SUPPLEMENTAL METHODS

Measurement of peritoneal macrophage apoptosis by Celigo plate imaging cytometer

For Celigo experiments, 0.1 ml containing 5 x 10^4 cells was seeded into 96 well plates for 30 min to allow macrophage adherence. Adherent macrophages were washed 2 times with media and treated with the indicated concentration of H_2O_2 (Sigma Aldrich) in IMDM plus 10% FBS for the indicated period of time. Anti-F4/80 and DAPI were added prior to being read serially using a Celigo S plate imaging cytometer (Celigo, Redwood City, CA).

Skin Transplantation

Tail skin from wild type or TIM-$4^{-/-}$ bm12 mice was transplanted onto ABM TCR transgenic mice. Skin allograft rejection was defined as the day on which 100% of the graft was necrotic.

Cytometer Configurations

In FITC painting experiments, PE fluorescence was acquired using yellow/green (561 nm) excitation and a standard PE bandpass emission filter in order to avoid excessive spillover generated by high levels of FITC using blue (488 nm) excitation. Live/Dead Blue and DAPI staining were acquired using UV (355 nm) excitation and a 425/50 bandpass emission filter. All other fluorochromes were read using standard excitation and emission parameters.

Immunohistochemistry and Immunofluorescence

Wild type and TIM-4$^{-/-}$ C57BL/6 heart allografts were harvested 3 or 7 days after transplantation into wild type BALB/c recipients. Tissues were snap-frozen in OCT tissue medium or formalin fixed and embedded in paraffin. Paraffin-embedded tissues were cut into 5 μm sections and stained with hematoxylin and eosin (H&E). Snap-frozen tissues were cut into 5 μm sections and stained with monoclonal rat anti-mouse CD169 (MOMA-1, Abcam, Cambridge, MA) and polyclonal goat anti-mouse TIM-4 (R&D Systems, Minneapolis, MN). Sections were subsequently stained with anti-rat Alexa fluor 488 and anti-goat Cy5. Slides were sealed with mounting medium containing DAPI (Vector Laboratories). Images were acquired on a Zeiss LSM 510 Meta (Carl Zeiss, Thornwood, NY).

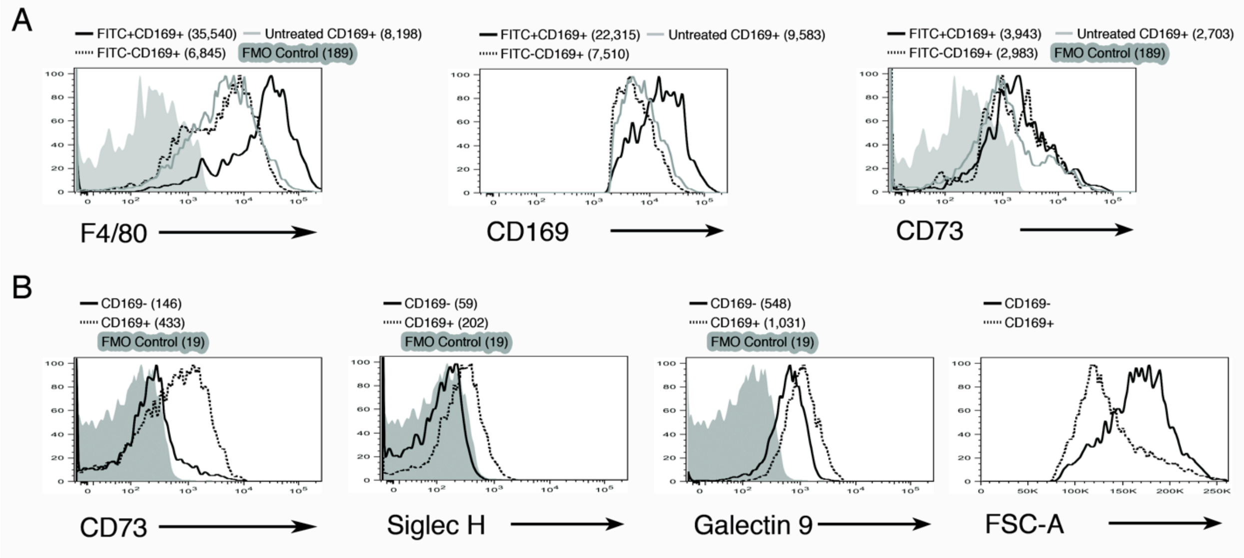
Generation of TIM-4$^{-/-}$ bm12 mice

Bm12 mice (TIM-4$^{+/+}$I-A$^{bm12/bm12}$) were crossed with C57BL/6 TIM-4$^{-/-}$ (TIM-4$^{-/-}$ I-A$^{b/b}$) mice(1). Mice homozygous for TIM-4$^{-/-}$ and the I-A^{bm12} (bm12) alleles (TIM-4$^{-/-}$ I-A$^{bm12/bm12}$) were derived by screening F2 progeny for TIM-4 deletion as previously described (44) and screening for the bm12 mutation by amplifying the region containing the three single nucleotide polymorphisms that define the bm12 mutation (Forward primer 5'- CATTACCTGTGCCTTAGAGATGG-3'; Reverse primer 5'-CATTCCTAATAAGCTGTGTGGATG-3'; Product size = 554 bp and subsequently digesting the product with restriction enzyme Sau3AI. The wild type allele contains a Sau3AI restriction site that yields two products of similar size (~250-300 bp) on a 2% agarose gel (Midsci, St. Louis, MO) whereas the bm12 allele has a mutated restriction site that does not permit digestion, yielding the original product of 554 bp.

T-suppressor Assay

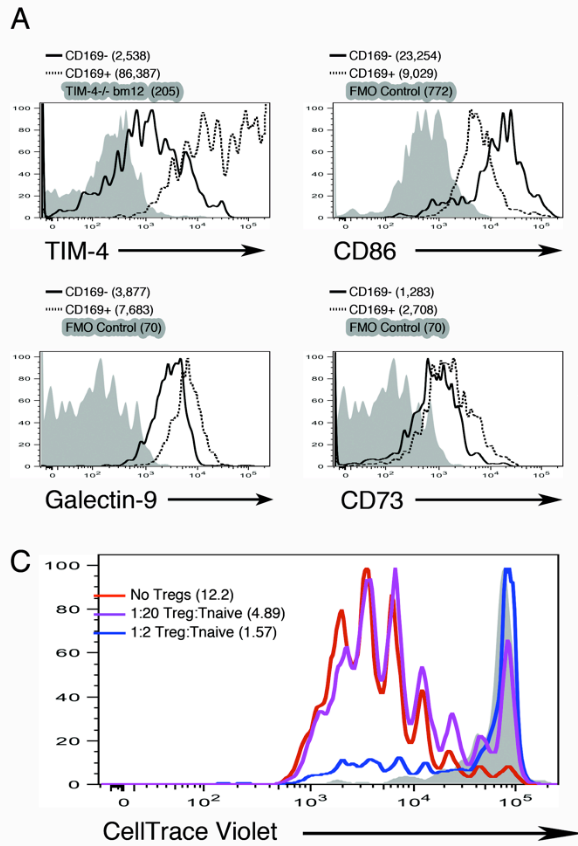
Unlabeled $CD4^+FoxP3/GFP^-$ ABM TCR transgenic T cells were stimulated with $DAPI^-$ $FITC^+CD11b^+Ly6C^-$ $CD169^+$ macrophages from FITC painted bm12 mice as described for CellTrace Violet labeled cells in MLR experiments. After 96 h of culture, $CD4^+FoxP3/GFP^+$ cells were FACS sorted and cultured at the indicated ratio with FACS sorted 5.0×10^4 CellTrace Violet labeled $CD4^+FoxP3/GFP^-$ ABM TCR transgenic T cells and 2.5×10^4 FACS sorted $CD90^-$ (T-depleted) splenocytes in IMDM media supplemented with 10% FBS and 20 ng/ml IL-2. Proliferation was analyzed after 96 hours, gated on $CD4^+Va2^+Vb8^+$ TCR transgenic T cells but excluding GFP^+ and CellTrace $Violet^-$ cells in order to exclude the added Tregs from analyses.

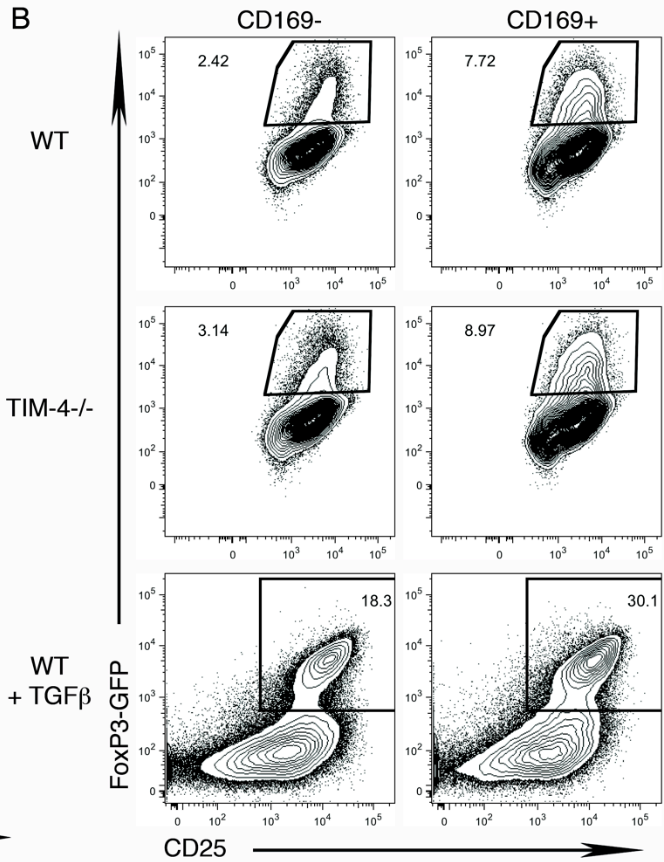
SUPPLEMENTAL FIGURES AND LEGENDS

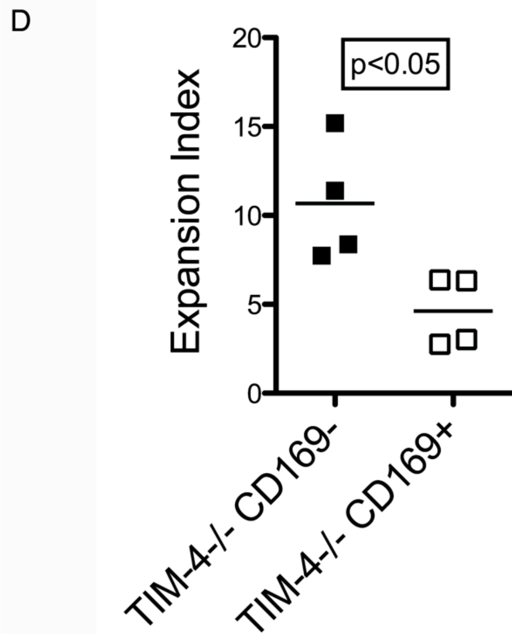


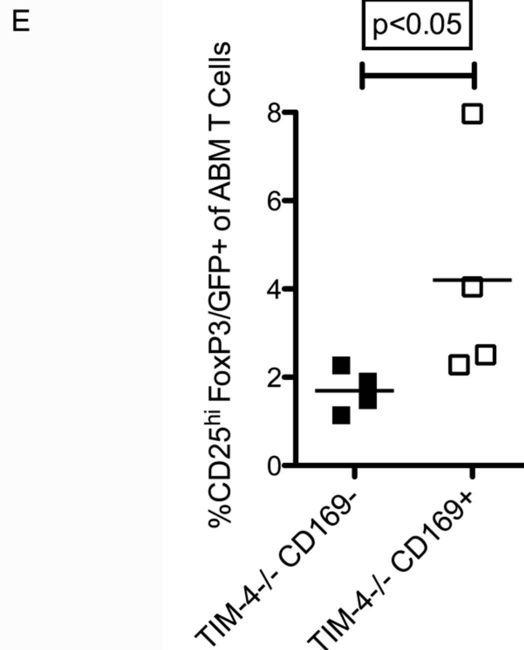
Supplemental Figure 1: CD169$^+$ Tissue-resident macrophages that migrate to the dLN express more CD73, siglec-H, and galectin-9 than CD169$^-$ APCs. (A) dLN cells from FITC painted and untreated C57BL/6 mice were stained and gated as described in **Figure 2.** The expression of the indicated markers on FITC$^+$CD11b$^+$Ly6C$^-$CD169$^+$ macrophages and FITC$^-$CD11b$^+$Ly6C$^-$CD169$^+$ macrophages from FITC painted C57BL/6 mice, and CD11b$^+$Ly6C$^-$CD169$^+$ macrophages from unpainted C57BL/6 mice, are shown. FITC$^+$CD11b$^+$Ly6C$^-$CD169$^+$ macrophages express higher levels of F4/80 and CD169, but similar levels of CD73, as compared to either FITC$^+$CD11b$^+$Ly6C$^-$CD169$^+$ macrophages from FITC painted mice or CD11b$^+$Ly6C$^-$CD169$^+$ macrophages from unpainted mice. **(B)** An extended phenotype of CD169$^+$ macrophages and CD169$^-$ APCs from the dLN of wild type C57BL/6 FITC painted mice is shown. Cells were stained and analyzed as described in **Figure 2A**. CD169$^+$ macrophages possess lower forward scatter properties and express higher levels of CD73, siglec-H, and galectin-9

as compared to $CD169^-$ APCs. Shaded histograms represent the FMO controls. MFIs are noted parenthetically.

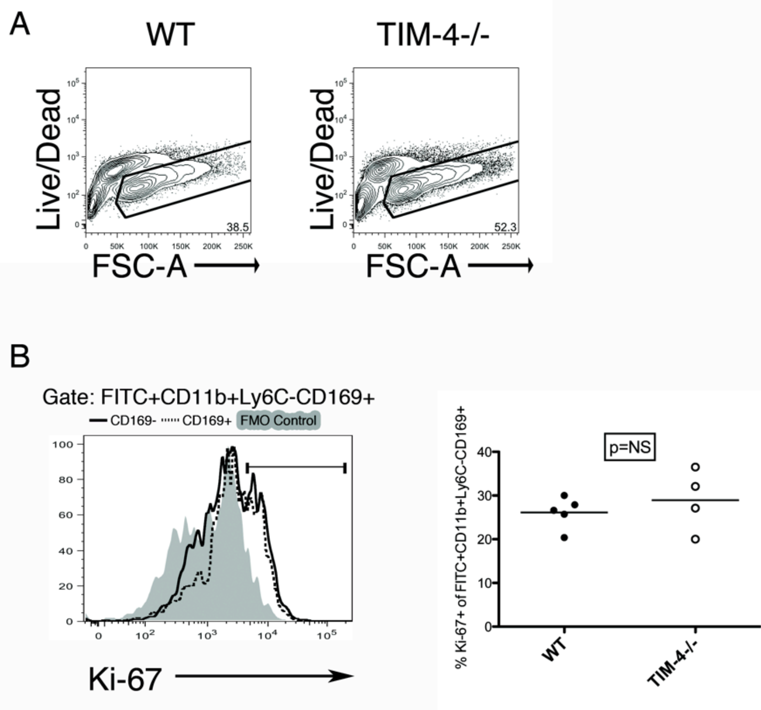

A
CD169- (2,538)
CD169+ (86,387)
TIM-4-/- bm12 (205)
TIM-4
CD169- (23,254)
CD169+ (9,029)
FMO Control (772)
CD86
CD169- (3,877)
CD169+ (7,683)
FMO Control (70)
Galectin-9
CD169- (1,283)
CD169+ (2,708)
FMO Control (70)
CD73
C
No Tregs (12.2)
1:20 Treg:Tnaive (4.89)
1:2 Treg:Tnaive (1.57)
CellTrace Violet


B
CD169-
CD169+
WT
2.42
7.72
TIM-4-/-
3.14
8.97
WT
+ TGFβ
18.3
30.1
FoxP3-GFP
CD25

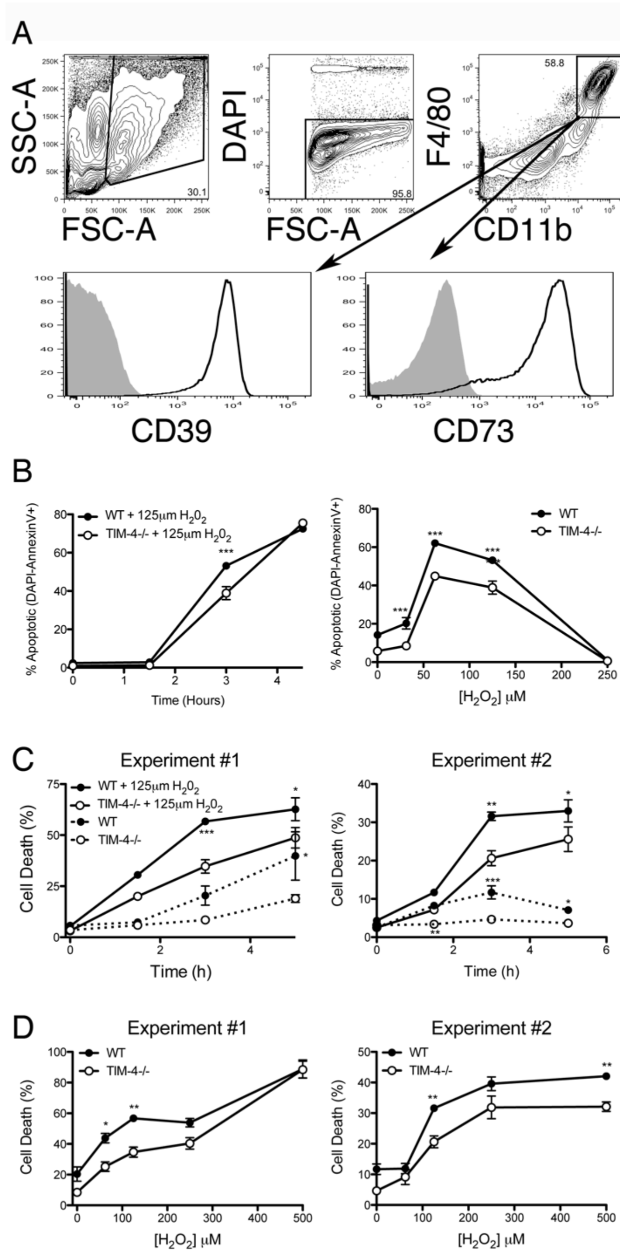

D
Expansion Index
p<0.05
TIM-4-/- CD169-
TIM-4-/- CD169+


E
%CD25hi FoxP3/GFP+ of ABM T Cells
p<0.05
TIM-4-/- CD169-
TIM-4-/- CD169+

Supplemental Figure 2: FoxP3/GFP$^+$ Tregs from CD169$^+$ macrophage stimulated cultures are immunoregulatory. (A) dLN cells from FITC painted bm12 mice were stained and gated as described in **Figure 2** for C57BL/6 dLN cells. The phenotype of FITC$^+$CD11b$^+$Ly6C$^-$CD169$^+$ relative to CD169$^-$ bm12 cells is similar to C57BL/6 mice in **Figure 2**. **(B)** Flow cytometry plots depict FoxP3/GFP reporter and CD25 expression in ABM TCR transgenic (CD4$^+$Vα2$^+$Vβ8$^+$) T cells stimulated with wild type **(top)** or TIM-4-/- **(middle)** CD169$^-$ **(left)** or CD169$^+$ **(right)** APCs in the presence **(bottom)** or absence of 5 ng/ml TGFβ as indicated. **(C)** Unlabeled CD4$^+$FoxP3/GFP$^+$ ABM TCR transgenic Tregs from CD169$^+$ macrophage stimulated MLR cultures were sorted and re-cultured with 2.5 x 10^4 sorted CD90$^-$ (T-depleted) bm12 splenocytes and CellTrace Violet labeled FoxP3/GFP$^-$ ABM TCR transgenic responder T cells at the indicated ratio for 96 hours. The shaded histogram represents the unstimulated control cells. Proliferation histograms for responder T cells are shown. Expansion indices are indicated parenthetically. **(D)** The expansion indices are plotted for ABM TCR transgenic T cells stimulated with TIM-4$^{-/-}$ APC populations. **(E)** The frequencies (mean +/- S.D.) of CD25^{hi}FoxP3/GFP$^+$ cells within the gated transgenic CD4$^+$Vα2$^+$Vβ8$^+$ population in TIM-4$^{-/-}$ APC stimulated cultures are graphed.

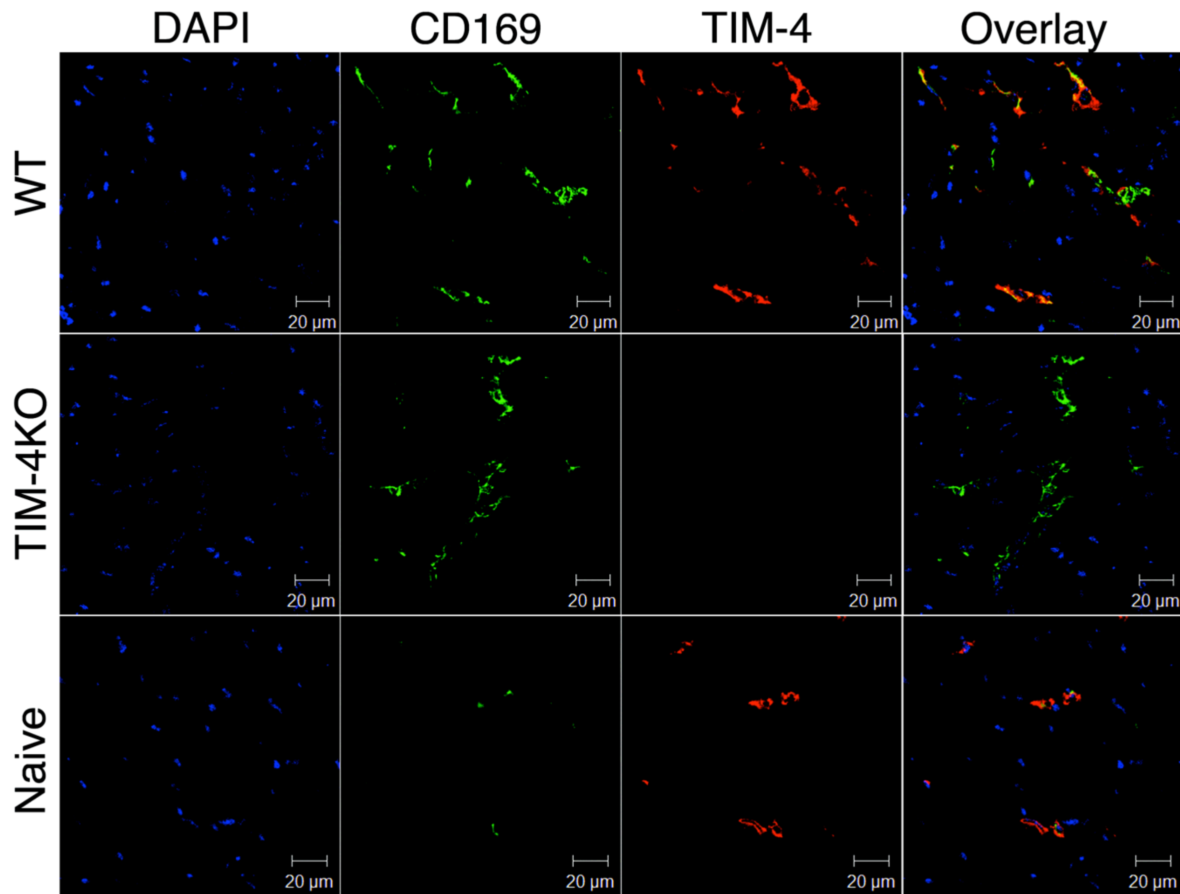


Supplemental Figure 3: The increase in dLN migrating CD169$^+$ macrophages in TIM-4$^{-/-}$ as compared to wild type hosts is due to an increase in viability and not proliferation. (A) We identified viable FITC$^+$CD11b$^+$Ly6C$^-$CD169$^+$ cells by excluding dying cells undergoing shrinkage (FSC-A) and dead cells staining with DAPI in wild type (left) or TIM-4$^{-/-}$ (right) FITC painted mice. **(B)** The percent of FITC$^+$CD11b$^+$Ly6C$^-$ CD169$^+$ dLN cells expressing the proliferation-specific antigen Ki-67 is similar in wild type (solid line and filled circles) and TIM-4$^{-/-}$ (dashed line and open circles) FITC painted mice, p=N.S. by Mann-Whitney t test.



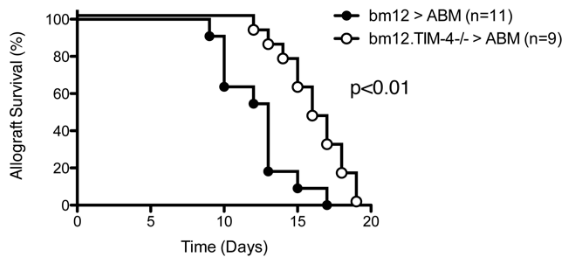
Supplemental Figure 4: TIM-4$^{-/-}$ peritoneal macrophages are resistant to peroxide induced apoptosis relative to wild type macrophages. (A) For peritoneal macrophage phenotype data, we identified viable peritoneal macrophages by size and light scattering properties (panel 1), viability (panel 2), and the expression of CD11b and F4/80 (panel 3). CD39 and CD73 expression on gated CD11b$^+$F4/80$^+$ cells is shown

(bottom). **(B)** Peritoneal macrophage apoptosis was measured by flow cytometry as described in **Figure 5C. (C&D)** C57BL/6 wild type (filled circles) or TIM-4$^{-/-}$ (open circles) peritoneal macrophages were plated in 96 well plates, stained with anti-F4/80-PE and DAPI, and cultured with (solid line) or without (dashed line) H_2O_2 (125 μM unless otherwise indicated). Using an F4/80^{+} macrophage gate, we calculated the frequency of dead (DAPI^{+}) macrophages on a Celigo plate imaging cytometer. Means +/- S.E.M. from each of two independent experiments are plotted according to time **(C)** or dose **(D)**. A two-way ANOVA revealed a significant difference between wild type (filled circles) and TIM-4$^{-/-}$ (open circles) groups for time ($p<0.001$) and dose ($p<0.0001$) in both experiments. For all panels, Bonferroni's post-test results are indicated as: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$**.**

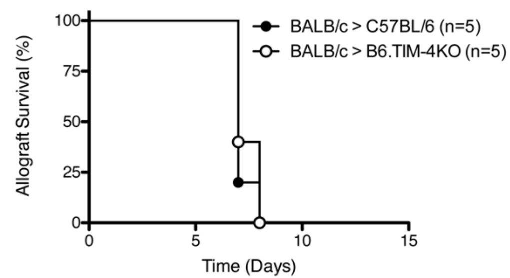


Supplemental Figure 5: CD169$^+$ macrophages in donor hearts are donor-derived and co-express TIM-4. Three days after transplantation into wild type BALB/c recipients, C57BL/6 wild type or TIM-4$^{-/-}$ hearts were removed, snap-frozen in OCT medium, sectioned at 5 µm, and stained with anti-CD169, anti-TIM-4, and DAPI. The naïve control heart was obtained from an untransplanted C57BL/6 mouse. CD169$^+$ macrophages in wild type but not TIM-4$^{-/-}$ hearts are TIM-4^{hi}, demonstrating that TIM-4^{hi}CD169$^+$ macrophages are donor tissue-resident macrophages.

A skin transplantation



B heart transplantation



Supplemental Figure 6: TIM-4$^{-/-}$ bm12 skin allograft survival in ABM TCR transgenic recipients is prolonged but wild type heart allografts exhibit comparable survival in wild type and TIM-4$^{-/-}$ C57BL/6 mice. (A) Wild type bm12 (filled circles) or TIM-4$^{-/-}$ bm12 (open circles) tail skin was transplanted onto ABM TCR transgenic mice. The survival of TIM-4$^{-/-}$ bm12 skin allografts was significantly prolonged as compared to wild type bm12 transplants; $p<0.01$ by log-rank Mantel-Cox analysis. **(B)** Wild type BALB/c hearts were transplanted into allogeneic wild type (filled circles) or TIM-4$^{-/-}$ (open circles) C57BL/6 recipients as described in **Figure 6**. The survival was comparable between both groups; p=N.S. by log-rank Mantel-Cox analysis.

SUPPLEMENTAL TABLES

Supplemental Table I: Real-time PCR primer sequences

Transcript	Forward Primer (5 to 3')	Reverse Primer (5' to 3')
GAPDH	AGGTCGGTGTGAACGGA TTTG	TGTAGACCATGTAGTTGAGGTCA
CD169	AGTGATAGCAACCGCTGGTTA	GCACAGGTAGGGTGTGGAAC
CD86	TCAATGGGACTGCATATCTGCC	CAGCTCACTCAGGCTTATGTTTT
CD80	ACCCCCAACATAACTGAGTCT	TTCCAACCAAGAGAAGCGAGG
TGFβ	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
Tbet	AGCAAGGACGGCGAATGTT	GGGTGGACATATAAGCGGTTC
GATA3	CTCGGCCATTCGTACATGGAA	GGATACCTCTGCACCGTAGC
FoxP3	CCCATCCCCAGGAGTCTTG	ACCATGACTAGGGGCACTGTA
TRIB1	GCTCGGCTCTTCAAGCAGATT	GCTTTCCAGTCTAAGCTGGGT
CD39	AAGGTGAAGAGATTTTGCTCCAA	TTTGTTCTGGGTCAGTCCCAC
CD73	GGACATTTGACCTCGTCCAAT	GGGCACTCGACACTTGGT
TIM-4	CACCTGGCTCCTTCTCACAA	TGATTGGATGCAGGCAGAGTT
galectin-9	ATGCCCTTTGAGCTTTGCTTC	AACTGGACTGGCTGAGAGAAC