induced by adoptive transfer of 10^7 BM cells + 3×10^7 splenocytes from C57BL/6 mice donors to B6D2F1 mice. Mice that received syngeneic (B6D2F1) BM cells and splenocytes did not develop any disease and were considered the control group. To pharmacologic 5-LO–inhibition, recipient mice were treated with zileuton by gavage (30 mg/kg, 12 h/12 h) on day 0 after transplant until the onset of GVHD clinical signs (WT → F1 + zil). At the onset of mortality, mice were killed, and the concentrations of IFN-γ, TNF, IL-17, IL-12, CCL2, CCL3, and CCL5 in the intestinal and hepatic homogenates were evaluated by ELISA for intestinal (A–D) and hepatic (E–H) cytokines and intestinal (I–K) and hepatic (L–N) chemokines. The results are representative of two independent experiments and are presented as means ± SEM ($n = 5$); * and #, $P < 0.05$ when compared with the control and WT → F1 + vehicle groups, respectively, using ANOVA with a multiple-comparison test.

Transplant of 5-LO–deficient cells or treatment with zileuton inhibits leukocyte accumulation resulting from the GVHD inflammatory response

In lymphoid organs, the frequency of $CD4^+$ and $CD8^+$ T cells in the spleen and BM of mice subjected to GVHD was greater than that in the control group (Fig. S3). The transplant of 5-LO–deficient leukocytes reduced the frequency of those cells in the spleen and BM (Fig. S3). The frequency of $CD4^+$ T cells and $CD8^+$ T cells in the spleen and BM was not reduced by zileuton treatment (Fig. S3). The transplant of 5-LO–deficient leuko-

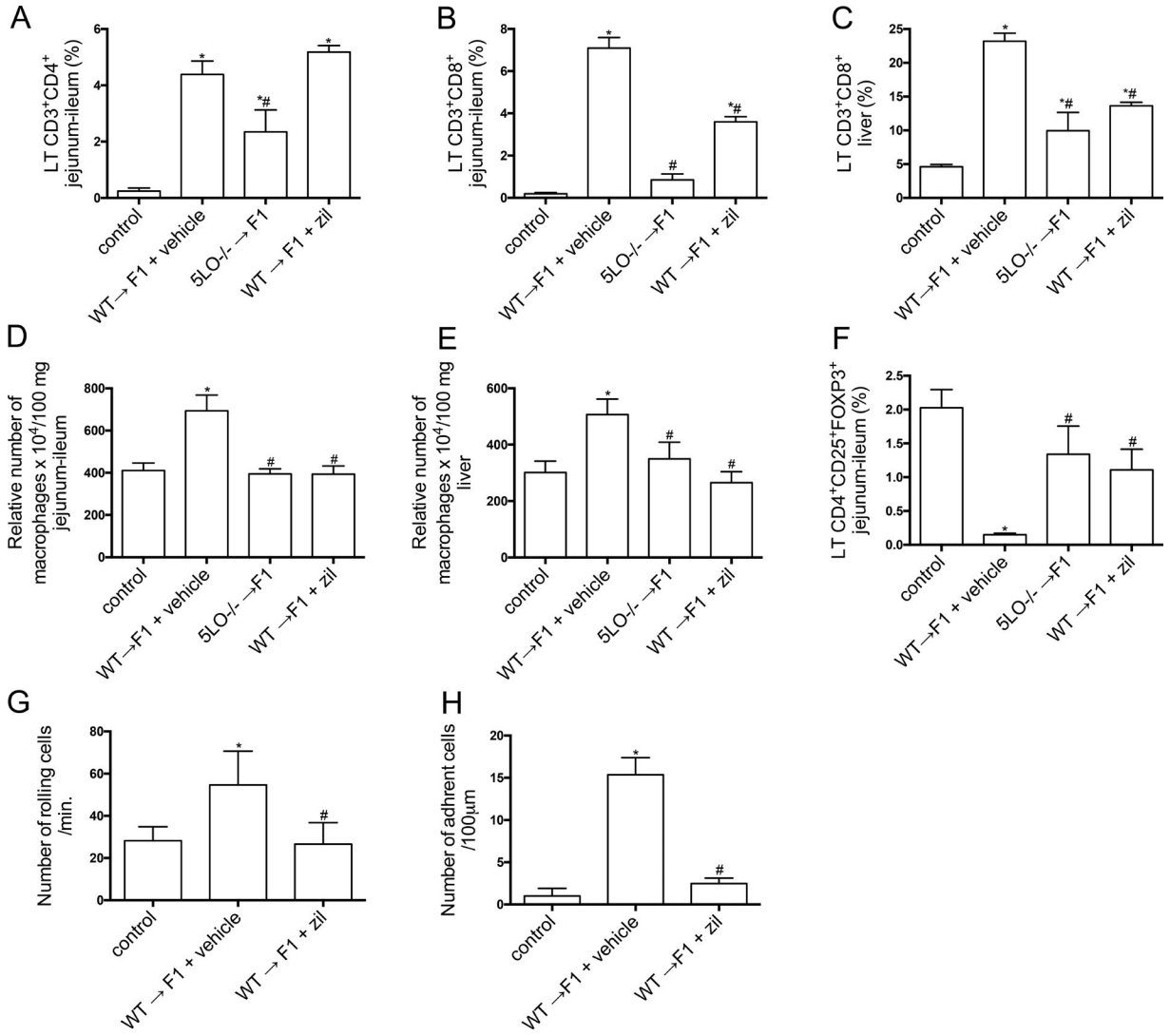

Figure 7. **5-LO–deficient leukocytes transplants or zileuton treatment inhibits leukocyte recruitment and remains intestinal T reg cells.** GVHD was induced by adoptive transfer of 10^7 BM cells + 3×10^7 splenocytes from WT SV129 (WT → F1 + vehicle group; WT → F1 + zil group) or 5-LO$^{-/-}$ SV129 (5-LO$^{-/-}$ → F1 group) mice donors to B6D2F1 mice. Mice that received syngeneic (B6D2F1) BM cells and splenocytes did not develop disease and were considered the control group. To pharmacologic 5-LO inhibition, recipient mice were treated with zileuton by gavage (30 mg/kg, 12 h/12 h) on day 0 after transplant until the onset of GVHD clinical signs. The percentage of intestinal T CD4$^+$ and T CD8$^+$ (A and B), hepatic T CD8$^+$ cells (CD3$^+$CD8$^+$; C); and intestinal Treg cells (CD4$^+$CD25$^+$FOXP3$^+$; F) were evaluated by flow cytometry. Macrophages were quantified in the jejunum–ileum (D) and liver (E) by enzymatic methods (NAG assay). The results are presented as means ± SEM (n = 4). The leukocyte recruitment was evaluated on day 10 after transplantation. The mice were anesthetized, and intestinal venules (±40 µm) were selected to count the numbers of rolling and adherent leukocytes by intravital microscopy. (G) The number of rolling cells/minute; (H) The number of adherent cells/100 µm. The results are representative of three independent experiments and are presented as means ± SEM (n = 4). * and #, $P < 0.05$ when compared with the control and WT → F1+ vehicle groups, respectively, using ANOVA with a multiple-comparison test.

cytes or zileuton treatment was able to inhibit the accumulation of macrophages in the spleen and BM (Fig. S3).

In our mouse model of GVHD, there was a greater frequency of CD4$^+$ and CD8$^+$ T cells in the jejunum–ileum of mice subjected to GVHD. Remarkably, the frequency of those cells was less in mice transplanted with 5-LO–deficient leukocytes (Fig. 7, A and B). Zileuton treatment also resulted in reduced frequency of CD8$^+$ T cells in the jejunum–ileum (Fig. 7 B). The frequency of CD4$^+$ T cells in the liver was similar for all groups at the onset of mortality (not depicted). However, there was a greater CD8$^+$ T cell frequency in the livers of mice that received WT leukocytes compared with the control group (Fig. 7 C). Moreover, there was a substantial reduction in the CD8$^+$ T cell frequency in the liver of 5-LO–deficient, leukocyte-transplanted mice or zileuton-treated mice, compared with WT, leukocyte-transplanted mice (Fig. 7 C). We also ob-

served an increase in the accumulation of macrophages in the jejunum–ileum and liver after induction of GVHD (Fig. 7, D and E). The accumulation of those cells was inhibited in the 5-LO–deficient, leukocyte-transplanted mice and in the mice treated with zileuton, with the reduced relative number of macrophages in the jejunum–ileum (Fig. 7 D) and liver (Fig. 7 E). Moreover, there was a reduction in regulatory T cells (T reg cells) in the mice subjected to GVHD, compared with the control group. The frequency of T reg cells in the 5-LO–deficient, leukocyte-transplanted mice and the zileuton-treated mice remained similar to that in the control group (Fig. 7 F). No significant changes were detected in the hepatic T reg cell frequency among all the groups (not depicted). The representative dot plots of the data presented in Fig. 7 are shown in the Fig. S4.

Finally, we observed an increase in the number of rolling and adherent cells in the vehicle-treated mice compared with the control group. Zileuton treatment reduced both the recruitment and the adhesion of leukocytes to the intestinal mesenteric vessels (Fig. 7, G and H).

To assess whether zileuton treatment was having any direct effect on the presentation of antigen by APCs or by proliferation of lymphocytes, we performed a mixed-lymphocyte reaction test in the presence and absence of that compound. There was significant proliferation of lymphocytes among the splenocytes isolated from WT mice when they were incubated in vitro with DCs isolated from F1 mice (F1 splenocytes + F1 DCs [negative control group], 5.3% ± 1.6 cell proliferation; WT splenocytes + F1 DCs + vehicle, 16.3% ± 0.7 cell proliferation; $n = 3$, $P < 0.01$). Treatment with zileuton did not affect lymphocyte proliferation (WT splenocytes + F1 DCs + zileuton at 40 μM, 17.0% ± 0.6 cell proliferation; $n = 3$), suggesting zileuton does not affect APCs directly or lymphocyte function in a mixed-lymphocyte reaction.

Impaired function of 5-LO decreased GVHD but did not interfere with chimerism or the graft-versus-leukemia (GVL) response

Next, we evaluated whether the absence of 5-LO could interfere with hematopoietic recovery, engraftment, and GVL maintenance. First, we assessed the frequency of $H2^{d+}H2^{b+}$ cells (a marker of B6D2 F1 cells) and $H2^{d+}$ cells (a marker of C57BL/6 and SV129 cells) in the spleen and BM. On day 10 after transplant, the control group presented mostly $H2^{d+}H2^{b+}$ cells in both the spleen and BM (Fig. 8, C, E, and H). 5-LO–deficient, leukocyte-transplanted mice and the WT, leukocyte-transplanted mice treated with zileuton and the WT, leukocyte-transplanted mice treated with vehicle exhibited a high frequency of $H2^{b+}$ cells and a low frequency of $H2^{d+}H2^{b+}$ cells in both the spleen and marrow (Fig. 8, A–H). These results indicated that neither 5-LO–deficient–leukocyte-transplant nor zileuton treatment interfered with BM reconstitution.

Our next step was to evaluate whether GVL would still occur after 5-LO pathway blockade. The control group, which received a syngeneic cell transplant plus tumor-cell injection, showed a frequency of 62% P815 GFP^+ cells in the spleen, demonstrating tumor growth in that organ (Fig. 8, I and J). The mice that received syngeneic cell transplant plus tumor cell injection and were treated with zileuton showed a reduced frequency of P815 GFP^+ cells, indicating the antitumoral effect of zileuton (Fig. 8, I and K). Mice subjected to allogeneic transplant plus tumor cell injection and treated with vehicle showed a reduction in the number of tumor cells (Fig. 8, I and L). The mice that received tumor cell injection and were transplanted with 5-LO–deficient leukocytes or were treated with zileuton maintained the ability to react against tumor cells after allogenic transplant (Fig. 8, I, M, and N).

DISCUSSION

Although numerous studies investigating the pathogenesis of GVHD have been performed, limited progress has been made regarding therapeutic strategies used in the management of the disease. Nevertheless, the role of the 5-LO pathway in GVHD has not been investigated. In the present study, we provide both genetic and pharmacologic evidence for a role of the 5-LO pathway in the initiation and progression of GVHD. The increase in 5-LO nuclear expression after allogenic transplant suggests the activation of this enzyme in GVHD. Many studies have shown that 5-LO is phosphorylated and translocates to the nuclear membrane during inflammatory processes (Rouzer and Samuelsson, 1987; Rouzer and Kargman, 1988; Wong et al., 1991; Woods et al., 1993; Fredman et al., 2014, 2016). The production of lipid mediators derived from the metabolism of arachidonic acid by 5-LO is determined by the intracellular localization of the enzyme. The presence of nuclear 5-LO favors the production of LTB_4.

GVHD is the main stem-cell transplant complication and is associated with high mortality, weight loss, and clinical signs, such as the loss of skin integrity, diarrhea, and occult blood in the faces. Herein, we could show for the first time, to our knowledge that 5-LO–deficient leukocyte transplant and zileuton treatment prevents GVHD-associated mortality and morbidity. These results were followed by a reduction in intestinal and hepatic injury in both 5-LO–deficient, leukocyte-transplanted mice and zileuton-treated mice. This was an important finding because ∼50% of patients who develop GVHD have bowel and liver injury, which is associated with GVHD morbidity (Robb and Hill, 2012). Moreover, LTB_4 levels in target organs were increased in GVHD mice, and there was a reduction in that leukotriene in mice that received 5-LO–deficient leukocytes and/or zileuton treatment, which could contribute to reduced hepatic and intestinal injury. Similarly, Li et al. (2014) observed that 5-LO pharmacologic inhibition with AA-861 (Sigma-Aldrich) improved the survival rate, prevented hepatocellular necrosis, and significantly reduced LTB_4 levels in the livers of rats in a model of acute liver failure induced by D-galactosamine, demonstrating the relevance of the 5-LO pathway in hepatic diseases.

Further, we demonstrate here that the selective antagonism of BLT1 with CP-105,696, but no antagonism of

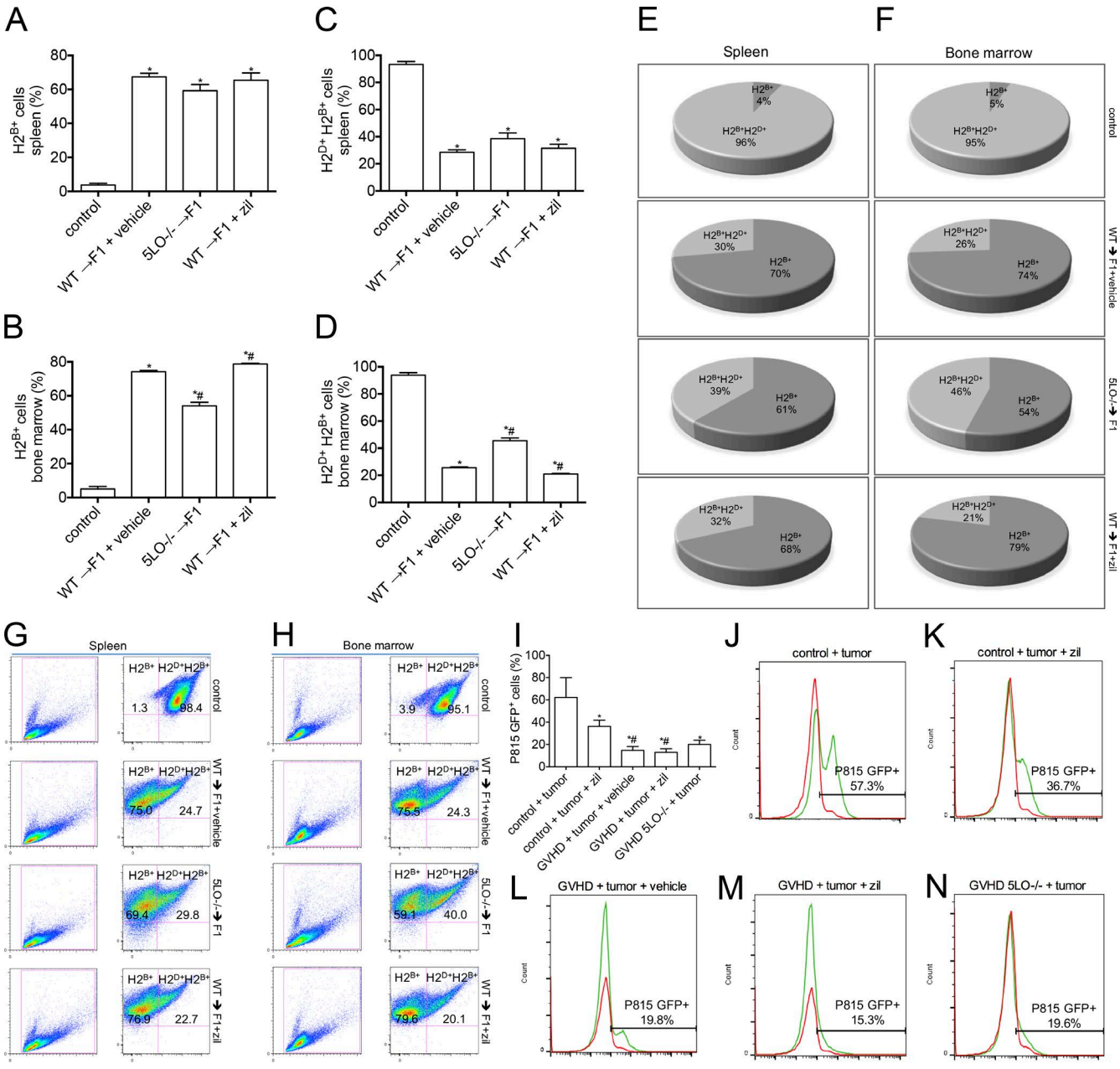

Figure 8. **5-LO–deficient leukocytes transplants or zileuton treatment decreased GVHD but did not interfere with chimerism and GVL response.** GVHD was induced by adoptive transfer of 10^7 BM cells + 3×10^7 splenocytes from WT SV129 (WT → F1 + vehicle group; WT → F1 + zil group); or 5-LO$^{-/-}$ SV129 (5-LO$^{-/-}$ → F1 group) mice donors to B6D2F1 mice. Mice that received syngeneic (B6D2F1) BM cells and splenocytes did not develop any disease and were considered the control group. To pharmacologic 5-LO inhibition, recipient mice were treated with zileuton by gavage (30 mg/kg, 12 h/12 h) on day 0 after transplant until the onset of GVHD clinical signs. At the onset of mortality, the mice were killed and the percentage of $H2^{d+}H2^{b+}$ cells (marker for B6D2F1 cells; C, D, and E–H) and $H2^{b+}$ cells (marker for C57BL/6 and SV129 cells; A, B, and E–H) in the spleen and BM were evaluated by flow cytometry. (G) Frequency of $H2^{b+}H2^{d+}$ in the spleen, control: 98.4%; WT → F1 + vehicle: 24.7%; 5LO$^{-/-}$ → F1: 29.8%; and WT → F1 + zileuton: 22.7%. (H) Frequency of $H2^{b+}H2^{d+}$ in the BM, control: 95.1%; WT → F1 + vehicle: 24.3%; 5LO$^{-/-}$ → F1: 40.0%; and WT → F1 + zileuton: 20.1%. To GVL response evaluation, on the same day as transplantation, all groups received i.v. infusion of 6,000 P815 GFP$^+$ tumor cells. After 5 d, the mice were sacrificed, and the percentage of GFP$^+$ P815 cells in the spleen was evaluated by flow cytometry (I–N). The results are representative of three independent experiments and are presented as means ± SEM ($n = 4$); * and #, $P < 0.05$ when compared with the control and WT → F1 + vehicle groups, respectively, using ANOVA with a multiple-comparison test.

the cys-leukotriene receptor 1 (CysLTR1) with montelukast, was able to protect mice from GVHD. However, the cys-leukotriene pathway is composed of three ligands (leukotriene C_4, leukotriene D_4, and leukotriene E_4) and three receptors (CysLTR1–3; Kanaoka and Boyce, 2004; Singh et al., 2010; Laidlaw and Boyce, 2012; Kanaoka et al., 2013), and montelukast antagonizes only one of them. Studies with gene-deficient mice have implicated CysLTR2 in the induction of bleomycin lung fibrosis (Beller et al., 2004) and CysLTR3 in the epithelium of the airway as a regulator of goblet cell development and function (Bankova et al., 2016). Therefore, it is possible that those two receptors may have contributed to the GVHD in our model. Studies in mice lacking specific biosynthesis of cys-leukotrienes would be necessary to rule out their participation. However, the pharmacologic blockade of BLT1 mimicked most of the beneficial effects of the 5-LO inhibitor or its absence, suggesting that the LTB_4 pathway is the major pathway downstream of 5-LO leading to injury in this system. Altogether, these results open new therapeutic possibilities for the clinical management of GVHD.

Pulmonary complications are responsible for ∼70% of morbidity and 30% of mortality after GVHD (Yousef et al., 2013). In humans, the disease tends to be more often chronic and characterized primarily by an obliterating bronchiolitis (Haddad, 2013; Xu et al., 2013; Yousef et al., 2013; Kambham et al., 2014). Regardless, we observed that pulmonary injury in our system was acute at onset and occurred at significant levels during the onset of lethality. Pulmonary injury was greatly prevented by treatment with zileuton and the LTB_4 antagonist, but not by the cys-Leukotriene receptor antagonist, montelukast. It is difficult to translate these acute findings to the chronic human situation, but these beneficial effects of blocking 5-LO and LTB_4 receptors add significantly to the overall beneficial effects of these treatments.

We also demonstrated that there was a reduction in $CD4^+$ and $CD8^+$ T cell frequency in the spleen and BM after 5-LO–deficient leukocyte transplant. Several studies have provided evidence that the spleen is a GVHD target organ (Schattenfroh et al., 1995; Kataoka et al., 2001; New et al., 2002). Splenic GVHD is characterized by lymphoid atrophy, focal necrosis, and an increase in proinflammatory cytokines and chemokines. This occurs because, after BM transplant, donor CD4 and CD8 cells first migrate to lymphoid organs and proliferate after contact with alloantigens and induce lymphocyte Fas/FasL-mediated apoptosis. Animal models of GVHD have also shown that BM is a GVHD target organ because this disease can affect hematopoiesis and lymphoid cells development (Müller et al., 2010; von Bonin and Bornhäuser, 2014; Yao et al., 2014; Szyska and Na, 2016). Similar results were obtained in patients with GVHD who showed extensive donor T cell infiltration and osteoblast reduction (Mensen et al., 2014). GVHD BM is associated with TNF and IL-1β production, which stimulates the increased expression of MHC class II and CD40, adhesion molecules, and increased vascular permeability to facilitate the migration and activation of innate immune cells and alloreactive T cells (Szyska and Na, 2016). After the proliferation of donor T lymphocytes in the spleen and BM, the migration of those cells to the intestine and the liver triggers a severe inflammatory response and injury in those peripheral organs (Schattenfroh et al., 1995; Kataoka et al., 2001; New et al., 2002). Thus, we hypothesized that the reduced number of CD4 and CD8 T cells in the spleen and BM of 5-LO–deficient, leukocyte-transplanted mice could contribute to early GVHD development, in turn, reducing activation of those cells and their subsequent migration to the other GVHD target organs. Corroborating our results of reduction in CD4 and CD8 T cells in the BM and spleen, we also observed a reduction in those cells in the intestine and liver of mice that received leukocytes deficient in 5-LO. Although zileuton did not reduce the frequency of CD4 T cells in the spleen, BM, or intestine, there was a reduction in the number of CD8 T cells in the liver and jejunum–ileum of zileuton-treated mice. These results are relevant because the maintenance of T lymphocytes in the liver and gut is associated with extensive cytokine production, the recruitment of other cell types, and tissue damage by cytotoxic lymphocytes, resulting in GVHD perpetuation and death if effective therapeutic strategies are not used to control the disease (Blazar et al., 2012; Markey et al., 2014). Overall, our findings showing that the reduced frequency of intestinal and hepatic CD4 and CD8 T cells after 5-LO blockade is consistent with the reduced injury of the jejunum–ileum and liver in mice that received 5-LO–deficient leukocytes or were treated with zileuton.

In a mixed leukocyte reaction, we found that incubation with zileuton failed to affect lymphocyte proliferation. These results suggest that there appears to be no dysfunction associated with the presentation of antigens and T cell responses associated with GVHD in the presence or absence of zileuton. Therefore, 5-LO does not appear to be relevant in the context of the APC function or the T cell responses under this limited in vitro setting. As discussed above, there was some inhibition of T lymphocyte migration to the spleen, the major systemic, secondary lymphoid organ, when 5-LO–deficient leukocytes were transplanted but not when zileuton treatment was given. Therefore, there does not appear to be direct inhibition of T cell function in this system. This is consistent with the maintained GVL response, which relies on effective systemic APC function and T cell responses. In contrast, there was inhibition of the recruitment of leukocytes to target organs.

We further demonstrated reduced macrophage accumulation in the spleen, BM, gut, and liver after 5-LO blockade. Macrophages are important in GVHD for their phagocytic function, for participation in antigen presentation to T lymphocytes, for expression of many proinflammatory receptors, and for production of inflammatory mediators, such as reactive oxygen species and cytokines (Fieren, 2012). Supporting our hypothesis, the study published by Li et al. (2014) showed that 5-LO inhibition improved acute liver failure,

and that was associated with reduced macrophage number. Another study by Luz et al. (2014) supports our findings of reduced macrophage infiltration in the intestine and liver of both 5-LO–deficient, leukocyte-transplanted mice and zileuton-treated mice. These authors showed that 5-LO inhibition by MK886 reduced recruitment of neutrophils and macrophages into the peritoneal cavity of mice stimulated with eotaxin, demonstrating the importance of the 5-LO pathway for the recruitment of those cells.

GVHD pathophysiology is associated not only with the accumulation of proinflammatory cells but also with persistent reductions in the number of T reg cells (Beres and Drobyski, 2013). Several studies have shown that the absence of T reg cells exacerbates GVHD and that infusion of those cells has an important role in modulating GVHD (Ermann et al., 2005; Beres and Drobyski, 2013). Brunstein et al. (2016) published a study showing that T reg cell infusion, in conjunction with umbilical cord stem cell transplantation in humans, decreased the incidence of acute GVHD. Thus, the steady T reg cell frequency in the jejunum–ileum after 5-LO–deficient leukocyte transplant may have contributed to the reduction in intestinal injuries in our study.

Cytokines and chemokines are key molecules in GVHD development. Our experiments showed reduced intestinal levels of the chemokine CCL3 and the cytokines IFN-γ, TNF, and IL-17 in mice that received 5-LO–deficient leukocytes. We have already shown the participation of CCL3 in GVHD in a previous study (Castor et al., 2010). CCL3-deficient donor cells and the pharmacologic blockade of the CCL3 receptor also reduce mortality and injury in graft-versus-disease target organs. Moreover, the absence of CCL3 reduced the number of CD4 and CD8 T cells infiltrating into the small intestine (Castor et al., 2010). Thus, the reduction of CCL3 after 5-LO blockade may also be contributing to the reduction of CD4 and CD8 T cells in the target organs shown here. The proinflammatory cytokines IFN-γ and TNF are the major contributors to GVHD development (Robb and Hill, 2012), and increased levels of these cytokines precede clinical GVHD signs (Koide et al., 1997), including severe weight loss, diarrhea, skin changes, and high levels of mortality (Piguet et al., 1987; Baker et al., 1996; Koide et al., 1997; Schroeder and DiPersio, 2011). Elevated levels of IFN-γ contribute to intestinal damage through direct cytotoxic effects and the activation of monocytes/macrophages to secrete lymphotoxins and TNF, enhancing organ damage (Robb and Hill, 2012). TNF also has an important role in GVHD pathophysiology by participating in APC activation and by directly stimulating cytotoxic T lymphocyte proliferation and mononuclear cell recruitment. Several studies have demonstrated the involvement of IL-17 in the pathogenesis of acute GVHD. Both IL-17–producing CD4 and CD8 T lymphocytes are associated with Th1 disease and increase tissue inflammation, which leads to the initiation and perpetuation of GVHD (van der Waart et al., 2014). We also observed that zileuton treatment reduced intestinal IL-12, CCL2, and CCL5 levels and the hepatic CCL2, IFN-γ, and IL-17 levels. Previous studies have shown that IL-12 neutralization in vivo improves acute GVHD by reducing NK cell activity in the spleen and by reducing IFN-γ production, which, in turn, causes less weight loss and reduced mortality (Williamson et al., 1996, 1997). CCL2 and CCL5 also have central roles in GVHD injuries. These chemokines drive the influx of leukocytes to GVHD target organs, contributing to the inflammatory response (Serody et al., 2000; Terwey et al., 2005; Wysocki et al., 2005). Thus, our results showing the importance of reducing proinflammatory cytokine and chemokine levels in terms of prevention of fatal GVHD are in agreement with those of previous studies, which proposed an association between reducing the inflammatory response mediated by cytokines and chemokines and GVHD protection after experimental allogeneic hematopoietic stem cell transplant (Piguet et al., 1987; Baker et al., 1996; Koide et al., 1997; Serody et al., 2000; Terwey et al., 2005; Wysocki et al., 2005; Castor et al., 2010; Schroeder and DiPersio, 2011; Robb and Hill, 2012). In addition, the reduced levels of the chemokines CCL2, CCL3, and CCL5 in zileuton-treated mice may also lead to reduced recruitment of inflammatory cells to target organs.

IL-10 is also an important anti-inflammatory cytokine in GVHD. Weber et al. (2014) showed that transplantation of IL-10–deficient leukocytes led to severe GVHD, increased the response of alloreactive T lymphocytes, and increased mortality. In another study, Lin et al. (2014) showed that the IL-10 produced by T reg cells was crucial in reducing the interaction time between conventional donor T cells and host DCs, resulting in the attenuation of GVHD. In this study, we demonstrated an increase in the intestinal IL-10 level in the mice that received 5-LO–deficient leukocytes. This result may be associated with the maintenance of intestinal T reg cell frequency and the reduced intestinal injury previously reported here. In the liver, the level of this cytokine was reduced in the GVHD mice and remained similar to the level in the control group in 5-LO–deficient, leukocyte-transplanted mice. Moreover, hepatic IL-10 maintenance in mice that received 5-LO–deficient leukocyte transplants could be responsible for the attenuated liver injury in those mice.

Several therapies that are effective in preventing GVHD usually lead to reduced GVL activity, which is the ability of donor-derived, infused lymphocytes to react against remaining leukemic cells, impairing their effective application. In this study, we observed that the transplant of 5-LO–deficient leukocytes or leukocytes from zileuton-treated mice did not interfere with chimerism or the GVL response. Altogether, our results suggest that 5-LO is associated with GVHD pathophysiology and that its absence or pharmacologic inhibition protects mice from GVHD. Thus, zileuton has a potential therapeutic application in GVHD treatment.

MATERIALS AND METHODS

Ethics statement

The animal care and handling procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee, and the study received prior approval from Animal Ethics Committee, of Universidade Federal de Minas Gerais (UFMG; protocol 135/13). Animals judged to be moribund were euthanatized with an overdose of anesthesia (100 µl of a mixture of ketamine [37.5 mg/ml] and xylazine [2.5 mg/ml], i.v.), which was counted as GVHD lethality. At the end of these experimental procedures, the remaining mice were also euthanized with an overdose of anesthetics. In all experiments, efforts were made to minimize suffering at all times.

Mice

8–12-wk-old C57BL/6, B6D2F1 (C57BL/6 X DBA/2), or BALB/c mice were obtained from the Centro de Bioterismo (UFMG). WT SV129 and SV129 5-LO–deficient were provided by J.S. da Silva (School of Medicine of Ribeirão Preto, São Paulo, Brazil) and maintained at our laboratory. All mice were housed under standard conditions in a temperature-controlled room (23 ± 1°C) on an automatic 12h/12h light/dark cycle. The mice had free access to commercial rodent food and water. The number of mice in each specific group is provided in the figure legend. One representative experiment with at least five mice per group of similar, independent experiments is shown in each figure.

Induction of GVHD

Induction of GVHD in B6D2F1 mice. Recipient B6D2F1 mice were irradiated at 9 Gy total-body radiation (source ^{60}Co) in two doses at 2-h intervals to minimize gastrointestinal toxicity and then given an i.v. infusion of 3×10^7 splenocytes and 1×10^7 BM cells from WT or 5-LO–deficient donors. The B6D2F1 mice that received splenocytes from B6D2F1 mice (B6D2F1 to B6D2F1) did not develop any disease and were considered the control group.

Induction of GVHD in C57BL/6 mice. Recipient C57BL/6 mice were irradiated with 9 Gy total-body radiation (source ^{60}Co) in two doses at 2-h intervals to minimize gastrointestinal toxicity and then given an i.v. infusion of 3×10^7 splenocytes and 1×10^7 BM cells from BALB/c mice. The C57BL/6 mice that received splenocytes from C57BL/6 mice (C57BL/6 to C57BL/6) did not develop any disease and were considered the control group.

Because of the toxicity of the high level of body irradiation, the recipient mice received an oral suspension of ciprofloxacin (70 mg/L) in their drinking water from 1 d before to 15 d after transplantation. The BM cells and splenocytes were isolated as previously described (Rezende et al., 2013; Bernardes et al., 2015). Recipient mice were monitored for survival and clinical GVHD symptoms and were sacrificed for blinded histopathologic and flow cytometric analysis as previously described (Rezende et al., 2013; Bernardes et al., 2015).

Treatment

Mice were transplanted with cells from WT mice and treated with zileuton (30 mg/kg) dissolved in 0.1% carboxymethylcellulose + 5% ethanol two times per day by oral gavage from day 0 until the onset of clinical symptoms. The WT → F1 + vehicle group received 0.1% carboxymethylcellulose + 5% ethanol two times per day.

The WT → F1 + CP-105,696 group received 3 mg/kg of CP-105,696 dissolved in 0.1% carboxymethylcellulose + 5% ethanol once a day. The WT → F1 + montelukast group received 10 mg/kg of montelukast dissolved in 0.1% carboxymethylcellulose + 5% ethanol once a day.

Assessment of GVHD

Recipient mice were evaluated clinically with a standard scoring system as previously described (maximum index = 14; Rezende et al., 2013; Bernardes et al., 2015). Scores were according to weight loss, posture (hunching), activity, fur texture, skin integrity, diarrhea, and fecal occult blood.

Confocal microscopy

On day 6 after transplant, mice were killed, and the spleen was prepared for immunofluorescence analysis with confocal microscopy. Images were obtained using a Ti microscope (Nikon) with a C2 confocal laser equipped with three different lasers (excitation at 405, 488, and 543 nm) and 450/50 nm (channel 1), 515/30 nm (channel 2), and 584/50 nm (channel 3) emission filters. The cells with nuclear-localized 5-LO were identified in cytocentrifuged slides by staining with a goat anti–5-LO primary antibody (1:100; Santa Cruz Biotechnology) plus an Alexa Fluor-488–conjugated anti-goat secondary antibody (1:100; Thermo Fisher Scientific). Nuclei were stained with DAPI (1 mg/ml; Cell Signaling Technology). Fluorescence intensity was measured offline using Volocity software (version 6.3; PerkinElmer).

Histopathology

A set of experiments was conducted to quantify the histopathologic parameters in the intestine, liver, and lung, which are important GVHD target organs. Tissue sections were processed for histologic analysis as described previously (Cooke et al., 1996; Castor et al., 2010) and evaluated by a pathologist. A numeric value was attributed to the changes observed in the intestinal layers (mucosal, lamina propria, muscular, and serosal), in the liver (degenerative alterations in the parenchyma), and in the lung (periluminal infiltrates around airways/vessels and pneumonitis alveolar/interstitial), and each animal received a score that was generated by summation of all observed changes (maximum index, 9 for intestine, 6 for liver, and 3 for lung). Histopathologic scores were determined for samples that were obtained from mice on day 10 after the transplant, which corresponded to the mortality phase of the disease. The target organs were also removed for histopathologic analysis 24 h after irradiation; significant pathologic changes were not detected at that time (data not shown).

Quantification of macrophage infiltration

The relative numbers of infiltrating macrophages in the intestine and liver were quantified by measuring the *N*-acetyl glucosaminidase (NAG) activity at onset mortality. A 100-mg portion of the small intestine was resuspended in 0.9% saline (4°C) with 0.15 vol/vol Triton X-100 (Merck & Company), homogenized, and centrifuged at 4°C for 10 min at 3,000 rpm. The supernatants were collected and assayed immediately for NAG activity at a 1:3 dilution, as described previously (Barcelos et al., 2004).

Quantification of cytokines and chemokines

The concentrations of cytokines and chemokines were quantified from intestinal or liver homogenates from animals at the onset of mortality. The tissues were mixed with PBS that contained antiproteases (0.1 mM PMSF), 0.1 nM benzethonium chloride, 10 mM EDTA, 20 Kallikrein inhibitor units, aprotinin A, and 0.05% Tween 20. The samples were centrifuged for 10 min at 10,000 rpm and 4°C. Dilutions of the supernatants in PBS (1:3) were immediately analyzed by ELISA. The cytokine and chemokine concentrations were measured according to the manufacturer procedures (R&D Systems), and the colorimetric reactions were analyzed with a spectrophotometer at a wavelength of 492 nm.

Intravital microscopy

GVHD was induced, and the mice were treated with zileuton or vehicle. On day 10 after transplantation, the mice were anesthetized, and the intestinal venules were exposed in a perfusion system with warm bicarbonate-buffered saline (pH 7.4). An intravital microscope (ECLIPSE 50i; Nikon) with a 20 objective lens was used to examine the mesenteric microcirculation. A digital camera (DS-Qi1MC; Nikon) was used to project the images onto a computer monitor, and the images were recorded for playback analysis with Nikon imaging software. 40–60 μm of intestinal venules were selected, and the numbers of rolling and adherent leukocytes were determined offline during the video-playback analysis. Rolling leukocytes were defined as those cells that moved at a velocity less than that of the erythrocytes within a given vessel. The flux of rolling cells was measured as the number of rolling cells that passed by a given point in the venule per minute. A leukocyte was considered to be adherent if it remained stationary for ≥30 s, and total leukocyte adhesion was quantified as the number of adherent cells in the intravascular space within an area of 100 μm.

Mixed leukocyte reaction and in vitro cell proliferation

First, splenocytes isolated from WT mice were labeled with 2 μM CFSE, at 37°C for 15 min. For mixed leukocyte reaction, WT splenocytes labeled with CFSE (5×10^5/well) were cocultured with DCs isolated from F1 mice by the CD11c immunomagnetic separation kit (10^5/well, Big Easy Magnet; STEMCELL Technologies) in triplicate in 96-well plates containing complete DMEM for 72 h. After 72 h, the cells were harvested, and the cell proliferation was measured by flow cytometric analysis of CFSE dilution in the following groups: F1 splenocytes + F1 DCs (negative control group), WT splenocytes + F1 DCs + vehicle, and WT splenocytes + F1 DCs + zileuton (40 μM).

GVL induction

The P815 mouse mastocytoma cell line (H-2 d;ATCC), which had been transduced with a lentiviral vector (elongation factor 1-GFP), was provided by A.C. Leal and M. Bonamino (Instituto Nacional do Câncer, Rio de Janeiro, Brazil). That cell line was maintained in RPMI-1640/10% FCS at 37°C and 5% CO and was used for GVL experiments in vivo. The protocols for irradiation and GVHD induction have been described previously. B6D2F1 recipients were injected i.v. with 5×10^4 GFP + P815 cells on day 0 of the transplantation. 10 d after tumor cell transplantation, the mice were sacrificed, and tumor cells were analyzed by flow cytometry and confocal microscopy. On day 10 after transplantation, mice were euthanized, and the lymph nodes and the spleen were prepared for flow cytometry analysis. Erythrocytes were lysed, and cells were centrifuged for 5 min at 350 *g*. 10^6 cells were resuspended in buffer containing 5% BSA and 0.01% sodium azide and fixed in a solution containing 2% formaldehyde in PBS. Flow cytometry was performed using a BD C6 Accuri, and 20,000 events were evaluated per sample. The presence of tumor cells was determined by assessing the frequency of GFP^+ cells using FlowJo software (Tree Star). The frequency of GFP^+ P815 cells was analyzed in tumor-bearing mice that received, or did not receive, zileuton treatment or were transplanted with 5-LO–deficient cells.

Flow cytometry

Cells were stained for extracellular molecular expression patterns using mAbs against mouse CD3 (APC–Cy5 conjugated), CD4 (Pacific Blue conjugated), CD8 (V450 conjugated), CD45R/B220 (PE conjugated), CD11b (PE–Cy5 conjugated), F4/80 (PE–Cy7 conjugated;V450 conjugated), Ly6G (APC conjugated), CD25 (APC conjugated), FOXP3 (PE conjugated), H-$2D^b$ (Alexa Fluor-647 conjugated; BioLegend), and H-$2K^b$/H-$2D^b$ (PE conjugated; BioLegend). All antibodies were purchased from BD. The frequency of positive cells was analyzed using a gate that included lymphocytes, granulocytes, and/or monocytes/macrophages. Limits for the quadrant markers were always set based on negative populations and isotype control antibodies. Cells were acquired with a BD FACSCanto II cytometer and analyzed using FlowJo 7.5.3 software. The frequency (percentage) of the analyzed population in the total acquired events was used in the construction of the graphs.

Enzymatic immune assay for LTB_4

Animals were sacrificed 10 d after transplantation, and the jejunum–ileum and liver were collected for the determination of LTB4 levels. The samples of jejunum–ileum and liver were homogenized in 500 μl of the appropriate buf-

fer containing protease inhibitors (Cunha et al., 2003). Hepatic and intestinal LTB4 levels were determined by enzyme immunoassay using a commercial kit (Biotrak; GE Healthcare), which was used according to the manufacturer's instructions. In brief, recombinant mouse LTB4 standards at various dilutions and the samples were added to a microliter plate precoated with mouse mAbs. Then, LTB4 acetylcholinesterase tracer and anti-LTB4 antiserum were added to each well. The plate was incubated at room temperature overnight. The plate was washed, 200 µl acetylcholinesterase substrate (Ellman's reagent) was added to each well, and the plate was incubated for 60–90 min for color development. The OD was determined at 405 nm, and the results were expressed as picograms of LTB4/sample, based on the standard curve.

Statistics

Data in the text are expressed as the means ± SEM. Comparisons among the groups were performed by unpaired Student's *t* test or ANOVA, followed by the Student-Newman-Keuls post hoc analysis. A log-rank test was used to compare the relevant survival curves. Statistical significance was set as $P < 0.05$, and all graphs and analysis were performed with GraphPad Software's Prism version 6.

Online supplemental material

Fig. S1 shows that the treatment with CP-105,696 improved clinical signs of GVHD, reduced intestinal and hepatic injury, and reduced production of proinflammatory cytokines and chemokines related to GVHD. Fig. S2 shows that treatment with zileuton or CP-105,696, but not montelukast, reduced lung injury associated with GVHD. Fig. S3 shows the effect of transplants with 5-LO–deficient leukocytes or treatment with zileuton on the recruitment of leukocytes to lymphoid organs after induction of GVHD in mice. Fig. S4 shows representative flow cytometry dot plots of the recruitment of $TCD4^+$, $TCD8^+$, and T reg cells to GVHD target organs.

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