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STAT3 expression in host myeloid cells controls GVHD severity

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Abstract

Professional antigen presenting cell (APCs) are important modulators of acute graft-versus-host disease (GVHD). Although dendritic cells (DCs) are most potent APC subset, other myeloid cells, especially macrophages (MFs) and neutrophils have recently been shown to play a role in the severity of GVHD. However, the critical molecular mechanisms that determine the functions of myeloid cells in GVHD are unclear. Signal transducer and activator of transcription 3 (STAT3) is a master transcription factor that plays a crucial role in regulating immunity. But its role in MF biology and in acute GVHD remains unknown. To determine the role of myeloid cell specific expression of STAT3 on the severity of acute GVHD, we utilized myeloid cell specific STAT3 deficient LysM-Cre/STAT3$^{fl/-}$ animals as recipients and donors in well-characterized experimental models of acute GVHD. We found that reduced expression of STAT3 in myeloid cells from the hosts, but not the donors, increased inflammation, donor T cell activation and exacerbated GVHD. Our data demonstrate that STAT3 in host myeloid cells, such as MFs, dampens acute GVHD.

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Keywords

bone marrow transplantation; graft-versus-host disease; STAT-3; myeloid cells

INTRODUCTION

Graft-versus-host disease (GVHD) is a major life threatening complications of allogeneic hematopoietic cell transplantation (allo-HCT). Approximately, 35–50% of patients suffer from acute GVHD despite prophylaxis with calcineurin inhibitors, which suppress the main effector cell of GVHD pathogenesis, allogeneic T cells[1, 2]. Therefore, new GVHD prophylaxis and treatment strategies are needed.

The biology of GVHD is complex. Antigen presenting cells (APCs), derived from both donor and host, play important roles in the development of acute GVHD by producing pro-inflammatory cytokines, such as TNF-alpha (TNF-α) and IL-6, as well as by directly stimulating donor T cells[3–9]. The complex role of dendritic cells (DCs), a potent subset of APCs, in GVHD pathogenesis is being increasingly appreciated[9]. But the effect of macrophages (MFs), another type of APC which infiltrate GVHD target organs[10, 11] on GVHD is less well-understood. In support of a role for MFs influencing GVHD, acute GVHD was reduced when macrophage recruitment to GVHD target organs was inhibited by CYM-5442, a sphingosine 1-phosphate 1(S1P1) receptor agonist[12], and the anti-GVHD properties of corticosteroids are likely due in part to inhibition of MF functions[13]. However, these studies did not distinguish the role of donor versus recipient-derived MFs on acute GVHD, which is important because both host and donor MFs are present early post allo-HCT due to the resistance of host MFs to transplant conditioning regimens[14]. In an effort to clarify this, Hashimoto et al, showed that deleting host MFs increases donor T cell expansion and exacerbates GVHD while colony stimulating factor (CSF)-1 expanded host MFs and reduced acute GVHD[15]. In contrast to host-derived MFs, CSF-1-dependent donor MFs exacerbate chronic GVHD[16]. However, it is unclear exactly how host versus donor MFs modulate GVHD, what macrophage molecular and signaling pathways are required for GVHD modulation, or how MFs affect allogeneic T cell responses.

Signal transducer and activator of transcription 3 (STAT3) is a member of the STAT family and plays a central role in regulating innate and adaptive immune responses[17, 18]. STAT3 signaling in T cells exacerbates both acute and chronic GVHD potentially due to its essential role for donor T cell activation and inflammatory T_H17 cell differentiation[19–26]. In addition, STAT3 signaling exacerbates GVHD by reducing the stability of naïve regulatory T (T_{reg}) cells and by limiting the expansion of induced donor T_{reg} cells[27]. In contrast, absence of STAT3 in donor T cells ameliorates chronic GVHD by reducing follicular helper T (T_{fh}) cells and increasing T follicular regulatory (T_{fr}) cells[28].

STAT3 activation is required for their immune regulatory functions and survival of MFs[29, 30]. Interestingly, IL-10 secretion by MFs driven by STAT3 signaling is crucial for inhibition of inflammatory responses[31, 32], whereas deficiency of STAT3 in MFs increases pro-inflammatory cytokine production in response to lipopolysaccharide (LPS) and helps create a T_H1 promoting milieu[33]. In DCs, STAT3 signaling is crucial for both DC activation

and induction of immune tolerance[34, 35]. Increased STAT3 expression in donor derived plasmacytoid DCs (pDCs) reduces acute GVHD[36]. STAT3 acetylation negatively regulates DC function by promoting indoleamine 2,3-dioxygenase (IDO) expression and ameliorates acute GVHD[37–40]. Taken together, these studies suggest that STAT3 signaling influences inflammation in a cell intrinsic and presumably context dependent manner.

To determine whether STAT3 contributes to the role of myeloid cells such as MFs in regulation of GVHD, we tested the influence of STAT3-deficient myeloid lineage cells generated from LysM-Cre/STAT3$^{fl/-}$ animals in experimental models of acute GVHD[33]. We found that the absence of STAT3 in host- derived but not donor-derived myeloid cells (i.e. MFs) exacerbates GVHD. These data suggest that STAT3 signaling in host myeloid cells such as MFs and PMNs mitigates the severity of acute GVHD.

RESULTS

LysM-Cre/STAT3$^{fl/-}$ animals showed reduced expression of STAT3 in macrophages (MF)

Host macrophages (MFs) regulate the severity of acute GVHD[9, 15]. STAT3 is a master regulator of MF dependent innate immune responses. But the role of STAT3 in professional APCs, specifically in MFs, in regulation of their allo-stimulatory functions is not known. To evaluate the role of STAT3 in MFs on allogeneic immune responses, we generated STAT3 deficient MFs by crossing B6 STAT3 hemizygous mice in which one STAT3 allele was floxed and the other was already ablated (STAT3$^{fl/-}$), with B6 mice expressing Cre recombinase under the control of the myeloid lineage (MFs and granulocytes) specific promoter, lysosome M (LysM-Cre)[41, 42]. We next confirmed that both mRNA and protein expression of STAT3 in peritoneal MFs from LysM-Cre/STAT3$^{fl/-}$ animals were decreased relative to wild type LysM-Cre/STAT3$^{flox/flox}$ (WT) animals (Figure 1A)[33]. STAT3 expression in bone marrow derived DCs (BMDCs) from LysM-Cre/STAT3$^{fl/-}$ animals was not reduced suggesting that, of myeloid-derived APCs, STAT3 expression was exclusively decreased in MFs from LysM-Cre/STAT3$^{fl/-}$ animals[33]. The MFs from both naïve WT and LysM-Cre/STAT3$^{fl/-}$ animals showed similar expression of the co-stimulatory molecules, such as CD40, CD80, PD-L1, and I-Ab (Figure 1B–C).

Reduced STAT3 expression in host myeloid APCs amplifies the severity of acute GVHD

Next, we determined whether reduced expression of STAT3 in host myeloid cells, specifically MFs but not in DCs, will modulate GVHD severity. To accomplish this, we utilized the well-established major histocompatibility complex (MHC) mismatched acute GVHD model, BALB/c into B6. Both B6-WT and LysM-Cre/STAT3$^{fl/-}$ animals were lethally irradiated and transplanted with bone marrow (BM) and splenic T cells from either syngeneic B6-WT or allogeneic BALB/c donors. Compared to allogeneic B6-WT animals, the allogeneic LysM-Cre/STAT3$^{fl/-}$ animals demonstrated significantly greater mortality ($p=0.02$) and increased clinical severity of GVHD (Figure 2A–B).

The increase in mortality and GVHD severity was associated with more severe histopathological damage of GVHD target organs, liver (Figure 2C–D) and gastrointestinal (GI) tract (Figure 2 E) on day 14 post-BMT compared with B6-WT allogeneic animals.

Next, in order to determine whether the impact on GVHD is due to differences in the survival between the STAT3 deficient WT macrophages after conditioning and BMT, we analyzed MF survival following similar conditioning protocol as above. To this end, wild-type Balb/c animals were lethally irradiated and then treated with either 20mg/kg of WP1066 (a STAT3 inhibitor)[56] or diluent immediately after conditioning and analyzed the MF recovery and survival from both spleen and peritoneal cavity. This dose has been shown to inhibit STAT3 in vivo[56]. Peritoneal and splenic CD11b+ MFs were then analyzed at 24 and 48 hours post irradiation. MF counts in non-conditioned animals were used as a baseline. No significant differences in MF survival were observed at either time points in MFs regardless of STAT3 inhibition (Figure 2 G–H). This approach suggests, but is not definitive, that STAT3 deficiency in MF does not alter their survivability after conditioning.

To rule out strain, model, and major MHC mismatch dependent artifacts, we utilized a multiple minor histocompatibility antigens (miHAs) –mismatched model of GVHD, C3H.sw into B6. Similar to the major MHC mismatched model, serum levels of IFN-γ (Supplementary Figure 1a) and IL-17A (Supplemental Figure 1b) were increased in the allogeneic LysM-Cre/STAT3$^{fl/-}$ recipient animals, and their livers and GI tracts exhibited greater GVHD specific histopathological damage (Supplemental Figure 1c-e). These data demonstrate that STAT3 signaling in host myeloid APCs regulates the severity of GVHD in a strain independent manner.

Deficiency of STAT3 in host MFs enhances donor T cell expansion after allogeneic BMT

We next explored the putative mechanisms for enhanced acute GVHD when STAT3 is deficient in host APCs of myeloid origin. In light of the role of STAT3 in attenuating pro-inflammatory responses by the MFs[33], we determined whether the absence of STAT3 in host myeloid APCs also enhanced T cell expansion and inflammation at day +14 after BMT, which is in the time range of the peak donor T cell response in this model. Consistent with this notion, compared to the WT recipients, the enhanced mortality of the allogeneic LysM-Cre/STAT3$^{fl/-}$ recipient animals was associated with increased donor CD4$^+$ and CD8$^+$ T cell expansion (Figure 3A) and increased serum levels of T cell derived pro-inflammatory cytokines, IFN-γ (Figure 3B) and IL-17A (Figure 3C) on day +14 after BMT. Furthermore, the expansion of CD4$^+$Foxp3$^+$ T_{reg} was not changed, but the effector T cells/regulatory T cell ratio (T_{Eff})/T_{reg}) was significantly increased in allogeneic LysM-Cre/STAT3$^{fl/-}$ compared to WT animals (Figure 3D–E). These data show that STAT3 signaling in host myeloid cells inhibits murine allogeneic T cell proliferation *in vivo* and reduced acute GVHD.

STAT3 deficient macrophages show enhanced stimulation of allogeneic T cells in vitro

Amongst the myeloid derived cells, MFs are bona fide APCs. Because in vivo STAT3 deficiency in host myeloid cells showed enhanced in vivo expansion of allogeneic T cells, we therefore reasoned that the absence of STAT3 in MFs (and not the other myeloid derived cells namely neutrophils) might be the main driver of donor T cell expansion and hence the cause of amplified GVHD. To determine the cell intrinsic effect of STAT3 deficiency in MF, we examined whether reduced expression of STAT3 in MFs from LysM-Cre/STAT3$^{fl/-}$ animals affects their ability to stimulate allogeneic T cell response *in vitro*. To this end,

peritoneal MFs were used as stimulators for co-cultured splenic T cells from either syngeneic B6 or allogeneic BALB/c animals in mixed lymphocyte reaction (MLR). We found that MFs from LysM-Cre/STAT3$^{fl/-}$ animals enhanced allogeneic T cell proliferation and production of IL-2, IFN-γ, and IL-17A but not IL-4 and IL-10 compared to WT MFs (Figure 4A–F). Importantly, BMDCs from LysM-Cre/Stat3$^{fl/-}$ animals showed equivalent allogeneic T cell responses to WT DCs *in vitro* (Supplemental Figure 2). These data show that STAT3 signaling in MFs inhibits allogeneic T cell responses and possibly T_H1 or T_H17 differentiation *in vitro* and suggest that the *in vivo* effects are from deficiency in host MFs.

STAT3 deficient macrophages exhibit enhanced innate immune responses

Allo-HCT conditioning causes tissue damage which results in the generation of damage- and pathogen-associated molecular patterns (DAMPs and PAMPs, respectively), such as LPS. DAMPs and PAMPs activate GVHD-promoting inflammation via pattern recognition receptor signaling, particularly in APCs[43–46]. Therefore, we next determined whether STAT3 expression alters APC responses to LPS. Consistent with previous observations, STAT3 deficient MFs showed enhanced production of IL-1β, IL-6 and TNF-α, and decreased production of IL-10 relative to WT MFs (Supplemental Figure 3, a-d) when stimulated with LPS (1μg/ml) for 16 hours[33]. In contrast, STAT3 deficient DCs produced similar levels of these cytokines compared to WT DCs upon LPS stimulation (Supplemental Figure 4 a-d). These data demonstrate increased LPS-stimulated innate immune responses in STAT3 deficient MFs which may contribute to their ability to aggravate acute GVHD.

STAT3 deficiency in donor myeloid cells is dispensable for acute GVHD severity

Donor APCs also contribute to GVH responses[8, 47–49]; therefore, we explored whether murine GVHD was affected by STAT3 signaling in donor myeloid cells. To test this, we utilized the well-established major MHC mismatched B6 into BALB/c acute GVHD model. Recipient BALB/c animals were lethally irradiated and transplanted with splenic CD90.2$^+$T cells from B6-WT animals and with T cell depleted BM (TCD-BM) from either B6-WT or LysM-Cre/STAT3$^{fl/-}$ animals. Survival, GVHD clinical score, serum pro-inflammatory cytokine levels, and donor T cell expansion except for IL-17A at day 14 after allo-BMT were similar between BALB/c animals that received either allogeneic WT or STAT3-deficient TCD-BM (Figure 5 A–J). These data indicate that STAT3 expression in donor myeloid cells is dispensable for regulation of the severity of acute GVHD.

DISCUSSION

Previous reports suggested that host myeloid derived APCs such as MFs and neutrophils aggravate acute GVHD[9]. For example, host MFs survive BMT conditioning, modulate donor T cell functions[14], infiltrate GVHD target organs[10, 11], and inhibiting their ability to migrate to GVHD target organs ameliorates GVHD[12]. In addition, dexamethasone ameliorates GVHD by reducing production of pro-inflammatory cytokines from host derived MFs[13], while decreasing the number of host MFs enhances donor T cell expansion and aggravates acute GVHD[15]. However, the molecular signaling mechanisms required for host myeloid derived APCs such as MF-dependent aggravation of acute GVHD are incompletely characterized. Herein we addressed this important question. We found that STAT3 limits the

ability of host myeloid cells to enhance GVHD presumably by limiting host macrophage and neutrophil mediated inflammatory innate immune responses and their intrinsic ability to stimulate allogeneic T cell proliferation.

Importantly, our data show that STAT3 expression in host MFs does not completely block GVHD. This is likely because other APCs, including DCs or neutrophils may be sufficient for induction of murine GVHD. In contrast, STAT3 in donor MFs has no effect on murine acute GVHD. These data suggest that host MFs, much like DCs, are critical APCs for regulating the severity of GVHD while not being mandatory for the induction of GVH responses when all other APCs are intact[9].

Thus our results are consistent with and expand on previous reports that host MFs, when expanded by administrating CSF-1, can decrease GVHD severity and mortality by modulating donor T cell function[15]. Our data points to STAT3 as being a critical molecule that regulates the ability of host MFs in mitigating GVHD responses. Future studies will address the downstream targets of STAT3 that are critical for negatively regulating host MFs after allogeneic BMT.

STAT3 expression in myeloid cells is required for modulating inflammatory responses to PAMPs *in vivo*[33, 34]. Therefore, we confirmed that STAT3 deficiency in MFs enhances their production of inflammatory cytokines when stimulate with LPS. In addition, we show that recipient animals deficient in STAT3 in their myeloid lineage showed aggravated GVHD associated with increased allogeneic T cell proliferation. Remarkably, we couldn't find any significant aggravation of GVHD when using STAT3 deficient donor myeloid cells. These data suggest that STAT3 expression in host MFs may play an important role in regulating allogeneic immune responses possibly by limiting inflammatory host macrophage innate immune responses and creating a milieu that is less advantageous for allogeneic T cell proliferation. However, our *in vitro* data suggest that MF intrinsic deficiency amplified their ability to stimulate allogeneic T cell proliferation and inflammatory cytokine secretion. These data, when taken in light of the lack of impact of STAT3 deficiency in donor MFs on GVHD, point to the notion that the alteration of the allo-stimulatory capacity of MFs might be a critical component for the aggravation of GVHD. The reasons for enhanced allo-stimulatory effects by STAT3 deficient macrophages remains to be explored. It may be secondary to changes in expression of PDL1 and/or other co-inhibitory markers or due to impact on other immune-regulatory pathways such as IL-10 in the macrophages. Furthermore, although the deficiency of STAT3 in host myeloid cells aggravated GVHD across multiple models, the magnitude of GVHD aggravation was greater in the major MHC mismatched (BALB/c into B6) model than in the MHC matched multiple miHAs mismatched (C3H.sw into B6) model of acute GVHD, indicating that the anti-GVHD effect of STAT3 in host myeloid cells, especially MFs may be dependent on the strength of allogenic stimulation. Overall, our data suggest that activating STAT3 in host myeloid cells, decreases allogeneic immune responses. Further efforts will focus on determining the molecular signaling events and the specific effects in MF versus those in neutrophils that are critical for this phenomenon.

We wish to point out certain caveats that are germane to our study. First, the LysM-Cre/STAT3$^{fl/-}$ are not complete knock-outs but are merely deficient (not absent) of STAT3 due to leakiness of the LyM *Cre* driver. Second, the deficiency of STAT3 in the LysM-Cre/STAT3$^{fl/-}$ animals is not limited to MFs, but also includes other myeloid derived cells, specifically neutrophils. Importantly, neutrophils also drive allogeneic T cell responses[50], therefore, our in vivo results are due the net effect of STAT3 deficiency in all host myeloid cells and we cannot formally rule out a role for STAT3-expressing neutrophils mitigating GVHD in the LysM-Cre/STAT3$^{fl/-}$ recipient animals.

We and others previously demonstrated that STAT3 negatively regulates DCs[39, 40, 51]. Interestingly, the HDAC inhibitor, vorinostat, which is currently being investigated in clinical trials, reduces GVHD by modulating DC functions via enhancing STAT3 acetylation thereby promoting the transcription of IDO, which is critical for the amelioration of acute GVHD by HDAC inhibitors[37–40, 52, 53]. Therefore, the activation and acetylation of STAT3 in host MFs, in addition to DCs, may be required for the anti-GVHD activity of HDAC inhibitors and suggests the intriguing possibility that HDAC inhibitors may skew host MFs to an anti-inflammatory phenotype after allo-HCT.

In conclusion, STAT3 expression in host, but not donor myeloid cells, regulates experimental acute GVHD severity and suggest that STAT3 inhibition in these cells could serve as a potential target for development of novel approaches to mitigate clinical GVHD.

MATERIALS and METHODS

Mice

Female BALB/c (H-2^{d}, CD45.2^{+}) and C57BL/6 (B6, H-2^{b+}, CD45.2^{+}) mice were purchased from Charles River Laboratories (Wilmington, MA). B6Ly5.2 (H-2^{b+}, CD45.1^{+}) mice were purchased from National Cancer Institute-Frederick (Frederick, MD). C3H.SW (H-2^{b+}, CD45.2^{+}) mice were purchased from Jackson Laboratory (Bar Harbor, ME). B6-background Stat3$^{fl/+}$ animals were provided by Dr. Akira (Osaka University) and have been described previously[54]. All animals were cared for according to regulations reviewed and approved by the University Committee on Use and Care of Animals of the University of Michigan, based on University Laboratory Animal Medicine guidelines.

STAT3 genotyping

Stat3$^{fl/+}$ females and Stat3$^{fl/+}$ males were crossbred and genotyped to obtained Stat3$^{fl/-}$ as previously described[33]. The mice were inbred and maintained by the University of Michigan Breeding Colony.

Bone marrow derived dendritic cells (DCs)

To obtain bone marrow derived DCs (BMDCs), bone marrow (BM) cells from B6-WT or LysM-Cre/Stat3$^{fl/-}$ animals were cultured with murine recombinant GM-CSF (20 ng/ml; PeproTech, Rocky Mill, NJ) for 7 days and harvested as described previously. BMDCs were isolated by using CD11c (N418) MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) on an autoMACS (Miltenyi Biotec).

STAT3 detection

Peritoneal macrophages (MFs, 1×10^6), purified BMDCs (1×10^6), and whole splenic cells (1×10^6) from naïve B6 or LysM-Cre/Stat3$^{fl/-}$ animals were washed with PBS and lysed with RIPA buffer. Cell lysates were subjected to western blot analysis using the following antibodies: STAT3 (Abcam), β-actin-HRP (Abcam), and Cre-lox (Imegenix) per the manufacturer's instructions.

STAT3 inhibitor study

WT Balb/c animals were irradiated (^{137}Cs source) with 800 cGy total body irradiation (TBI). Immediately after irradiation, mice were treated via intraperitoneal injection with a single dose (20 mg/Kg) of WP1066 (Sigma Aldrich, St. Louis, MO) STAT3 inhibitor dissolved in DMSO/polyethylene glycol (PEG) 300 (1:4, v/v) **(REF)**. Control mice were treated with only diluent. At 24 and 48 hours post injection mice were euthanized and peritoneal (20 ml wash) and whole splenic CD11b+ cells were analyzed. Cells were analyzed on an Attune NxT Acoustic Focusing Flow Cytometer (Life Technologies, Carlsbad, CA).

Quantitative real-time PCR

Total RNA was obtained by phenol/chloroform separation. *Gapdh* (forward: 5′-GACATGCCGCCTGGAGAAAC-3′; reverse 5′-AGCCCAGGATGCCCTTTAGT-3′) was used as the control house-keeping gene. The primers for Stat3$^{fl/fl}$ detection were: forward 5′-GGGGTGAGAGTTACCGTGAA-3′ and reverse 5′-CACACACACACAAGCCATCA-3′. Real-time PCR was performed with a SYBR green PCR mix (ABI biosystems, Waltham, MA) in a Realplex Eppendorf Real-Time PCR instrument (Eppendorf, Hamburg, Germany).

FACS analysis

Flow cytometry was performed as previously described. Briefly, to analyze phenotypes of peritoneal MFs, isolated cells were suspended in FACS wash buffer (2% BSA in PBS) and stained with conjugated monoclonal antibodies (FITC-, PE-, or APC). Anti-mouse monoclonal antibodies (mAb) for CD11b (M1/70), F4/80(BM8), I-Ab (AF6-120.1), CD40 (3/23), CD80 (16-10A1), Gr-1 (RB6-8C5), PD-L2 (TY25), and B220 (RA3-6B2) were purchased from Biolegend (San Diego, CA). PE-conjugated anti-PD-L1 mAb (M1H5) was purchased from eBioscience (San Diego, CA). After staining, cells were washed with FACS wash buffer and fixed with FACS Lysing Solution (BD Biosciences). Samples were analyzed on a C6 cytometer (BD bioscience). To analyze donor T cell expansion after allo-BMT, isolated spleen cells were processed as above and stained with CD4 (RM4-4), CD8a (53-6.7), CD90.2 (53-2.1), CD229.1 (30C7), CD45.1 (A20), and CD45.2 (104) mAbs purchased from Biolegend. For intracellular staining of Foxp3, cells were washed with permeabilization buffer (eBioscience) and stained with PE-conjugated anti-Foxp3 mAb (FJK-16s; eBioscience) for 30 min at 4°C. The cells were then washed with FACS wash buffer and analyzed on a C6 cytometer (BD bioscience).

Bone marrow transplantation

Host animals were irradiated (^{137}Cs source) with 800-1100 cGy total body irradiation (TBI) on day -1 before allo-BMT. Donor BM cells were harvested from the femur and tibia. Where indicated, T cells were magnetically depleted from the BM (TCD-BM) using mouse CD90.2-microbeads and MACS™ LS columns (Miltenyi Biotec). Splenic T cells were magnetically isolated by using mouse CD90.2-microbeads and MACS™ LS columns. T cells purity was checked by flow cytometry and adjusted accordingly. Syngeneic or allogeneic BM (either whole or TCD-BM) and T cells were infused through the tail vein. Host mice were housed in sterilized micro-isolator cages and maintained on acidified water (pH<3) for three weeks after allo-BMT, as described previously[55]. Survival was monitored daily, and clinical GVHD was assessed weekly. All animal studies were performed per the Institutional Animal Care and Use Committee guidelines of the University of Michigan.

Mixed lymphocyte reaction (MLR)

Splenic T cells from B6- or BALB/c-WT animals (magnetically separated by MACS using CD90.2 microbeads) were used as responders and B6-WT or B6- LysM-Cre/Stat3$^{fl/-}$ derived BMDCs or peritoneal MFs were used as stimulators. 1×10^5 T cells and irradiated (20Gy) 2.5×10^3 BMDCs or 1×10^5 MFs were co-cultured on 96-well U-bottom plates for 72 hours. The incorporation of ^{3}H-thymidine (1μCi/well) by proliferating T cells during the final 16 hours of co-culture was measured by a Betaplate reader (Wallad, Turku, Finland).

ELISA

IFN-γ, TNF-α, IL-6, Il-17A, and IL-10 were measured in culture supernatants or mouse serum by ELISA with specific anti-mouse mAbs for capture and detection. The appropriate standards were purchased from BD Systems (TNF-α and IL-6) or BD OptEIA (IFN-γ, IL-17A and IL-10). Assays were performed according to the manufacturer's protocol and read at 450 nm using a microplate reader (Molecular Devices).

Histology

Formalin-preserved livers and GI tracts were embedded in paraffin, cut into 5mm-thick sections and stained with hematoxylin and eosin (H&E) for histological examination. Slides were scored in a blind fashion by Dr. C Liu using a semi-quantitative scoring system that assess the abnormalities known to be associated with GVHD.

Statistical analysis

Student's t-test was used for *in vitro* data, and the Wilcoxon rank test was used to analyze survival data. $p < 0.05$ was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- STAT3 negatives regulates macrophage responses
- Expression of STAT3 in host derived myeloid cells such as macrophages and neutrophils restrains allo-immune responses and reduces GVHD severity
- Expression of STAT3 in donor derived myeloid cells is dispensable for GVHD severity

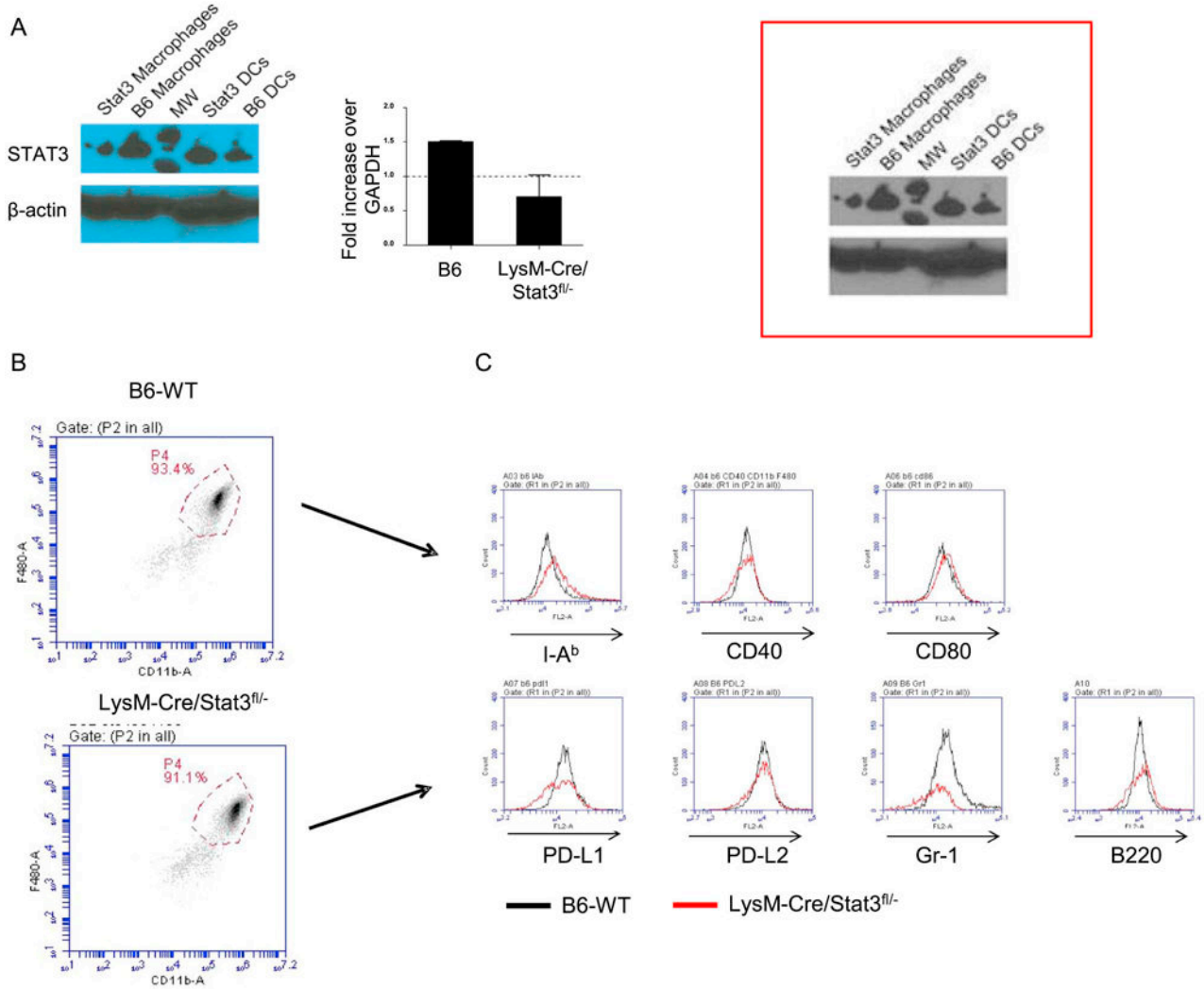

Figure 1. LysM-Cre/STAT3$^{fl/-}$ aanimals showed reduced expression of STAT3 in macrophages (MF)

(A)Western blot showing total STAT3 (top) and β-actin (middle) for B6 macrophages (MFs), LysM-Cre/STAT3$^{fl/-}$ MFs, B6-WT dendritic cells (DCs) and LysM-Cre/STAT3$^{fl/-}$ DCs. (bottom) Relative increase in RNA expression of STAT3 in both B6-WT and LysM-Cre/STAT3$^{fl/-}$ MFs by qRT-PCR. (B)Representative dot-plots and gates of peritoneal CD11b^{+}F4/80^{+} cells from B6-WT or LysM-Cre/STAT3$^{fl/-}$ animals are shown. (C) Representative expression levels of MHC class II (I-Ab), CD40, CD80, PD-L1, PD-L2, Gr-1 and B220 on CD11b^{+}F4/80^{+} peritoneal MFs from B6-WT or LysM-Cre/STAT3$^{fl/-}$ animals are shown. All error bars show the mean ± SEM.

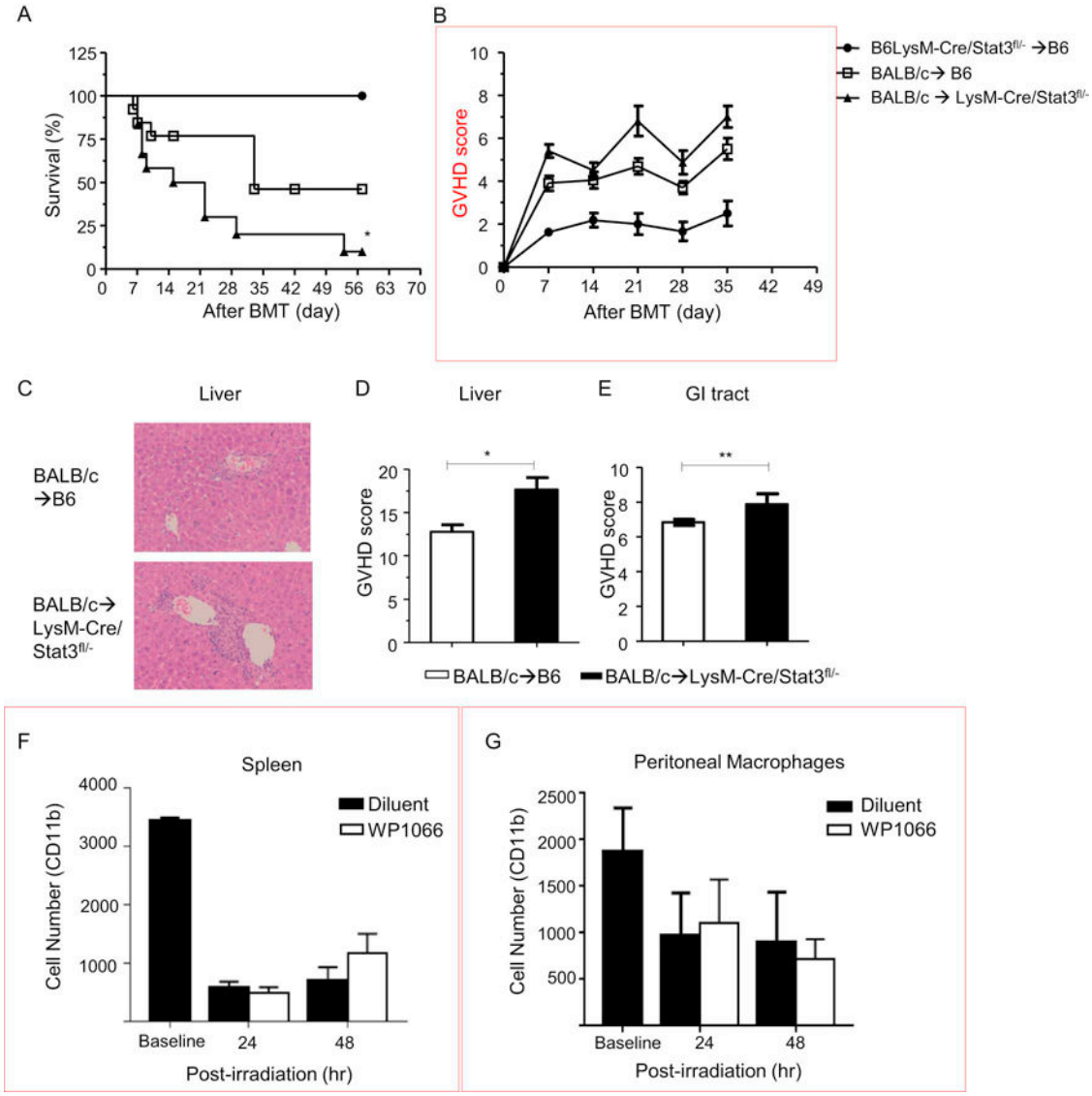

Figure 2. Reduced STAT3 expression in host myeloid APCs amplifies the severity of acute GVHD

Host B6-WT and B6-LysM/Cre STAT3$^{fl/-}$ animals were lethally irradiated (11 Gy) on day −1 and infused with 2 × 10^6 CD90.2$^+$ T cells along with 5 × 10^6 bone marrow (BM) cells from either syngeneic B6 or allogeneic BALB/c animals on day 0. (A) Survival (n=12–16 per group). Data are combined from three experiments with similar results. (B) GVHD clinical score. (C) Representative hematoxylin and eosin (H&E) stained images of liver on day 14 after allo-BMT are shown. (D and E) Histopathological GVHD scores from liver (D) and gastrointestinal (GI) tract (E) on day 14 after allo-BMT (n=12 per group, pooled from two experiments). Balb/c-WT mice were irradiated (8 Gy) and then immediately i.p. injected with 20 mg/Kg WP1066 STAT3 inhibitor or diluent. At 24 and 48 hours after injections mice were euthanized and whole spleens (F) and peritoneal (G) macrophages and were harvested (n=3 per group). Cells were analyzed for CD11b+ cells within the myeloid gate. All error bars show the mean ± SEM. **p<0.01, *p<0.05.

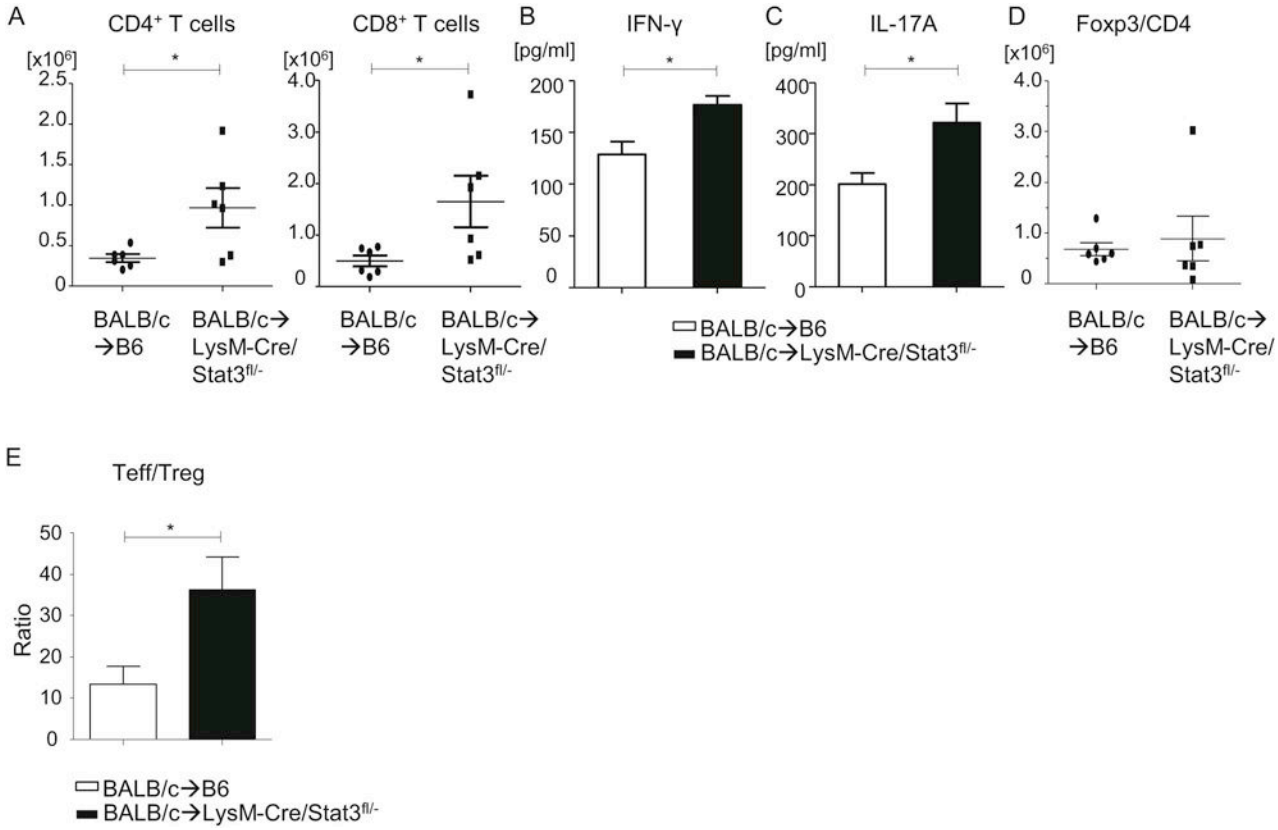

Figure 3. Deficiency of STAT3 in host MFs enhances donor T cell expansion after allogeneic BMT

Host B6-WT and B6-LysM/Cre STAT3$^{fl/-}$ animals were lethally irradiated (11 Gy) on day −1 and infused with 2 × 10^6 CD90.2$^+$ T cells along with 5 × 10^6 bone marrow (BM) cells from either syngeneic B6 or allogeneic BALB/c animals on day 0. (A) To evaluate donor T cell (H-2k^{d+}CD4$^+$ aor H-2k^{d+}CD8$^+$) expansion, spleen cells from B6-WT or B6 LysM/Cre STAT3$^{fl/-}$ animals were harvested on day 14 after allogeneic bone marrow transplantation (allo-BMT), stained, and analyzed by flow cytometry (n=6 per group, pooled from two experiments). (B and C) Serum was collected from recipients on day 14 and IFN-γ (B) and IL-17A (C) levels were determined by ELISA. (n=6 per group, pooled from two experiments). (D and E) Donor CD4$^+$Foxp3$^+$ regulatory T cell (T_{reg}) expansion (D) and the ratio of T_{Eff} cells (CD4$^+$FoxP3$^-$ and CD8$^+$FoxP3$^-$) to T_{reg} cells (E) on day 14 after allo-BMT are shown. All error bars show the mean ± SEM. *p<0.05.

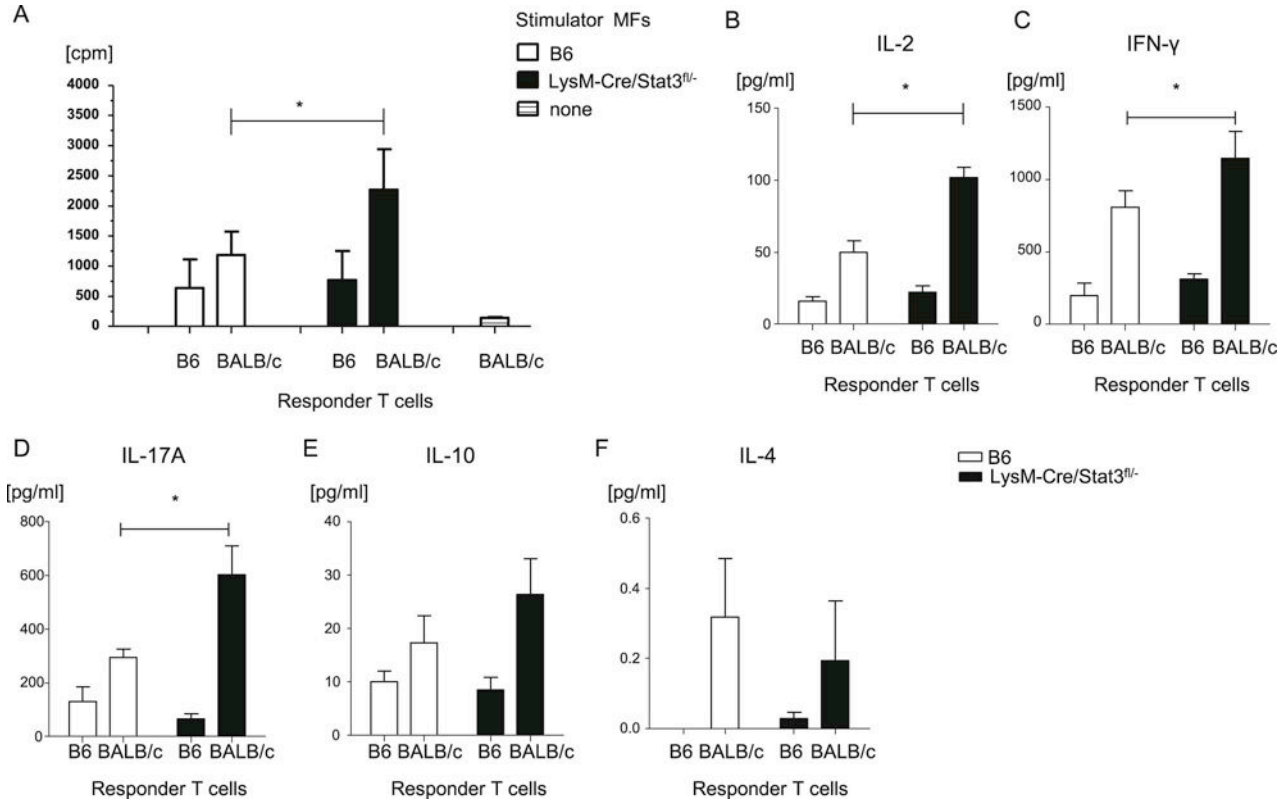

Figure 4. STAT3 deficient macrophages show enhanced stimulation of allogeneic T cells in vitro

(A) Peritoneal MFs from B6-WT and LysM-Cre STAT3$^{fl/-}$ animals were used as stimulators in an MLR with T cells from either syngeneic B6 or allogeneic BALB/c animals and analyzed for T-cell proliferation via ^{3}H-thymidine incorporation at 72 h. (B-F) Supernatants from MLR cultures were collected at 72 h and analyzed for IL-2 (B), IFN-γ (C), IL-17A (D), IL-10 (E), and IL-4 (F) by ELISA. The data are representative of three independent experiments. Error bars show the mean ± SEM. * $p<0.05$.

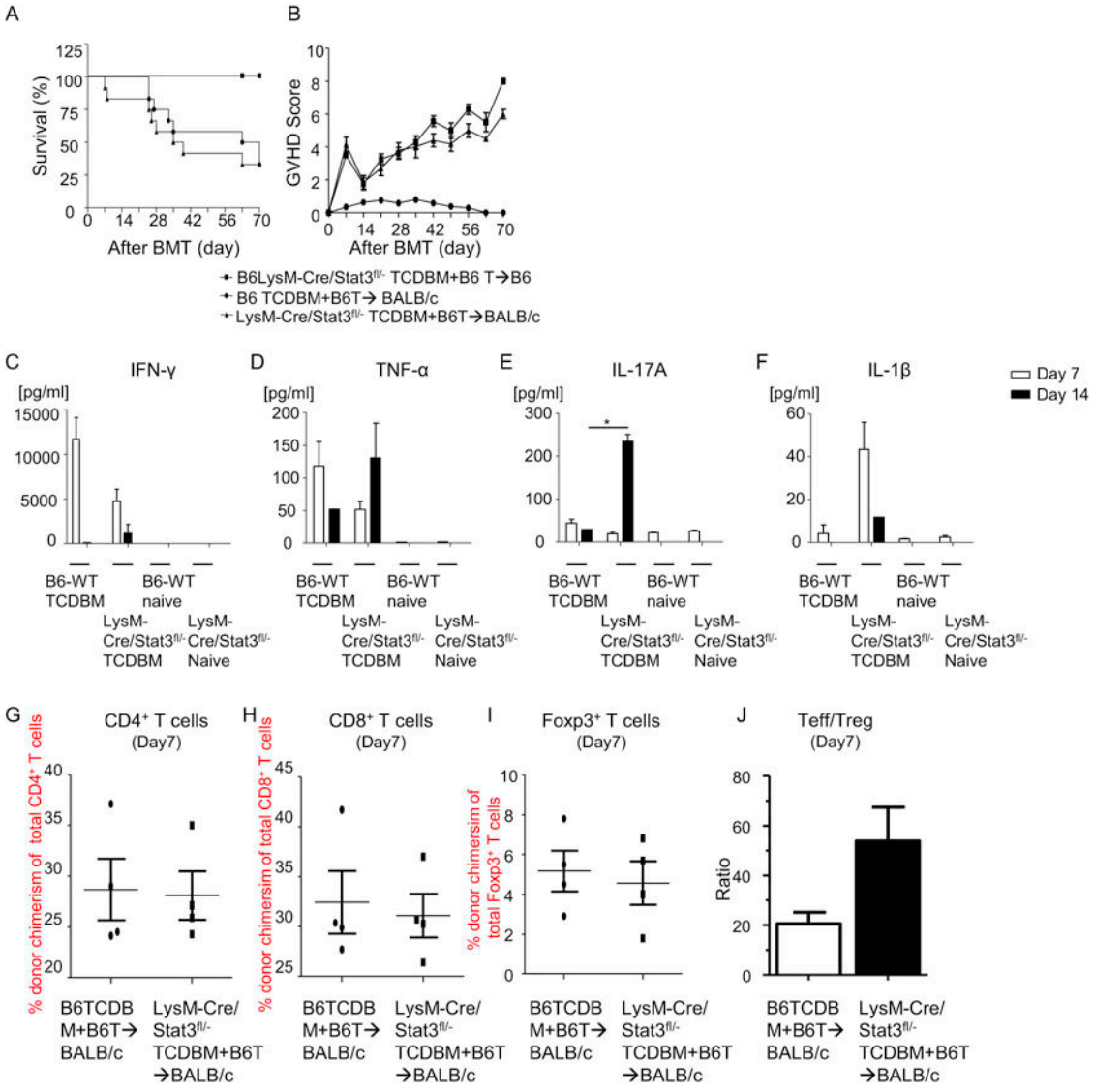

Figure 5. STAT3 deficiency in donor myeloid cells is dispensable for acute GVHD severity

syngeneic controls were irradiated (8 Gy) on day −1 and infused with 5 × 10^6 TCD-BM cells along with 1 × 10^6 CD90.2$^+$ T cells from B6-LysM-Cre/STAT3$^{fl/-}$ animals on day 0 (■, n=12). Allogeneic host BALB/c animals were irradiated as above and infused with B6-WT 2×10^6 CD90.2$^+$ T cells along with 5×10^6 TCD-BM from either WT-B6 (•, n=12) or B6-LysM/Cre STAT3$^{fl/-}$ animals (▲, n=12). The recipients were monitored for (A) Survival and (B) GVHD clinical scores on a weekly basis. Data are combined from three experiments with similar results. (C–F) Serum was collected from recipients on day 7 and day14 after allo-BMT and cytokine levels were determined by ELISA for IFN-γ (C), TNF-α (D), IL-17A (E), and IL-1β (F). *p<0.05. (G-I) Spleen cells from B6-WT or B6 LysM/Cre STAT3$^{fl/-}$ animals were harvested on day 7 after allo-BMT, stained, and analyzed by flow cytometry. Donor T cell (H-2k^{b+}CD4$^+$ a(G) or H-2k^{b+}CD8$^+$ (H)) expansion, donor CD4$^+$Foxp3$^+$ T_{reg}cell expansion (I), and the ratio of T_{Eff} cells (CD4$^+$FoxP3$^-$ and

$CD8^+FoxP3^-$) to T_{reg} cells (J) on day 7 after allo-BMT are shown (n=4 per group, pooled from two experiments). Error bars show the mean ± SEM.