Targeting Sirt-1 Controls Graft-versus-Host Disease by Inhibiting T-cell Allo-Response and Promoting Treg Stability in Mice

Anusara Daenthanasanmak[1], Supinya Iamsawat[1], Paramita Chakraborty[2], Hung D. Nguyen[1], David Bastian[1], Chen Liu[3], Shikhar Mehrotra[2], and Xue-Zhong Yu[1,4*]

[1]Department of Microbiology and Immunology; [2]Department of Surgery, Hollings Cancer Center, Medical University of South Carolina, Charleston, SC; [3]Department of Pathology and Laboratory Medicine, Rutgers-Robert Wood Johnson Medical School, New Brunswick, NJ; [4]Department of Medicine, Medical University of South Carolina, Charleston, SC.

*Corresponding Author:

Dr. Xue-Zhong Yu, MD, MS

Professor, Department of Microbiology and Immunology

HCC350, MSC 955

Medical University of South Carolina

86 Jonathan Lucas Street, Charleston, South Carolina 29425-5090, USA.

Phone: 843-792-4756

E-mail: yux@musc.edu

Supplemental Methods and Figures

Graft versus leukemia mouse models

For MLL-AF9-GFP model, 2×10^4 Mixed Lineage Leukemia (MLL) including myeloid leukemia–expressing CD11b and GFP were used.[21] For P815 model, 5×10^3 P815-fluc mastocytoma/mouse were injected intravenously together with BM alone or BM plus T cells. MLL-AF9-GFP tumor growth was monitored in mice peripheral blood and determined for CD11b$^+$ (myeloid marker) GFP$^+$ double positive cells by flow cytometry. P815-fluc tumor growth was measured weekly with bioluminescent imaging (BLI) using Xenogen-IVIS ® 200 *in vivo* Imaging System (Perkin-Elmer). For treatment group, Ex-527 was administered at 2 mg/kg/day/mouse by i.p. daily at day 0 of allo-BMT for three weeks.

Chronic GVHD mouse models

A major MHC-mismatched (B6 to BALB/c) or major MHC-matched but miHA mismatched mouse models (B10.D2 to BALB/c) mouse models were used as previously described.[20,22] Briefly, BALB/c recipients were lethally irradiated at 700 cGy and transplanted i.v. with 5×10^6 TCD-BM cells plus 0.5×10^6 CD25-depleted splenocytes from B6 donors or 5×10^6 whole splenocytes B10.D2 donors. The recipients were monitored for survival, body weight loss, and clinical scores. For Ex-527 or PBS administration, recipient mice were injected i.p. with 2 mg/kg/mouse daily at day 0 of allo-BMT for four weeks or injected on day 28 post allo-BMT for three weeks. On day 50 or 60 after BMT, recipient spleens were harvested for T and B-cell analysis.

T cell purification and T-cell depleted bone marrow

T cells were purified from spleen and lymph nodes by negative selection with magnetically labeled biotin-conjugated antibodies against CD25 (clone PC61.5), CD45R (clone RA3-B2), CD49b (clone DX5), CD11b (clone M1/70), and Ter-119 (clone Ter-119); all antibodies were from eBioscience) and anti-biotin microbeads (Miltenyi Biotec). Bone marrow (BM) was extracted from donor tibia and femurs and T cells were depleted from BM (TCD-BM) by incubated with anti-Thy1.2 Abs (clone 30H12, Bio X-cell) for 30 min at 4°C followed by rabbit complement incubation (RC-100, Immunocor GTI diagnostics, Inc.) at 37°C for 45 min.

Antibodies and flow cytometry

The following antibodies were used for cell surface staining: anti-CD4 (clone RM4-5), anti-CD8 (clone 53-6.7), anti–CD45.1 (clone A20), anti-CD229.1 (clone 30C7), anti-CD11b (clone M1/70) were purchased from BD Biosciences. Anti–H-2K^{b} (clone AF6-88.5.5.3), anti-CD25 (clone PC61.5), anti–B220 (clone RA3-6B2), anti-CXCR5 (clone SPRCL5) and anti–PD-1 (clone J43), anti-CD86 (clone GL1) were purchased from eBioscience. Anti-CD138 (clone 281-2) and anti-I-Ab (clone AF6-120.1) were purchased from Biolegend. To measure intracellular cytokines, cells were stimulated for 4 hours at 37°C with PMA (100 ng/ml; Sigma-Aldrich) and ionomycin (100 ng/ml; Sigma-Aldrich) in the presence of GolgiStop (BD Biosciences). Fixation and permeabilization were performed using Cytofix/Cytoperm Plus (BD Biosciences) or FoxP3/Transcription Factor Staining Buffer Kit, followed by staining with the appropriate antibodies, including anti–

IFN-γ (clone XMG1.2), anti–IL-17 (clone TC11-18H10.1), anti-Foxp3 (clone FJK-16s), anti-IL-21 (clone FFA21) and anti-BCL6 (BCL-DWN) were purchased from eBioscience. Anti–IL-4 (clone 11B11), anti-TNFα (MP6-XT22), anti-pSTAT5 (Clone 47/STAT5, pY694) were purchased from BD Biosciences. LIVE/DEAD yellow cell staining kit (catalog L-34968) and CFSE (catalog C1157) were purchased from Invitrogen. Stained cells were analyzed using BD LSR II (BD Biosciences) and FlowJo (Tree Star). Serum cytokine levels in recipient mice were quantified by using a cytometric bead assay according to the manufacturer's instructions (BD Biosciences).

In vitro T-cell polarization

$CD4^+CD25^−$ cells isolated from WT or Sirt-$1^{-/-}$ donors were stimulated with allogeneic DCs or syngeneic APCs with 1 μg/mL anti-mouse CD3ε (clone 145-2C11). 10 ng/ml mIL-12 and 1 ng/ml mIFN-γ were used for Th1 polarization. For western blot analysis, polarized cells were pre-treated with 2 μM Trichostatin A (TSA, Sigma Aldrich) for 45 min before harvested to induce basal protein acetylation. For Th17 polarization, cultures were supplemented with 2 ng/mL TGF-β, 10 ng/mL IL-6 and 10 μg/mL anti-IFN-γ mAb. For iTreg generation, 5 ng/ml IL-2, 5 ng/ml TGF-β, and 40 nM retinoic acid (RA) were included in cell culture. All cytokines were purchased from PeproTech. On day 3, cells were harvested for phenotype and stained for flow cytometry.

In vitro and *in vivo* mixed lymphocyte reactions

T-cell-depleted CD25 were purified from WT or Sirt-$1^{-/-}$ spleens and lymphnodes and labeled with CFSE (Invitrogen, Molecular Probes Inc.). For *in vitro* experiments, 0.2×

10^6 T cells were cocultured with 0.6×10^6 T cell–depleted splenocytes for 5 days in the presence of DMSO or 10 μg/ml Ex-527 (Selleckchem) or 10 μg/ml p53 inhibitor (Pifithrin-μ, Selleckchem) or in combinations. To measure intracellular cytokines, cells were stimulated for 4 h at 37°C with PMA plus ionomycin and GolgiStop was added for the last 2 h of incubation. For *in vivo* experiments, 2×10^6 CFSE labeled T cells were injected i.v. into lethally irradiated BALB/c recipient mice for 3 days. Recipient spleens were harvested, T cells were stained and analyzed for percentages of CFSE dilution (gated on donor H-2^{b+}) and cytokine production by flow cytometry.

***In vitro* and *in vivo* iTreg stability assay**

Allogeneic-CD4 iTregs were generated as previously described.[23] Briefly, purified $CD4^+CD25^-$ T-cell were co-cultured with enriched allogeneic DCs at 10:1 ratio in the presence of 5 ng/ml IL-2, 5 ng/ml TGF-β, and 40 nM retinoic acid (RA) for 5 days. iTregs were enriched from bulk culture using positive-selection with CD25 microbeads (Miltenyi Biotech). To test their stability *in vitro*, CD4 iTregs were enriched and co-cultured with allogeneic APCs at 1:3 ratio in the presence of IL-2 alone (iTreg favored condition) or IL-2 plus IL-12 (Th1 favored condition). On day 3, iTregs were harvested and analyzed for Foxp3 expression. For *in vivo* assay, lethally irradiated BALB/c mice were adoptively transferred with $5\text{x}10^6$ $Rag1^{-/-}$ BM and 0.5-$1\text{x}10^6$ CD4 iTregs ($Ly5.2^+$). Three days later, CD25-depleted T-cells isolated from C57BL/6 (Ly5.1) congenic mice were labeled with CFSE and injected i.v. to recipient mice to induce GVHD. Stability of iTregs and IFN-γ^+ $Ly5.1^+$ T cells were analyzed on day 7 and day 14 after allo-BMT.

Lymphocyte isolation from recipient liver

Liver of recipient mice were harvested on day 14 after allo-BMT. The organs were homogenized and passed through a 100-μm cell strainer. Pellets were resuspended in PBS, overlaid on Ficoll (Corning), and centrifuged at 500 g for 20 minutes. Lymphocytes were recovered from the interface after centrifugation. For intracellular cytokines staining, cells were stimulated for 4 h at 37°C with PMA plus ionomycin and GolgiStop was added for the last 2 h of incubation. After 4 h, cells were stained and analyzed by flow cytometry.

Histological analysis

Samples of skin, liver, small intestine, large intestine and lung were harvested from transplanted recipients on day 14 after transplant and immediately fixed in 10% formalin and washed with 70% ethanol. Samples were then embedded in paraffin, cut into 6-μm thick sections, and stained with H&E. Data were presented as individual GVHD target organ. All slides for GVHD analysis were coded and read in a blinded fashion. For Trichrome Staining, 6-μm cryo-sections were stained with a Masson tri-chrome staining kit (Sigma-Aldrich) for detection of collagen deposition. Collagen deposition was quantified on trichrome- stained sections as a ratio blue staining area to total staining area by using ImageJ 1.51s (National Institutes of Health, USA) analysis tool.

DNA methylation assay

Genomic DNA was isolated from enriched WT or Sirt-1$^{-/-}$ CD4iTregs using Blood & Tissue Genomic DNA Extraction kit (Qiagen). Extracted genomic DNAs were converted

by the EZ DNA Methylation Kit (Zymo Research). Antisense strands of bisulfite-treated DNA were then subjected to PCR for amplification of CNS2.[26] The PCR products obtained were cloned into the pCR2.1-TOPO vector (Thermo Fisher Scientific), and 14 individual clones from each sample were sequenced with M13-reverse primers. Sequencing results for methylation status were analyzed for 12 CpG positions in the CNS2 region by using quantification tool for methylation analysis (http://quma.cdb.riken.jp/).

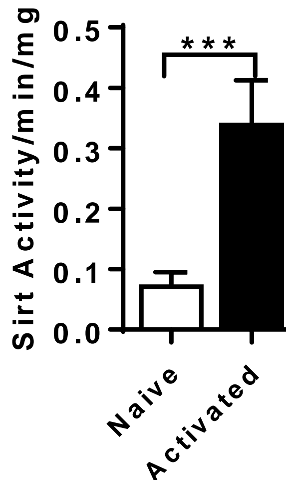
Human Sirt-1 activity

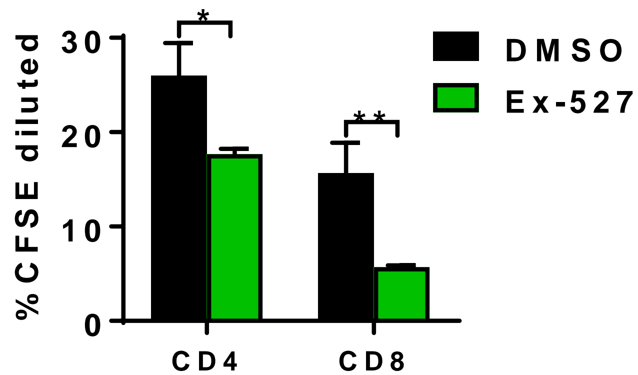
Naïve human T cells were isolated from PBMCs of HLA-A*02-negative healthy donor using Pan T cell isolation kit following the manufacturer's protocol (Miltenyi Biotec). Allogeneic DCs were generated from $CD14^+$ monocytes isolated from PBMCs of A*02-positive donor using CD14 isolation beads (Miltenyi Biotec). Monocytes were kept in the presence of recombinant human GM-CSF and IL-4 (50 ng/ml each, Peprotech) for 5 days. On day 5, allogeneic DCs were matured with 10 µg/ml LPS for 24 h. Purified human T cells were co-cultured with matured allogeneic DCs at 10:1 ratio for further 5 days in the presence of DMSO or 10 ug/ml Ex-527. On day 5, nuclear extraction of activated T cells was prepared for Sirt activity assay[27] using the universal Sirt activity assay kit (Colorimetric) following the manufacturer's protocol (Abcam). For human MLR, T cells labeled with CFSE were used for measuring CFSE dilution by flow cytometry.

Western blotting

Whole-cell lysates were collected using lysis buffer supplemented with the protease inhibitor cocktail solution according to the manufacturer's protocol (MCL-1, Sigma-Aldrich). Cell lysates were electrophoresed in precast polyacrylamide gel and blotted onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Inc.). After blocking with 5% nonfat dry milk, the membranes were incubated with primary antibodies overnight at 4°C, followed by washing and adding of a horseradish conjugated secondary antibody for 1 h. Blots were treated with ECL Western Blotting Substrate (Thermo Fisher Scientific) and visualized by exposing to photographic film. All antibodies were from Cell Signaling Technology; Acetyl-p53 (K379), p53 (1C12), Acetylated lysine (9441) and Histone H3 (96C10). Monoclonal anti-β-actin antibody (AC-74) was purchased from Sigma-Aldrich.

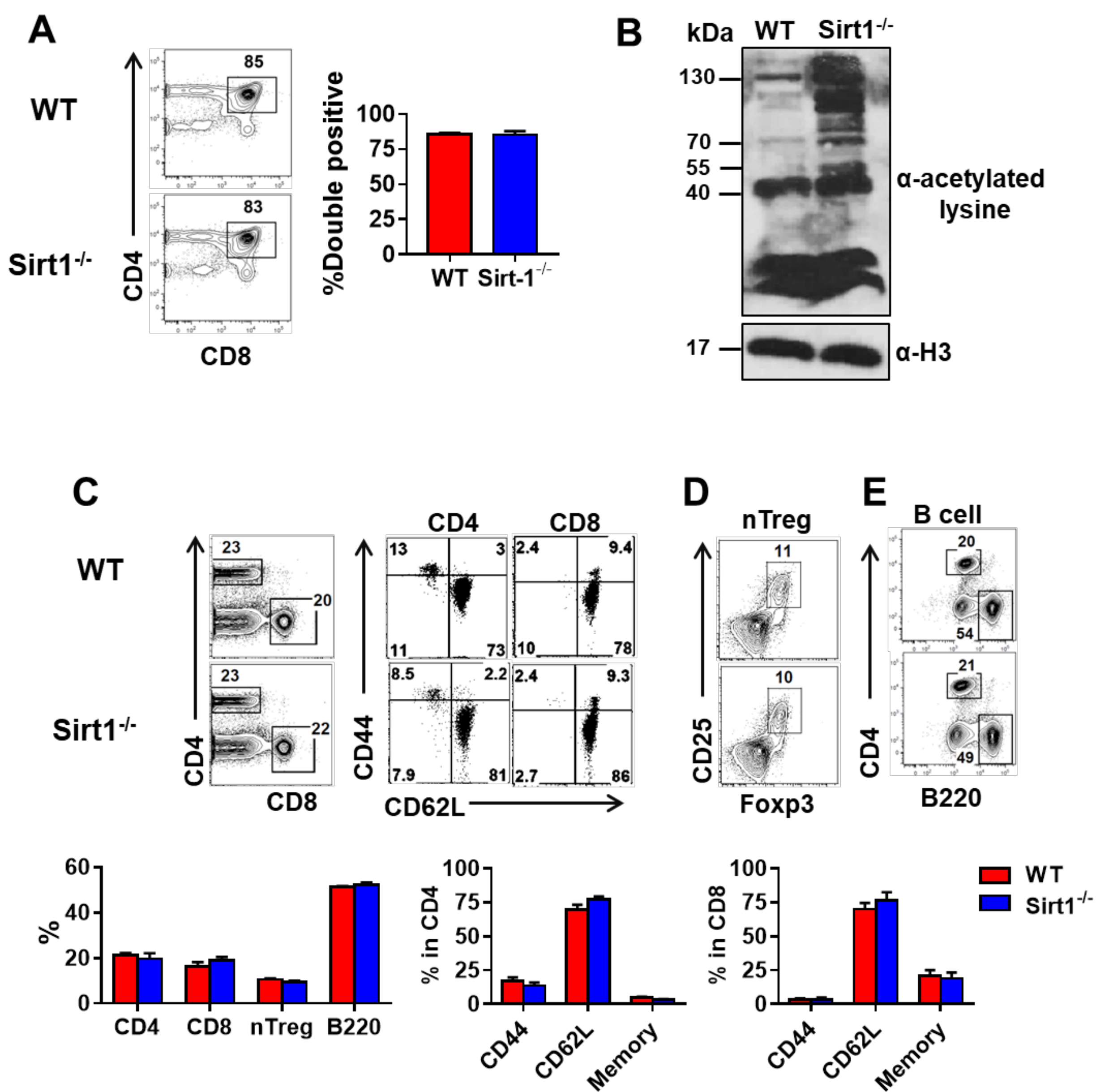
Supplemental figure 1





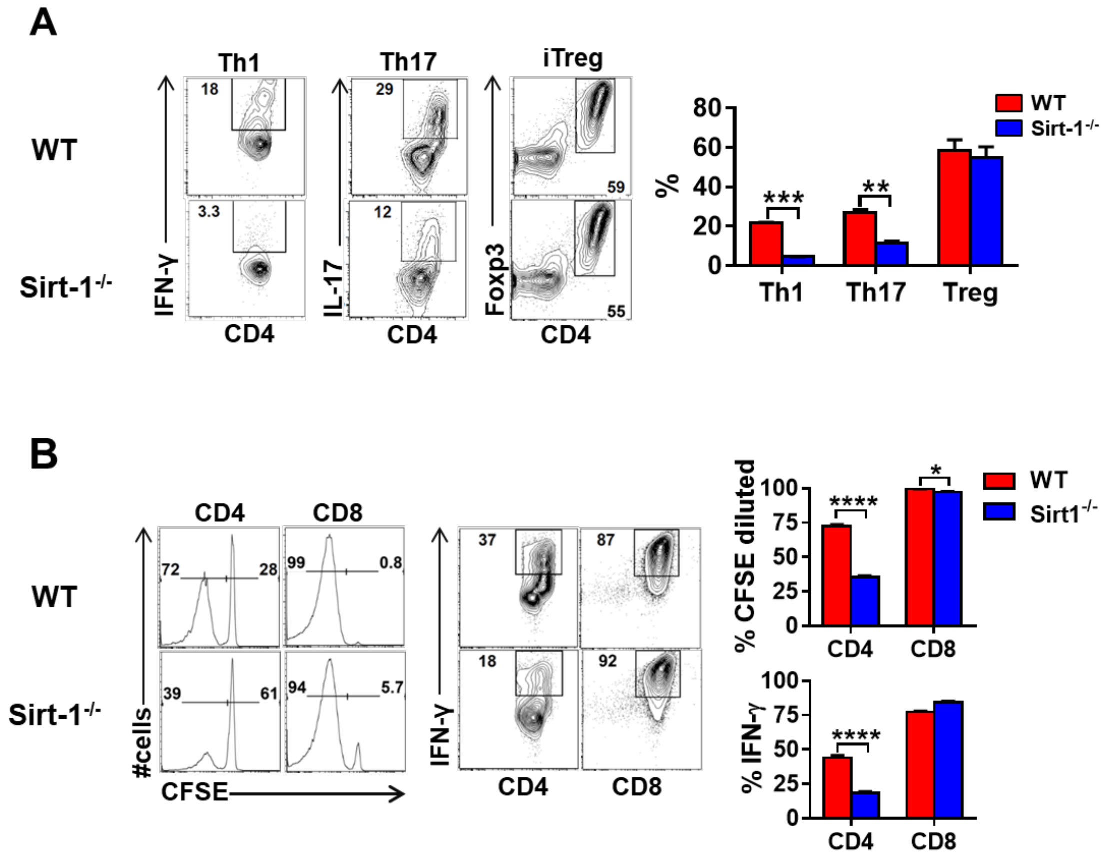
Supplemental figure 1. (A) Sirt-1$^{-/-}$ activity of activated human T cells compared with naïve T cells. Purified human T cells were incubated with allogeneic DCs. On day 5, Sirt activity/min/mg from nuclear extraction of naïve or activated T cells were measured (n=4). **(B)** Purified human T cells were stimulated with allogeneic DCs in the presence of DMSO or Ex-527 at 10 µg/ml for 5 days. Percentages of CFSE diluted T cells measured on day 5 (n=3). Two-tailed-Student's *t*-test was performed to determine statistical analysis, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Supplemental figure 2



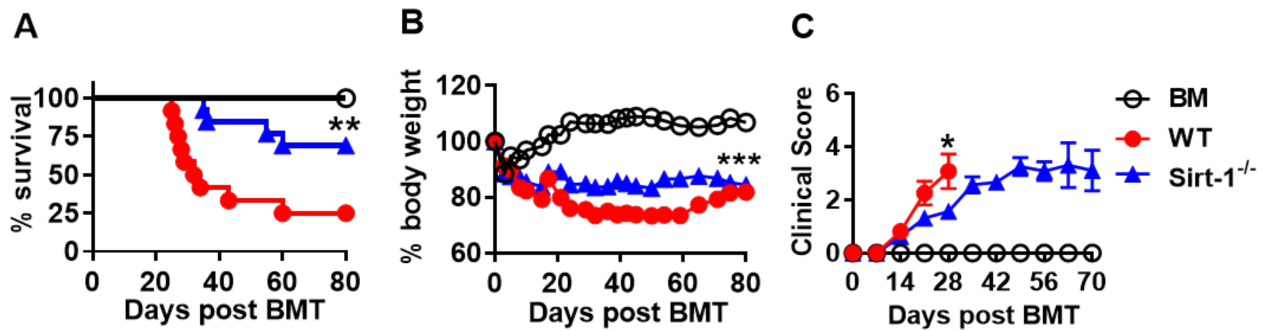
Supplemental figure 2. Phenotypes of immune cells from WT B6 or Sirt-1$^{-/-}$ mice. **(A)** Percentages of double positive-T cells in thymus**.** **(B)** Detection of total acetylation in thymocytes. **(C)** Percentages of CD4, CD8 and T-cell subsets. **(D)** Frequency of nTregs **(E)** B cells in spleen (n=3).

Supplemental figure 3

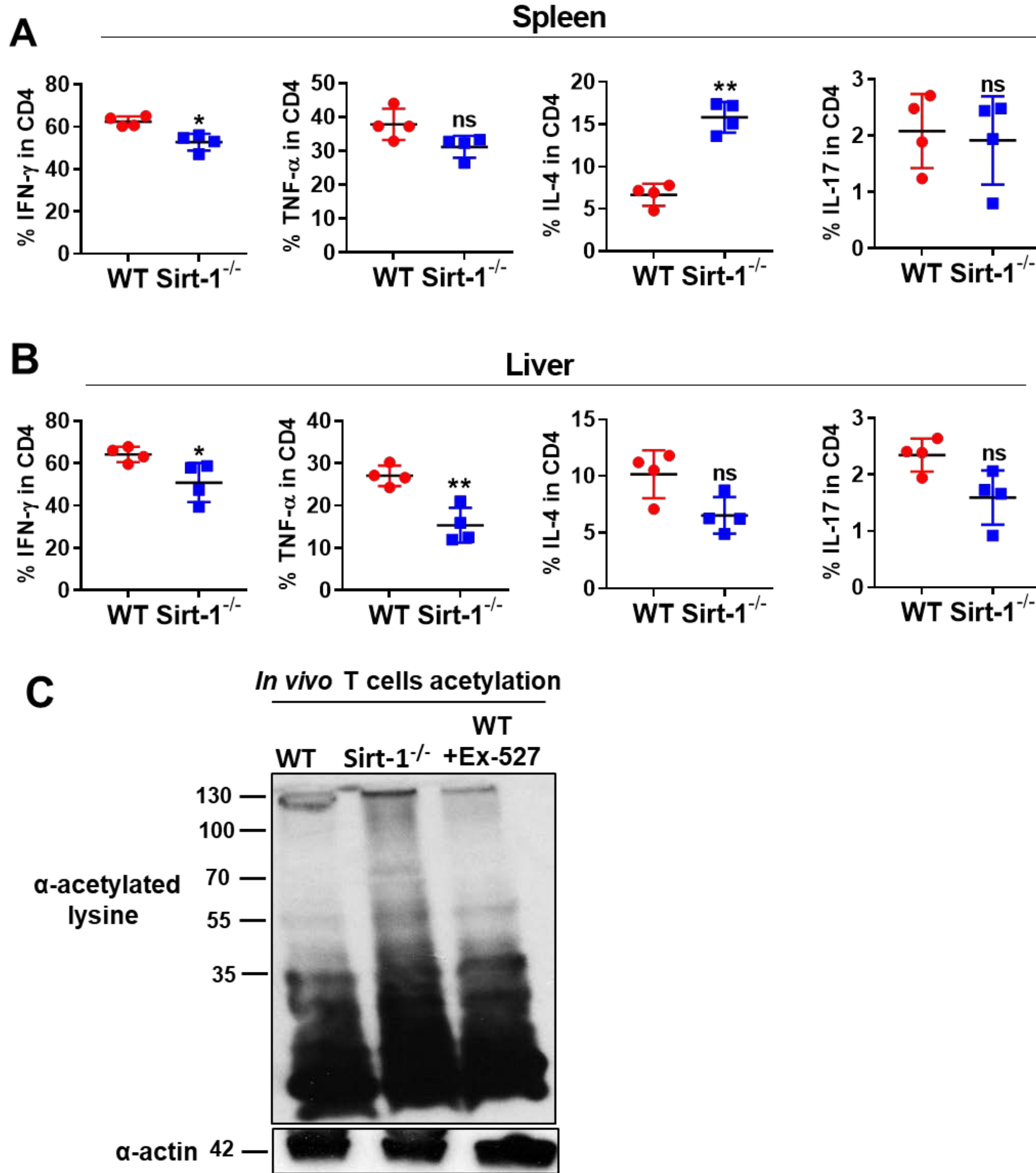


Supplemental figure 3. *In vitro* T-cell polarization and mixed lymphocyte reactions (A) Purified CD4 T cells from WT and Sirt-1$^{-/-}$ donors were stimulated with allogenic DCs under different polarizing conditions; Th1 (10 ng/ml mIL-12 and 1 ng/ml mIFN-γ) or Th17 (2 ng/mL TGFβ, 10 ng/mL IL-6 and 10 µg/mL anti-IFN-γ mAb) or iTreg subsets (5 ng/ml IL-2, 5 ng/ml TGF-β, and 40 nM retinoic acid). **(B)** Total T cells isolated from WT or Sirt-1$^{-/-}$ donors were co-cultured with allogeneic APCs for 5 days, and donor T cells were analyzed for percentages of CFSE dilution and IFN-γ production (n=3), Two-tailed Student's *t*-test was used for statistical analysis. *$p< 0.05$, **$p< 0.01$, ***$p < 0.001$, ****$p< 0.0001$.

Supplemental figure 4

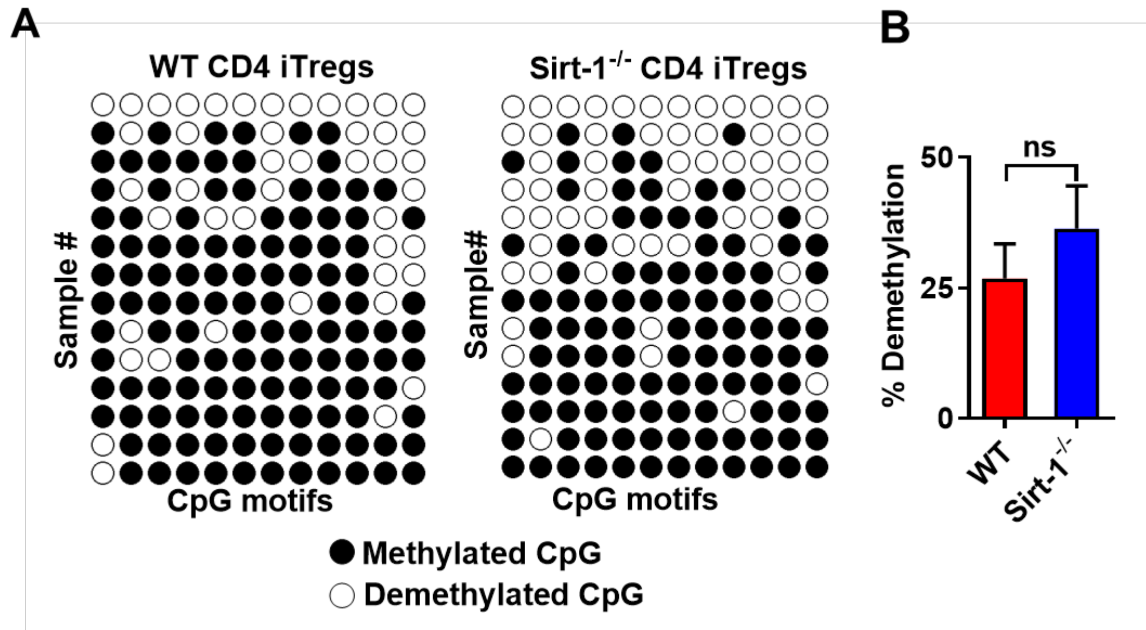


Supplemental figure 4. aGVHD in MHC-matched, B6 to BALB.b model. Lethally irradiated BALB.b (1100 cGy, split dose) mice were transplanted with 5 × 10^6 TCD-BM plus 2.5 × 10^6 CD25-depleted T cells/mouse from WT B6 or Sirt-$1^{-/-}$ donors. Survival **(A)**, body weight loss **(B),** and clinical scores **(C)** were monitored for 80 days (n=10). The log-rank (Mantel-Cox) test and two-tailed Student's *t*-test was used for statistical analysis, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.



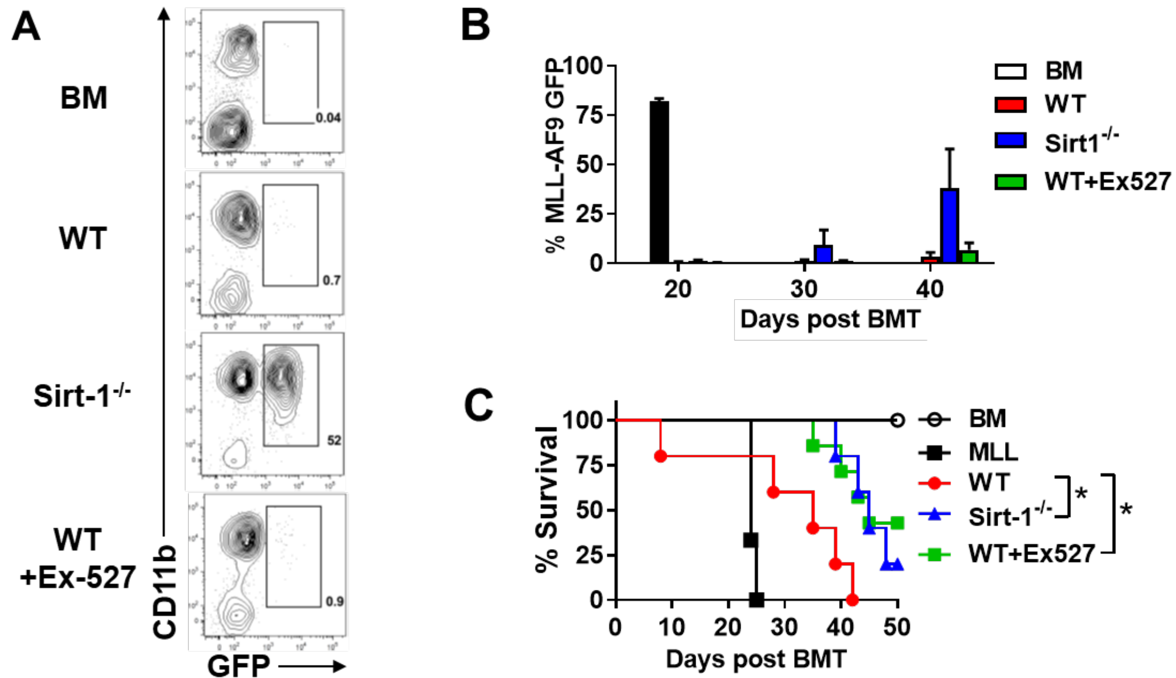
Supplemental figure 5. Effects of Sirt-1 on cytokine production and acetylation of donor T cells *in vivo* after allo-BMT. Two weeks after allo-BMT (B6 to BALB/c model), the spleen and liver of the recipients as indicated were harvested and analyzed. **(A-B)** Average percentages of IFN-γ, TNF-α, IL-4 and IL-17 expressions on gated donor $CD4^+$ T cells from spleen and liver are shown, (n = 4 mice/group). Two-tailed Student's *t*-test was used for statistical analysis. *$p < 0.05$, **$p < 0.01$. **(C)** Global acetylation in T cells isolated from recipient spleens were shown 14-day after allo-BMT.

Supplemental figure 6



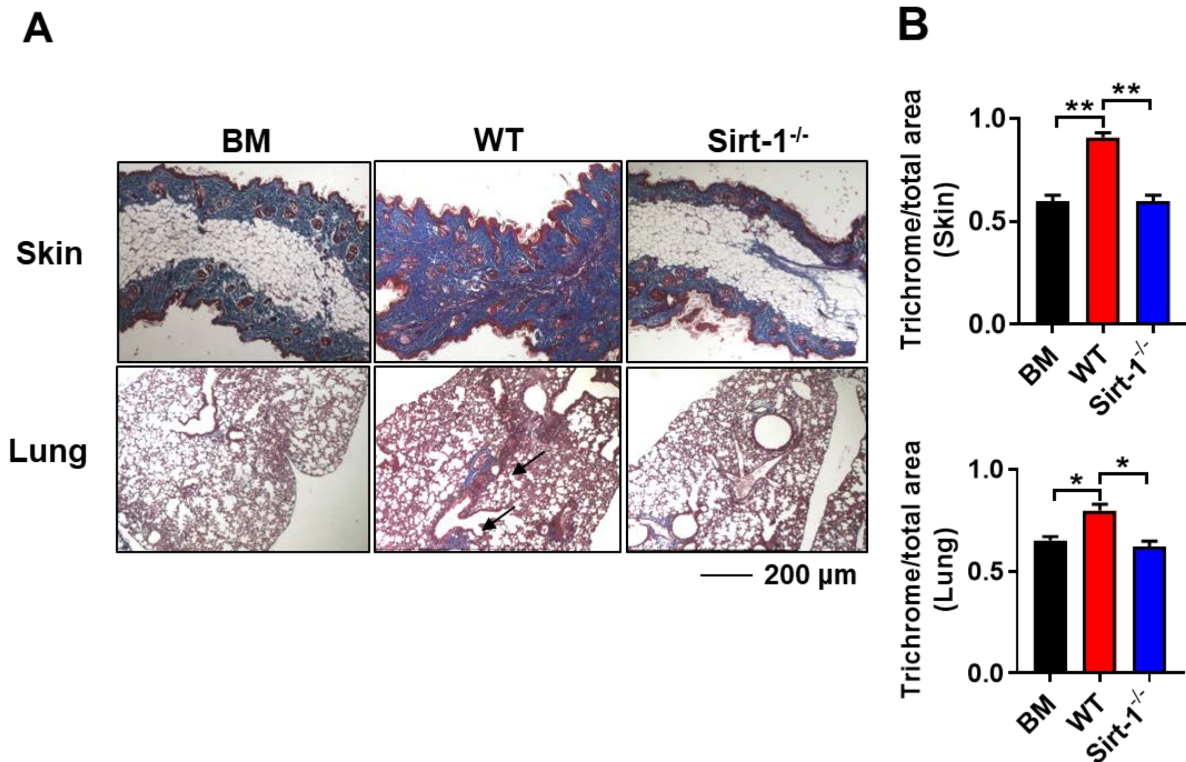
Supplemental figure 6. Effects of Sirt-1 on Foxp3 methylation in iTregs. (A) $CD4^+$ T cells from male WT (C57BL/6) and $Sirt1^{-/-}$ mice were co-cultured with allogeneic DCs under iTreg polarizing condition. On day 5, $CD4^+CD25^{hi}$ (CD4 iTregs) were enriched and subjected to bisulfite sequencing for demethylation pattern of CNS2 region analysis. Methylation status of individual 12 CpG motifs in the anti-sense strands was shown by white (demethylation) or black (methylation) colors. **(B)** Bar graph shows average percentages of demethylation comparing WT CD4 iTreg and $Sirt1^{-/-}$ CD4 iTreg (n=14 clones/group). Two-tailed Student's *t*-test was used for statistical analysis.

Supplemental figure 7



Supplemental figure 7. Pharmacological inhibition of Sirt-1 with Ex-527 alleviates aGVHD while preserving the GVL activity. Lethally irradiated (700 cGy) BALB/c mice were transplanted with 5×10^6 TCD-BM alone or plus 0.7×10^6 total T cells with nTregs isolated from WT or Sirt-$1^{-/-}$ donors or administered with 2 mg/kg/mouse Ex-527 daily for 3 weeks. **(A)** Representative dot plots of %MLL-AF9-GFP cells ($CD11b^+GFP^+$) from peripheral blood of recipient mice on days 40 are shown **(B)** Bar graph shows average percentages of MLL-AF9-GFP in mice peripheral blood on day 20, 30 and 40. **(C)** **%**Survival (n = 5 mice/group). The log-rank (Mantel-Cox) test was used for statistical analysis of the survival curve, $^*p < 0.05$.

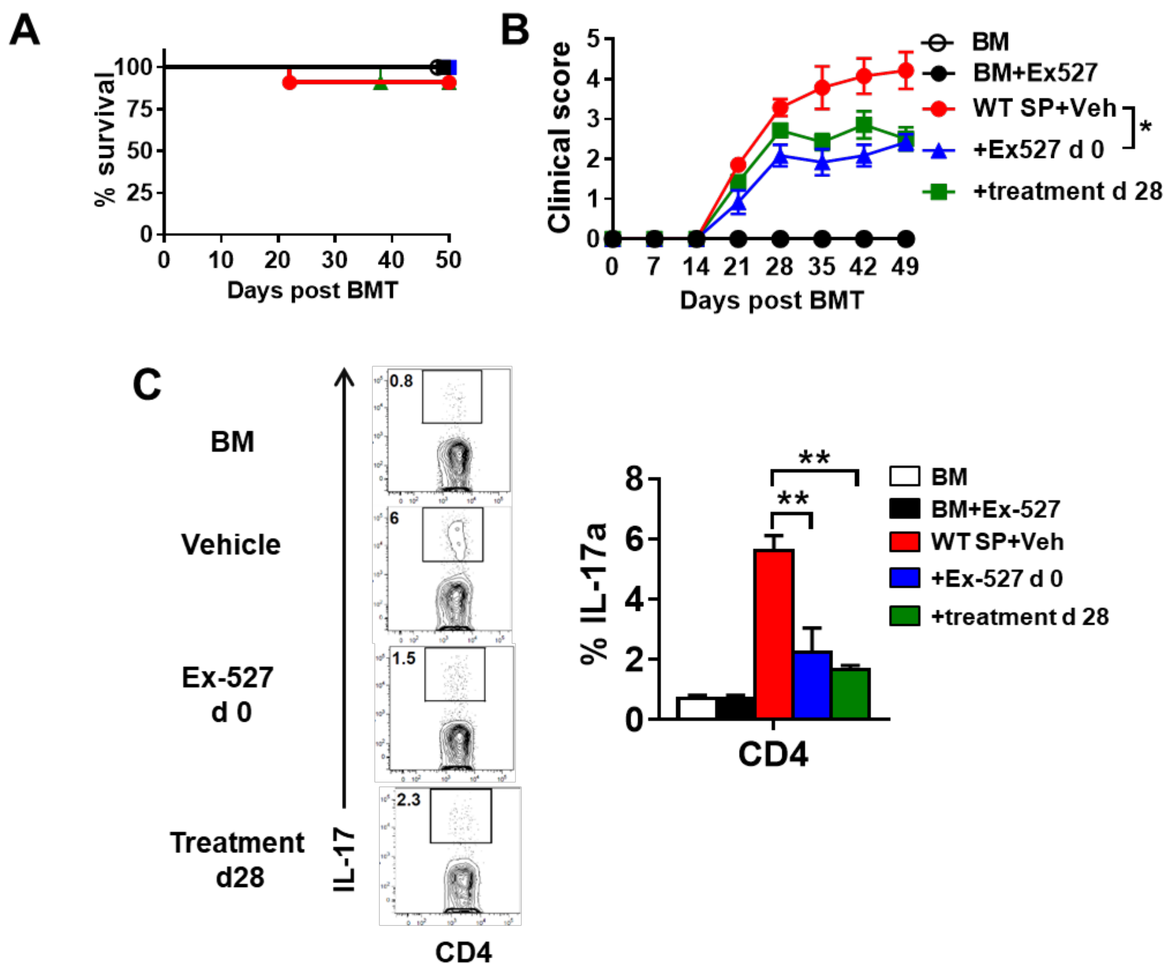
Supplemental figure 8



Supplemental figure 8. Collagen deposition in skin and lung.

Lethally irradiated BALB/c mice were transplanted with 5 × 10^6 TCD-BM or plus 0.5 × 10^6 $CD25^-$ splenocytes from WT or Sirt-$1^{-/-}$ B6 donors. Recipient skin and lung were harvested on day 60 after allo-BMT and processed for Masson's trichrome staining. Representative images of trichome staining from skin and lung are shown. Collagen deposition in skin and lung was qualified using ImageJ as ratio of trichome area to total area of the tissue, (n = 4 mice/group). Two-tailed Student's *t*-test was used for statistical analysis, *$p < 0.05$ and **$p < 0.01$.

Supplemental figure 9



Supplemental figure 9. Effect of Ex-527 treatment on cGVHD. Lethally irradiated (700 cGy) BALB/c mice were transplanted with 5 × 10^6 TCD-BM plus 0.5x10^6 splenocytes per mouse isolated from B6 WT mice (B6 to BALB/c). The recipients were injected i.p. with PBS or 2 mg/kg/mouse/day Ex-527 on day 0 for four weeks or starting on day 28 post-transplant for three weeks **(A)** Survival **(B)** cGVHD clinical scores were monitored **(C)** Analysis of IL-17 expressions on gated donor CD4 T cells in recipient spleen on day 50, (n = 5 mice/group). Two-tailed Student's *t*-test was used for statistical analysis, *$p < 0.05$, **$p < 0.01$.