Supplemental Information

STIM1 controls T cell-mediated immune regulation and inflammation in chronic infection

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Supplemental Materials and Methods

Calcium measurements

Murine $CD4^+$ and $CD8^+$ T cells isolated from the spleens of WT and *Stim1*^{CD4} mice or human $CD4^+$ and $CD8^+$ T cells from the cord blood or peripheral blood lymphocytes (PBL) from healthy donors (HD) and PBL from a SOCE-deficient patient (PAT) with an ORAI1 p.R91W mutation (1) were loaded with 2 μM Fluo-4-AM for 30min at room temperature and incubated with αCD4 and αCD8 antibodies. Measurements of intracellular $[Ca^{2+}]_i$ were conducted using a LSRII. Baseline $[Ca^{2+}]_i$ was acquired in 0 mM Ca^{2+} Ringer solution containing (in mM) 155 NaCl, 4.5 KCl, 3 $MgCl_2$, 10 D-glucose, 5 Na-HEPES. After 30s, cells were stimulated with 1 μM of the sarco/endoplasmic reticulum ATPase (SERCA) inhibitor thapsigargin (TG)(EMD Millipore). After 300s, Ringer solution containing 2 mM $CaCl_2$ was added to cells for a final extracellular $[Ca^{2+}]$ of 1 mM. $[Ca^{2+}]_i$ was calculated as F/F_0 where F is Fluo-4 fluorescence emission at 506 nm and F_0 is baseline Fluo-4 fluorescence emission before TG stimulation (0-30s).

iTreg suppression assays

For suppression assays, naïve $CD4^+CD62L^+CD25^-$ T cells were isolated from the spleens of WT or *Stim1*^{CD4} mice and stimulated in vitro with αCD3/αCD28 and 2.5 ng/ml TGFβ. After 72h, cells were cultured in RPMI-1640 medium supplemented with 20 U/ml recombinant human IL-2. On day 7, $CD4^+CD25^+$ cells were sorted and co-incubated at a 1:1:1 ratio with naïve $CD4^+$ T cells from $CD45.1^+$ WT mice labeled with 1 μM CFSE and bone marrow-derived macrophages (BMDM) from WT mice. Cells were cultured in the presence of 0.5 μg/ml αCD3 and analyzed for CFSE dilution in $CD45.1^+$ T cells after 4 and 5d. For investigating the role of STIM1 in iTreg mediated suppression of myeloid cells, in-vitro differentiated WT or STIM1-deficient iTreg cells (see above), were co-incubated over night with WT BMDM in the presence of 0.5 μg/ml αCD3 in a 1:1 ratio. After 16h, 1μg/ml LPS was added to cell cultures to activate macrophages. After 24h, supernatants were harvested and the production of IL-12p40 was determined by ELISA (eBioscience).

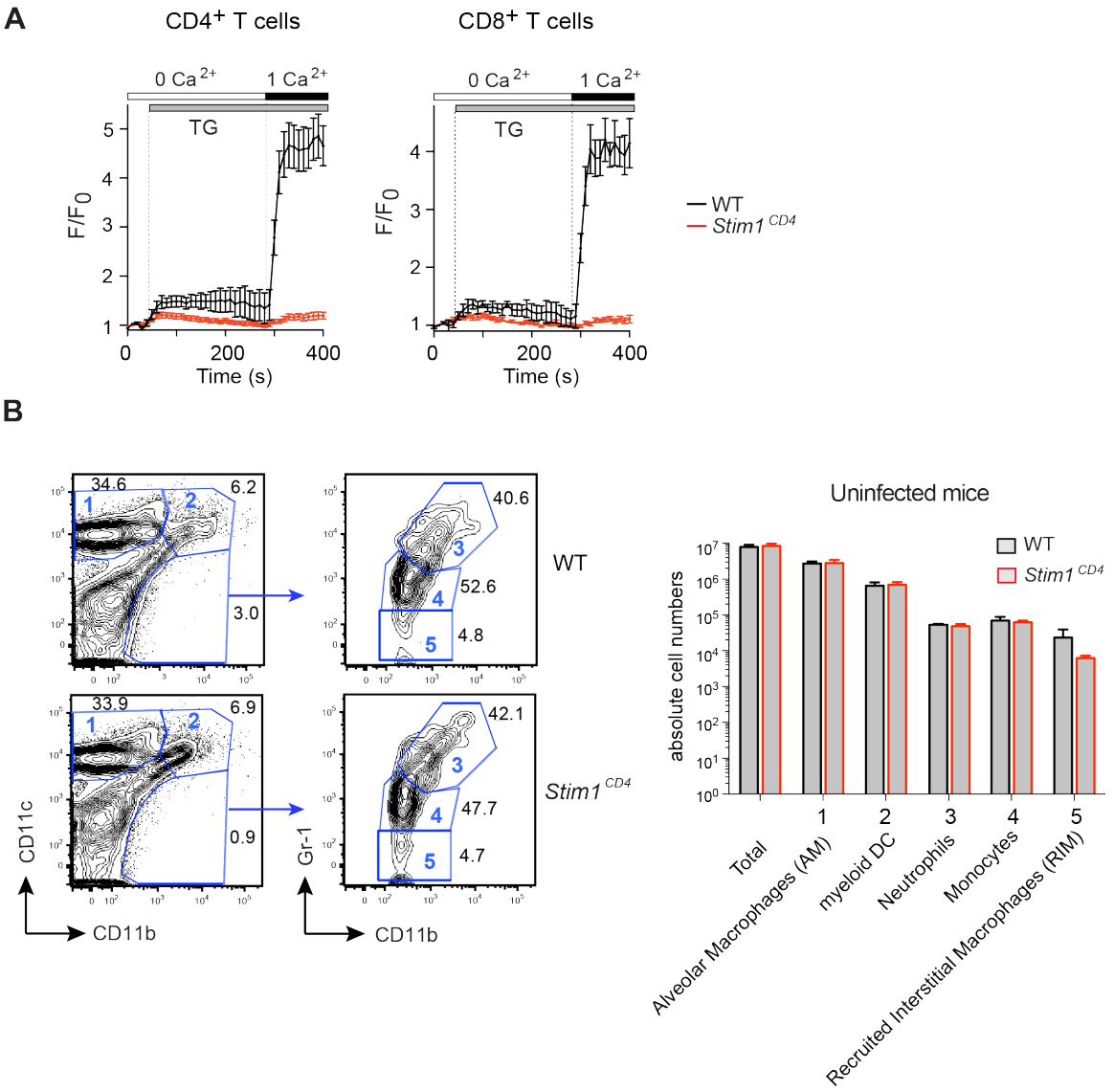
Supplemental Reference

1. Feske, S., Y. Gwack, M. Prakriya, S. Srikanth, S. H. Puppel, B. Tanasa, P. G. Hogan, R. S. Lewis, M. Daly, and A. Rao. 2006. A mutation in Orai1 causes immune deficiency by abrogating CRAC channel function. *Nature* 441:179-185

Supplemental Table I

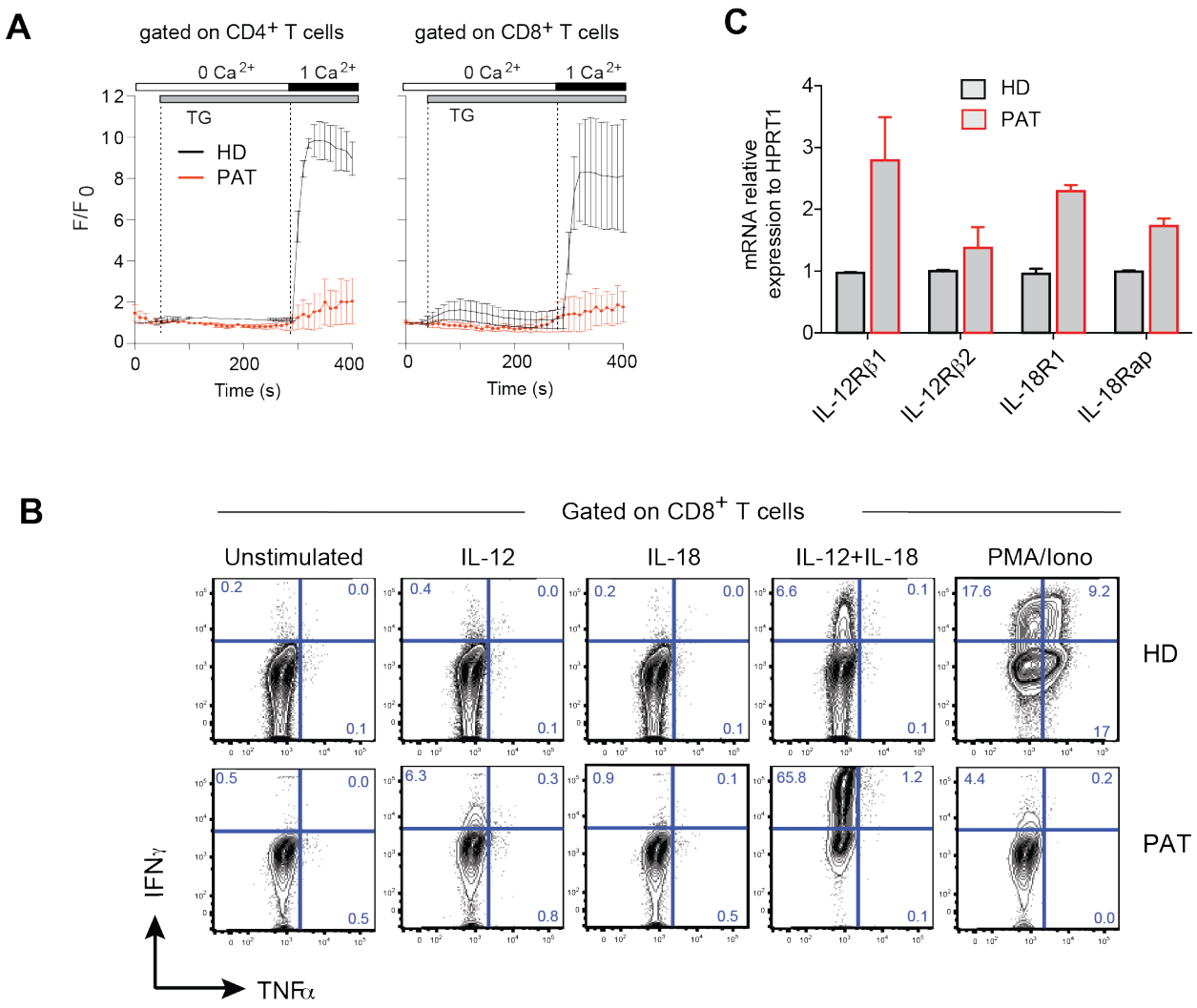
Gene name	Forward primer	Reverse primer
Mouse		
Cd4	CAAGCGCCTAAGAGAGATGG	CTTCTGCAACCTCCAGCATC
Cd8	TCAAGACGGCCCTTTCTCAGT	TCCCTGTCCCAAAGACCATCT
Fas	TATCAAGGAGGCCCATTTTGC	TGTTTCCACTTCTAAACCATGCT
Fasl	CTGGGTTGTACTTCGTGTATTCC	TGTCCAGTAGTGCAGTAGTTCAA
Noxa	GCAGAGCTACCACCTGAGTTC	CTTTTGCGACTTCCCAGGCA
Human		
CD4	CTTGGTCCCAAAGGCTTCTT	AGATTCTGGGAAATCAGGGC
CD8	GTCTCCCGATTTGACCACA	GGGTCCTTCTCCTGTCACTG
FAS	TCCTCAATTCCAATCCCTTG	GCATCTGGACCCTCCTACCT
FASLG	GGACCTTGAGTTGGACTTGC	GCACACAGCATCATCTTTGG
IL12RB1	TGAGTCTGCATCCGGATATG	TCCTCTTCCTCTTCCTGCTG
IL12RB2	AGAATGTTGTCCATGCCCTC	CAGTTCTCTGCCCACTCGTA
IL18R1	GGTCCAAGAAGAACCGGAAC	GCGTGCATTTCCTTCATCAT
IL18RAP	ATCCACTACGATTCGGTTGC	CAGTCTCAGCTGCCAAAGTG
NOXA	AAGTTTCTGCCGGAAGTTCA	GCAAGAACGCTCAACCGAG

Supplemental Figures



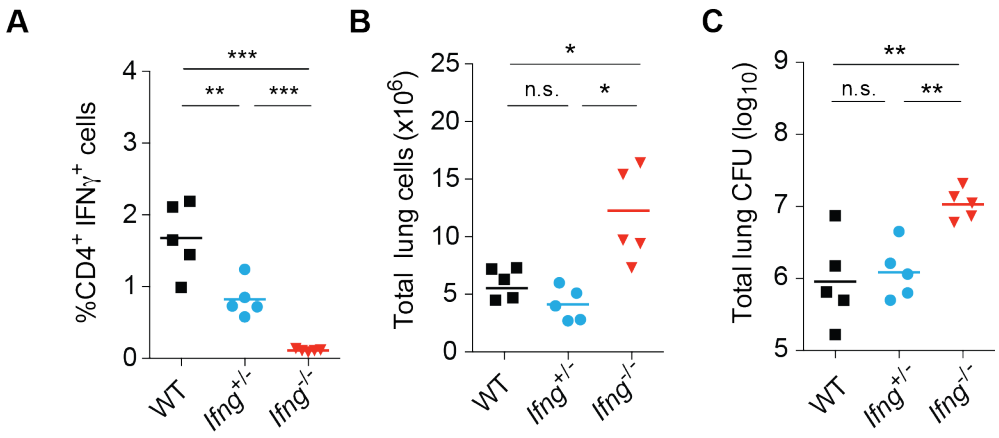
Supplemental Figure 1. Uninfected *Stim1*^{CD4} mice lack signs of pulmonary inflammation despite defective SOCE in T cells.

(**A**) Store-operated Ca^{2+} influx (SOCE) in $CD4^+$ and $CD8^+$ T cells isolated from the spleen of uninfected *Stim1*^{CD4} mice or WT littermates and analyzed by flow cytometry. Fluo-4 loaded T cells were stimulated with 1 μM thapsigargin (TG) in Ca^{2+} free buffer followed by re-addition of Ca^{2+} (final concentration of 1 mM). Graphs represent the mean±SEM of Fluo-4 fluorescence (F) normalized to baseline fluorescence (F_0, at 0-30s) from 6 mice per group. (**B**,**C)** Myeloid cell populations in the lungs of uninfected WT and *Stim1*^{CD4} mice analyzed by flow cytometry after staining for CD11b, CD11c and Gr-1. Gating strategy and absolute numbers of alveolar macrophages (AM, 1), myeloid dendritic cells (mDC, 2), neutrophils (3), monocytes (4), recruited interstitial macrophages (RIM, 5) from three mice per group. Statistical significance was calculated by Student's t-test.



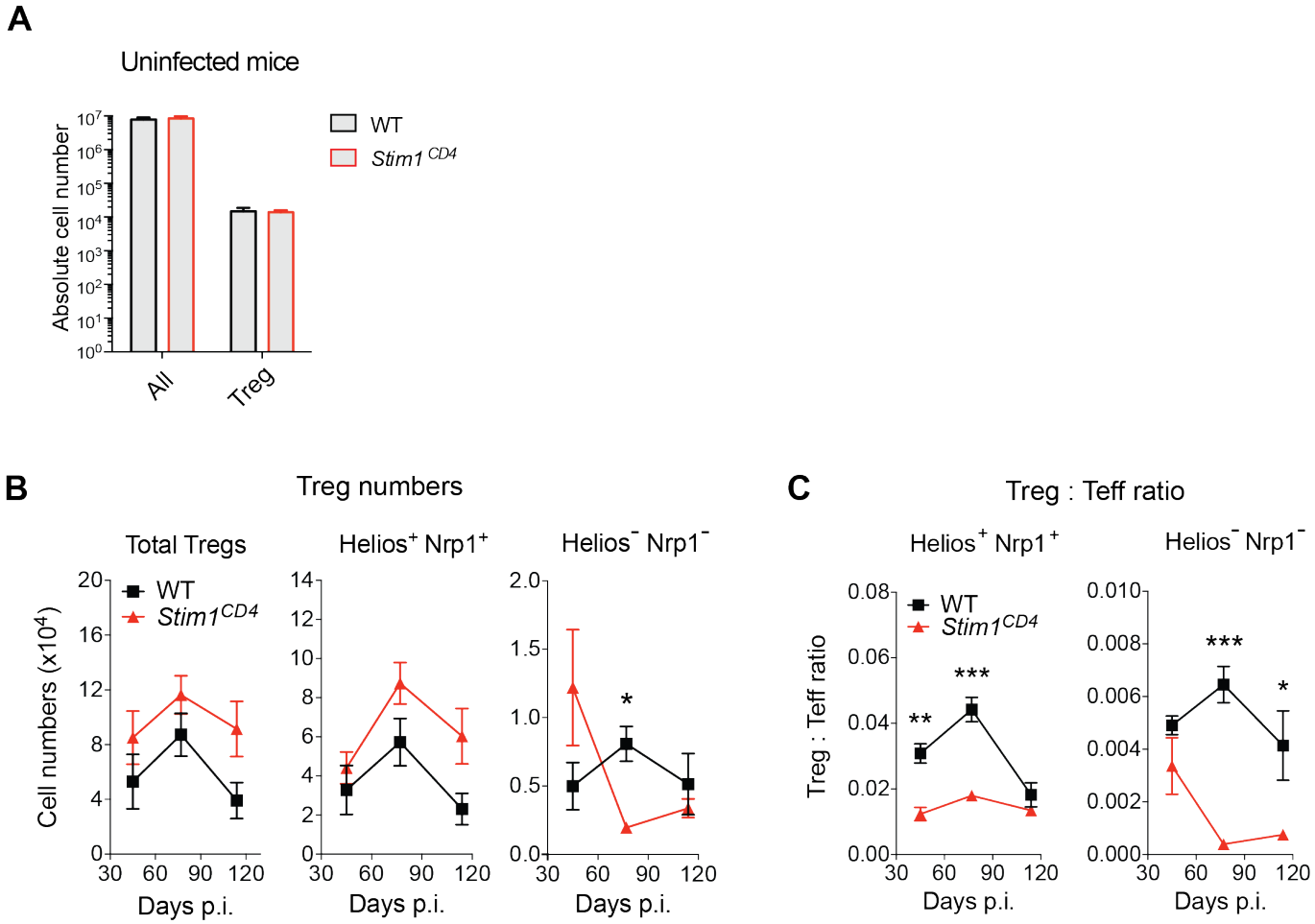
Supplemental Figure 2. IL-12- and IL-18-induced IFNγ production by T cells from human patients and *Mtb*-infected mice is independent of SOCE.

(**A**) SOCE in $CD4^+$ and $CD8^+$ T cells isolated from PBL of a SOCE-deficient patient with ORAI1 p.R91W mutation (PAT) (1) and a healthy donor (HD) was analyzed by flow cytometry. Fluo-4 loaded T cells were stimulated with 1 μM thapsigargin (TG) in Ca^{2+}-free buffer followed by re-addition of Ca^{2+} (final concentration 1 mM). Graphs represent the mean ± SEM of Fluo-4 fluorescence (F) normalized to baseline fluorescence (F_0, at 0-30 sec) of one experiment done in technical duplicates. (**B**) $CD8^+$ T cells from the ORAI1-deficient patient (PAT) and cord blood cells from a HD were stimulated with recombinant IL-12p70, IL-18, IL-12p70 plus IL-18 or with PMA/ionomycin for 6h and analyzed for IFNγ and TNFα production by flow cytometry. Contour plots represent results of two independent experiments. (**C**) Expression of IL-12R and IL-18R subunits in PBMC from the ORAI1-deficient patient (PAT) and a HD analyzed by quantitative RT-PCR. Results shown are representative of one experiment done in technical triplicates. Data are supplementary to **Figures 3F, 5F and 7B**.



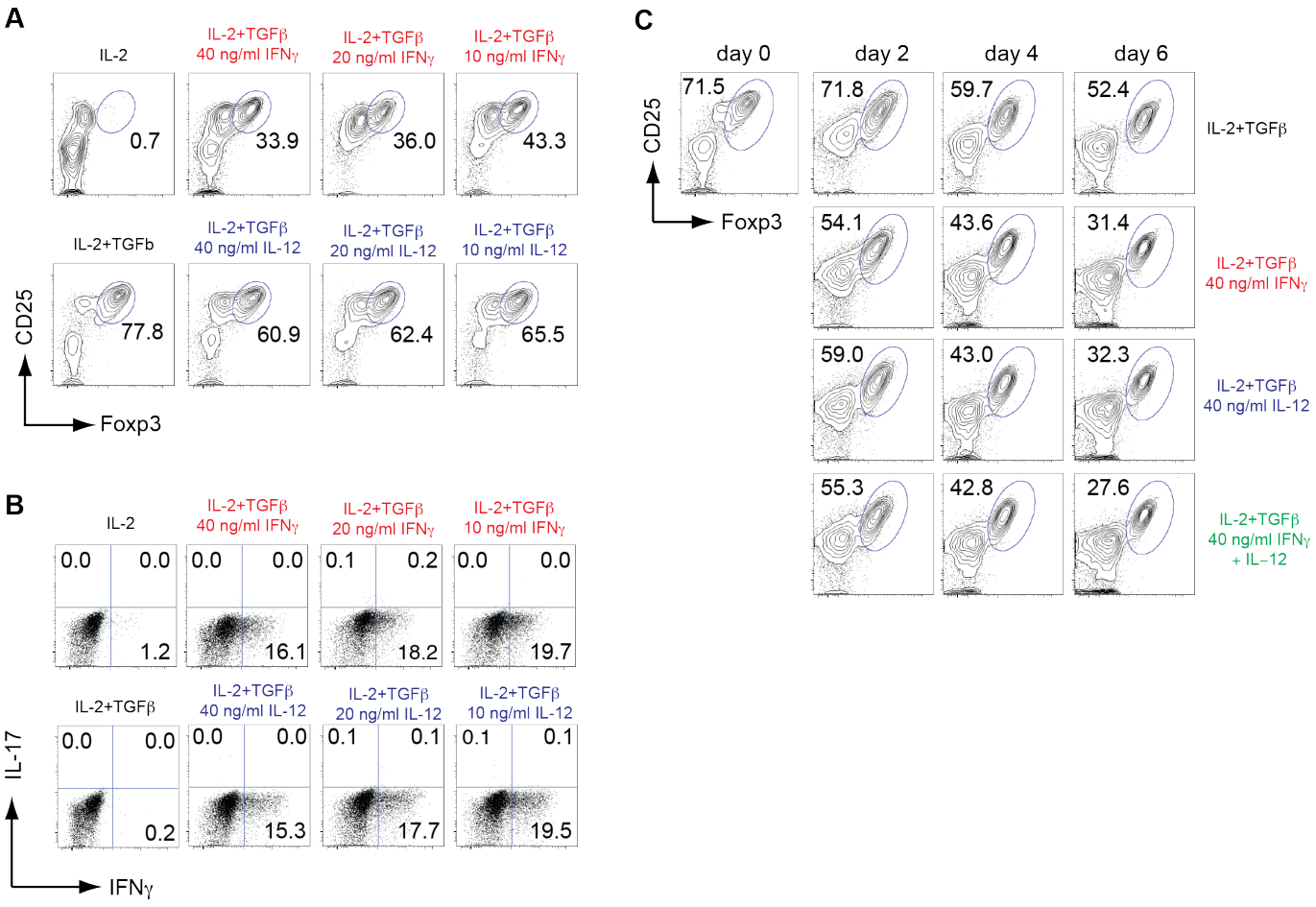
Supplemental Figure 3. Haploinsufficient *Ifng*$^{+/-}$ mice control *Mtb* growth normally during acute pulmonary *Mtb* infection despite reduced IFNγ production by CD4^{+} T cells.

C57BL/6 (WT), *Ifng*$^{+/-}$ and *Ifng*$^{-/-}$ mice (5 per group) were infected with *Mtb* H37Rv (100 Colony Forming Units/mouse) via the aerosol route and their lungs were harvested 35d later. (**A**) Frequency of CD4^{+} IFNγ^{+} T cells in the lungs quantified without ex vivo re-stimulation by intracellular cytokine staining and flow cytometry. (**B**) Quantification of total live lung cell numbers by trypan blue exclusion method. (**C**) Enumeration of total lung Colony Forming Units (CFU) of *Mtb* by plating lung supernatants on 7H11 agar. Statistical significance was calculated using Student's t test. (*p<0.05; **p<0.01; ***p<0.005; n.s., not significant).



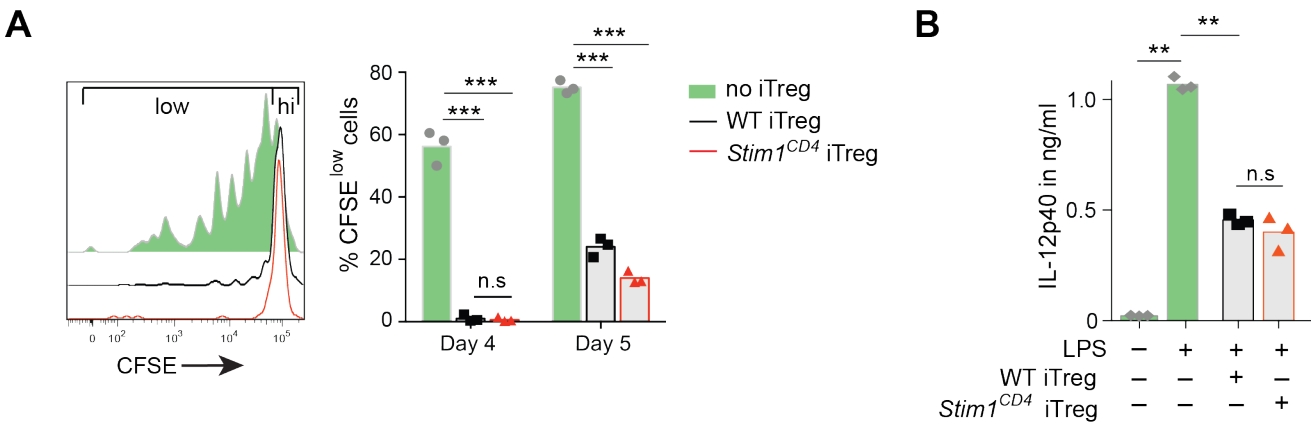
Supplemental Figure 4. Lack of STIM1 results in reduced iTreg cell numbers and iTreg:Teff ratios during chronic *Mtb* infection.

(**A**) Numbers of total cells and $CD4^+CD25^+Foxp3^+$ Treg cells in the lungs of uninfected WT and *Stim1*^{CD4} mice were analyzed by flow cytometry. For Treg cells, the same gating strategy was used as in **Fig. 6A**. Bar graphs represent cell numbers from 3 mice per group. (**B**) Absolute numbers all Treg, $Helios^+Nrp1^+$ nTreg and $Helios^-Nrp1^-$ iTregs cells in the lungs of WT and *Stim1*^{CD4} mice following *Mtb* infection. (**C**) Ratios of $Helios^+Nrp1^+$ nTreg and $Helios^-Nrp1^-$ iTregs cells to $Foxp3^-CD4^+$ effector T cells (Teff) in the lungs of WT and *Stim1*^{CD4} mice over the course of *Mtb* infection. Statistical significance was calculated using Student's t test. (*p<0.05; **p<0.01; ***p<0.005). Data are supplementary to **Figure 6A-C**.



Supplemental Figure 5. IFNγ and IL-12 impair the differentiation and maintenance of iTreg cells in vitro.

(**A,B**) Effects of IFNγ and IL-12 on iTreg cell differentiation. Naïve $CD4^+$ T cells isolated from WT mice were stimulated with αCD3/αCD28 and cultured under iTreg polarizing conditions with TGFβ and IL-2 for 3d in the presence or absence of 10-40 ng/ml IFNγ or 10-40 ng/ml IL-12. Cells were analyzed by flow cytometry for (**A**) the frequency of $Foxp3^+CD25^+$ iTreg cells and (**B**) the production of IL-17 and IFNγ after re-stimulation of cells with PMA/ionomycin for 6 h. (**C**) Effects of IFNγ and IL-12 on iTreg cell maintenance. Naïve $CD4^+$ T cells were differentiated into iTreg cells for 3d as described above. Cells were subsequently cultured in IL-2 + TGFβ (Control) or IL-2 + TGFβ supplemented with IFNγ, IL-12, or IFNγ + IL-12 as indicated for 2-6d. The frequency of $Foxp3^+CD25^+$ iTreg cells was analyzed by flow cytometry. Contour and dot plots in A-C are representative of 4 mice per group and supplement **Figure 6D** and **E**.



Supplemental Figure 6. STIM1 is not required for the suppressive function of iTreg cells in vitro. (**A,B**) Suppressive function of in vitro differentiated iTreg cells. (**A**) Naïve $CD4^+$ T cells were isolated from *Stim1*^{CD4} and WT mice, stimulated with plate-bound αCD3/αCD28 and differentiated into iTreg cells for 6d in vitro in the presence of TGFβ. $CD4^+CD25^+$ T cells (Treg) were enriched by cell sorting and co-incubated at a 1:1:1 ratio with CFSE-labeled naïve $CD4^+$ T cells from $CD45.1^+$ WT mice (T_{naive}) and bone marrow derived macrophages (BMDM) from WT mice in the presence of 0.5 µg αCD3. CFSE dilution in T cells was measured at days 4 and 5 after stimulation; representative histograms and frequencies of $CFSE^{low}$ $CD45.1^+$ T cells from 3 mice per genotype. (**B**) iTreg cells and BMDM were generated as described in A and co-cultured at a 1:1 ratio in the presence of LPS. Levels of IL-12p40 produced by BMDM were measured after 24h in cell culture supernatants. Each dot represents one mouse, bar graphs represent means. Statistical significance was calculated using Student's t-test. (*p<0.05; **p<0.01; ***p<0.005; n.s., not significant). Data are supplementary to **Figure 7.**