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Supplemental Information

Airway Memory CD4^{+} T Cells Mediate Protective Immunity against Emerging Respiratory Coronaviruses

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Supplemental Figure 1

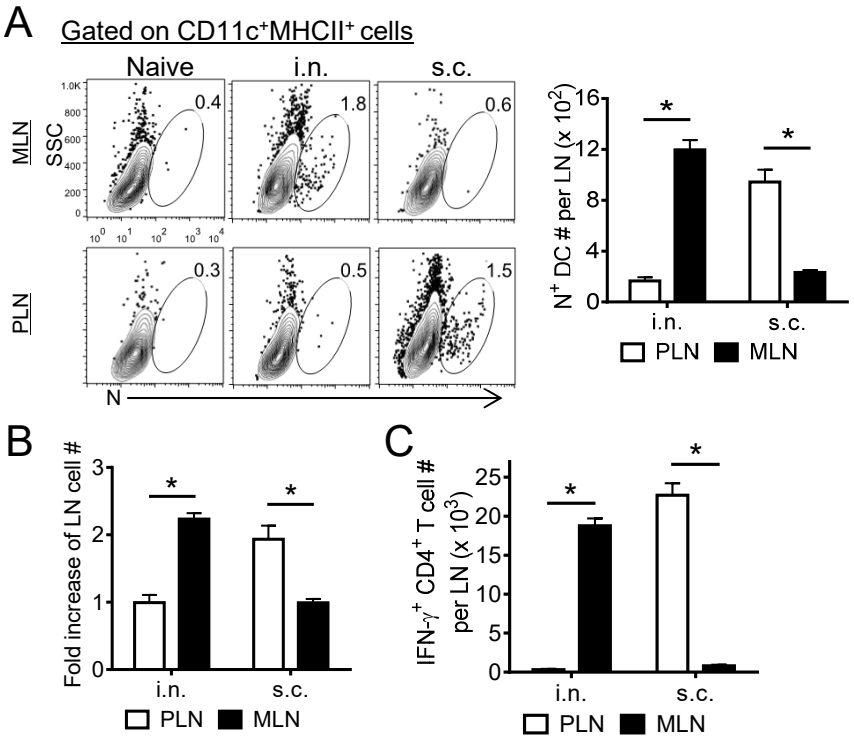

Figure S1 related to Figure 1. VRP-SARS-N infected DCs presented antigen in the regional DLNs. (**A**) To determine DC migration from vaccinated lung or footpad to DLNs, mice were vaccinated with VRP-SARS-N i.n. or s.c. After 18 hours, single cell suspensions were prepared from MLNs or PLNs. Cells were labeled with 1:1000 diluted anti-SARS-N mouse serum, followed by Alexa488-goat anti mouse IgG. The numbers represent the percentage of N^+ DC population (left) and total N^+ DC numbers per LN (right). (**B**) Fold increase of cell numbers in the DLNs at 18 hours post vaccination. (**C**) Cells from DLN were prepared at day 5 post vaccination and stimulated with N353 peptide in the presence of BFA. Cells were then stained for intracellular IFN-γ. IFN-γ^+ SARS-N353-specific $CD4^+$ T cell numbers per LN are shown. *n*= 3 mice/group. **P* values of <0.05. Error bars represent SEM. Data are representative of 2 independent experiments.

Supplemental Figure 2

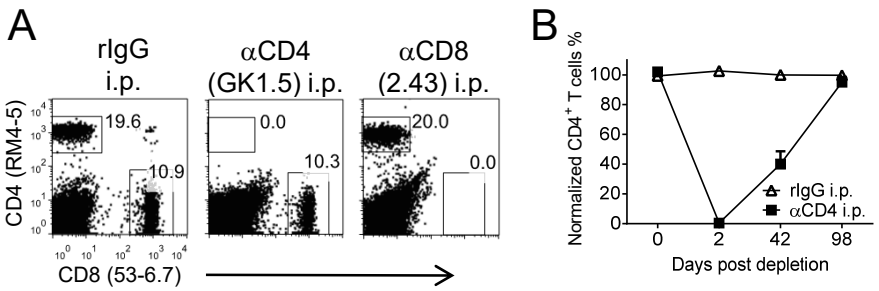

Figure S2 related to Figures 1 and 5. T cell depletion. (**A, B**) For systemic depletion of $CD4^+$ or $CD8^+$ T cells, mice were injected intraperitoneally (i.p.) with 1 mg anti-CD4 antibody (GK1.5) or 500 μg anti-CD8 antibody (2.43) at day -2 and day 0, respectively. Whole lungs were harvested at day 1 (**A**), or blood was harvested at indicated time points (**B**). Error bars represent SEM.

Supplemental Figure 3

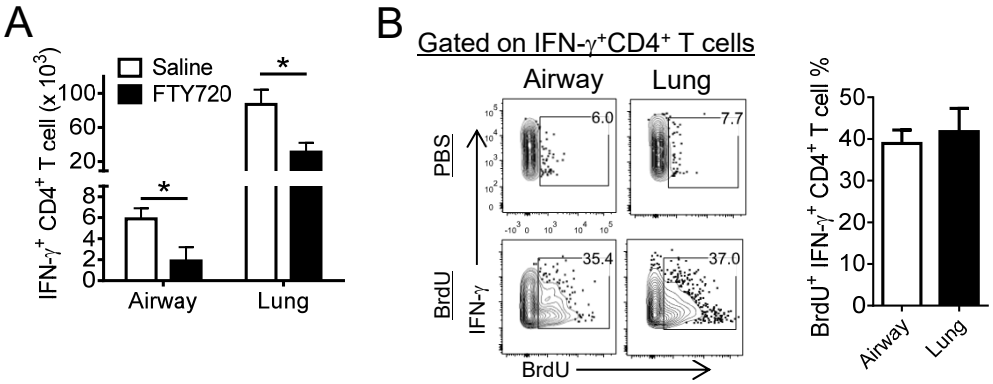

Figure S3 related to Figure 2. Recruitment from lymphoid organs and *in situ* proliferation contribute to protective CD4⁺ T responses in the respiratory tract. VRP-SARS-N i.n. vaccinated mice were treated with FTY720 (4 mg/kg) in saline i.p. daily starting one day prior to infection with 500 PFU SARS-CoV. (**A**) Cells from airways and lungs were stimulated with SARS-N353 peptide day 4 p.i. Numbers of IFN-γ⁺ CD4⁺ T cells are shown. (**B**) 1 mg BrdU in 100 μl PBS was delivered to FTY720 treated mice i.n. at day 4 p.i. After 4 hours, mice were sacrificed and cells from airways and lungs were stimulated with SARS-N353 peptide. Frequencies of BrdU⁺IFN-γ⁺ CD4⁺ T cells are shown. *n*= 3 mice/group. Error bars represent SEM. Data are representative of 3 independent experiments.

Supplemental Figure 4

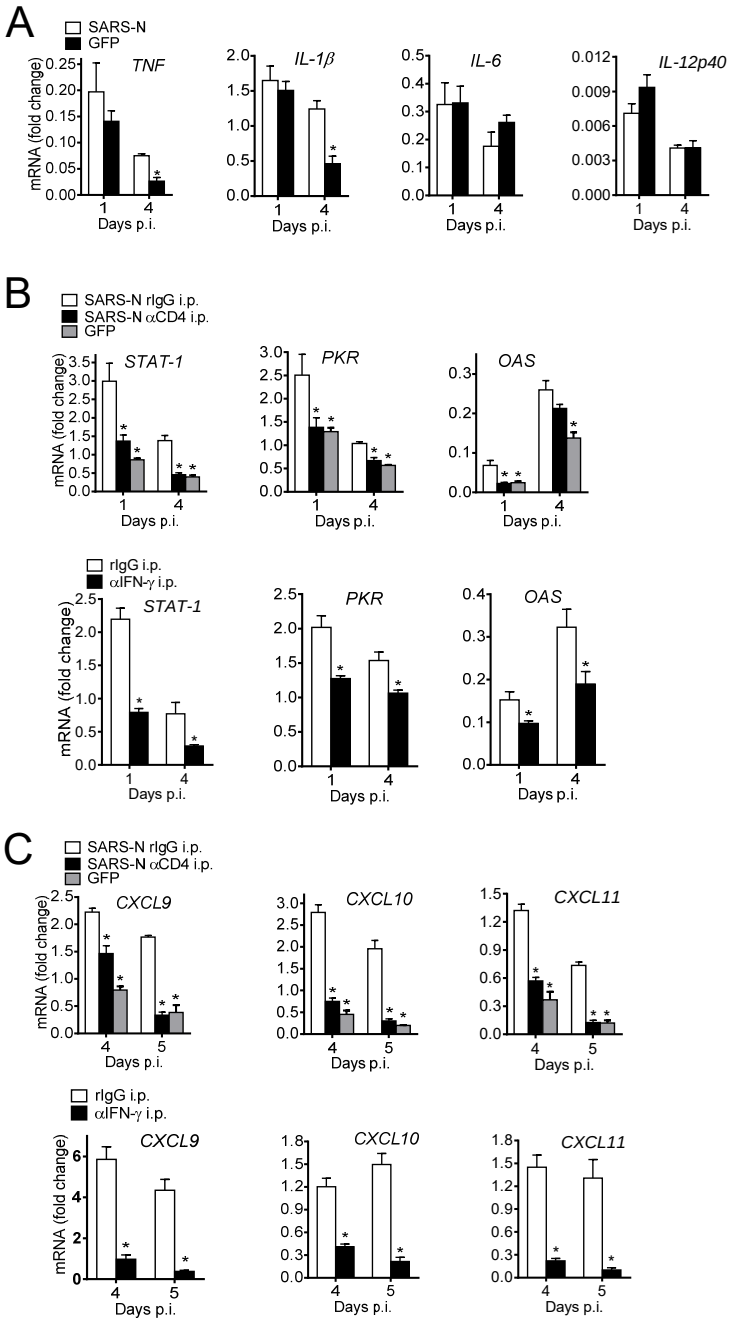

Figure S4 related to Figures 3 and 5. Pro-inflammatory cytokine mRNA levels. (**A, B, C**) Vaccinated mice were treated with anti-CD4 antibody, IFN-γ neutralizing antibody, or control antibody i.p. before SARS-CoV infection. Lungs were harvested at the indicated time points p.i. mRNA levels in the lungs were measured. *n*= 3-6 mice/group/time point. **P*<0.05. Error bars represent SEM. Data are representative of 2 independent experiments.

Supplemental Figure 5

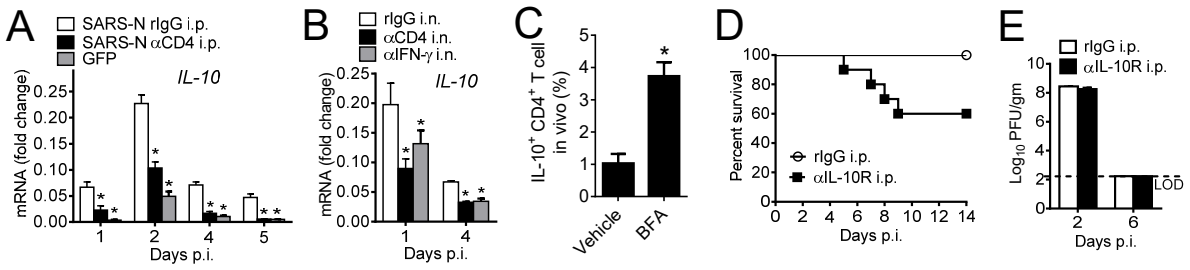

Figure S5 related to Figure 2. IL-10R blockade increased mortality in SARS-CoV infected mice. (**A, B**) VRP-SARS-N vaccinated mice were treated with anti-CD4 antibody (GK1.5) or rIgG i.p. (**A**) or anti-CD4 antibody (GK1.5) or anti-IFN-γ (XMG1.2) or rIgG i.n. (**B**). Mice were then infected with 500 PFU SARS-CoV. Lungs were harvested at indicated time points and IL-10 mRNA levels measured. *n*= 3-6 mice/group/time point. **P* values of <0.05. Error bars represent SEM. Data are representative of 2 independent experiments. (**C**) For *in vivo* ICS, VRP-SARS-N vaccinated mice were treated with BFA i.n. or vehicle at day 6 p.i. After 6 hours, lung-derived $CD4^+$ T cells were analyzed for IL-10 expression. $IL\text{-}10^+$ SARS-N353-specific $CD4^+$ T frequency is shown. Error bars represent SEM. (**D**) VRP-SARS-N vaccinated mice were treated i.p. with anti-IL-10R antibody (clone 1B1.3A), or equivalent doses of rIgG. Mice were infected with SARS-CoV. *n*= 5, rIgG i.p.; *n*= 10, αIL-10R i.p. (**E**) To obtain virus titers, mice were treated with antibodies as in (**D**). Titers are expressed as PFU/g tissue. n= 3 mice/group/time point. Error bars represent SEM. Data are representative of 2 independent experiments.

Supplemental Figure 6

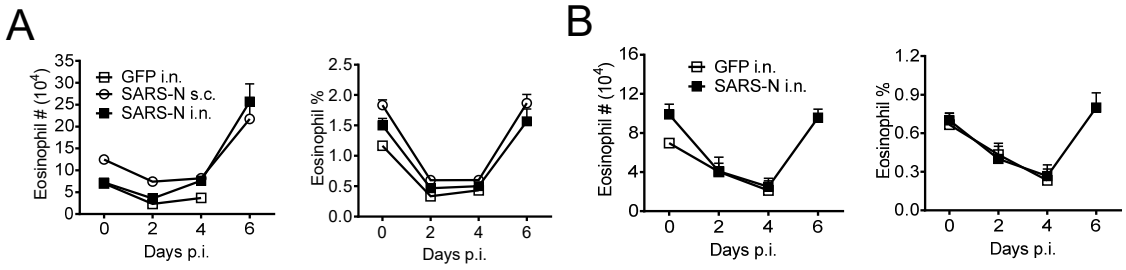

Figure S6 related to Figure 1. Eosinophils in SARS-CoV infected lungs. 6 week-old (**A**) and 12 month-old (**B**) BALB/c mice were vaccinated with VRP-SARS-N or VRP GFP. Mice were infected with 500 PFU (**A**) or 100 PFU (**B**) SARS-CoV. At indicated time p.i., cells from lungs were prepared and stained for eosinophils ($CD45^+CD11c^+SiglecF^+$). Most of the mice in the GFP group died by day 6. Data from days 0-4 are shown. *n*= 3-4 mice/group/time point. Error bars represent SEM. Data are representative of 2 independent experiments.

Supplemental Figure 7

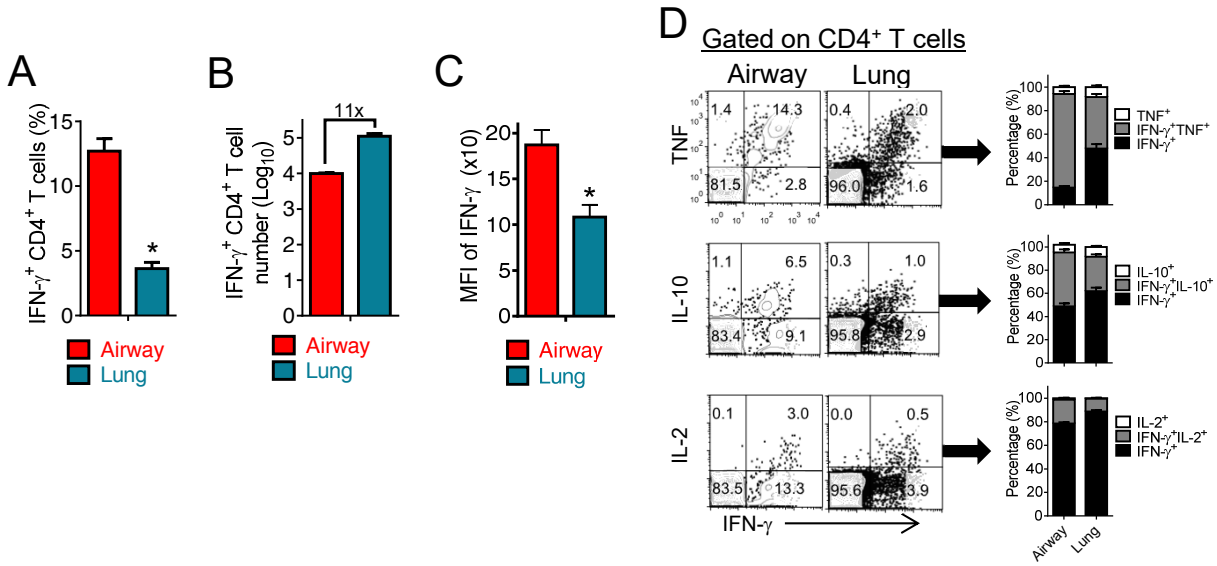

Figure S7 related to Figure 6. Airway-derived N350-specific CD4⁺ T cells are superior effector cells in MERS-CoV infected mice. Vaccinated mice were infected with MERS-CoV at the indicated times. Cells from airway and lung were stimulated with MERS-N350 peptide. Frequency (**A**), numbers (**B**) and mean fluorescence intensities (MFI) of IFN-γ (**C**) of IFN-γ⁺ CD4⁺ T cells or cells expressing IFN-γ and TNF, IL-10 or IL-2 (**D**) are shown. *n*= 3-4 mice/group. Error bars represent SEM. Data are representative of 3 independent experiments.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice, virus and cells. Specific pathogen-free 6 week and 12 month old BALB/c mice were purchased from the National Cancer Institute and Charles River Laboratories International. HLA-DR2 (DRB1*1501), HLA-DR3 (DRB1*0301) and HLA-DQ8 (DQA1*0301/DQB*0302) transgenic mice were produced as previously described(Cheng et al., 1996; Koehm et al., 2007; Kong et al., 1996). Mice were maintained in the Animal Care Facility at the University of Iowa. All protocols were approved by the University of Iowa Institutional Animal Care and Use Committee. The EMC/2012 strain of MERS-CoV (passage 8, designated MERS-CoV) was provided by Drs. Bart Haagmans and Ron Fouchier *(*Erasmus Medical Center*)*. Mouse-adapted SARS-CoV (MA15) was a kind gift from Dr. Kanta Subbarao (N.I.H., Bethesda, Maryland)(Roberts et al., 2007). SARS-CoV and MERS-CoV were passaged once on Vero E6 or Vero 81 cells and titered on the same cell line. A20 cells were used as antigen presenting cells. All work with SARS-CoV and MERS-CoV was conducted in the University of Iowa Biosafety Level 3 (BSL3) Laboratory.

Venezuelan Equine Encephalitis Replicon particles (VRPs), recombinant vaccinia virus and mouse immunization. VRPs expressing the SARS-CoV, MERS-CoV or HKU4 nucleocapsid proteins (N) were constructed as previously described(Scobey et al., 2013; Zhao et al., 2014). Mice were primed and boosted (6-7 weeks after priming) with $1x10^5$ Infectious Units (IU) of VRP-SARS-N, VRP-SARS-S or VRP-GFP in the left footpad in 20 μl PBS or intranasally (i.n.) in 50 μl PBS after light anesthesia with isoflurane. Recombinant vaccinia virus expressing SARS-CoV N protein was generated as previously described(Castro and Perlman, 1995). Mice were immunized intravenously (i.v.) with $1x10^6$ PFU rVV-N or irrelevant rVV in 200 μl PBS or i.n. in 50 μl PBS after light anesthesia with isoflurane**.** For sera transfer, sera were

obtained from vaccinated mice 2-4 weeks after boosting. Mice were challenged with SARS-CoV or MERS-CoV 4-6 weeks post boosting.

Virus titers. Virus was titered on Vero E6 cells or Vero 81 cells as previously described (Zhao et al., 2014).

Histology and immunohistochemistry. Lungs were removed, fixed in zinc formalin, and paraffin embedded. Sections were stained with hematoxylin and eosin for histological analysis.

Flow cytometry. The following anti-mouse monoclonal antibodies were used: CD4 (RM4-5; GK1.5); CD8α (53-6.7; 2.43); CD90.2 (53-2.1); IFN-γ (XMG1.2); TNF (MP6-XT22); IL-2 (JEH6-5H4); IL-10 (JES-2A5); CD16/32 (2.4G2); CD11a (M17/4); CD27 (LG.7F9); CD44 (IM7); CD49d (R1-2); CD69 (H1.2F3); CD103 (2E7); CD127 (eBioSB/199); Ly6C (AL-21); CXCR3 (CXCR3-173); CCR4 (2G12); CCR5 (HM-CCR5); CD11c (HL3); Siglec F (E50-2440); I-A/E (M5/114.15.2); CCR7 (4B12); CD45 (30-F11); CD40 (HM40-3); CD80 (16-10A1); CD86 (GL1); T-bet (eBio4B10); CD107a (eBio1D4B); CD107b (ABL-93); Granzyme B (16G6), all antibodies were from BD Bioscience, eBioscience or Biolegend.

For surface staining, 10^6 cells were blocked with 1 μg anti-CD16/32 antibody and 1% rat serum, and stained with the indicated antibodies at 4^{o}C, except for those stained with CCR7, which were stained at 37^{o}C.

For *in vitro* intracellular cytokine/protein staining, 1×10^6 cells/well were cultured in 96-well dishes at 37^{o}C for 5-6 hours in the presence of 2.5-10 μM peptide (BioSynthesis Inc., Lewisville, TX), brefeldin A (BFA, BD Biosciences) and antigen presenting cells (A20 cells). Cells were then labeled for cell surface markers, fixed/permeabilized with Cytofix/Cytoperm Solution (BD Biosciences) and labeled with anti-intracellular cytokine/protein antibodies.

To assess functional avidity, cells were stimulated with graded doses of the relevant peptide pulsed onto A20 cells and examined for IFN-γ production. The frequency of $CD4^+$ T cells producing IFN-γ at each concentration of peptide was measured and expressed as a percentage of the maximum response detected. Data were fit to sigmoidal dose–response curves and used to calculate the amount of peptide needed to reach a half-maximum response (EC_{50}).

For *in vivo* intracellular cytokine staining, infected mice were treated with 50 μg BFA (Sigma-Aldrich) or vehicle (DMSO) i.n**.** 6 h later, lung-derived lymphocytes were analyzed directly *ex vivo* for IFN-γ or IL-10 expression as described previously(Hufford et al., 2011).

For N^+ rDC staining, cells from MLNs were labeled for cell surface markers, fixed/permeabilized with Cytofix/Cytoperm Solution (BD Biosciences) and labeled with 1:1000 diluted anti-N serum, followed by A488-Goat anti mouse IgG.

For MHC class II tetramer staining, cells were stained with 8 μg/ml APC-conjugated $I\text{-}A^b$ /N353 tetramers (obtained from the National Institutes of Health/National Institute of Allergy and Infection Diseases MHC Tetramer Core Facility, Atlanta, GA) in complete RPMI 1640 media for 1 h at 37°C. Cells were then incubated with surface and intracellular markers.

All flow cytometry data were acquired on a BD FACSCalibur or BD FACSVerse and analyzed using FlowJo software (Tree Star, Inc.).

In situ CFSE staining. CFSE (Molecular Probes) (8 mM) was administered i.n. (50 μl/mouse) 6 hours before infection(Legge and Braciale, 2003).

In vivo cytotoxicity assay. *In vivo* cytotoxicity assays in the lungs were performed and analyzed on day 6 p.i., as previously described(Barber et al., 2003; Zhao et al., 2009).

Cytokine and chemokine RNA levels in infected lungs using qRT-PCR. RNA was extracted from infected lungs using Trizol (Invitrogen), and used as a template for cDNA synthesis (Invitrogen). qRT-PCR was performed using a previously described set of primers (Zhao et al., 2011).

Peptide immunization and T cell proliferation assay. Mice were immunized subcutaneously with N peptides (100 μg) emulsified in CFA and sacrificed 10 days later; draining lymph nodes were removed and stimulated with peptides *in vitro*. The results are presented as stimulation indices (cpm of test sample/cpm of the control).

FTY720 and BrdU treatment. Vaccinated mice were treated with FTY720 (4 mg/kg) (Cayman, Ann Arbor, MI) in saline i.p. daily starting one day prior to SARS-CoV infection. 1 mg BrdU (BD) in 100 μl PBS was delivered i.n. to FTY720-treated mice at day 4 p.i., 4 hours before sacrifice. Intracellular detection of BrdU was performed using reagents and protocols provided by the manufacturer (BD).

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