Supplemental Materials File

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Other Supplementary Materials for this manuscript include the following:

Data S1 to S9
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Data S3 – DEGs in vivo mouse MAIT cell activation
Data S4 – Overlap of upregulated DEGs between in vitro and in vivo stimulation
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Data S6 – DEGs B6 immunized versus *Il18rap*$^{-/-}$ immunized
Data S7 – DEGs B6 immunized versus *Tnfrsf1a*$^{-/-}$*Tnfrsf1b*$^{-/-}$ immunized
Data S8 – DEGs B6 immunized versus *Ifnar*$^{-/-}$ immunized
Data S9 – Overlap of upregulated DEGs in B6 ChAdOx1 versus knockout mice or naive

Materials and Methods

Viral vectors

E1/E3-deleted replication-incompetent recombinant Ad5-GFP (VP:PFU ratio batch 1: 34, batch 2: 15, batch 3: 21), ChAdOx1-GFP (VP:PFU ratio batch 1: 118, batch 2: 13, batch 3: 78, batch 4: 73), ChAdOx1-HCV-GT1-6_D_TM-Ii+L (*17*) (VP:PFU ratio 95), ChAdOx1-nCoV-19 (VP:PFU ratio 51), ChAd63-GFP (VP:PFU ratio 107), ChAd63-OVA (VP:PFU ratio batch 1: 144, batch 2: 27), and Ad5-LacZ (VP:PFU ratio 21) vectors were produced by the Jenner Institute Viral Vector Core Facility at the University of Oxford, as previously described (*24*). The ChAdOx1 MenB.1 used in human volunteers (VP:PFU ratio 96) was produced at the Clinical Biomanufacturing Facility at the University of Oxford, as previously described (*25*). E1/E3-deleted replication-incompetent recombinant Ad6 (VP:PFU ratio 95), Ad24 (VP:PFU ratio not calculated), Ad35 (VP:PFU ratio 124), and ChAd68 (AdC68; VP:PFU ratio 100) vectors were produced by ReiThera SRL (Rome, Italy); ChAdN13 (VP:PFU ratio not calculated) was produced by Nouscom, SRL (Rome, Italy), as previously described (*26*). Vectors were propagated in HEK293 cells, except for ChAdOx1 MenB.1 produced by the Clinical Biomanufacturing Facility, which was propagated in PER.C6 cells, and isolated by $CsCl_2$ ultracentrifugation.

Phylogenetic analysis

Analysis of adenoviral polymerase genes was performed to generate a phylogenetic tree. Analysis shown was performed with MEGA X (*27*) using a bootstrap-confirmed (500 replicates) maximum likelihood tree constructed from the aligned polymerase nucleotide sequences. Numbers close to branches represent confidence values from the bootstrap consensus tree. Tree display was performed with iTOL (*28*).

Human PBMCs and selection of cell populations

Fresh blood from healthy human volunteers was collected in EDTA-coated Vacutainer tubes (BD Biosciences) under the "Gastrointestinal Illness in Oxford: prospective cohort for outcomes, treatment, predictors and biobanking" (Ref: 11/YH/0020) ethics, or blood from anonymized healthy donors was collected from the NHS Blood and Transplant Service. Written informed consent was received from all blood donors. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation, as previously described (*29*).

CD8 MicroBeads (Miltenyi Biotec) were used to enrich for MAIT cell populations. CD14 MicroBeads and CD123 MicroBeads (Miltenyi Biotec) were used to deplete (or enrich) monocytes and plasmacytoid DCs (pDCs), respectively. All kits were used as per the manufacturer's instructions.

Vaccinated human volunteers

PBMCs and plasma were collected from healthy volunteers aged 18-50 enrolled in the clinical trial ISRCTN trial number: ISRCTN46336916. Written informed consent was received from all trial participants. Samples were collected 1 day prior to a boosting immunization with 5×10^{10} vp of ChAdOx1 MenB.1 (antigen from *Neisseria meningitidis* group B) and 1 or 14 days after.

Mice and tissue processing

JAX^{TM} C57BL/6J mice (aged 6-10 weeks) were purchased from Charles River. *Mr1*$^{-/-}$ mice (*16*) (kindly provided by Dr. M. Salio and Professor V. Cerundolo, University of Oxford; MGI ID: 3664578), *Ifnar*$^{-/-}$ mice (kindly provided by Professor J. Rehwinkel; MGI ID: 5584125), *Il18rap*$^{-/-}$

mice (*Il18rap*$^{tm1a(KOMP)Wtsi}$ mice from the MRC Harwell Institute; MGI ID: 5766748), and *Tnfrsf1a*$^{-/-}$*Tnfrsf1b*$^{-/-}$ (B6.129S-*Tnfrsf1a*^{tm1Imx}*Tnfrsf1b*^{tm1Imx}/J purchased from Jackson Labs, cat. no. 003243) were bred in house and used at 6-10 weeks of age. Sex and age were matched between groups. All animals were housed in specific pathogen-free conditions at the Biomedical Services Building (University of Oxford) or the Wellcome Centre for Human Genetics (University of Oxford). For indicated experiments, C57BL/6J and *Mr1*$^{-/-}$ mice were co-housed for ≥28 days to normalize the microbiome between strains (*19*). All work was performed under UK Home Office license PPL P874AC0F0, P040D93C, or P9804B4F1 in accordance with the UK Animal (Scientific Procedures) Act 1986. All work was performed by trained and licensed individuals.

Animals were immunized intramuscularly in the hind legs with the indicated dose of Ad5-GFP, ChAdOx1-GFP, ChAdOx1-HCV-GT1-6_D_TM-Ii+L, ChAdOx1-nCoV-19, or ChAd63-OVA, as previously described (*30*). Mouse blood was collected from the lateral tail vein, and PBMCs were isolated by lysis of red blood cells using a 1X ACK solution. Lymph nodes were processed as described previously (*31*). Liver tissue was processed as described previously (*32*).

Genotyping

Genotyping of *Mr1*$^{-/-}$ mice was performed by PCR and gel electrophoresis, as previously described (*16*) with slight modification. DNA was extracted from splenocytes using the Qiagen DNeasy Blood and Tissue Extraction kit per the manufacturer's instructions. For testing of wildtype *Mr1* the MR1 5′ 8763-8783 (5′-AGC TGA AGT CTT TCC AGA TCG-3′) and MR1 9188-9168 rev (5′-ACA GTC ACA CCT GAG TGG TTG-3′) primers were used. For mutant *Mr1* the MR1 5′ 8763-8783 (5′-AGC TGA AGT CTT TCC AGA TCG-3′) and MR1 10451-10431 (5′-GAT TCT GTG AAC CCT TGC TTC-3′) primers were used. Primers (Sigma-Aldrich) were used at 10 μM

and QuantiFast SYBR Green Mastermix (Qiagen, cat. no. 204054) was used. Thermocycler conditions were: Step 1: 95°C for 5 min, Step 2: 94°C for 30 s, Step 3: 60°C for 30 s, Step 4: 72°C for 30 s, Step 5: repeat Step 2-4 35-times, Step 6: 72°C for 5 min, Step 7: hold at 22°C. PCR products were run on a 2% agarose gel and imaged on a GelDoc-It (UVP Imaging).

In vitro MAIT cell stimulation assays

For in vitro stimulation of human PBMCs with Ad vectors, fresh PBMCs were used. For in vitro stimulation of human PBMCs with cytokines, fresh or freeze-thawed PBMCs were used with equivalent outcomes. For Ad vector stimulations, a previously described protocol (*10*) was used with slight modifications. One million whole PBMCs or cell subset-depleted PBMCs were added to a 96-well U-bottom plate. Ad vectors were added at an MOI of 10^3 vp, unless indicated otherwise.

For cytokine stimulations, a previously-described protocol (*33*) was used with slight modifications. Briefly, 10^6 whole PBMCs or cell subset-depleted PBMCs were added to a 96-well U-bottom plate, and for isolated $CD14^+$ monocytes and $CD8^+$ T cells, $1\text{-}2\times10^5$ cells were used. For the transwell assay, a 0.3-μm 96-well transwell plate (Corning) was used. Isolated $CD8^+$ T cells (2×10^5) were added to the lower chamber, and 10^6 PBMCs were added to the upper chamber. Recombinant human IFN-α2a (Sigma-Aldrich, cat. no. SRP4594), IL-12p70 (R&D Systems, cat. no. 10018-IL), IL-18 (R&D Systems, cat. no. 9124-IL), and TNF (R&D Systems, cat. no. 210-TA) were all used at a final concentration of 50 ng/ml. Anti-TNFR2 agonist antibody (clone: MR2-1, Hycult Biotech, cat. no. HM2007) was used at a concentration of 2.5 μg/ml.

If applicable, inhibitors (listed below) were added immediately prior to addition of vectors or cytokines. Samples were mixed and incubated at 37°C in 5% CO_2.

For measurements of MAIT cell activation, Brefeldin A (final concentration of 5 μg/ml; BioLegend, cat. no. 420601) was added after 20 hours, and samples were collected after an additional 4 hours incubation (total stimulation time of 24 hours). For experiments where cytokine secretion or characteristics of cell transduction were assessed, Brefeldin A was not added and samples were collected after 24 hours.

Blocking and inhibitory reagents

The following reagents were used: mouse IgG1 isotype control antibody (10 μg/ml; clone: MOPC-21, BioLegend, cat. no. 400102), mouse IgG2a isotype control antibody (10 μg/ml; clone: MOPC-173, BioLegend, cat. no. 400202), anti-MR1 antibody (10 μg/ml; clone: 26.5, BioLegend, cat. no. 361102), anti-IL-12p70 antibody (10 μg/ml; clone: 24910, R&D Systems, cat. no. MAB219), anti-IL-15 antibody (10 μg/ml; clone: 34559, R&D Systems, cat. no. MAB2471), anti-IL-18 antibody (10 μg/ml; clone: 125-2H, R&D Systems, cat. no. D044), anti-IFNAR2 (10 or 25 μg/ml; clone: MMHAR-2, Merck Chemicals, cat. no. 407395), B18R (1 or 10 μg/ml; ThermoFisher, cat. no. 34-8185-86), CA-074-Me (*34*) (0.01 to 10 μM; Merck Chemicals, cat. no. 205531), MCC950 (*35*) (0.01 to 10 μM; Sigma-Aldrich, cat. no. 5381200001), elevated extracellular K^+ ion concentration (*36*) (5 to 30 mM KCl; Sigma-Aldrich, cat. no. P3911) , Z-YVAD-FMK (*37*) (0.1 to 100 μM; R&D Systems, cat. no. FMK005), vedolizumab (10 μg/ml; anti-α4β7 integrin antibody; Takeda Pharmaceuticals), adalimumab (10 μg/ml; anti-TNF antibody; AbbVie Inc), and etanercept (10 μg/ml; TNFR2-Fc fusion protein; Pfizer; kind gift of Dr. H. Al-Mossawi, University of Oxford).

Quantification of cytokines and chemokines

For quantification of cell culture supernatant, the following ELISA kits were used: Human TNF Quantikine ELISA kit (R&D Systems, cat. no. DTA00D), IL-18 Human ELISA Kit (MBL International, cat. no. 7620), Human IL-12p70 Quantikine ELISA kit (R&D Systems, cat. no. D1200), and VeriKine Human IFN-α Multi-Subtype ELISA Kit (PBL Assay Science, cat. no. 41110) as per the manufacturer's instructions. All data were collected on a FLUOstar OPTIMA plate reader (BMG LABTECH). For multiplex analysis of IFN-α-stimulated monocytes, the Proteome Profiler Human XL Cytokine Array Kit (R&D Systems, cat. no. ARY022B) was used as per the manufacturer's instructions, and the membrane was developed for 10 min on an SRX-101A Film Processor (Konica Corporation). For analysis of plasma cytokines in vaccinated human volunteers, the LEGENDplex Human Inflammation Panel (13-plex) (BioLegend, cat. no. 740808) was used as per the manufacturer's instructions. For all assays, samples were diluted as appropriate to fall within the dynamic range of the assay. Samples were freeze-thawed a maximum of one time.

Generation of MR1 tetramers

Human and murine MR1/5-OP-RU and MR1/6-FP monomers were provided by the NIH Tetramer Facility (Emory University). Tetramers were generated using Phycoerythrin (PE)-Streptavidin and Brilliant Violet 421 (BV421)-Streptavidin (BioLegend, cat. nos. 405245 and 405225, respectively) following the NIH Tetramer Facility's guidelines.

Surface and intracellular flow cytometry staining of human PBMCs

Staining antibodies, clones, and concentrations are listed in Table S1. MR1 tetramer staining was performed in FACS buffer (PBS + 0.05% BSA + 1% EDTA) for 40 min at room temperature.

Surface staining was for 15 min (viability dye only) or 30 min (surface antibodies) at 4°C. Samples were washed twice in FACS buffer, and cells were fixed and permeabilized for 20 min at 4°C using Cytofix/Cytoperm (BD Biosciences). Samples were subsequently washed twice in Perm/Wash buffer (BD Biosciences). Intracellular staining was performed for 30 min at 4°C. Following intracellular staining, samples were washed twice in Perm/Wash buffer, and stored in FACS buffer at 4°C until analyzed on a flow cytometer. Following staining for FACS, cells were resuspended in PBS + 0.05% BSA, and cells were sorted on a BD Aria II directly into 1 ml of TRIzol (Ambion), immediately snap frozen on dry ice, and stored at −80°C.

IFN-γ FluoroSpot on human PBMCs

Precoated plates (Mabtech FSP-010308-10) were blocked before addition of 50 µl/well peptide pools consisting of 15-mer sequences with 11 amino acid overlaps and covering the sequence of the MenB.1 vaccine antigen. The peptides were dissolved in 100% DMSO (Sigma) and arranged in a single pool. Concentration was adjusted to 0.6 mg/ml and used in the FluorSspot assay at a final concentration of 3 µg/ml of each peptide. DMSO and ConA (Sigma) were used respectively as negative and positive controls. After defrosting, 50 µl/well of PBMCs were added to peptide wells at a concentration of 4×10^6 cells/ml in triplicate and incubated overnight at 37°C, 5% CO_2, and 95% humidity. Detection of spots was carried out according to the manufacturer's instructions (Mabtech) and analyzed using an AID iSPOT ELISPOT reader (AID-diagnostika, Germany).

Characterization and sorting of murine MAIT cells

Staining antibodies, clones, and concentrations are listed in Table S2. Following cellular isolation (above), MR1 tetramer staining was performed in FACS buffer for 40 min at room temperature.

Cells were washed twice and surface staining was performed in FACS buffer for 20 min at 4°C. After surface staining, samples were washed twice in FACS buffer, and cells were fixed and permeabilized for 20 min at 4°C using Cytofix/Cytoperm (BD Biosciences, cat. no. 554722). Cells were subsequently washed twice in Perm/Wash buffer (BD Biosciences, cat. no. 554723). Intracellular staining was performed for 30 min at 4°C. After two additional washes, cells were stored in FACS buffer at 4°C until analyzed on a flow cytometer. For FACS, cells were resuspended in PBS + 0.05% BSA. Cells were sorted on a BD Aria II directly into 1 ml of TRIzol (Ambion, cat. no. 15596026), immediately snap frozen on dry ice, and stored at −80°C.

Peptide stimulation and intracellular cytokine staining of mouse splenocytes

Peptide stimulation of mouse splenocytes was performed as previously described (*31*, *38*). The following peptides were used at a final concentration of 1 or 2 μg/ml: 15-mer peptides of the HCV genotype 1b (overlapping by 11 amino acids), 15-mer peptides of the SARS-CoV-2 S1 and S2 proteins (overlapping by 11 amino acids), D8V peptide (from β-Galactosidase), or SIINFEKL (from Ovalbumin). Brefeldin A (final concentration of 5 μg/ml; BioLegend) and anti-CD107a were added at the time of peptide addition. Staining antibodies, clones, and concentrations are listed in Table S2. Following stimulation, cells were washed once in FACS buffer and surface staining was performed for 30 min at 4°C. After surface staining, samples were washed twice in FACS buffer, and cells were fixed and permeabilized for 20 min at 4°C using Cytofix/Cytoperm (BD Biosciences). Cells were subsequently washed twice in Perm/Wash buffer (BD Bioscience). Intracellular staining was performed for 30 min at 4°C in Perm/Wash buffer. Following intracellular staining, samples were washed twice in Perm/Wash buffer, and stored in FACS buffer at 4°C until analyzed on a flow cytometer.

RNA extraction and RNA-sequencing library preparation

RNA extraction was performed as previously described (*39*). RNA was subsequently submitted to the Oxford Genomics Centre (Wellcome Centre for Human Genetics, University of Oxford) for library preparation using the Smart-seq2 protocol (*40*). Libraries were sequenced on an Illumina HiSeq 4000 with 75 bp paired-end reads.

RNA-sequencing data analysis

Library trimming and read mapping was done using a CGAT-core-based pipeline (*41*). Read quality was assessed using FastQC (v. 0.11.9). Adaptor trimming was performed using Trimmomatic (v. 0.39). Pseudoalignment was performed using Kallisto (v. 0.46.1) (*42*). Mapping was done to genome build GRCh38 for humans and GRCm38 for mice with ERCC spike-ins added. Post-mapping read counts were between 1.7×10^7 and 4.4×10^7 reads. Differential expression analysis was performed in RStudio (v. 1.1.456) using R (v. 3.6.2), and DESeq2 (v. 1.26) (*43*). Genes were pre-filtered to remove genes with a count <10 across all samples, and ERCC reads were removed. The DESeq object was created with stimulation condition as the factor. Lfcshrink was performed using type = 'normal'. DEGs were defined as $\log_2$ fold-change>1 and adjusted $P<0.05$. For comparison of DEGs between mouse and human, mouse gene symbols were converted to human gene symbols using the Ensembl dataset from biomaRt (v. 2.42.0) and matched on Ensembl gene ID. All genes without matching human annotations were subsequently removed. GSEA (*12*) was performed using fgsea (v. 1.12) (*44*) with the MSigDB Reactome Collection (gsea-msigdb.org). pheatmap (v. 1.4.3), ggplot2 (v. 3.2.1), ggrepel (v. 0.8.1), ggfortify

(v. 0.4.8), VennDiagram (v. 1.6.20), gplots (v. 3.0.1.2), and ComplexHeatmap (v. 2.2.0) were used for data visualization.

Data analysis and statistics

All flow cytometry data were acquired on a BD Fortessa Flow Cytometer (BD Biosciences) and processed in FlowJo v. 9.9.6 (FlowJo, LLC). For analysis of the Human XL Cytokine Array, the developed film was scanned (Canon C-EXV), images were converted to grayscale, and pixel density was quantified using ImageJ (v. 1.51). Only membrane spots visible to the naked eye were considered to be positive. All data was analyzed in Prism v. 8.3.0 (GraphPad). For analysis of vaccinated human volunteers, a non-parametric paired Wilcoxon rank-sum test was used. For analysis of in vitro stimulations, unpaired *t* tests were used for comparison of two groups. A repeated-measures one-way ANOVA with Dunnett correction for multiple comparisons was used, or a mixed-effects model with Dunnett correction for multiple comparisons was used if there were different numbers of data points in each group. For analysis of dose response curves, a test for linear trend was performed. For analysis of in vivo mouse data, a one-way ANOVA with Dunnett correction for multiple comparisons, a two-way ANOVA, or an unpaired *t* test was performed, as appropriate. For analysis of SARS-CoV-2 spike protein-specific and OVA-specific T cell responses, background subtraction of cytokine production from unstimulated wells was performed. For all tests, $P<0.05$ was considered statistically significant, and $P<0.1$ was considered a trend with the exact *P* value reported.

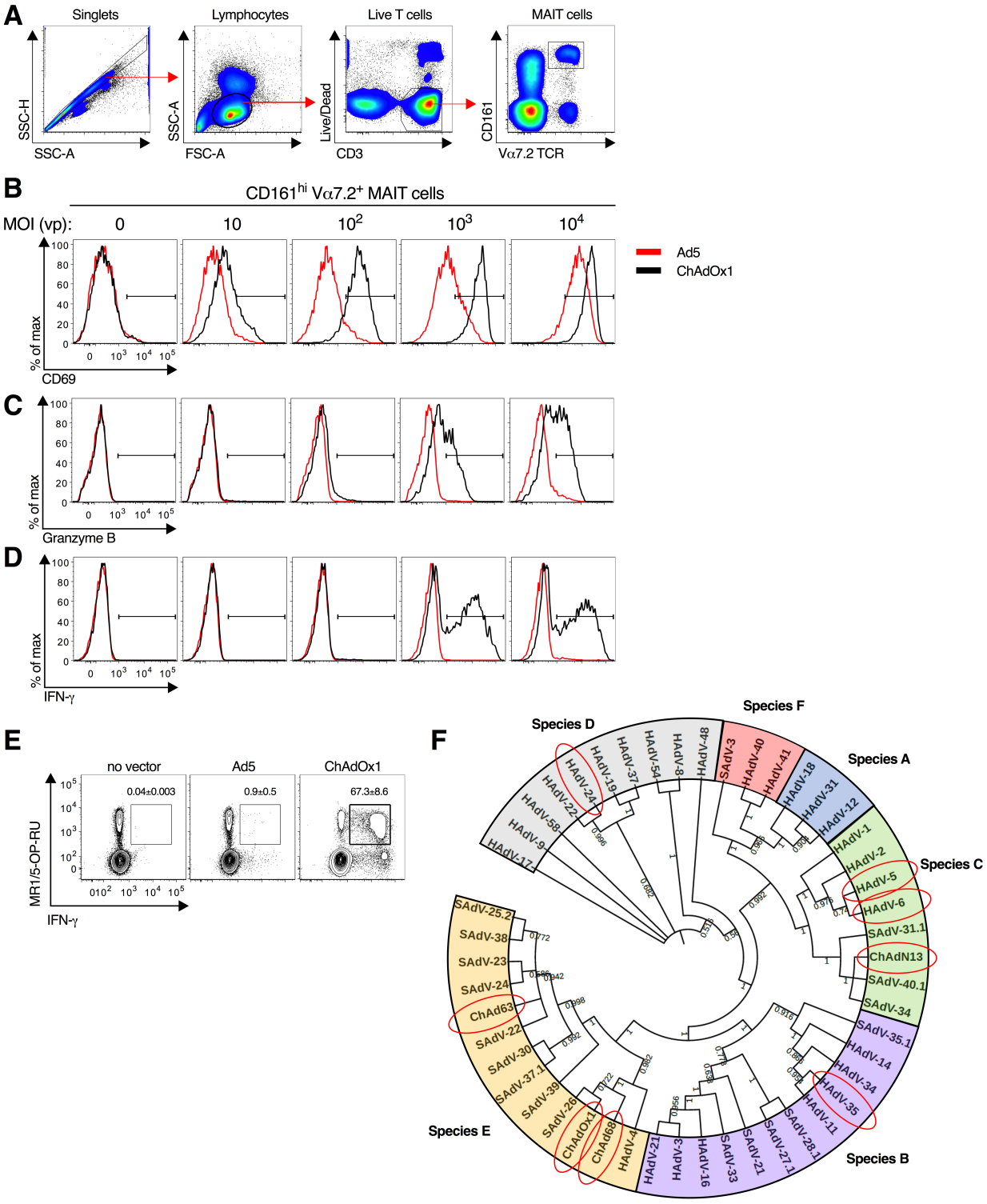

Fig. S1. (A) Gating scheme for the identification of MAIT cells (CD161^{hi}Vα7.2^{+} T cells) in human PBMCs. **(B to D)** Human PBMCs were stimulated with Ad5 or ChAdOx1 at increasing MOIs (0

to 10^4 vp), and CD69 **(B)**, granzyme B (GzmB) **(C)**, and IFN-γ **(D)** expression were measured on MAIT cells after 24 hours. Representative flow cytometry plots are shown. **(E)** Fresh human PBMCs (*n*=2; one experiment) were stimulated for 24 hours with Ad5 or ChAdOx1, and IFN-γ production by MAIT cells identified using MR1/5-OP-RU tetramers (gated on live $CD3^+$ lymphocytes) was assessed after 24 hours. Mean ± SEM are shown. **(F)** Phylogenetic analysis of adenoviral polymerase genes. Vectors used in the current study are circled in red.

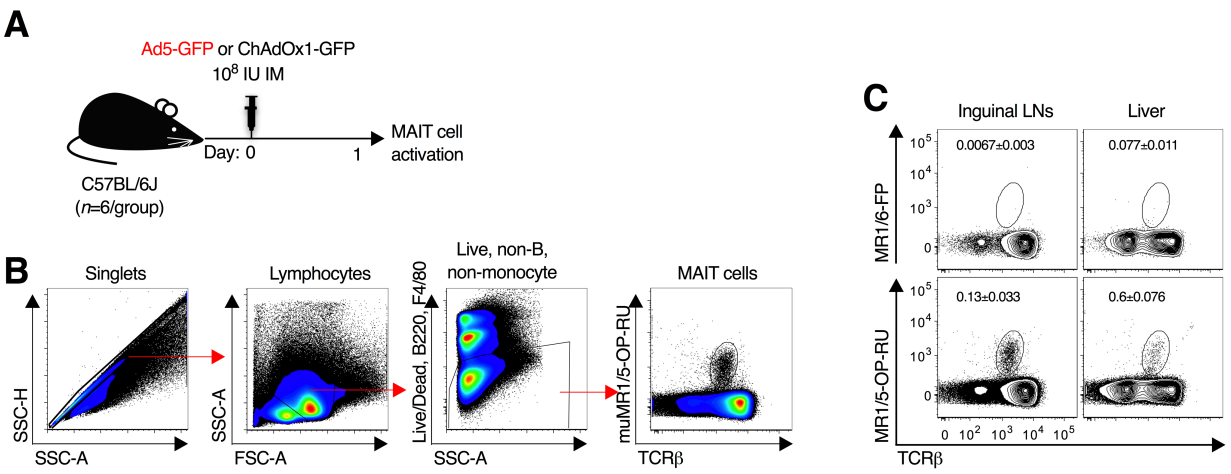

Fig. S2. (A) C57BL/6J mice were immunized with 10^8 IU of either Ad5-GFP or ChAdOx1-GFP, and MAIT cell activation was measured after 24 hours. **(B)** Gating scheme for the identification of MAIT cells in mice using MR1 tetramers. **(C)** Representative flow cytometry plots of the frequency of MAIT cells in the inguinal lymph nodes (LNs) and liver of C57BL/6J mice (n=12; two experiments). Mean ± SEM are shown.

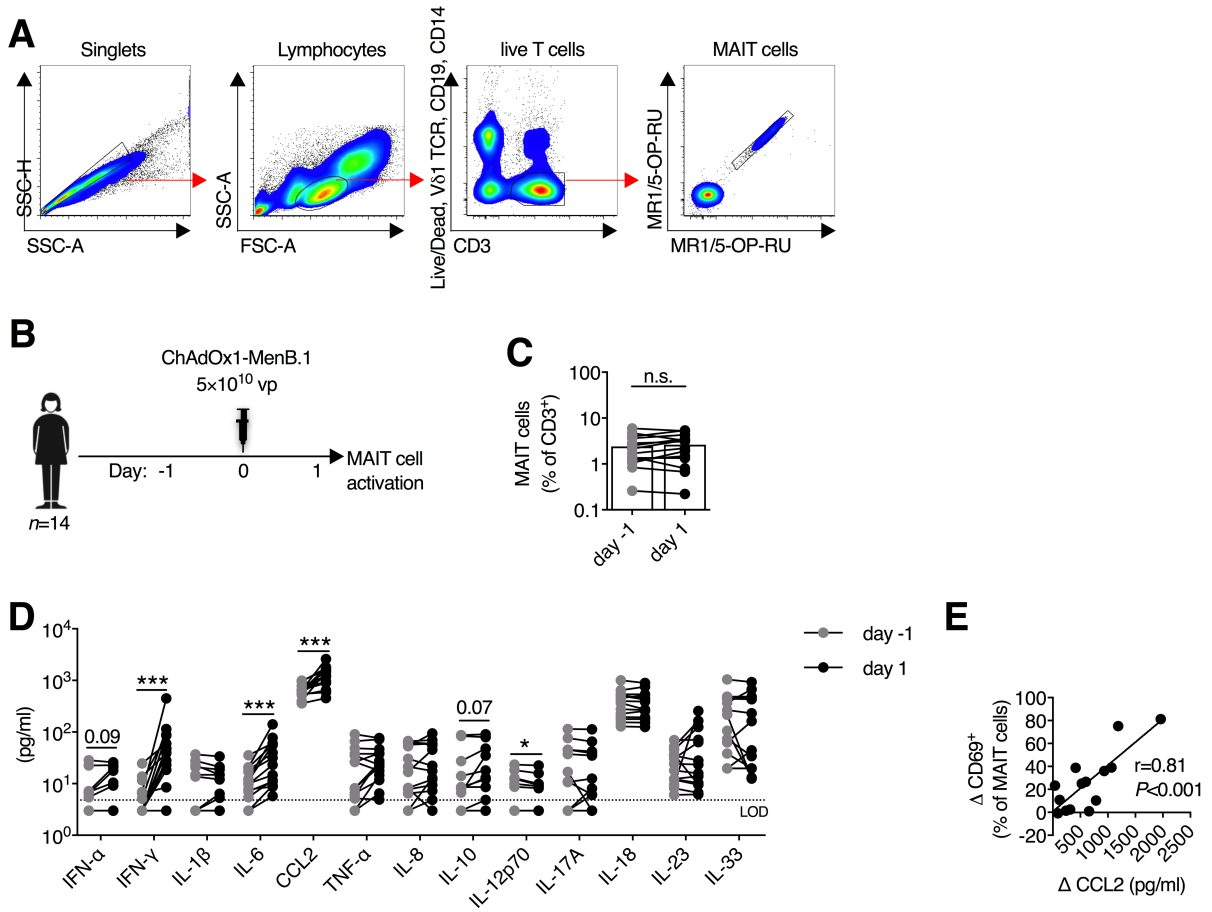

Fig. S3. (A) Gating scheme for the identification of MAIT cells (MR1/5-OP-RU$^+$ T cells) in PBMCs of healthy human volunteers immunized with ChAdOx1 MenB.1. **(B)** Healthy human volunteers (n=14) were immunized with a 5×10^{10} vp dose of ChAdOx1 MenB.1. **(C)** Frequencies of MAIT cells in peripheral blood 1 day pre- and 1 day post-immunization. **(D)** Concentration of plasma cytokine levels in healthy human volunteers (n=14) 1 day pre- and 1 day post-immunization with 5×10^{10} vp of ChAdOx1 MenB.1. **(E)** Pearson correlation of change in CCL2 chemokine level following vaccination and the change in expression of CD69 on MAIT cells. *,

P<0.05; ***, *P*<0.001; Wilcoxon rank-sum test. Symbols indicate individual donors, and group mean is shown.

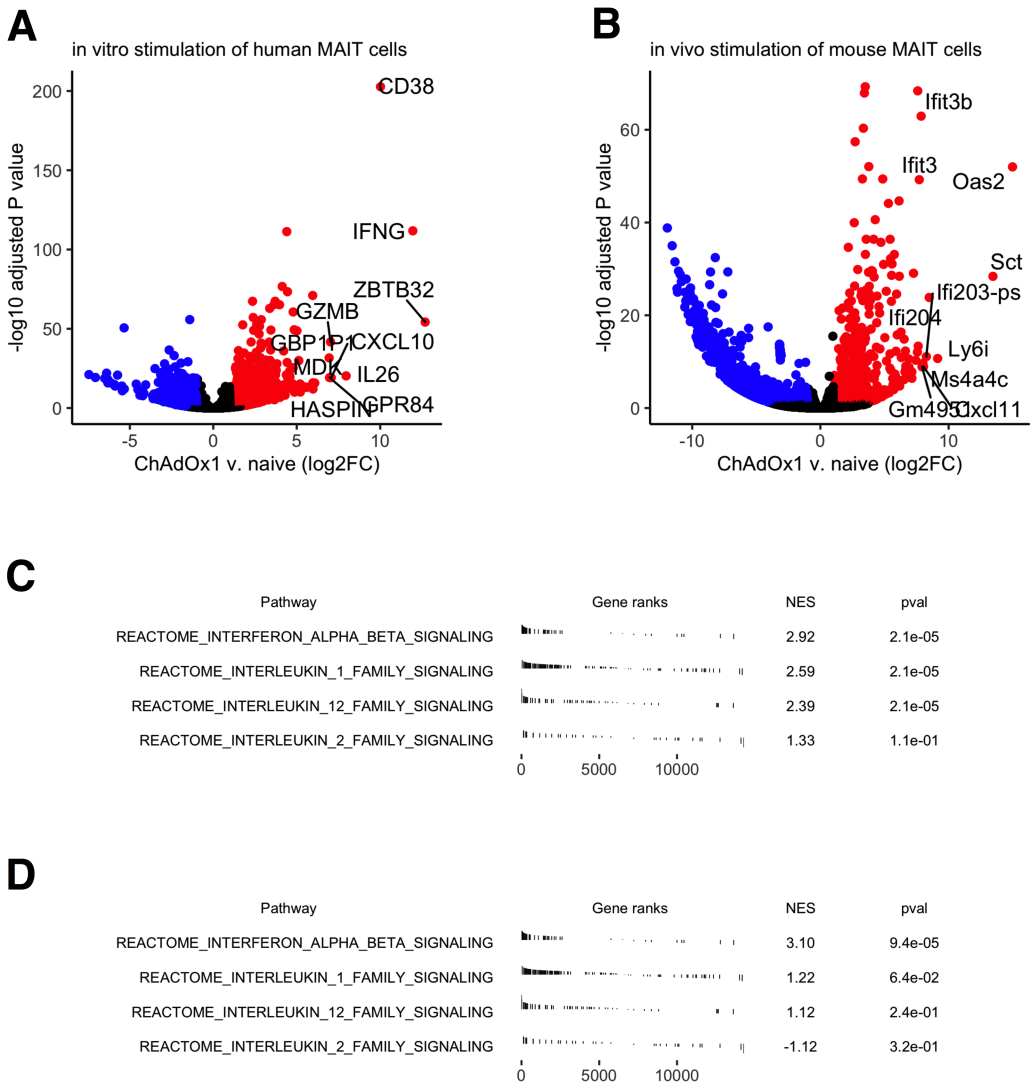

Fig. S4. (A and C) Gene expression analysis of MAIT cells isolated from human PBMCs stimulated in vitro for 24 hours with ChAdOx1-GFP or left unstimulated (n=4 per condition; one experiment). Volcano plot of differentially expressed genes ($\log_2$ FC>1, adjusted P<0.05). The top 10 upregulated genes are annotated **(A)**. Selected cytokine signaling pathways from the Reactome database enriched in MAIT cells post-vaccination by Gene Set Enrichment Analysis **(C)**. **(B and D)** Gene expression analysis of murine MAIT cells isolated from the draining inguinal LNs of C57BL/6J mice immunized intramuscularly 24 hours prior with 10^8 IU of ChAdOx1-GFP, or left immunized (n=5 per condition; one experiment). Volcano plot of differentially expressed genes

($\log_2$ FC>1, adjusted P<0.05). The top 10 upregulated genes are annotated **(B)**. Selected cytokine signaling pathways from the Reactome database in MAIT cells post-vaccination by Gene Set Enrichment Analysis **(D)**.

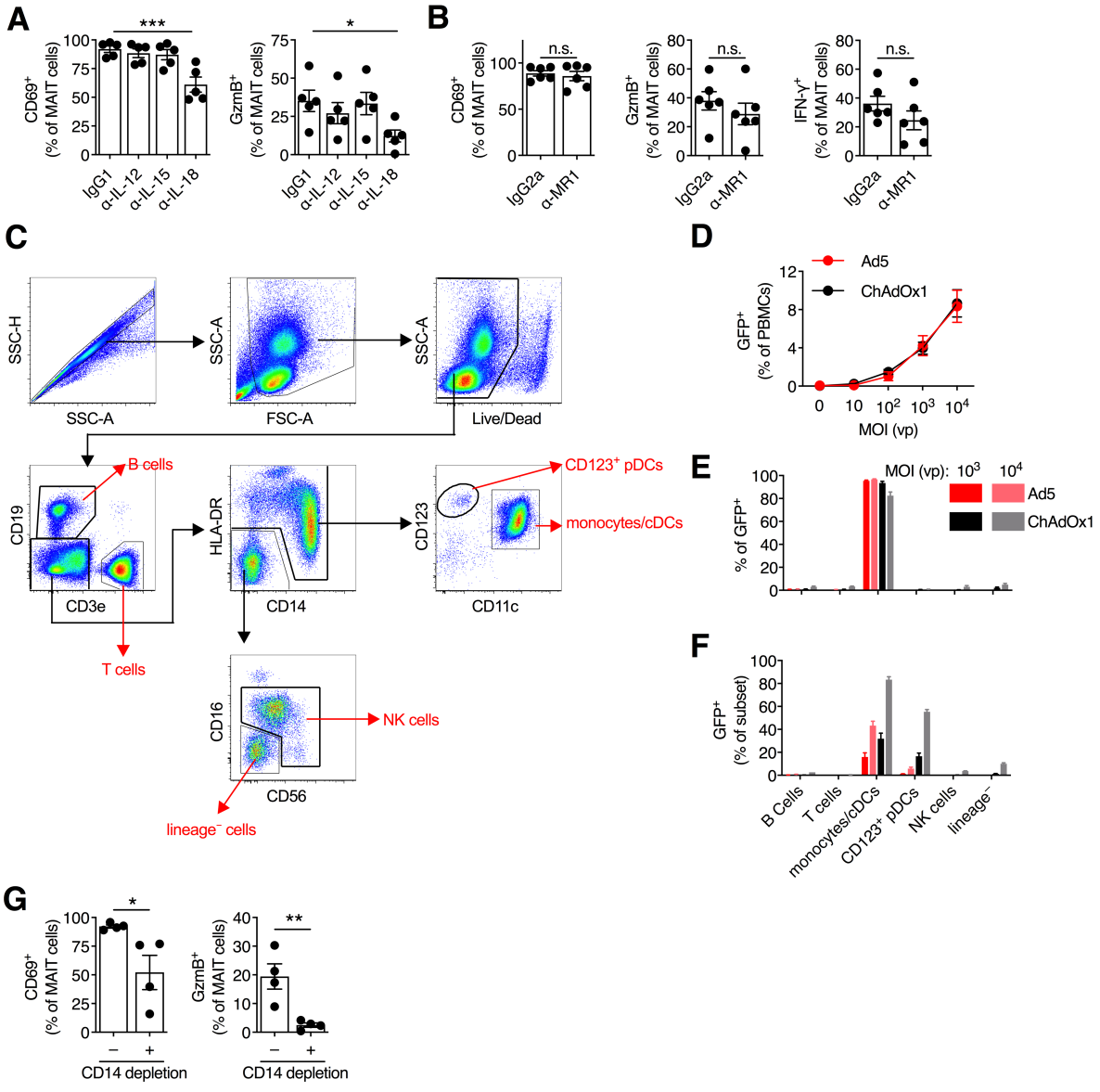

Fig. S5. (A) Human PBMCs (*n*=5; two experiments) were treated with anti-IL-12p70, anti-IL-15, or anti-IL-18 antibodies (10 μg/ml) immediately prior to stimulation with ChAdOx1-GFP. MAIT cell CD69 and granzyme B (GzmB) expression were measured after 24 hours. **(B)** Fresh human PBMCs were treated with anti-MR1 antibody (10 μg/ml, *n*=6; two experiments) immediately prior to stimulation with ChAdOx1-GFP. MAIT cell CD69, GzmB, and IFN-γ expression were measured after 24 hours. **(C to F)** Fresh human PBMCs were stimulated for 24 hours with either

Ad5-GFP or ChAdOx1-GFP at the indicated MOI, and the fraction of each PBMC immune subset that was GFP^+ was assessed. **(C)** Gating scheme for the identification of major immune cell subsets within human PBMCs. Fraction of all PBMCs (*n*=9; four experiments) that are GFP^+ **(D)**, fraction of all GFP^+ cells (*n*=3; one experiment) that are in each of the indicated subsets **(E),** and fraction of each subset (*n*=3; one experiment) that are GFP^+ following transduction with the indicated dose of vector **(F)**. **(G)** PBMCs were depleted of $CD14^+$ monocytes or left untreated as a control (*n*=8; two experiments) and stimulated with ChAdOx1-GFP. MAIT cell CD69 and GzmB expression were measured after 24 hours. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. Repeated-measures one-way ANOVA with Dunnett correction **(A)**, or unpaired *t* test **(B and G)**. Symbols indicate individual donors, and mean ± SEM are shown.

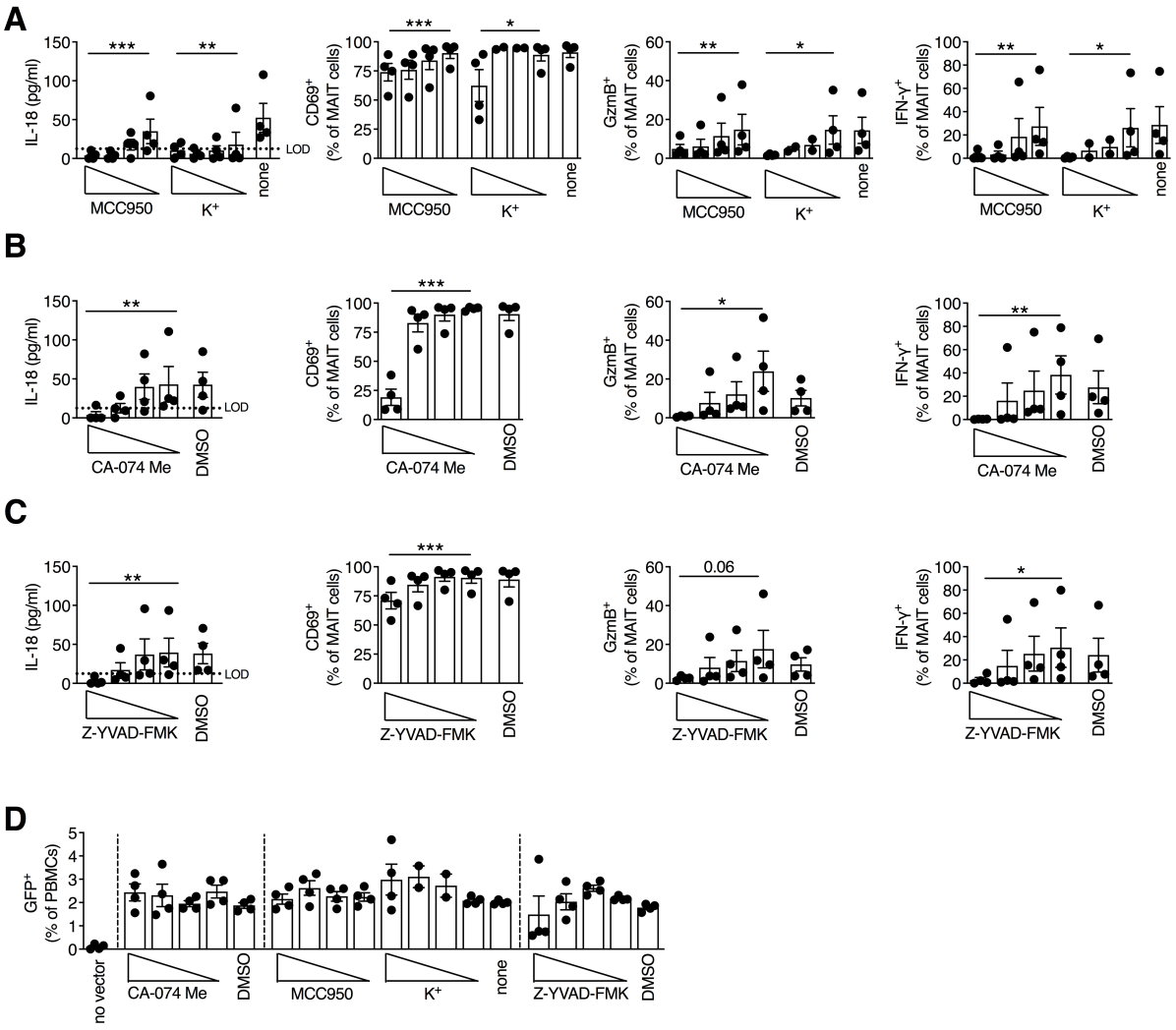

Fig. S6. (A) The NLPR3 inhibitors (MCC950 (0.01-10 μM) and elevated extracellular K^+ (5-30 mM)), **(B)** Cathepsin B inhibitor (CA-074-Me (0.01-10 μM)), or **(C)** Caspase 1 inhibitor (Z-YVAD-FMK (0.1-100 μM)) were added immediately prior to stimulation of PBMCs with ChAdOx1-GFP. IL-18 concentration, or MAIT cell CD69, granzyme B (GzmB), and IFN-γ production were measured after 24 hours (*n*=4; two experiments). **(D)** Frequency of GFP^+ PBMCs (*n*=4; two experiments) at 24 hours following transduction with ChAdOx1-GFP in the presence of the indicated dose of CA-074 Me, MCC950, extracellular K^+ ions, or Z-YVAD-FMK. *, $P<0.05$;

**, $P<0.01$; ***, $P<0.001$. Repeated-measures one-way ANOVA with test for linear trend. Symbols indicate individual donors. Mean ± SEM are shown.

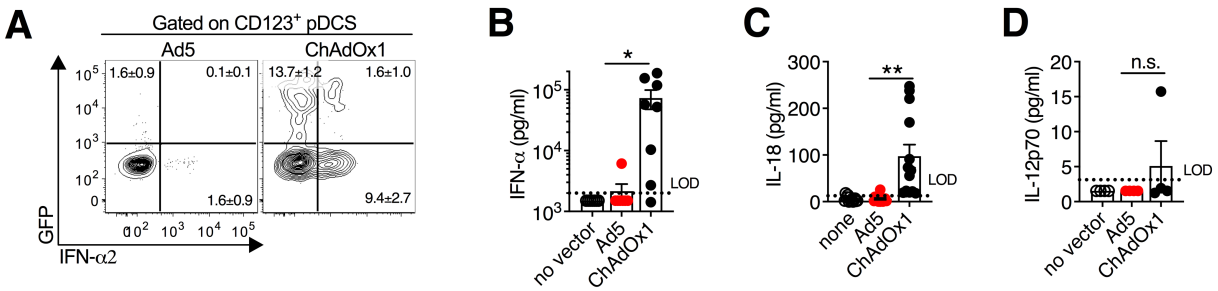

Fig. S7. (A) Expression of IFN-α2 on $CD123^+$ pDCs ($HLA\text{-}DR^+CD11c^-CD14^-CD16^-CD19^-CD3^-$ live cells) 24 hours after stimulation with Ad5-GFP or ChAdOx1-GFP. Data are representative of *n*=4 donors (two experiments). **(B-D)** Concentration of IFN-α (*n*=7-8; 4 experiments) **(B)**, IL-18 (*n*=13; six experiments) **(C)**, and IL-12p70 (*n*=4; 2 experiments) **(D)** in cell culture supernatants following 24 hour stimulation of fresh PBMCs with Ad5 or ChAdOx1. *, $p<0.05$; **, $p<0.01$. Unpaired *t* test. Symbols indicate individual donors. Mean ± SEM are shown.

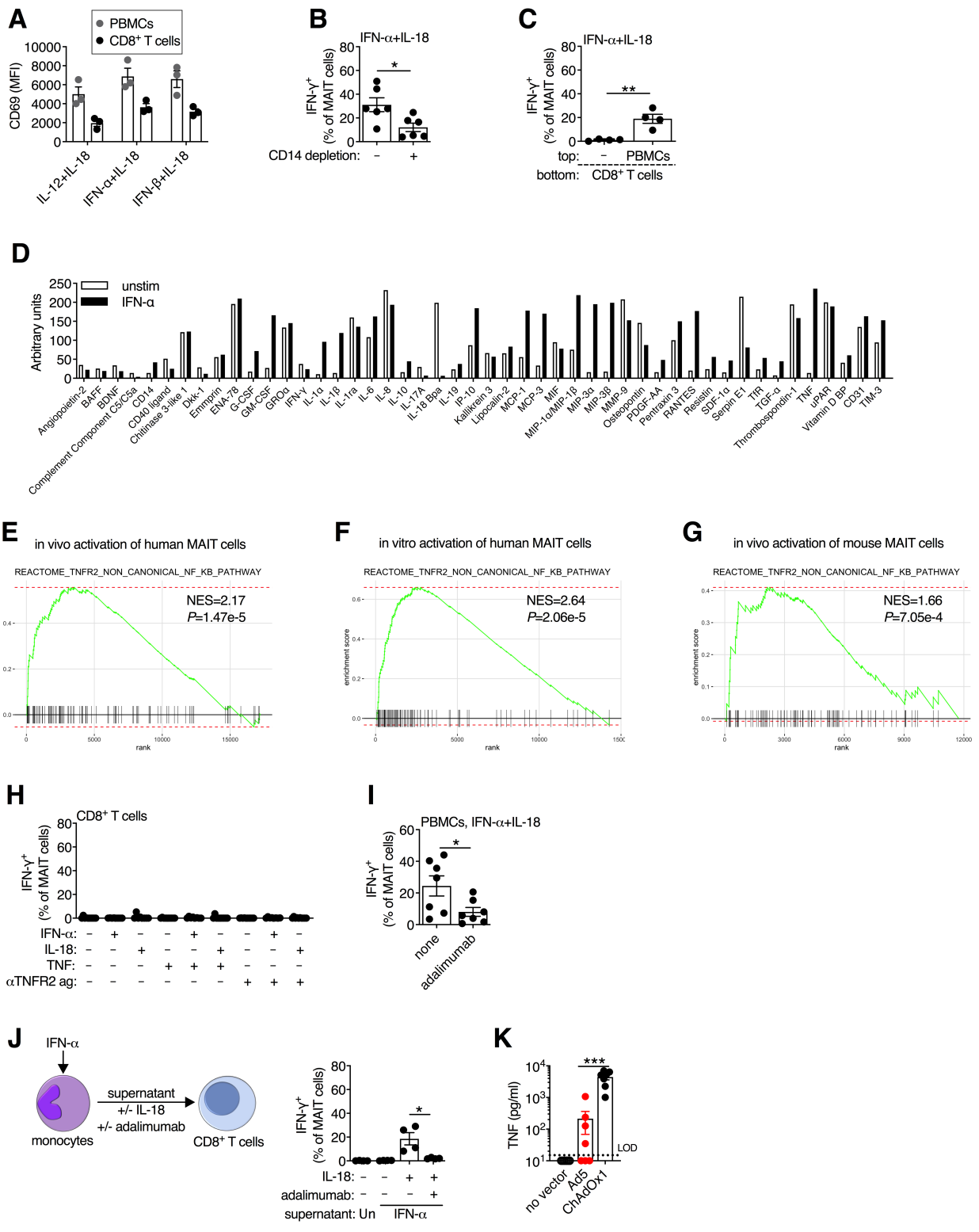

Fig. S8. (A) Unfractionated PBMCs or purified CD8$^+$ T cells (positive selection by CD8 MicroBeads) were stimulated with the indicated cytokines (50 ng/ml), and MAIT cell CD69

expression was measured after 24 hours (*n*=3; one experiment). **(B)** Unfractionated PBMCs or $CD14^+$ monocyte-depleted PBMCs were stimulated with IFN-α + IL-18 (50 ng/ml), and MAIT cell IFN-γ production was measured after 24 hours (*n*=6; two experiments). **(C)** Using a 0.3-μm transwell system, purified $CD8^+$ T cells were placed in the lower chamber and the upper chamber was loaded with either autologous PBMCs or left cell-free (*n*=4; one experiment). MAIT cell IFN-γ production was assessed at 24 hours following stimulation with IFN-α + IL-18 (50 ng/ml). **(D)** CD14-purified monocytes (*n*=1, in duplicate; one experiment) were stimulated with IFN-α (50 ng/ml) or left untreated. After 24 hours, supernatants were collected and used fresh for immunoblotting. Relative protein concentration was calculated by quantifying pixel density. **(E-G)** Gene Set Enrichment Analysis of the Reactome_TNFR2_non_canonical_NF_KB_pathway in MAIT cells from ChAdOx1-vaccinated human volunteers **(E)**, human MAIT cells from PBMCs stimulated in vitro with ChAdOx1 **(F)** or murine MAIT cells isolated 1 day post-immunization from the draining inguinal LNs of mice immunized with ChAdOx1 **(G)**. **(H)** Purified $CD8^+$ T cells (*n*=10; four experiments) were stimulated with single or double combinations of IFN-α, IL-18, TNF, or anti-TNFR2 agonist antibody (cytokine at 50 ng/ml and agonist antibody at 2.5 μg/ml). MAIT cell IFN-γ production was measured after 24 hours. **(I)** PBMCs (*n*=7; two experiments) were stimulated with IFN-α + IL-18 (50 ng/ml), and adalimumab (anti-TNF antibody; 10 μg/ml) was added immediately prior cytokine addition. MAIT cell IFN-γ production was measured after 24 hours. **(J)** Purified monocytes (*n*=4; one experiment) were stimulated with IFN-α (50 ng/ml), or left untreated, and after 24 hours supernatants were transferred ± IL-18 (50 ng/ml) and ± adalimumab (anti-TNF antibody; 10 μg/ml) to autologous purified $CD8^+$ T cells. MAIT cell IFN-γ production was measured at 24 hours. **(K)** Concentration of TNF in cell culture supernatants of PBMCs 24 hours after stimulation with Ad5 or ChAdOx1 (*n*=7; two experiments). *, $P<0.05$; **,

P<0.01; ***, *P*<0.001. Unpaired *t* test. Symbols indicate individual donors. Mean ± SEM are shown.

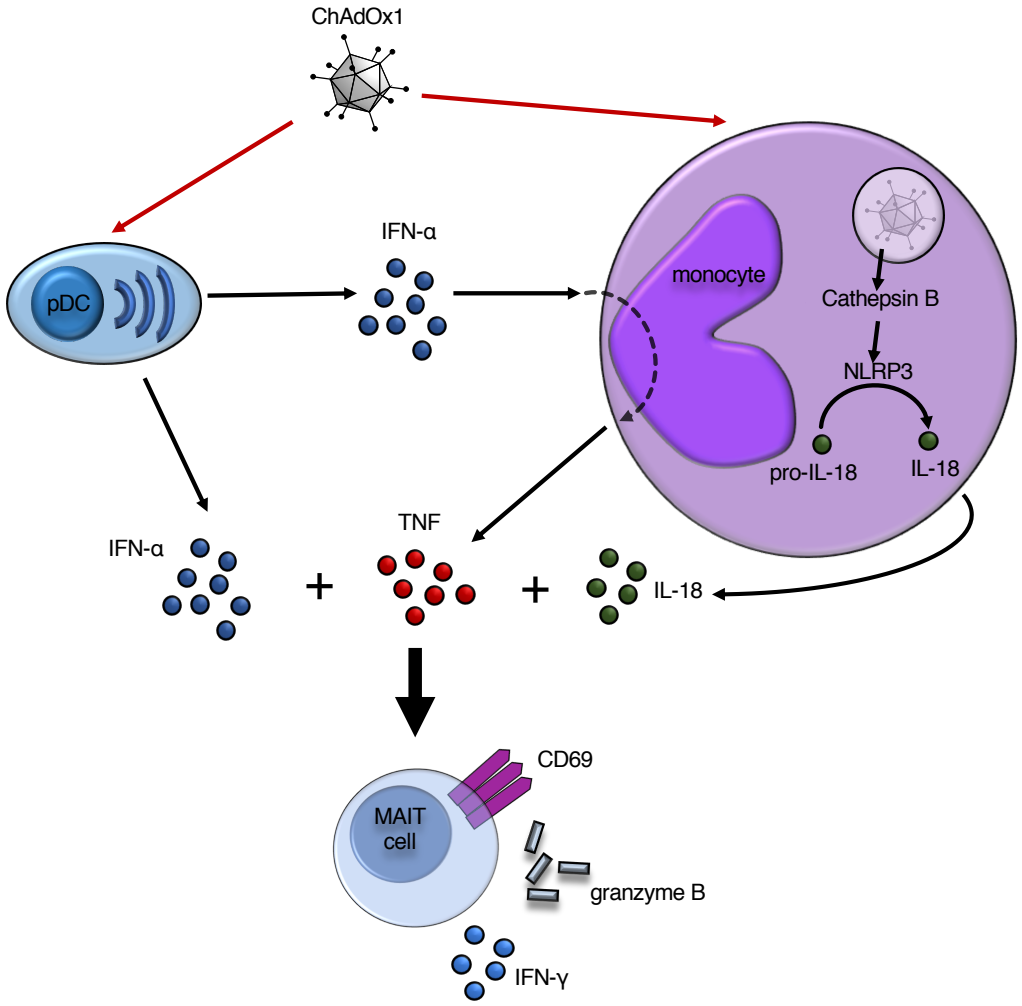

Fig. S9. Model for how ChAdOx1 (and other stimulatory Ad vectors) activates MAIT cells. Activation involves two pathways: (1) ChAdOx1 transduces pDCs and triggers the production of IFN-α, which in turn drives TNF production by monocytes, and (2) ChAdOx1 transduces monocytes and induces IL-18 production by activating the NLRP3 inflammasome. The combination of IL-18, IFN-α, and TNF act on MAIT cells to induce expression of CD69, granzyme B, and IFN-γ.

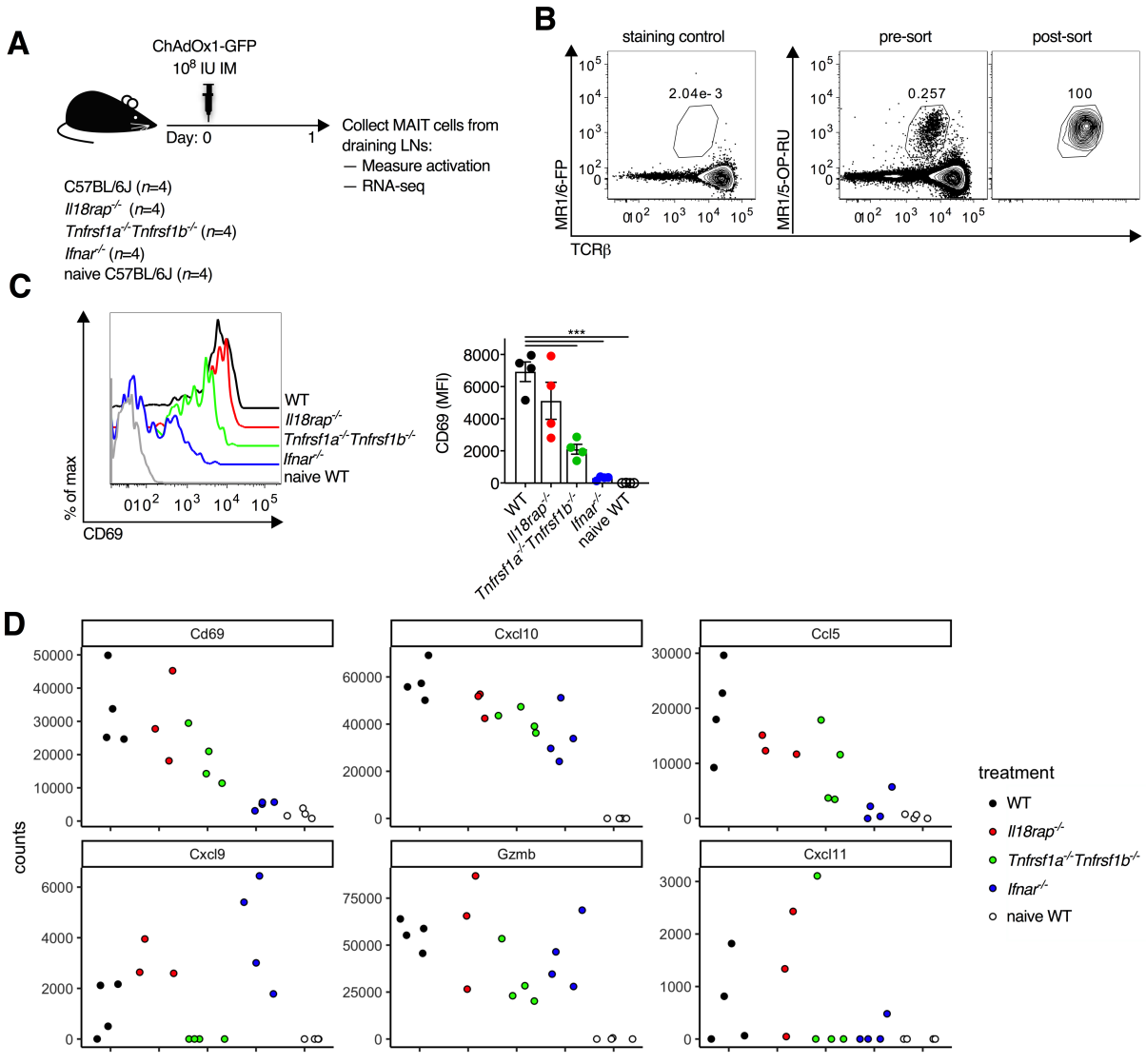

Fig. S10. **(A-D)** C57BL/6J (*n*=4), *Il18rap*$^{-/-}$ (*n*=4), *Tnfrsf1a*$^{-/-}$*Tnfrasf1b*$^{-/-}$ (*n*=4), or *Ifnar*$^{-/-}$ (*n*=4) were immunized intramuscularly with 10^8 IU of ChAdOx1-GFP (one experiment). Naive C57BL/6J mice (*n*=4) were used as a control. After 24 hours, MAIT cells (MR1/5-OP-RU$^+$ T cells) were isolated from the draining inguinal LNs and sorted for RNA-sequencing **(A)**. The sequencing library from one *Il18rap*$^{-/-}$ mouse failed QC, and was removed from further analysis. **(B)** Representative plot of post-sort purity of murine MAIT cells used for RNA-sequencing. **(C)** Representative plot and group average of CD69 expression on murine MAIT cells from the

draining inguinal LNs (n=4 per group; representative of two experiments). **(D)** RNA read counts for the indicated genes that are differentially expressed in MAIT cells from immunized and naive C57BL/6J mice. ***, P<0.001. One-way ANOVA with Dunnett correction **(C).** Symbols indicate individual mice. Mean ± SEM are shown.

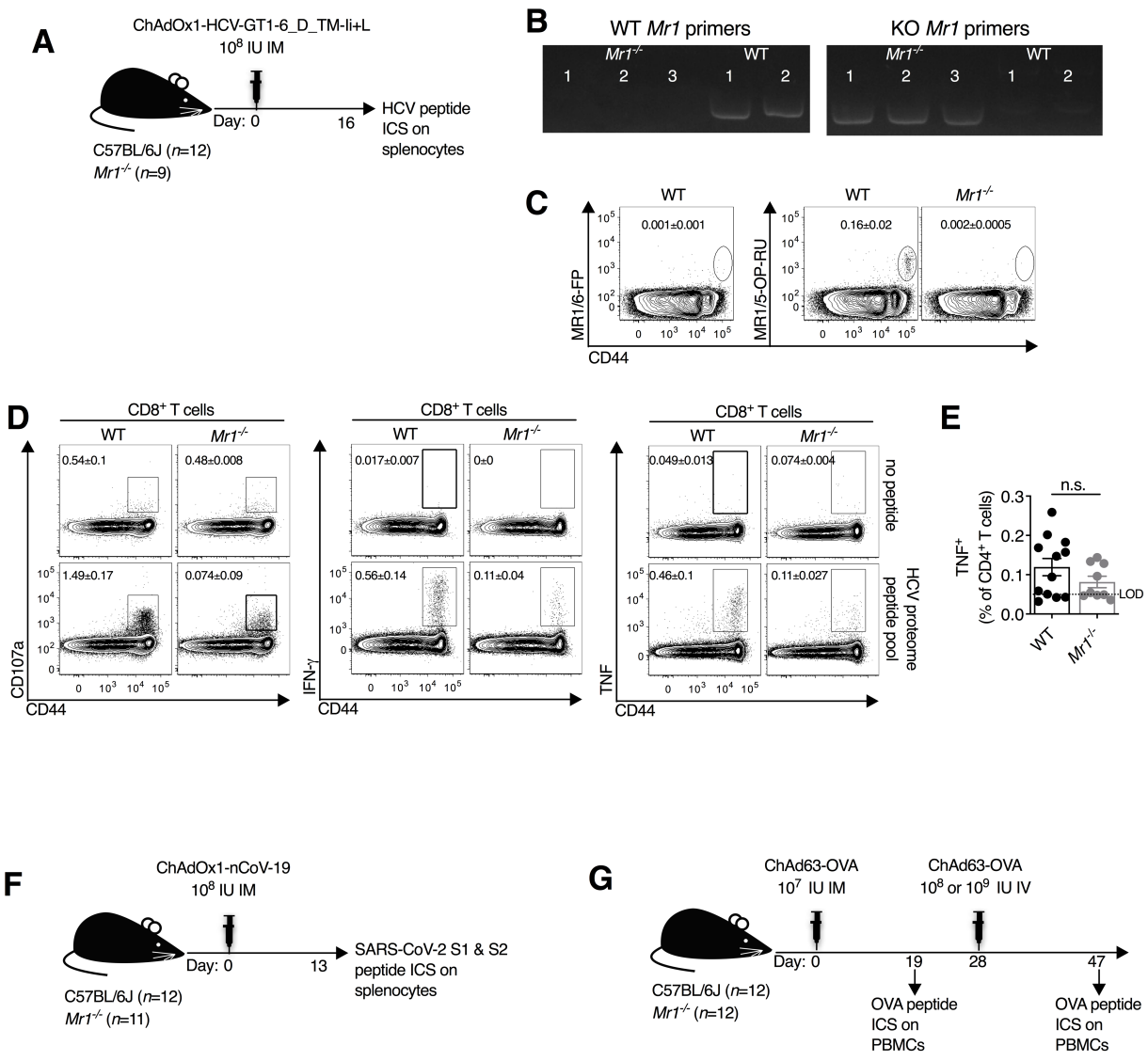

Fig. S11. (A to E) C57BL/6J (*n*=12) or *Mr1*$^{-/-}$ (*n*=9) mice were immunized intramuscularly with 10^8 IU of ChAdOx1 expressing a HCV-GT1-6_D_TM-Ii+L transgene, and on day 16 post-immunization splenocytes were collected (two experiments) **(A)**. **(B and C)** Genotyping of wild-type C57BL/6J and *Mr1*$^{-/-}$ mice by PCR for the *Mr1* gene **(B)** and confirmation of the absence of MAIT cells in the inguinal LNs by flow cytometry **(C)**. **(D)** Representative flow cytometry plots of CD107a, IFN-γ, or TNF expression on CD8$^+$ T cells following peptide stimulation. **(E)** Group averages for TNF production by CD4$^+$ T cells following 5-hour stimulation with an overlapping

peptide pool of the HCV genotype 1b proteome. Two-way ANOVA. Symbols indicate individual animals, dotted line indicates level of expression in absence of peptide stimulation, and mean ± SEM are shown. **(F)** C57BL/6J (*n*=12) or *Mr1*$^{-/-}$ (*n*=11) mice were immunized intramuscularly with 10^8 IU of ChAdOx1 expressing an nCoV-19 transgene (encoding the spike protein from SARS-CoV-2), and on day 13 post-immunization splenocytes were collected (two experiments). **(G)** C57BL/6J (*n*=12) or *Mr1*$^{-/-}$ (*n*=12) mice were immunized intramuscularly with 10^6 IU of ChAd63 expressing an ovalbumin (OVA) transgene, and boosted intravenously with either 10^8 or 10^9 IU of ChAd63-OVA (two experiments). OVA-specific T cell responses were measured in blood 3 weeks post-prime and 3 weeks post-boost.

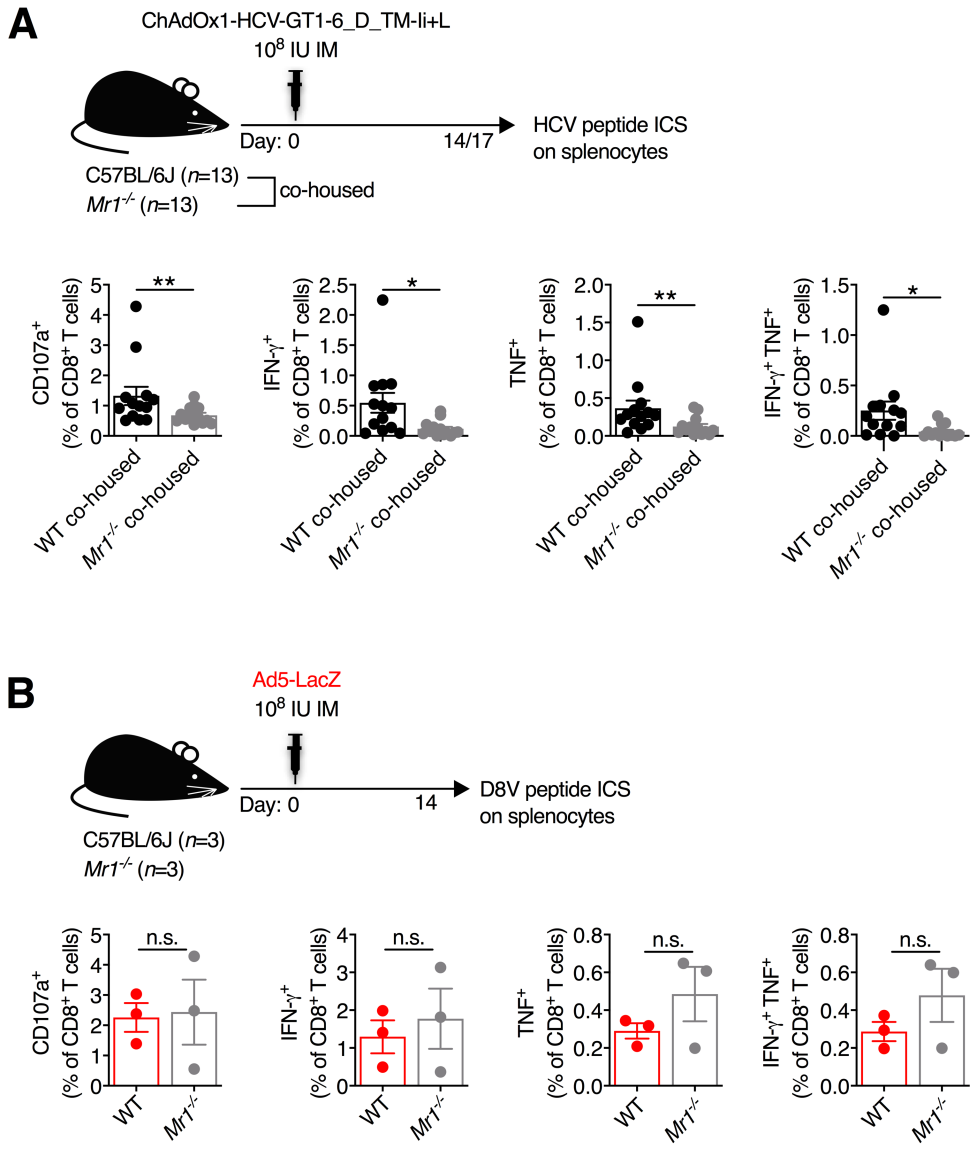

Fig. S12. (A) C57BL/6J (n=13) and *Mr1*$^{-/-}$ (n=13) mice were co-housed for ≥28 days prior to intramuscular immunization with 10^8 IU of ChAdOx1 expressing a HCV-GT1-6_D_TM-Ii+L transgene, and on day 14 or 17 post-immunization splenocytes were collected for ICS (two experiments). Representative plots and group averages for frequencies of CD107a$^+$, IFN-γ$^+$, TNF$^+$, or IFN-γ$^+$ TNF$^+$ CD8$^+$ T cells are shown following a 5-hour stimulation with an overlapping peptide pool of the HCV genotype 1b proteome. **(B)** C57BL/6J (n=3) or *Mr1*$^{-/-}$ (n=3) mice were immunized intramuscularly with 10^8 IU of Ad5 expressing a LacZ (β-galactosidase) transgene,

and on day 14 post-immunization, splenocytes were collected for ICS (one experiment). Group average for frequency of $CD107a^+$, IFN-γ^+, TNF^+, or IFN-γ^+TNF^+ $CD8^+$ T cells following a 5-hour peptide stimulation are shown. **, $P<0.01$; *, $P<0.05$. Two-way ANOVA **(A)** or unpaired *t* test **(B)**. Symbols indicate individual animals. Mean ± SEM are shown.

Table S1. Human flow cytometry reagents

Marker	Clone	Fluorophore	Dilution	Staining step	Company	Catalog number
MR1/5-OP-RU tetramer	n/a	PE or BV421	1 in 200	Surface	NIH Tetramer Facility	n/a
Fixable viability dye	n/a	Near-IR	1 in 400	Surface	Life Technologies	10154363
SYTOX Green Dead Cell Stain	n/a	SYTOX Green	1 in 6000	Surface (FACS)	ThermoFisher	S34860
DAPI solution	n/a	DAPI	1 in 1000	Surface (FACS)	ThermoFisher	62248
CD11c	B-Ly6	PE-CF594	1 in 100	Surface	BD Biosciences	562393
CD19	HIB19	FITC, PE-Cy7, or APC-Cy7	1 in 200	Surface	BioLegend	302206, 302216, 302218
CD16	3G8	BV711 or AF700	1 in 50	Surface	BioLegend	302044, 302026
HLA-DR	G46-6	BV421 or AF700	1 in 100	Surface	BioLegend	307636, 307626
CD123	6H6	BV510	1 in 100	Surface	BioLegend	306022
CD56	5.1H11	APC-Cy7	1 in 100	Surface	BioLegend	362512
CD14	M5E2	BV421 or BV650	1 in 100	Surface	BioLegend	301830, 301836
Vδ1 TCR	REA173	FITC	1 in 100	Surface	Miltenyi Biotec	130-118-362
CD69	FN50	PE-Dazzle594	1 in 100	Surface or Intracellular	BioLegend	310942
CD161	191B8	PE	1 in 50	Intracellular	Miltenyi Biotec	130-113-593
Vα7.2 TCR	3C10	PE-Cy7	1 in 50	Intracellular	BioLegend	351712
CD3ε	OKT3 or UCHT1	BV785	1 in 100	Intracellular	BioLegend	317330, 300472
IFN-γ	B27	Pacific Blue	1 in 100	Intracellular	BioLegend	506526
Granzyme B	GB11	APC or AF647	1 in 200	Intracellular	ThermoFisher or BD Biosciences	GRB05, 561999
IFN-α2	7N4-1	PE	1 in 50	Intracellular	BD Biosciences	560097

Table S2. Mouse flow cytometry reagents

Marker	Clone	Fluorophore	Dilution	Staining step	Company	Catalog number
MR1/5-OP-RU tetramer	n/a	PE	1 in 200	Surface	NIH Tetramer Facility	n/a
MR1/6-FP tetramer	n/a	PE	1 in 200	Surface	NIH Tetramer Facility	n/a
Fixable viability dye	n/a	Near-IR	1 in 400	Surface	Life Technologies	10154363
DAPI dead cell stain	n/a	DAPI	1 in 1000	Surface (FACS)	ThermoFisher	62248
CD8α	53-6.7	AF700 or FITC	1 in 100	Surface	BioLegend	100706, 100724
CD4	RM4-5	BV650	1 in 100	Surface	BioLegend	100555
CD44	IM7	BV785	1 in 100	Surface	BioLegend	103059
CD107a	1D4B	FITC	1 in 100	Surface	BioLegend	121606
B220	RA3-6B2	APC-Cy7	1 in 100	Surface	BioLegend	103224
F4/80	BM8	APC-Cy7	1 in 100	Surface	BioLegend	123118
TCRβ	H57-597	BV605	1 in 100	Surface	BioLegend	109241
CD69	H1.2F3	PE-Dazzle594	1 in 100	Surface	BioLegend	104536
IFN-γ	XMG1.2	BV711 or APC	1 in 100	Intracellular	BioLegend	505836, 505810
TNF	MP6-XT22	PE-Cy7	1 in 100	Intracellular	BioLegend	506324
Granzyme B	GB11	PE	1 in 200	Intracellular	ThermoFisher	GRB04

Data S1. DEGs in vivo human MAIT cell activation (separate file)

Differentially expressed genes (log_2 FC>1 and adjusted P<0.05) between MAIT cells isolated from the peripheral blood of human volunteers (n=14) 1 day prior to and 1 day following immunization with 5×10^{10} vp of ChAdOx1.

Data S2. DEGs in vitro human MAIT cell activation (separate file)

Differentially expressed genes (log_2 FC>1 and adjusted P<0.05) between MAIT cells isolated from human PBMC culture (n=4) after 24-hour stimulation with ChAdOx1-GFP (MOI=10^3 vp), or left untreated.

Data S3. DEGs in vivo mouse MAIT cell activation (separate file)

Differentially expressed genes (log_2 FC>1 and adjusted P<0.05) between MAIT cells isolated from the inguinal lymph nodes of C57BL/6J mice 24 hours after immunization with 10^8 IU of ChAdOx1-GFP (n=5), or from naïve controls (n=5).

Data S4. Overlap of upregulated DEGs between in vitro and in vivo stimulation (separate file)

Overlap of the upregulated genes between Data S1-S3. Mouse genes were converted to human gene annotations, as outlined in the methods section.

Data S5. DEGs B6 immunized versus B6 naïve (separate file)

Differentially expressed genes (log_2 FC>1 and adjusted P<0.05) between MAIT cells isolated from the inguinal lymph nodes of C57BL/6J mice (n=4) 24 hours after immunization with 10^8 IU of ChAdOx1-GFP, or from naïve controls (n=4).

Data S6. DEGs B6 immunized versus *Il18rap*$^{-/-}$ immunized (separate file)

Differentially expressed genes (log_2 FC>1 and adjusted P<0.05) between MAIT cells isolated from the inguinal lymph nodes of C57BL/6J mice (n=4) or *Il18rap*$^{-/-}$ mice (n=3) 24 hours after immunization with 10^8 IU of ChAdOx1-GFP.

Data S7. DEGs B6 immunized versus *Tnfrsf1a*$^{-/-}$*Tnfrsf1b*$^{-/-}$ immunized (separate file)

Differentially expressed genes (log_2 FC>1 and adjusted P<0.05) between MAIT cells isolated from the inguinal lymph nodes of C57BL/6J mice (n=4) or *Tnfrsf1a*$^{-/-}$*Tnfrsf1b*$^{-/-}$ mice (n=4) 24 hours after immunization with 10^8 IU of ChAdOx1-GFP.

Data S8. DEGs B6 immunized versus *Ifnar*$^{-/-}$ immunized (separate file)

Differentially expressed genes (log_2 FC>1 and adjusted P<0.05) between MAIT cells isolated from the inguinal lymph nodes of C57BL/6J mice (n=4) or *Ifnar*$^{-/-}$ mice (n=4) 24 hours after immunization with 10^8 IU of ChAdOx1-GFP.

Data S9. Overlap of upregulated DEGs in B6 ChAdOx1 versus knockout mice or naive (separate file)

Overlap of the differentially upregulated genes between MAIT cells from immunized and naïve C57BL/6J, and between immunized C57BL/6J mice and each of the knockout strains (*Il18rap*$^{-/-}$,

Tnfrsf1a$^{-/-}$*Tnfrsf1b*$^{-/-}$, and *Ifnar*$^{-/-}$). Only genes that are contained within the immunized versus naïve C57BL/6J comparison (i.e. genes differentially expressed in response to vaccination) are included in the overlap analysis.