SUPPLEMENTARY APPENDIX

Myeloid differentiation factor 88 signaling in donor T cells accelerates graft-*versus*-host disease

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Supplemental methods

Assessment of GVHD and GVL. In GVL experiments, 1×10^3 P815 cells (H-2d) were i.v. injected to BMT recipients on day 0. Survival after BMT was monitored daily, and the clinical GVHD scores and pathological GVHD scores were assessed. The cause of each death after BMT was determined by a postmortem examination to be either GVHD or tumor death. Leukemia death was defined by the occurrence of either hepatosplenomegaly, macroscopic tumor nodules in the liver and/or spleen, or hind-leg paralysis. GVHD death was defined as the absence of leukemia and by the presence of clinical signs of GVHD, as assessed by clinical GVHD score.

Histological analyses. The sections of the liver, small intestine, and colon were stained with hematoxylin and eosin (H&E). Pictures from tissue sections were taken at room temperature using a digital camera (DP20; Olympus, Tokyo, Japan) mounted on a microscope (BX50; Olympus). Images were acquired using at 10× /0.40 numeric aperture (NA) or a 40×/0.90 NA objective, depending on the desired magnification. Pathological GVHD was assessed using a semiquantitative scoring system.

In vivo bioluminescent imaging (BLI). Recipient mice were injected with 5×10^3 P815 cells firefly luciferase transduced P815 cells (P815-luc) on day 0 and BLI was performed weekly after allo-BMT. Recipient mice were intraperitoneally injected with 25 mg/kg VivoGlo™ Luciferin (Promega, Madison, WI) 5 minutes before imaging, and the whole body bioluminescent signal intensity was determined using IVIS® Spectrum CT imaging system (Caliper Life Sciences, Hopkinton, MA).

Flow cytometric analysis. For intracellular cytokine staining, cells were incubated for 4 hours with a leukocyte activation cocktail and BD GolgiPlug (BD Biosciences, San Jose, CA) at 37°C. Then the cells were permeabilized with a BD Cytofix/Cytoperm solution (BD Biosciences) and stained with mAbs against interferon-γ (IFN-γ), IL-4, and IL-17 listed in Table S1. Tregs were stained with PE-conjugated anti-Foxp3 mAbs (eBioscience, San Diego, CA) after permeabilization using Foxp3 transcription factor staining buffer set (eBioscience). Dead cells were identified as cells stained with 4'6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, OR) or Zombie Aqua™ Fixable Viability Dye (BioLegend, San Diego, CA). To detect apoptotic cells, cells were labeled with Annexin V-FITC in Annexin V binding buffer (Biolegend). The cells were analyzed using a FACSCantoII flow cytometer (BD Biosciences) and sorting was performed with a FACAriaII cell sorter. Data were analyzed by using a FlowJo v9.0 software (Tree Star, San Carlos, CA).

RNA extraction. Total RNA was extracted from sorted $CD4^+$ or $CD8^+$ T cells, or peritoneal macrophages, using ISOGEN II (Nippon Gene, Tokyo, Japan) and subjected to quantitative PCR (Q-PCR) as described in supplemental methods.

Q-PCR. Total RNA extracted from purified cells was subjected to reverse transcription. Reverse transcription was conducted using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo life science, Osaka, Japan). Q-PCR was performed on the ABI StepOnePlus system using TaqMan Fast Advanced Master Mix and the primers and fluorescent probe sets (Sigma-Aldrich, the sequences are listed in the Table S2) specific for mouse TLR2, TLR4, TLR5, TLR7, TLR9, IL-1R, IL-18R, and IL-33R. The reactions were performed in a 96-well plate in triplicate and relative expression to 18S rRNA was calculated using the ΔCt values.

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Table S1. List of primary antibodies used in flow cytometry

Target Ag	Clone	Fluorochrome	Supplier	Catalog#
TCRβ	H57-597	FITC	BD Biosciences	553171
TCRβ	H57-597	APC	BD Biosciences	554413
H-2Kd	SF1-1.1	FITC	BD Biosciences	553565
H-2Kd	SF1-1.1	Biotin	BD Biosciences	553564
CD4	GK1.5	PE-Cy7	BioLegend	100422
CD8a	53-6.7	BV510	BioLegend	100752
CD11b	M1/70	PerCP-Cy5.5	BD Biosciences	561114
CD44	PE	IM7	BD Biosciences	561860
CD62L	FITC	MEL-14	BD Biosciences	553150
TLR1	eBioTR23	PE	eBioscience	12-9011-80
TLR2	T2.5	PE	eBioscience	12-9024-80
TLR6	418601	PE	R&D Systems	FAB1533P
TLR7	polyclonal	PE	Imgenex	IMG-665D
IFN-γ	XMG1.2	APC	BioLegend	505810
IL-4	11B11	Alexa Fluor 647	BioLegend	504110
IL-17A	TC11-18H10	PE	BD Biosciences	559502
BrdU	Bu20a	APC	BioLegend	339808
Foxp3	FJK-16s	PE	eBioscience	12-5773-82

Table S2. List of primer and probe sequences used in Q-PCR

Gene		Sequence
18S rRNA	Forward	5'- GCTCTTTCTCGATTCCGTGGG-3'
	Reverse	5'-ATGCCAGAGTCTCGTTCGTTATC-3'
	Probe	5'-CTCCACCAACTAAGAACGGCCATGCACC-3'
Tlr2	Forward	5'- GCTTCACTTCTCTGCTTTTCGTTC-3'
	Reverse	5'- CATCCTCTGAGATTTGACGCTTTG-3'
	Probe	5'- TCTCTGGAGCATCCGAATTGCATCACCG-3'
Tlr4	Forward	5'- ATGGCACTGTTCTTCTCCTGC-3'
	Reverse	5'- CTGAGTTTCTGATCCATGCATTGG-3'
	Probe	5'- TGACACCAGGAAGCTTGAATCCCTGCAT-3'
Tlr5	Forward	5'- CGAGTGAGGTCAGTCCTGGA-3'
	Reverse	5'- GTCTGGAGAGGCTCATGCTAAG-3'
	Probe	5'- CGGCAAGCATTGTTCTCCCAAGCGG-3'
Tlr7	Forward	5'- CTGTCTCAGAGGACTCCATCTATAG-3'
	Reverse	5'- GTCAGAGATAGGCCAGGATCATC-3'
	Probe	5'- TGCCTTCAAGAAAGATGTCCTTGGCTCC-3'
Tlr9	Forward	5'- CTCTGAGAGACCCTGGTGTGG-3'
	Reverse	5'- GTCCTTCGACGGAGAACCATG-3'
	Probe	5'- ACATCATTCTCTGCCGCCCAGTTTGTCA-3'
IL-1R1	Forward	5'- AGGGACAGACCTGTTATCCTGAG-3'
	Reverse	5'- GACGTTGCAGATCAGTTGTATCATT-3'
	Probe	5'- CTGGGTCAGCTTCGATCGTCTCATTCCG-3'
IL-18R1	Forward	5'- GCCAACGAAGAAGCCATAGACA-3'
	Reverse	5'- GAGGCGAGAACAAGCACAGT-3'
	Probe	5'- ACTCCTCCTGTAAAGACATGGCCTGGGA-3'
IL-33R	Forward	5'- CCAAGAGCAAGACCAGGTGC-3'
	Reverse	5'- GACTCATGTTCACCATCAGCTTC-3'
	Probe	5'- ATCCACACCGTCGCCTGATTGACTTGC-3'

Table S3. List of synthetic TLR ligands used in culture

TLR ligands	Corresponding TLR	Final concentration	Supplier	Catalog#
Pam3CSK4	TLR1/2	1 μg/ml	InvivoGen	tlrl-kit1mw
LPS	TLR4	1 μg/ml	InvivoGen	tlrl-kit1mw
Flagellin	TLR5	1 μg/ml	InvivoGen	tlrl-kit1mw
ssRNA40	TLR7	5 μg/ml	InvivoGen	tlrl-kit1mw
R848	TLR7/8	2 μg/ml	InvivoGen	tlrl-r848-5
ODN1826	TLR9	5 μM	InvivoGen	tlrl-kit1mw

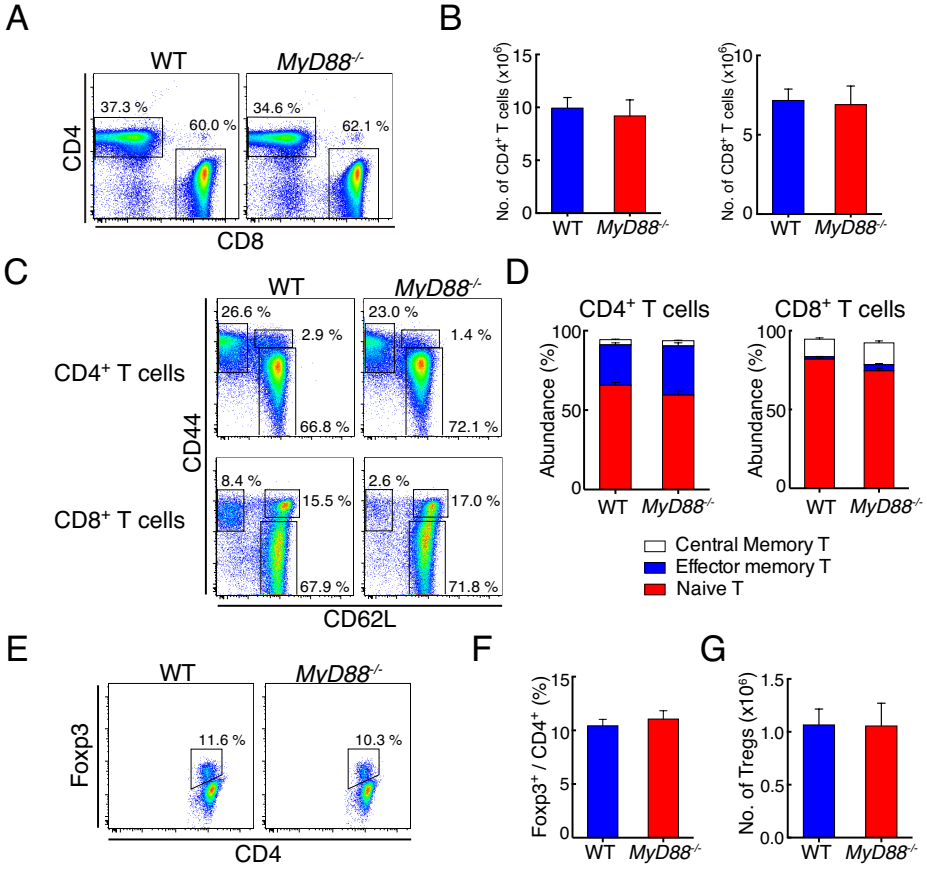

Figure S1. T cell development is intact in *MyD88*$^{-/-}$ mice.

Representative dot plots **(A)** and absolute numbers of splenic CD4^{+} and CD8^{+} T cells **(B)** from 8-weeks-old WT or *MyD88*$^{-/-}$ B6 mice are shown as means ± SE. Representative dot plots **(C)** and fractions **(D)** of naïve (red bars), central memory (white bars), and effector memory T cells (blue bars) from WT or *Myd88*$^{-/-}$ B6 mice shown as means ± SD. Representative dot plots **(E)**, frequencies **(F)**, and absolute numbers **(G)** of splenic Tregs shown as means ± SE. Data from two similar experiments were combined (n = 12 / group).

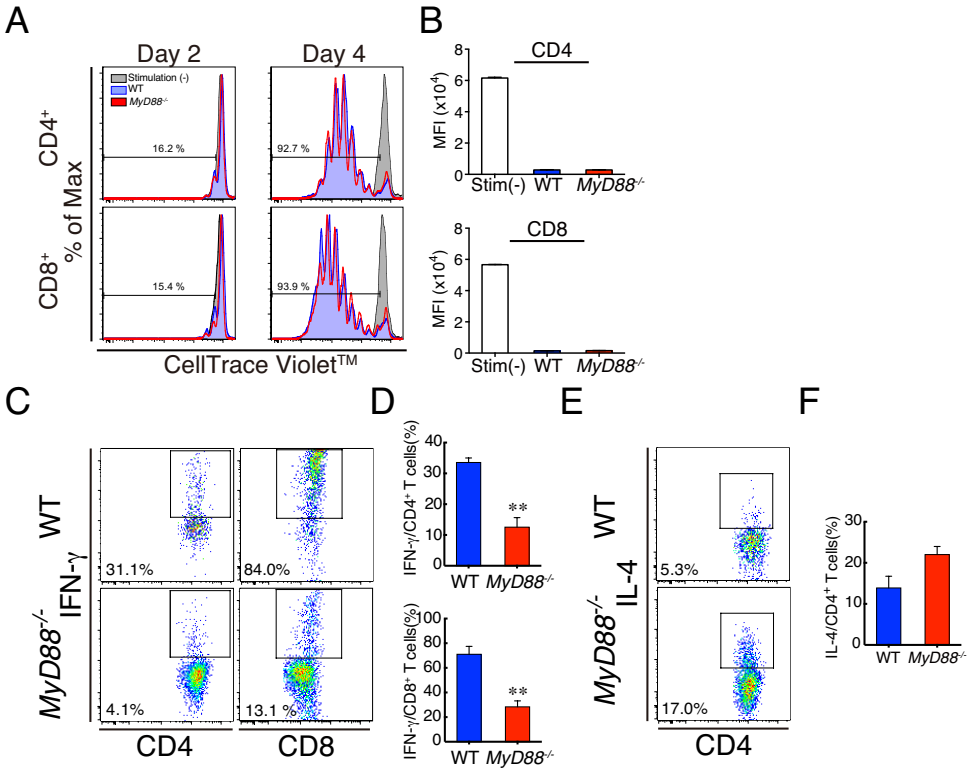

Figure S2. MyD88 regulates the T cell differentiation in a T-cell intrinsic manner.

Sorted 5 × 10^4 T cells from B6 mice were labeled with CellTrace Violet and incubated with Dynabeads coated with anti-CD3/CD28 mAbs for 4 days. Cell proliferation was assessed 48 and 96 hours later. Representative histograms (**A**) and MFI of CellTrace Violet (**B**, means ± SE) from one of two similar experiments were shown (n= 4/group). Productions of IFN-γ (**C,D**) and IL-4 (**E,F**) were studied after 96-hour stimulation. Representative dot plots **(C,E)** and frequency **(D,F**, means ± SE**)** of cytokine producing T cells from two similar experiments are combined (n= 6/group). **P<0.01.