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Supplemental Information

Differential Roles of the Calcium Ion Channel TRPV4 in Host Responses to *Mycobacterium tuberculosis* Early and Late in Infection

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Figure S1 Related to Figure 1

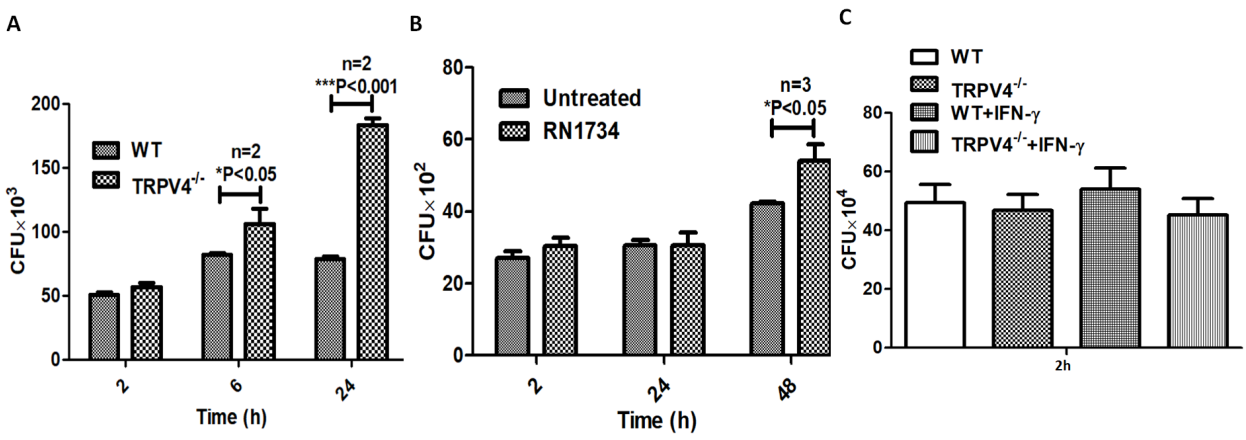

Figure S1.

(A) BMDM from WT and *Trpv4*$^{-/-}$ macrophages were infected with *M. smegmatis* (Msm) at a MOI 1 and intracellular bacterial counts were assessed by CFU at indicated time points p.i. **(B)**RAW264.7 macrophages were treated with the Trpv4 inhibitor, RN1734 (10μM), prior to *M. tuberculosis* infection of a MOI1. Intracellular mycobacterial counts were assessed at indicated time points by CFU assay. **(C)** BMDM from WT and *Trpv4*$^{-/-}$ mice were infected with *M. tuberculosis* at MOI 5 and the numbers of phagocytosed *M. tuberculosis* were assessed by CFU at 2h p.i. Data represent the mean ± SEM from 2 - 3 (n) independent experiments. Statistical analysis was performed with Two Way ANOVA Bonferroni posttests. *P<0.05, ***P<0.001.

Figure S2 Related to Figure 2

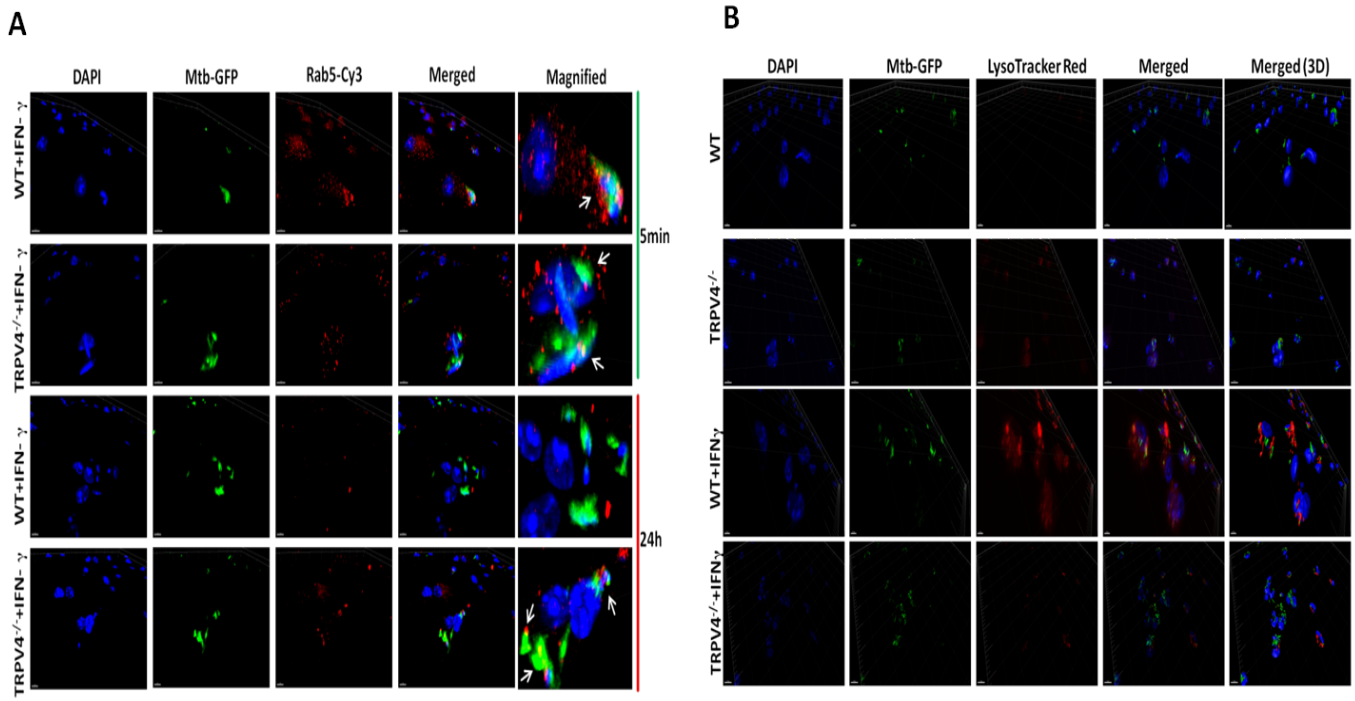

Figure S2

(A) Images showing different image channels for nuclei, *M. tuberculosis* and Rab5 as shown in Fig. 2A. **(B)** Images showing different image channels for nuclei, *M. tuberculosis* and lysotracker as shown in Fig. 2C.

Figure S3 Related to Figure 4 and Figure 5

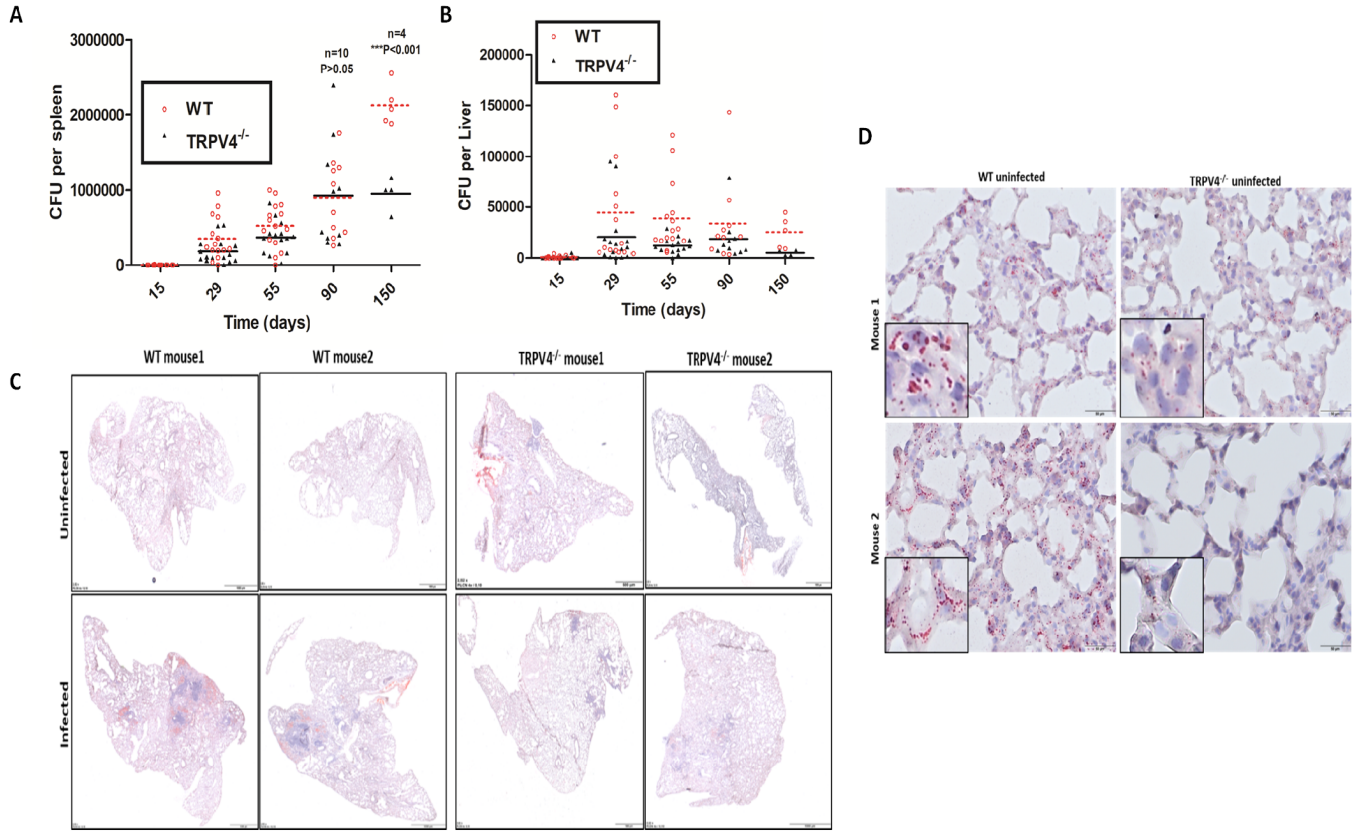

Figure S3

(A), (B) *M. tuberculosis* counts from spleen and liver of individual *M. tuberculosis* infected mice at different time points p.i. Data shown are from 3 independent experiments and each point represents one mouse. Statistical analysis was performed with Two Way ANOVA Bonferroni posttests. ***P<0.001. **(C)** Complete lung section of uninfected and *M. tuberculosis* infected mice showing oil red O stained lipid bodies (pink) at day 55 p.i. **(D)** Tissue section showing oil red O positive lipid bodies in uninfected WT and *Trpv4*$^{-/-}$ mice. Nuclei = DAPI/blue.

Figure S4 Related to Figure 5

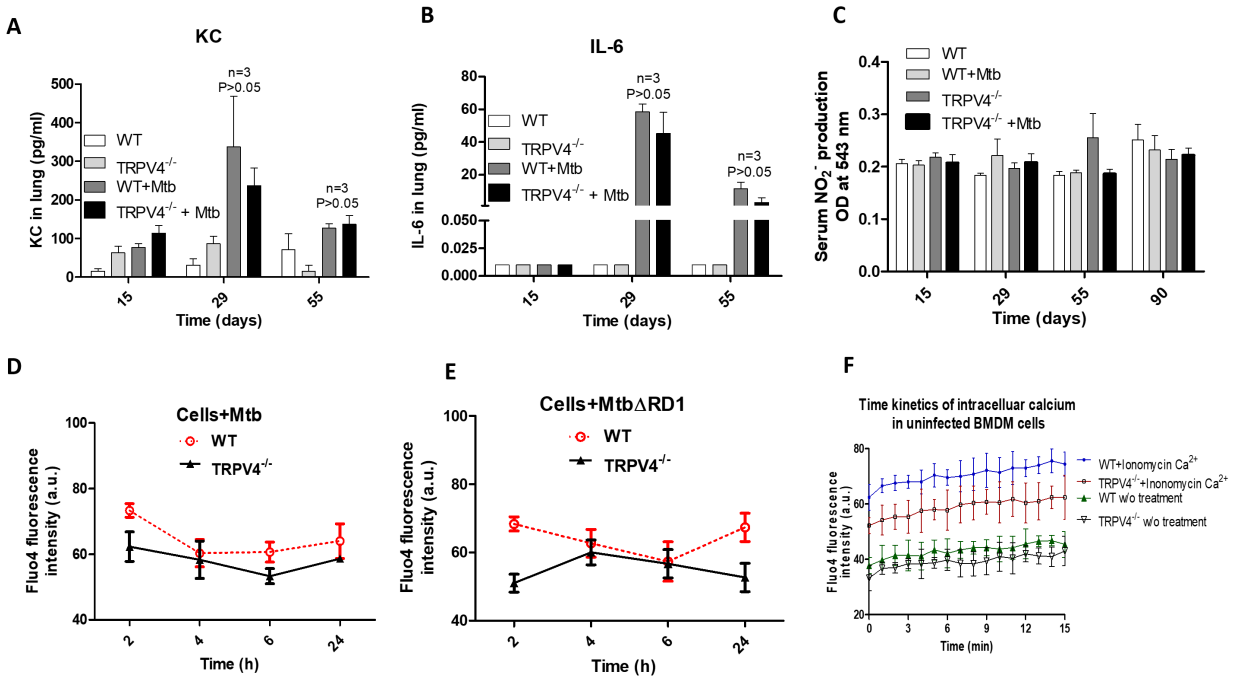

Figure S4

(A), (B) Expression of KC and IL-6 in lung lysates from WT and *Trpv4*$^{-/-}$ mice at different time points p.i. **(C)** Nitrite production was measured in lung lysates prepared from *M. tuberculosis* infected WT and *Trpv4*$^{-/-}$ mice at indicated time points using Nitrate reductase and Griess reagent. The NO_2^- production was represented as the absorbance of Griess reagent at 543nm. **(D,E)** Intracellular calcium levels in *M. tuberculosis* or MtbΔRD1 infected WT and *Trpv4*$^{-/-}$ BMDM was measured by incubating the cells with permeable Fluo4AM (4µg/ml) dye for 30 minutes in dark. Fluorescence intensity of Fluo4AM was measured at Ex/Em of 494/506 nm using Biotek multiplate reader. **(F)** Intracellular calcium level in resting WT and TRPV4$^{-/-}$macrophages in presence of ionomycin Ca^{2+}.Statistical analysis was performed with Two Way ANOVA Bonferroni posttests.

Transparent Methods

Statement

All experiments and methods were performed in accordance with institutional guidelines and regulations. Animal studies were approved by the Ministry of Energy, Agriculture, Environment, Nature and Digitization of the state of Schleswig-Holstein, Germany [V 242-71197/2017(14-2/18)]. Experiments performed with primary human cells were reviewed and approved by the Ethics Committee of the University of Lübeck, Germany (#13-032). Study with human TB patient samples were approved by the Ethics Committee of the University of Lübeck, Germany (Ethical Approval No.14-032 and #18-194).

Bacterial strains, Cells and Reagents

M. tuberculosis H37Rv, green fluorescent protein (GFP) tagged *M. tuberculosis* (Mtb-GFP), Discosoma Red fluorescent protein (DsRed) expressing *M. tuberculosis*, Region of Difference-1 (RD1) deleted *M. tuberculosis* (MtbΔRD1), MtbΔRD1-GFP and *M. smegmatis* were cultured as described previously (Dallenga et al., 2017). To stabilize GFP and Ds-Red expression, the bacteria were grown in 7H9 media supplemented with hygromycin-B (50 µg/ml), kanamycin (20 µg/ml) and hygromycin-B (50 µg/ml), respectively. Primary antibodies directed against Trpv4 (#ACC034, Alomone lab), Rab5 (#GTX108605, GeneTex), neutrophil elastase (#51-862, proSci-ELAN)were used in this study. Secondary antibodies goat anti rabbit-cy3 (#111-165-144) were procured from Jackson Immunoresearch, UK.

Murine alveolar and peritoneal macrophages from both male and female mice were isolated from broncho-alveolar and peritoneal lavages obtained from C57BL/6J WT or *Trpv4*-knockout (*Trpv4*$^{-/-}$) mice as described previously (Schneider et al., 2014). The isolated cells were suspended

in DMEM medium containing 10 % heat inactivated fetal bovine serum (FBS) and 2 mM L-glutamine, plated (1×10^5 cells per well) on 96-well tissue culture plates and incubated in 5% CO_2 at 37^0Cfor at least 3 hours to allow macrophages to adhere.

Bone marrow derived macrophages (BMDM) from both male and female WT or *Trpv4*$^{-/-}$ mice were harvested by flushing femurs and tibias as described previously (Herbst et al., 2011). Harvested cells were differentiated in DMEM containing 10% heat inactivated FBS, 2 mM L-glutamine and L929 cell supernatant (20% v/v) as a source of colony stimulating factor for one week at 37^0C and 5% CO_2.

Animal model, *M. tuberculosis* infection and CFU assay

Trpv4$^{-/-}$mice were generated on a C57BL/6J background as described previously (Liedtke and Friedman, 2003). Both WT and *Trpv4*$^{-/-}$mice were bred and housed under specific pathogen free (SPF) condition at the Research Center Borstel-Leibniz Lung Center (RCB). Animal studies were approved by the Ministry of Energy, Agriculture, Environment, Nature and Digitization of the state of Schleswig-Holstein, Germany [V 242-71197/2017(14-2/18)].

For aerosol infection, 6-8 weeks old both male and female mice were infected with *M. tuberculosis*-H37Rv (100 CFU) using an aerosol chamber (GlasCol, USA). After aerosol challenge, bacterial load in lung, spleen and liver was determined at different time points by mechanical disruption of organs in 0.1% v/v tween 80 in milliQ water containing a protease inhibitor cocktail (Roche Diagnostics). Ten-fold serial dilutions of organ homogenates in 0.01% v/v tween 80 and 0.05% w/v albumin were plated onto Middlebrook 7H11 agar plates and colonies were counted after 3-4 weeks of incubation at 37^0 C.

Macrophage infection assay

Bacterial cultures in mid-exponential phase were pelleted, washed with 1X PBS (pH 7.4). Bacterial clumps were removed by passing five times through a 27G blunt needle. *M. tuberculosis*-H37Rv culture at a final optical density of 0.1 at 600 nm (OD_{600}), which corresponds to 5×10^7 bacteria ml^{-1} was prepared with DMEM. 1×10^5 macrophages were infected at MOI 1 or otherwise as indicated. For activation of macrophages, cells were incubated overnight with 500 units/ml of IFN-γ prior to the infection. After 2 h of infection, cells were washed 3 times with 1X PBS to remove any extracellular bacteria. At different time points p.i., cells were lysed with ice cold 0.5% triton X-100, serially diluted with 1X PBS and plated on 7H11 agar plates. *M. tuberculosis* colonies were enumerated after 3 weeks of incubation at 37^0 C.

Isolation of human monocyte-derived macrophages

Human monocyte-derived macrophages (hMDM) from both male and female donors were generated from peripheral blood mononuclear cells (PBMCs) (purity consistently >92%) by elutriation and differentiated after 7 days in Teflon bag cultures in the presence of 10 ng/ml recombinant human M-CSF (VueLife 72C; Cellgenix, Freiburg, Germany) as described previously(Reiling et al., 2001). All experiments performed with primary human cells were reviewed and approved by the Ethics Committee of the University of Lübeck, Germany (#13-032).

Quantitative Real-time PCR

Total RNA was isolated from macrophages (0.2 $X10^6$) using Trizol (peqGOLDTriFast™, USA) according to the manufacturer's instructions. cDNA was synthesized using Maxima First Strand cDNA Synthesis Kit (Thermo Fisher). Gene-specific primers and TaqMan probes (Roche Applied Science, Germany) were designed usingUniversal Probe Library (UPL) assay design center (ProbeFinder Version 2.45). qRT-PCR was performed using the LightCycler 480 Probe Master

Kit and the LightCycler 480 II system (Roche Applied Science)(Neumann et al., 2010). Crossing point values of target and reference gene were determined by the second derivative maximum method. Relative gene expression was calculated using E-Method (https://www.nature.com/app_notes/nmeth/2006/062706/full/nmeth894.html).

Human TB patient samples

All experiments performed with primary human macrophages or the staining of human lung tissues derived from patients suffering from TB, which underwent surgery for lung tissue resection as part of the anti-TB therapy, were reviewed and approved by the Ethics Committee of the University of Lübeck, Germany (Ethical Approval No.14-032 and #18-194). Lung sections were obtained from both male and female TB patients. Figure 6A,C are samples from male patient and Figure 6B and Figure 7C are samples from female TB patients.

Immunofluorescence microscopy

WT and *Trpv4*$^{-/-}$BMDM (1×10^5) were seeded on coverslips and infected with *M. tuberculosis* -GFP or *M. tuberculosis* ΔRD1-GFP (MOI 5) as described above. At specific time points, cells were fixed overnight with 4% PFA, washed with 1X PBS, permeabilized with 0.5 % saponin (#558255, Calbiochem) for 10 minutes and then blocked with 10 % goat serum. Cells were then incubated with primary antibodies (Rab5 -5µg/ml; TRPV4- 4µg/ml; Neutrophil elastase- 5µg/ml) for 1h at room temperature followed by washing andfluorophore-conjugated secondary antibody (Goat anti rabbit-cy3 -2.5µg/ml) in dark for 1h each step. DAPI (#D1306, Thermofisher) staining was performed at 1:1000 dilution and incubated in dark for 7 minutes. After mounting, cells were observed under fluorescence microscope (Nikon eclipse Ti) and confocal microscope (Leica TCS SP5). Confocal images were analyzed with IMARIS life-science software. Fluorescence intensities were quantified using Fiji ImageJ software.

Phagosomal pH measurement

Phagosomal pH was measured by ratiometric fluorescence microscopy (Nunes et al., 2015). To this end, *Escherichia coli* (*E. coli*) was first grown till mid-exponential phase and fixed with 4% PFA for 1 h followed by labelling with fluorescence isothiocyanate (FITC) (0.1mg/ml). WT and *Trpv4*$^{-/-}$ BMDM (1×10^5 cells) were incubated with FITC labelled *E.coli* (MOI 10) and live cell imaging was performed for 45 minutes at 37^0C and 5% CO_2 growth conditions using confocal microscopy. FITC signal was captured at excitation wavelength of 488 nm and 458 nm. Fluorescence property of FITC is quenched by acidic pH when excited at approximately 488 nm but not when excited at 458 nm. The ratio of 488/458 was used to determine the pH of the phagosome. Finally, the observed ratio value was converted into pH value by using an equation X=17.5035×{-(y-10.5627)/(y-5843007)}^0.0761506748 with the help of a standard curve obtained from *E. coli*-FITC 488/458 ratio at different pH (where X=pH value and y=ratio of 488/458). In total, 15 phagosomes were analyzed from three individual biological replicates.

Lysotracker staining

BMDM (1×10^5/well) were seeded onto cover slips and infected with *M. tuberculosis*-GFP (MOI 5) for 2 h and washed. After 48 h of further incubation, cells were washed and incubated with DMEM containing the acidotropic dye, lysotracker Red (100nM; L12492, Invitrogen), for 1h in dark. Cells were fixed with 4% PFA and observed under confocal microscope with excitation/emission of 647/668nm for lysotracker red.

Cell death analysis

BMDM (1×10^5/well) were infected with WT or ΔRD1 *M. tuberculosis* and necrotic cell death was measured using Sytox green (S7020, Thermo) as per the manufacturer's instruction. Apoptotic cell

death was studied using CellEvent Caspase3/7 green detection kit (C10423, Invitrogen) as per manufacturer's instruction. Briefly, BMDM (1×10^5) were seeded on a four chambered tissue culture disc, treated with IFN-γ overnight and then infected with *M. tuberculosis*-DsRed at MOI 5. Three hours p.i., cells were washed with 1x PBS to remove extracellular bacteria followed by addition of caspase3/7 green detection reagent (10μM in DMEM media). Live cell imaging was performed till 72 h p.i. using biostation IMq imaging system (Nikon).

Determination of NO_2^- production

IFN-γ activated WT and *Trpv4*$^{-/-}$BMDM (1×10^5) were infected with *M. tuberculosis*-H37Rv at MOI 5 for 24 h before supernatants were harvested and developed using Griess reagent and measured at 543nm.

For the measurement of nitrite produced under *in-vivo* conditions, lung lysates and serum were collected from *M. tuberculosis* exposed WT and *Trpv4*$^{-/-}$mice. For serum, blood was collected from inferior vena cava of mice at different time points p.i. and serum was separated using Z-gel tubes (Sarstedt; 41.1500.005) followed by centrifugation at 6000 g for 5 minutes. 40 µl of serum or lung lysate samples were incubated with 40 µl of reduction reagent (NADPH 1mg ml^{-1}; FAD 5mM; KH_2PO_4 0.5M, pH 7.5 with 0.25U of nitrate reductase-N7265-Sigma) at 37^0C for 2 h (Petricevich et al., 2000). After incubation, 80 µl of Griess reagent was added and incubated for 15 minutes in dark. OD was measured at 543nm.

Western blot analysis

Macrophage cells (1×10^6) were infected with WT and ΔRD1 *M. tuberculosis* at MOI 5. Then cells were harvested, lysed in RIPA buffer and electrophoresed to SDS-PAGE and western blotting as described previously(Mohanty et al., 2016). Membranes were then incubated overnight with the

primary anti-Trpv4 antibody (Alomone lab; ACC-034), washed, further incubated with HRP conjugated secondary antibody for 2 h at RT and developed and imaged using chemiluminescence and the ChemiDoc Imaging system (Bio-Rad), respectively.

Immunohistochemistry and Histopathology

Lungs from *M. tuberculosis* infected WT and *Trpv4*$^{-/-}$mice were harvested at different time points, incubated with 4% PFA overnight for fixation and 4 µm tissue sections were prepared. For cryo-sections, PFA fixed tissues were incubated with 1X PBS overnight at 4^0C, transferred to increasing concentrations of saccharose (5-20%) and embedded in 20% saccharose/ tissuetek (2:1). 4 µm cryo-sections were prepared with Leica cryostat at -20^0C.

Paraffin sections were used for immunohistochemistry by deparaffination of tissue sections and antigen retrieval in citrate buffer (pH 6.0). Endogenous peroxidase was inhibited with H_2O_2 and blocking was done with 10% serum followed by overnight incubation with primary antibody at 4 ^{0}C. Tissue sections were washed 3 times in 1X PBS followed by incubation with biotin conjugated secondary antibody (2.6 µg/ml or 1:500 goat-anti rabbit #111066047, Jackson Labs) for 45 minutes at RT and avidin-biotin complex for 45 minutes at RT (Vectastain Elite ABC-Peroxidase Kit, #VC-PK-6100-KI01). Tissue sections were developed in DAB followed by nuclei staining with hematoxyline. Similar staining procedure was used for immunohistochemistry analysis of human patient suffering from TB lung tissues. For *M. tuberculosis* counts, tissue sections were stained with Ziehl-Neelsen followed by nuclei staining with hematoxylin. Slides were embedded in Entellan.

For lipid staining, lung cryo-sections were washed 3 times with 1X PBS followed by incubation with Oil Red O stain for 30 minutes at room temperature. After incubation, slides were washed 3 times with 1X PBS and the nuclei were counterstained in hematoxylin. Slides were

embedded in Kaiser's Glycerin Gelatine and observed by light microscopy (Olympus BX41). Images were captured with CellSens standard Olympus software.

Cytokine and chemokine analysis

Cytokines and chemokines in mouse lung lysate and serum were analyzed using U-plex MSD kits (#K15069L) and the assay was performed according to the manufacturer's instructions.

Intracellular calcium measurement

BMDM ($1x10^5$ cells per well) from WT and *Trpv4*$^{-/-}$ mouse were seeded in a 96 well plate. Cells were treated with 4µM Ionomycin, 2mM $CaCl_2$ or infected with *M. tuberculosis*-H37Rv or *M. tuberculosis*ΔRD1 with a MOI 5. At indicated time points, media were removed and cells were washed twice with 1xPBS. Cells were then incubated with Fluo-4AM (4µg/ml) for 30 minutes at 37 ^{0}C in dark, and the fluorescence intensity was obtained at Ex/Em of 494/506 nm using Biotek multiplate reader.

Statistical analysis

Data are presented as mean ± standard error mean (SEM). Two-way and One-way analysis of variance was used to determine statistical significance between groups where *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. All statistical significances between the experimental groups are marked.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
TRPV4	Alomone lab	ACC034
Rab5	GeneTex	GTX108605
Neutrophil elastase	proSci-ELAN	51-862
Goat anti rabbit-cy3	Jackson immunoresearch	111-165-144
Goat-anti rabbit biotin conjugated	Jackson immunoresearch	111-066-047
Goat-anti rabbit HRP conjugated	Jackson immunoresearch	111-035-045
Bacterial and Virus Strains		
M. tuberculosis H37Rv	Schaible lab, Research Center Borstel, Germany	N/A
M. tuberculosis H37Rv GFP	Tanya Parish, Center for Global Infectious Disease Research Seattle	N/A
M. tuberculosis H37Rv ΔRD1	Suzanne M. Hingley-Wilson, William R. Jacobs	N/A
M. tuberculosis H37Rv DsRed	Schaible lab, Research Center Borstel, Germany	N/A
M. smegmatis mc^2155	Schaible lab, Research Center Borstel, Germany	N/A
E. coli	Schaible lab, Research Center Borstel, Germany	N/A
Biological Samples / Cell line		
Human TB patient lung sample	Luebeck University, Germany	Ethical Approval No.14-032 and #18-194
RAW264.7 mouse macrophages	A. Sonawane lab, India	N/A
Chemicals, Peptides, and Recombinant Proteins		
7H11 agar media	Difco	283810
DMEM	Pan Biotech	P04-03600
RPMI	Pan Biotech	P04-17500

Kanamycin	Roth	T832.1
Hygromycin-B	Pan Biotech	P06-08020
FBS	Pan Biotech	P30-3306
L-glutamine	Pan Biotech	P04-80100
Tween-80	Roth	4780
Protease inhibitor cocktail	Roche	4693132001
DAPI	Thermofisher	D1306
TritonX-100	Roth	3051.3
Trizol Tri Reagent	Zymo Research	R2050-1-200
Lysotracker red	Invitrogen	L12492
Caspase 3/7 detection kit	Invitrogen	C10423
FITC	Sigma	F7250
RN1734	Sigma	R0658
Recombinant murine IFN-γ	Peprotech	315-05
Flou4AM	Goswami lab, NISER, India	N/A
Ionomycin	Goswami lab, NISER, India	N/A
TB Carbolfuchsin	BD	212518
Mayer's Haematoxylin	Roth	T865.2
Eosin	Roth	7089.2
Entellan	VWR	1079610500
Sucrose	Roth	9286.1
Tissue tek	Leica	020108926
Methylbutane	VWR	720-0821
Oil Red O	Sigma	O0625
Nitrate reductase	Sigma	N7265
NADPH	Sigma	N7265-2UN
FAD	Sigma	F6625-10MG
KH_2PO_4	Merck	1.04873.0250
DAB for immunohistochemistry	Sigma	D4293-50SET
Avidin Biotin Complex kit	Vector lab	VC-PK-6100-KI01

Critical Commercial Assays		
MSD kit for cytokine analysis	Meso Scale Discovery	K15069L
Experimental Models: Organisms/Strains		
TRPV4$^{-/-}$ mouse	Liedtke *et al.* 2003	N/A
Oligonucleotides		
Human TRPV4 forward primer for mRNA expression CTCTTCATGATCGGCTACGC	Roche	Probe-54
Human TRPV4 reverse primer for mRNA expression ACACCTTCATGTTGGCACAC	Roche	Probe-54
Human HPRT forward primer for mRNA expression TGACCTTGATTTATTTTGCATACC	Roche	Probe-73
Human HPRT reverse primer for mRNA expression CGAGCAAGACGTTCAGTCCT	Roche	Probe-73
Software and Algorithms		
Imaris 7	Bitplane	http://www.bitplane.com/imaris/imaris
GraphPad Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/
Graphical abstract	BioRender	Created with BioRender.com

Supplemental References

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