

Supplementary Information

Blockade of TIM-1 on the donor graft ameliorates graft-versus-host disease following hematopoietic cell transplantation

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Supplementary Materials and Methods

Allogeneic bone marrow transplantation

BALB/c recipient mice received lethal total body irradiation (TBI; 200kV x-ray source; Kimtron) consisting of two doses of 4.0 Gy 4 hours apart. 5.0×10^6 TCD-BM cells from either B6 WT or B6 TIM-1^{-/-} mice were injected via tail vein together with 1.0×10^6 B6 WT or B6 TIM-1^{-/-} Tcon, depending on the transplant system. In some experiments, mice were treated with PtdSer (40µg/mouse) i.p. (P7769, Sigma), on the same day as HCT.

In the transplant models, when we looked for the role of *i*NKT cells, 25,000 B6 WT, TIM-1^{-/-}CD4⁺ *i*NKT or WT B6 *i*NKT cells pre-activated with anti-TIM-1 mAb (3B3) were also co-injected on day 0. For the A20 and BCL₁ tumor models, tumor cells were injected via tail vein on day 0 together with TCD-BM. For the GVHD score weight, fur, skin, activity and posture were evaluated and a score from 0 to 2 was assigned to each characteristic. Histopathology of gut, liver and skin tissue, as well as serum collection for cytokine analysis was performed on day +9 post HCT.

CD4⁺ *i*NKT cells isolation and *in vitro* preactivation with 3B3 anti-TIM-1 mAb

CD4⁺ *i*NKT cells were isolated as previously described ²². Briefly, splenocytes from WT and TIM-1^{-/-} knockout mice underwent B cells depletion using CD45R (B220) MicroBeads (Miltenyi Biotec). They were later on stained with PBS-57-CD1d tetramer PE and enriched with anti-PE Microbeads (Miltenyi Biotec).

For the *in vitro* preactivation with the anti-TIM-1 mAb (3B3) experiment, *i*NKT cells were further sorted to purity on a FACSaria II cell sorter based on TCRβ⁺, CD4⁺ and PBS-57 CD1d staining were incubated with the anti-TIM-1 or isotype control mAb (10ug/ml) at 37°C for 60min.

Mouse *in vitro* cell stimulation assays

For analysis of cell proliferation, murine WT B6 Tcons were resuspended in PBS and stained with CellTrace Violet cell proliferation kit (Life Technologies) for 5 minutes

at 37°C. Immediately after staining, cells were washed 3 times in ice-cold RPMI 1640 (Mediatech) plus 10% FCS and finally resuspended in PBS. CellTrace Violet labeled Tcons (responders) were cultured with BALB/c irradiated (30 Gy) splenocytes (stimulators) at a ratio of 1:2 in the presence or absence of anti-TIM-1 mAb (3D10). In separate conditions, CellTrace Violet labeled WT or TIM-1^{-/-} knockout B6 Tcons were stimulated with anti-CD3/CD28 dynabeads (ThermoFisher) at 1:2 ratio. Similarly, WT *i*NKT cells were stained with CellTrace Violet and cultured with dendritic cells and α -galactosylceramide (AdipoGen) or with anti-CD3/CD28 dynabeads at 1:2 ratio. Data were collected using a BD LSR2 flow cytometer (BD Biosciences) and data were analyzed using FlowJo 10.0.7 software (Tree, Star, Ashland, OR). WT and TIM-1^{-/-} *i*NKT cells were sorted to purity and activated with anti-CD3/CD28 dynabeads at 1:1 ratio for 72 hours. Culture supernatant was collected and analyzed by multiplex assay (Luminex).

Human *in vitro* cell stimulation assays

Monocyte-derived immature dendritic cells were generated from peripheral blood mononuclear cells (PBMCs, Stanford Blood Center) using CD14⁺ MACS microbead enrichment (Miltenyi biotec, Auburn, CA). The CD14⁺ cells were cultured in 37 °C and 5% CO₂ for 7 days in Roswell Park Memorial Institute (RPMI) medium (ThermoFisher Scientific, Waltham, MA) supplemented with 250 IU/mL interleukin (IL)-4, 800 IU/mL granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN), L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (HyClone; GE Healthcare Life Sciences, South Logan, UT), and 5 % human AB serum (ThermoFisher). The DCs were irradiated (30 Gy) and were co-cultured with allogeneic responder PBMCs for 7 days at a ratio of 1:5. In separate conditions, responder PBMC were stimulated with anti-CD3/CD28 dynabeads (ThermoFisher) at a 1:1 ratio. The responder PBMCs were labelled with CellTrace Violet (ThermoFisher). Data were collected using a BD LSR2 flow cytometer (BD

Biosciences) and data were analyzed using FlowJo 10.0.7 software (Tree, Star, Ashland, OR)

Flow cytometry

For flow cytometry we purchased from Southern Biotech (Birmingham, AL), BD Biosciences (San Jose, CA), eBioscience (San Diego, CA), R&D Systems, (Minneapolis, MN), and Biolegend (San Diego, CA) the following anti-mouse antibodies: CD4 (GK1.5), CD8 (53-6.7), CD25 (PC61), FoxP3 (FJK-16s), PDCA-1 (927), H-2Kb (AF6-88.5), H-2Dd (34-2-12), CD19 (6D5). PBS-57-CD1d tetramer phycoerythrin (PE; National Institutes of Health). Anti-idiotypic BCL₁ antibody was purified from a hybridoma secreting rat IgG2a (Dutt S. et al, Blood, 2011). The antibody was conjugated with Alexa Fluor 488 for fluorescence-activated cell sorting (FACS). We used the Fixable Viability Dye eFluor® 506 (eBioscience) for dead cell staining. Analysis was performed on a LSR II (Becton-Dickinson, San Jose, CA). The following anti-human antibodies were used: APC/Fire 750-conjugated anti-human CD3 (UCHT1), PerCP/Cy5.5-conjugated anti-human CD8a (RPA-T8), Alexa Fluor 700-conjugated anti-human CD4 (RPA-T4, Brilliant Violet 605-conjugated anti-human CD3 (UCHT1), PE-conjugated anti-human programmed death-1 (PD-1, EH12.2H7), APC-conjugated anti-human CD25 (M-A251), PE-CF594-conjugated anti-human CCR7 (150503), PE-Cy7-conjugated anti-human CD45RA (HI100), Aqua amine (All from Biolegend, San Diego, CA). Fixable viability dye eFluor 506 (eBioscience) was used for identification and removal of dead cells.

Quantitative Real-time reverse transcription-PCR (qRT-PCR)

Gut tissue was collected into a RNA stabilization buffer and was cut into small pieces and homogenized before loaded onto a QIAshredder spin column (Qiagen). RNA from gut was extracted using the RNeasy Mini kit according to manufacturer's instructions (Qiagen). The qRT-PCR was performed using iTaq™ Universal SYBR Green One-Step Kit (Bio-Rad) on a 7900HT Fast Real Time PCR system (Applied Biosystems) using the following cycling parameters (95°C for 2min, followed by 40

cycles for 95 °C for 15s and 60 °C for 1min). A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. GAPDH were used as the reference gene and the relative levels of gene expression were calculated by the $2^{\Delta\Delta Ct}$ Method.

Microarrays

Total RNA was extracted, using an RNeasy Micro Kit (Qiagen) and its quality was assessed by and Agilent 2100 Bioanalyzer. Total RNA (from 500pg-2ng) was reverse transcribed into cDNA followed by in vitro transcription use GeneChip™ WT Pico (Affymetrix, Santa Clara, CA). Labeled cRNAs were hybridized to (Affymetrix) according to the manufacturer's protocol, and the chips were scanned using a GeneChip Scanner 3000 GeneChip™ Mouse Gene 2.0ST- this is a whole transcript design with probes at 3' as well as exon. Catalog number: 902118 (Affymetrix). Background correction, normalization and estimation of gene expression was performed using the Robust Multiarray Average (RMA) method in R/Bioconductor (www.bioconductor.org). Following aggregation, the samples were re-normalized using quantile normalization.

Statistical analysis

Differences in animal survival (Kaplan–Meier survival curves) were analyzed with the log-rank test. Weight variation and GVHD score were analyzed with a two-way ANOVA test, using Graphpad Prism. For the human MRL data a paired t-test between treated and untreated PBMCs was performed. All other comparisons were performed with the two-tailed Student *t* test, and $p < 0.05$ was considered statistically significant.

Table S1

Gene	Description	Fold change
<i>Ccne1</i>	Cyclin E1	1
<i>E2f1</i>	E2F transcription factor 1	1
<i>Cdc6</i>	Cell division cycle 6	1.24
<i>Pcna</i>	Proliferating cell nuclear antigen	1
<i>Rfc4</i>	Replication factor (activator 1) 4	1
<i>Dhft</i>	Dihydrofolate reductase	-1.91
<i>Rrm2d</i>	Ribonucleotide reductase M2	1.05
<i>Rad51</i>	RAD51 homolog	1
<i>Top2a</i>	Topoisomerase (DNA) II alpha	1.18
<i>Ccna2</i>	Cyclin A2	1.02
<i>Ccnf</i>	Cyclin F	1
<i>Ccnb1</i>	Cyclin B1	1
<i>Plk4</i>	Polo-like kinase 4	1
<i>Pttg1</i>	Pituitary tumor-transforming gene 1	1
<i>Rad21</i>	RAD21 homolog	-1.05
<i>Ccng2</i>	Cyclin G2	1
<i>Cdkn3</i>	Cyclin-dependent kinase inhibitor 3	1
<i>Ctla-4</i>	Cytotoxic T-lymphocyte-associated protein 4	1
<i>Cd69</i>	CD69 antigen	-1.04
<i>Il2</i>	Interleukin 2	1
<i>Pdcd1</i>	Programmed cell death 1	-1.21
<i>Sell</i>	Selectin lymphocyte (CD62L)	1
<i>Iag3</i>	Lymphocyte activation gene 3	-1.63

Table S1

No difference is observed in the expression of genes associated with cell cycle and activation.

List of genes associated with cell cycle and T cell activation as well as fold difference observed between anti-TIM1 (3D10) and isotype treated groups. RNA was isolated from whole gut.

Table S2

Inflammation of intestine	p-value	Activation z-score	# molecules
AGR2, ALOX15, ANXA1, APOA4, ATG16L1, C3, CCL11, CCL25, CCR9, CD274, CD44, CEACAM1, CFH, CKB, CKMT1A/CKMT1B, EDN1, ERN2, FAAH, GCNT3, IL18, LTBR, LY96, MEP1A, MERTK, mir-103, MME, MMP7, MUC2, NR1H4, NR1I2, NR5A2, PLS1, POR, PPARG, Retnla, RETNLB, RIPK2, SLC22A4, SLPI, TLR1, TLR2, TLR4, TNFRSF1B, TP53INP1, VNN1	1.39E-05	-0.930	45

Table S2

Inflammation of the intestine was downregulated in the anti-TIM-1 antagonistic mAb (3D10) treated group.

List of genes associated with inflammation of the intestine based on Ingenuity Pathway Analysis. Genes shown in black are upregulated in the anti-TIM-1 (3D10) treated group as opposed to the isotype control. Genes shown in grey are downregulated in the anti-TIM-1 (3D10) treated group as opposed to the isotype control.

Figure S1

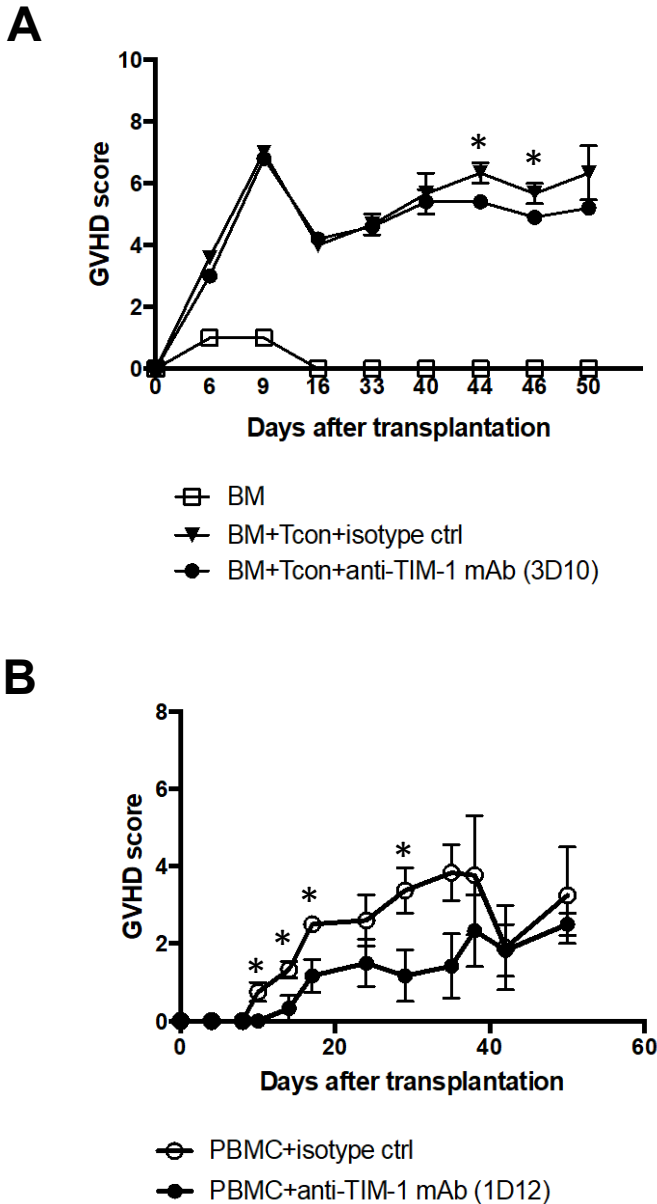


Figure S1. Anti-TIM-1 treated mice present reduced GVHD score.

(A) GVHD score of allogeneic transplanted BALB/c recipient mice treated with either B6 Tcon (▼), or Tcon and anti-TIM-1 mAb (●). Control mice that received TCD-BM only are shown (□). (●). Pooled data from two independent experiment (n=10/group) are shown.

(B) GVHD score of non-obese diabetic (NOD)-severe combined immunodeficient (*scid*) *IL2ry^{null}* mice transplanted with only Hu-peripheral blood mononuclear cells (PBMCs) (○), or PBMCs and anti-human TIM-1 mAb (1D12) (●). For GVHD score pooled data of two independent experiments are shown (n=8 mice/group) with statistical significance between groups. For GVHD the two-tailed Student's t test was used. Error bars indicate SEM. * $p \leq 0.05$

Figure S2

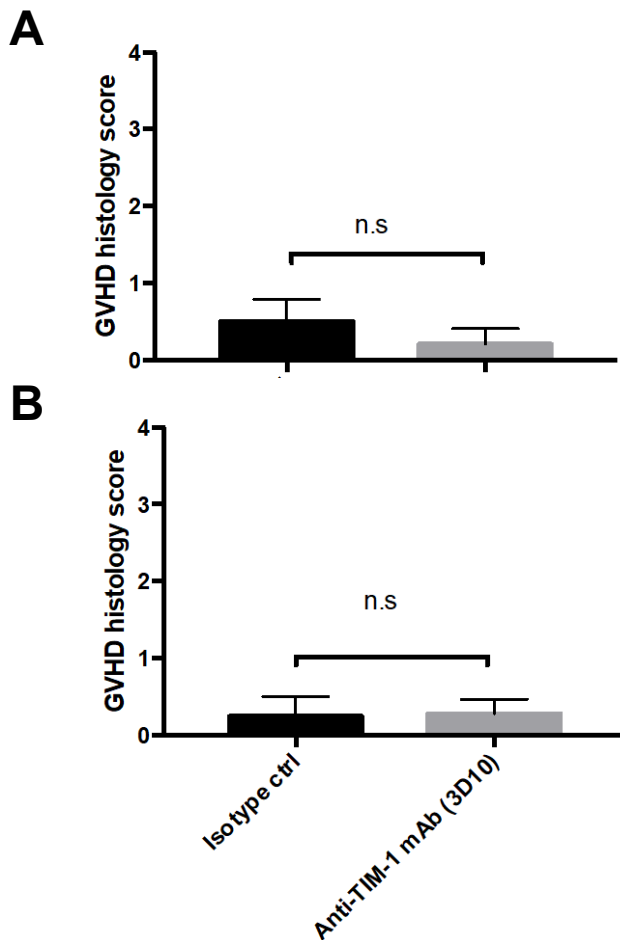


Figure S2. No differences in live or skin histopathology were observed in mice treated with anti-TIM-1 mAb (3D10). GVHD histology score as assessed by a histopathologist in a blinded fashion (n=10/group). **(A)** Liver was assessed for bile duct injury (manifest by nuclear hyperchromasia, nuclear crowding, infiltrating lymphocytes, and cytoplasmic vacuolation) and inflammation (infiltration with lymphocytes, neutrophils, and eosinophils). Disease was scored between 0 and 4 based on the number of involved tracts and the severity of disease in each tract (0, none; 1, few involved tracts with mild involvement; 2, numerous involved tracts but with only mild disease; 3, injury in the majority of tracts; 4, severe involvement of most tracts).

(B) Skin sections were scored on the basis of the following criteria: epidermis (0, normal; 1, foci of interface damage in <20% of section with occasional necrotic keratinocytes; 2, widespread interface damage in >20% of section); dermis (0, normal; 1, slightly altered with mild increased collagen density; 2, marked increased collagen density); inflammation (0, none; 1, focal infiltrates; 2, widespread infiltrates); s.c. fat (0, normal; 1, reduced number of normal adipocytes; 2, serous fat atrophy); and follicles (0, normal number of hair follicles, ~5 per linear millimeter; 1, between 1 and 5 follicles per linear millimeter; 2, <1 follicle per linear millimeter)

Figure S3

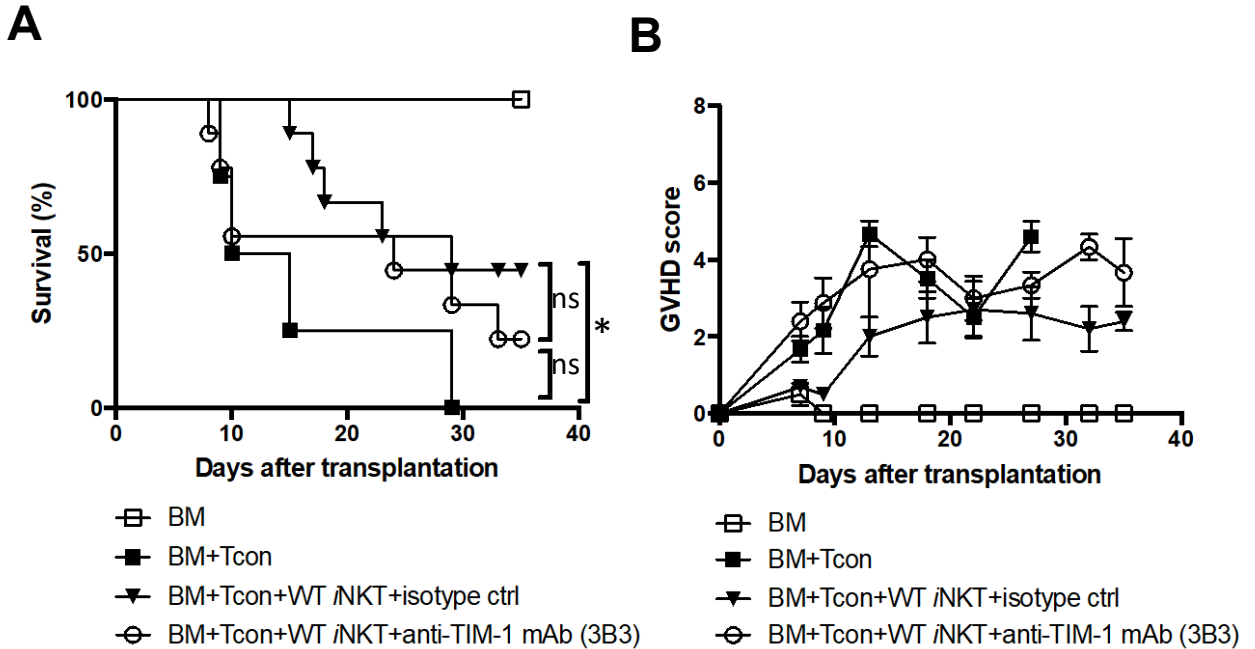


Figure S3. (A) Overall survival and **(B)** GVHD score of BALB/c recipient mice after injection of B6 Tcon alone (■), B6 Tcon and B6 *i*NKT cells (25,000/mouse) incubated with isotype control mAb (▼), B6 Tcon and B6 *i*NKT cells incubated with anti-TIM-1 agonist mAb (3B3)(○). GVHD scores for control mice that received only TCD-BM are shown (□). Pooled data of two independent experiments are shown (n=9 mice/group). The log-rank test was used for statistical evaluation of mouse survival (Kaplan-Meier survival curves). For GVHD the two-tailed Student's t test was used. Error bars indicate SEM. ns= not significant * $p \leq 0.05$.

Figure S4

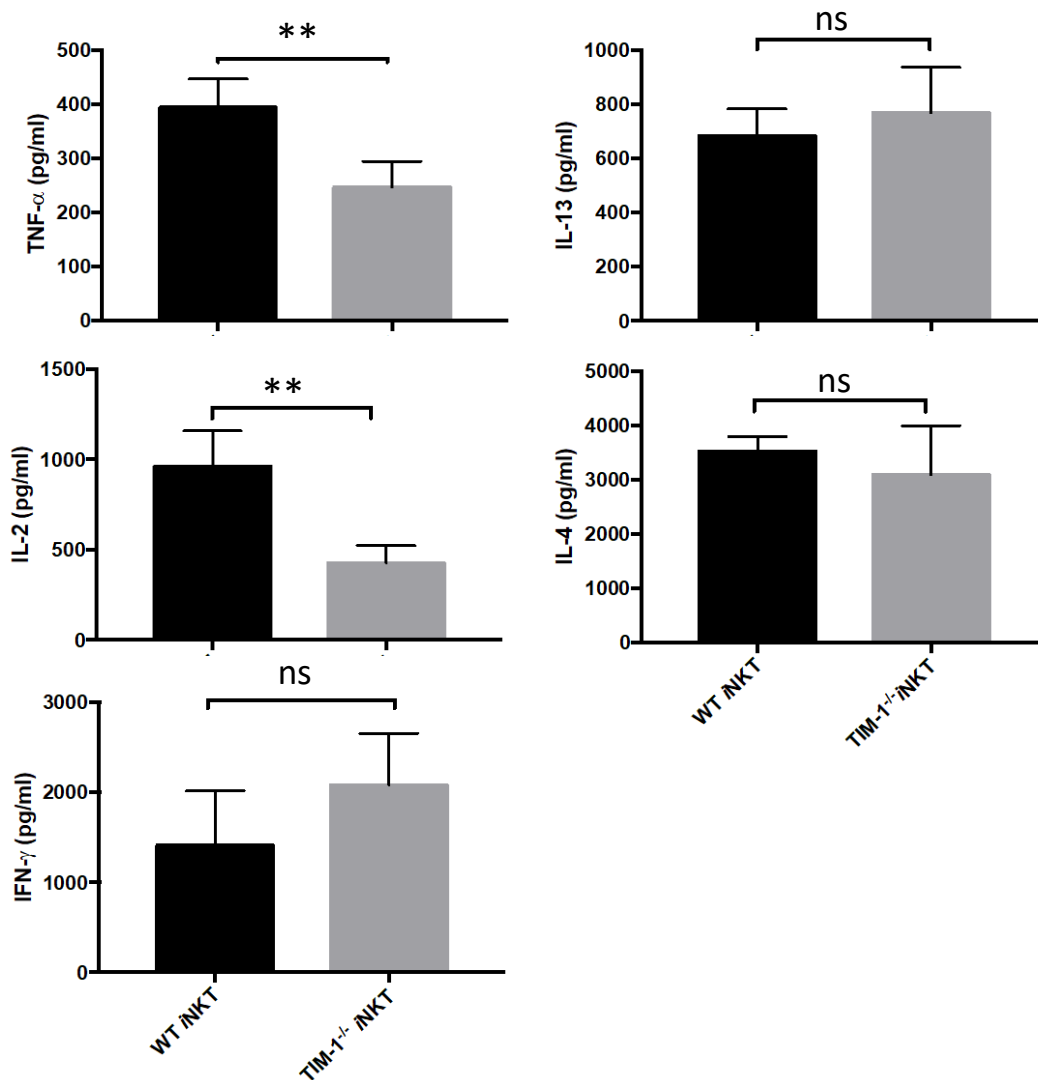


Figure S4. Reduced levels of proinflammatory cytokines TNF- α , and IL-2 were detected in the supernatant of the TIM-1^{-/-} knockout iNKT cells compared to WT iNKT. Cytokine levels in the supernatant of sorted WT and TIM-1^{-/-} iNKT cells that were stimulated *ex vivo* with anti-CD3/CD28 dynabeads beads (1:1 ratio) for 72hrs, as assessed by Luminex. Error bars indicate SEM. Pooled data from 2 independent experiments. For statistical analysis the two-tailed Student's t test was used. ** $p \leq 0.01$

Figure S5

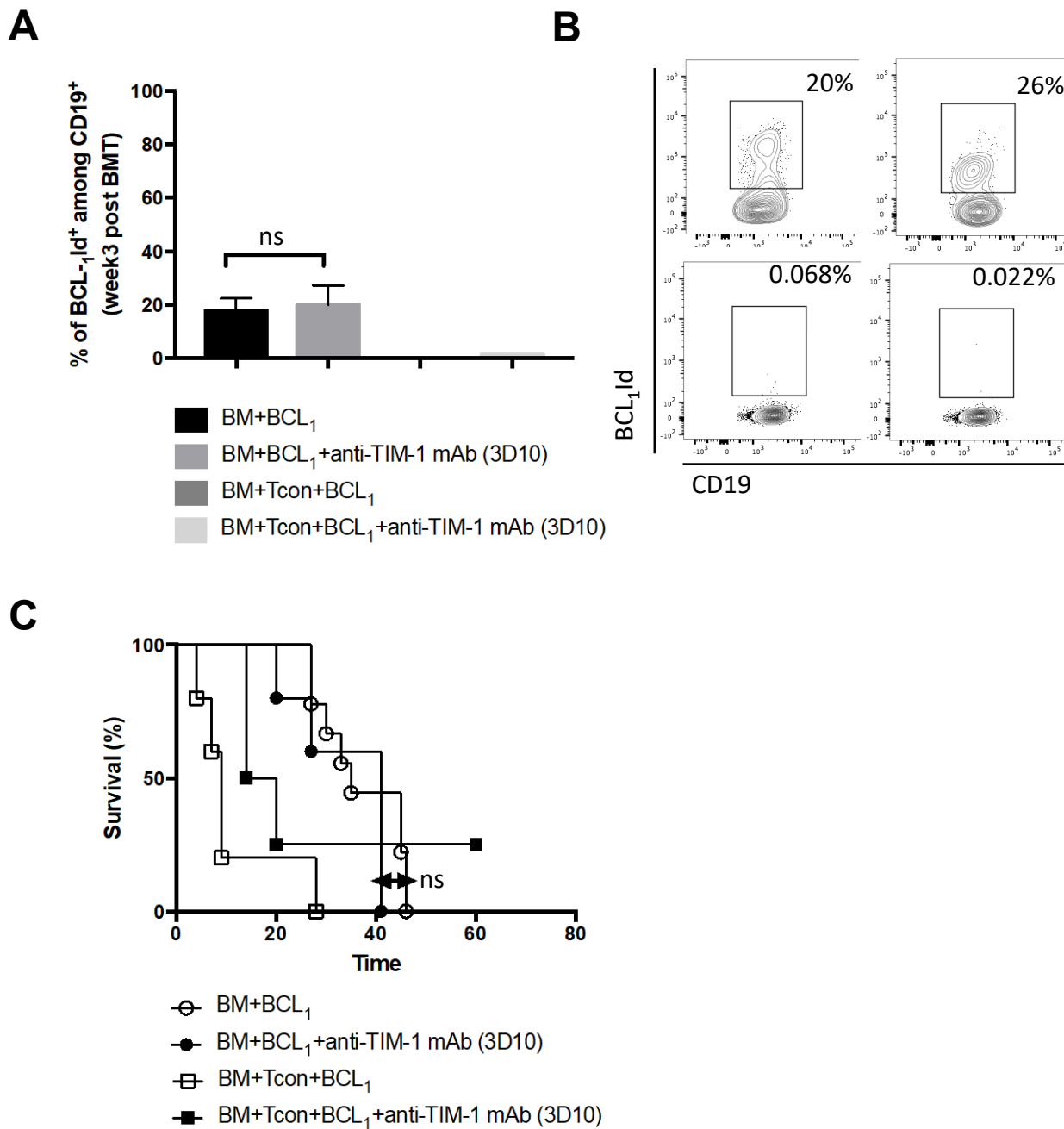


Figure S5. Antagonistic anti-TIM-1 does not interfere with GVT effect in the BCL₁ tumor model

(A) Tumor burden in mice infused intravenously with 500 BCL₁ leukemia tumor **(B)** Representative flow cytometry plots of BCL₁⁺ cells among CD19⁺ cells ; top left: BM+ BCL₁, top right: BM+ BCL₁+anti-TIM-1 (3D10) bottom left: BM+Tcon+ BCL₁ and bottom right: BM+Tcon+ BCL₁+anti-TIM-1 (3D10) **(C)** Survival of BALB/c recipient mice when transplanted with B6 TCD and either sham infusion (●), sham infusion and anti-TIM-1 mAb (3D10; 400 μg per mouse given i.p.) (●), Tcons and isotype (□), or Tcons and anti-TIM-1 mAb (3D10; 400 μg per mouse given i.p.) (■). Pooled data of two independent experiments are shown (n=8 mice/group). The log-rank test was used for statistical evaluation of mouse survival (Kaplan-Meier survival curves), with comparison of BM+ BCL₁ to BM+ BCL₁+ anti-TIM-1 mAb (3D10) shown. Error bars indicate SEM. For statistical analysis the two-tailed Student's t test was used, ns=not significant

Figure S6

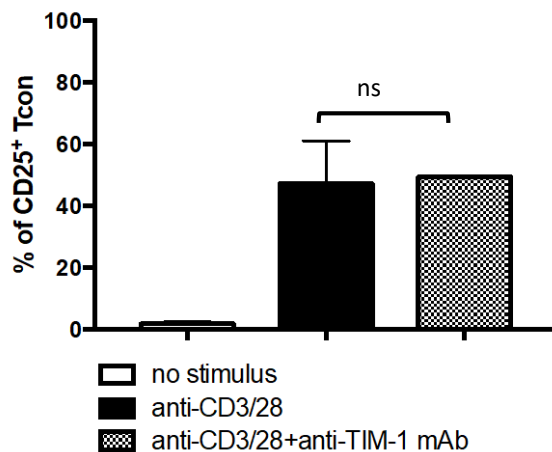


Figure S6. Treatment with antagonistic anti-TIM-1 mAb does not affect CD25 expression on Tcons *in vitro*. TIM-1 blockade does not affect CD25 expression of murine T cells stimulated with anti-CD3/anti-CD28 dynabeads at 1:2 ratio, in the presence or absence of anti-TIM-1 mAb (3D10; 20 μ g/ml). Pooled data of two independent experiments are shown (n=7/group). Error bars indicate SEM. For statistical analysis the two-tailed Student's t test was used, ns=not significant

Figure S7

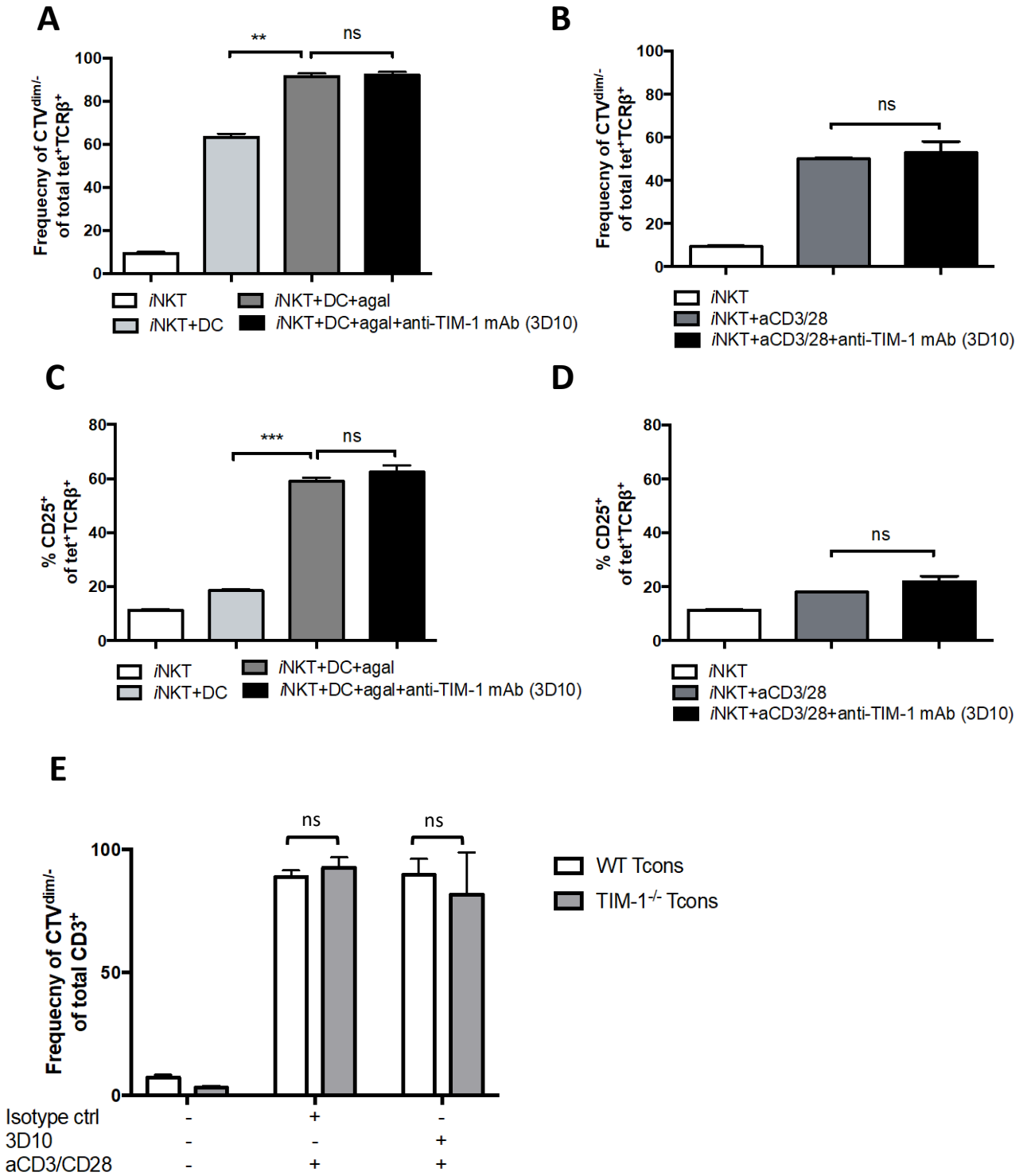


Figure S7. Treatment with antagonistic anti-TIM-1 mAb does not affect *i*NKT cell proliferation *in vitro*. (A) TIM-1 blockade (anti-TIM-1 mAb, 3D10; 20 μ g/ml) does not affect murine *i*NKT cells proliferation upon dendritic cells and α -galactosylceramide or anti-CD3/anti-CD28 stimulation as shown in (B), neither CD25 upregulation under neither experimental conditions (C) and (D) respectively. (E) WT B6 and TIM-1^{-/-} knockout T cells were labeled with CellTrace Violet and stimulated with anti-CD3/anti-CD28 dynabeads at 1:2 ratio. No difference in proliferation was observed between WT and TIM-1^{-/-} knockout Tcons. Pooled data of two independent experiments are shown (n=8/group). Error bars indicate SEM. For statistical analysis the two-tailed Student's t test was used. ** $p \leq 0.01$, *** $p \leq 0.001$, ns=not significant

Figure S8

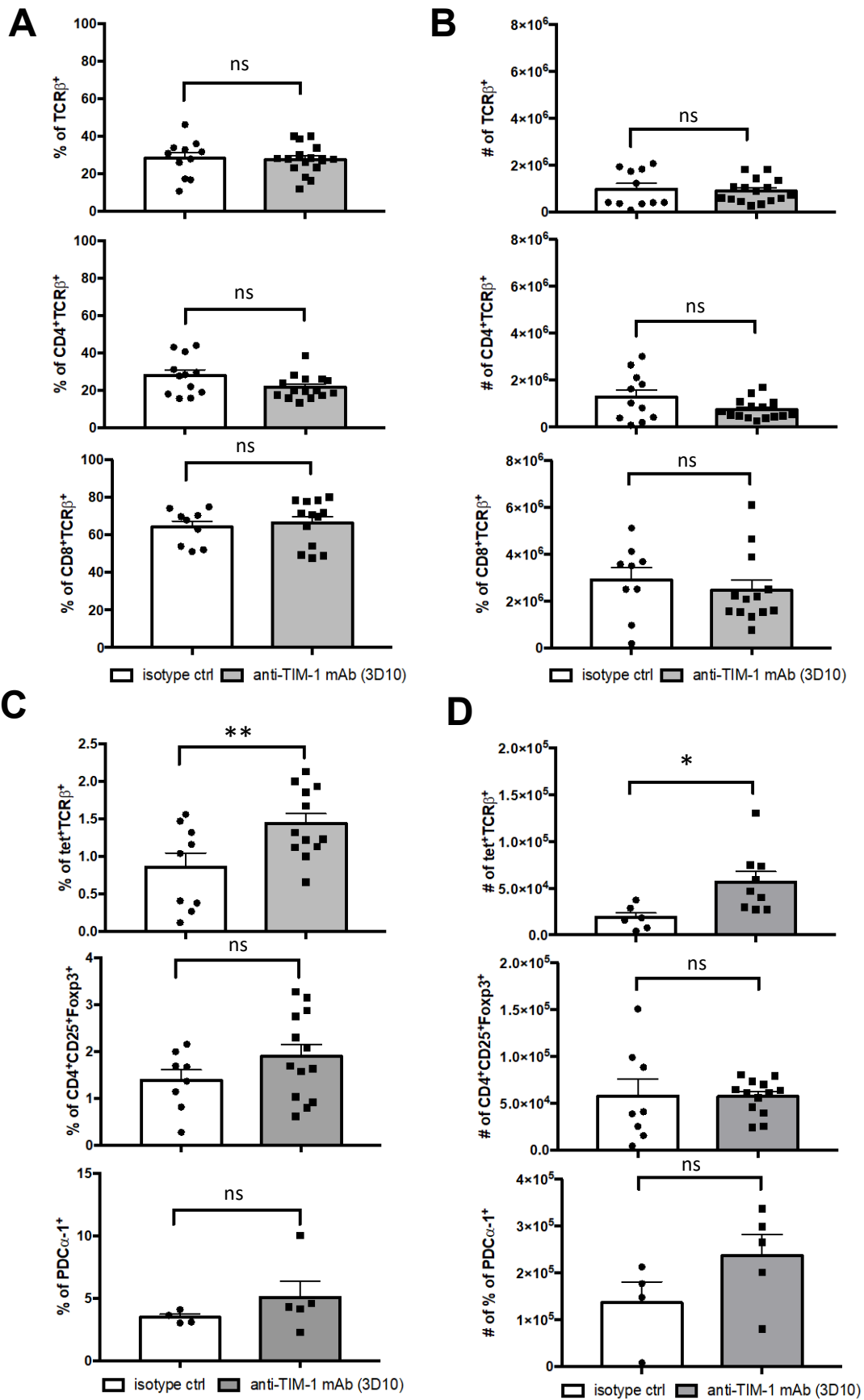
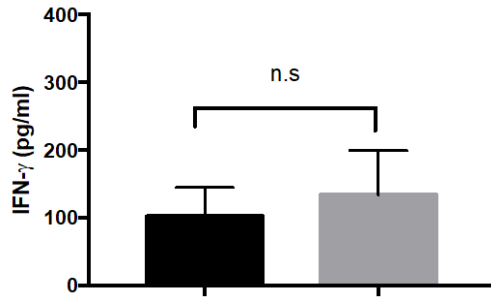


Figure S8. *i*NKT cells are significantly increased in the spleen of mice treated with the anti-TIM-1 mAb. (A) Relative percentages and **(B)** absolute numbers of TCR β^+ , CD4 $^+$ TCR β^+ , CD8 $^+$ TCR β^+ , calculated, and **(C)** relative and **(D)** absolute numbers of tet $^+$ TCR β^+ , CD4 $^+$ CD25 $^+$ Foxp3 $^+$ and PDC α -1 $^+$ at day 9 after transplantation from spleens of BALB/c recipient mice treated with isotype control (white bars) or anti-TIM-1 mAb (3D10) (grey bars). PBS-57-loaded murine CD1d tetramer (tet $^+$) was used to identify *i*NKT cells.

Figure S9

A



B

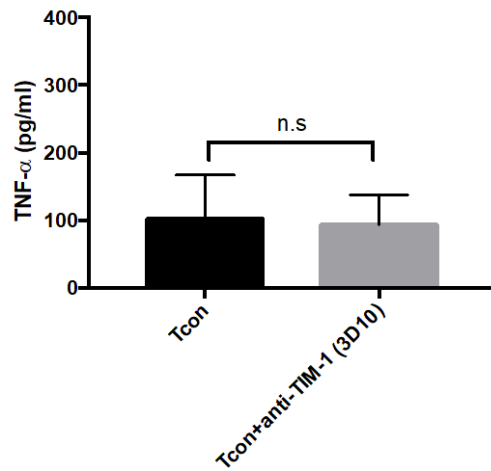
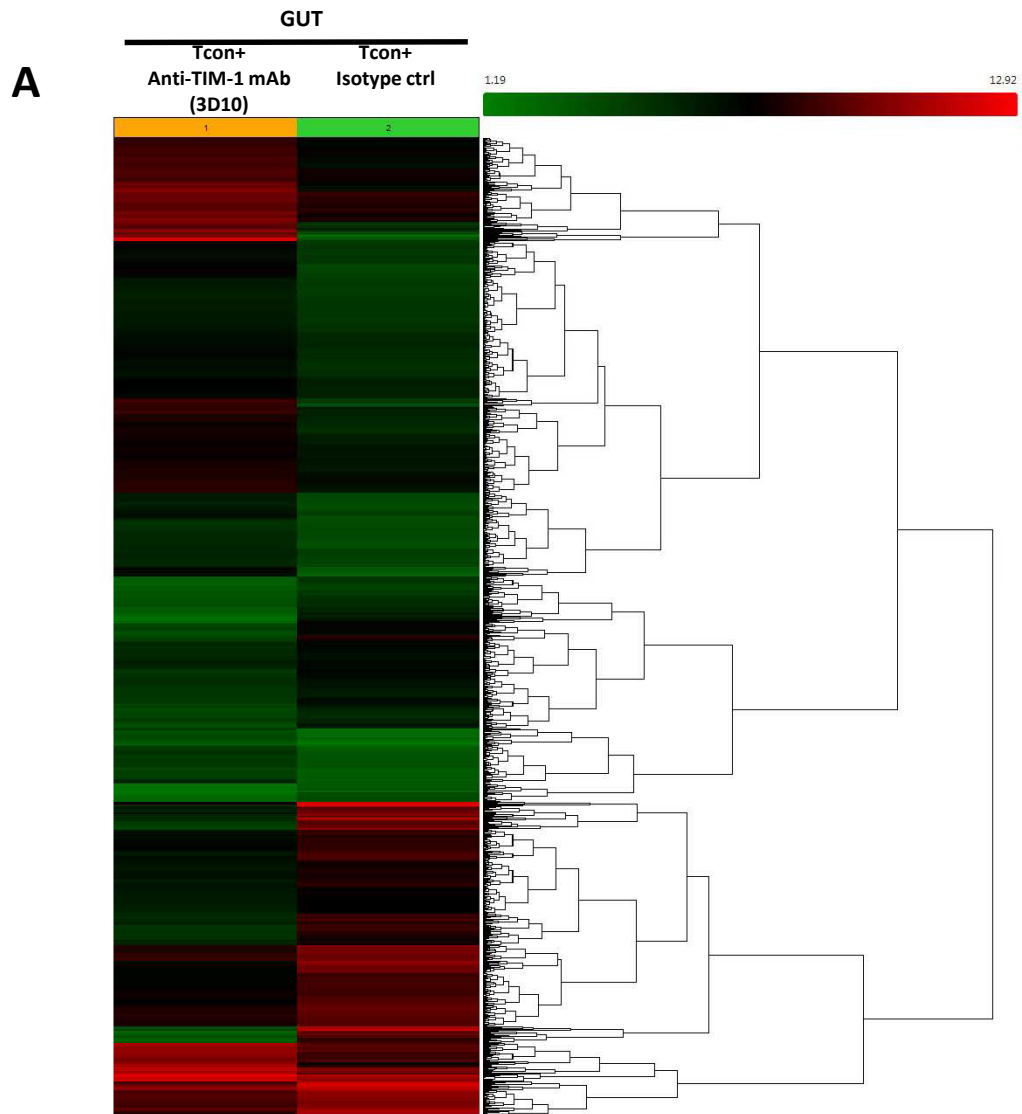


Figure S9. No differences were detected on serum cytokine levels. (A) IFN- γ and **(B)** TNF- α levels in the serum in mice that received Tcons, Tcons+ anti-TIM-1 mAb (3D10) on day +9 post HCT were analyzed by a multiplex assay (Luminex)

Figure S10



B

Upstream regulator	Tcons+ Anti-TIM-1 mAb	Tcons
IL-10-RA	+	-
IL-4	+	-
IL-13	+	-

Figure S10. Microarray analysis. (A) Microarray heat map of whole gut cells isolated at day 9 after transplantation from BALB/c recipient mice treated with isotype control or anti-TIM-1 mAb (3D10). Red color indicates upregulated genes and green color indicates downregulated genes. **(B)** Upstream regulators were identified after pathway analysis using IPA (Ingenuity Pathway Analysis) between the anti-TIM-1 mAb (3D10) and isotype control treated groups.