Science Advances
AAAS

Supplementary Materials for

Lung-innervating nociceptor sensory neurons promote pneumonic sepsis during carbapenem-resistant *Klebsiella pneumoniae* lung infection

Prabhu Raj Joshi *et al.*

Corresponding author: Pankaj Baral, baral@ksu.edu

Sci. Adv. **10**, eadl6162 (2024)
DOI: 10.1126/sciadv.adl6162

This PDF file includes:

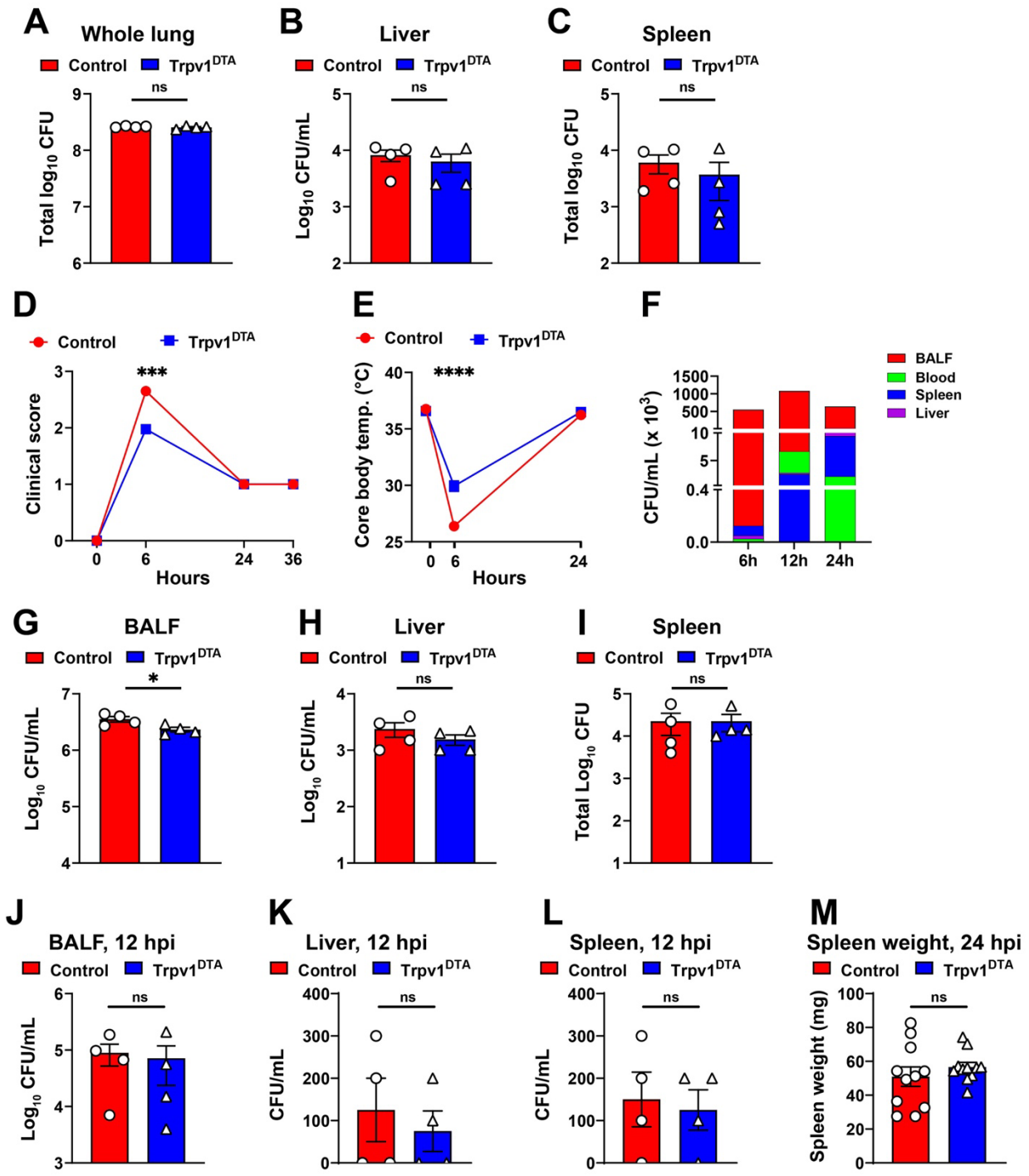

Figure S1: CRKP load at 50 minutes post-infection, clinical score and core body temperature after heat-killed CRKP challenge, CRKP dissemination pattern (0, 6, 12 and 24 hpi), hvKp clearance (24 hpi), CRKP load (12 hpi) and spleen weight (24 hpi) (Related to Figure 1)

(A to **C)** Colony forming units (CFUs) recovered from the whole lung (A), liver (B) and spleen (C) homogenates from Trpv1^{DTA} (n=4) and littermate control mice (n=4) after 50 minutes of intranasal infection with CRKP (10^9 CFU/mouse). (**D** to **E**) Clinical score (D) and core body

temperatures (E) of control and Trpv1^{DTA} mice after intranasal challenge with heat-killed CRKP bacteria (10^9 CFUs/mouse). Data in (D) to (E) involve the Trpv1^{DTA} and littermate control mice from at least one independent experiments (n=4-8 mice/group). (**F**) Bacterial load in BALF, blood, spleen and liver of wild type mice at 6h, 12h and 24h post-infection with CRKP. Data in (F) involves four mice for each time point. (**G to I**) The number of CFUs in BALF (G), liver (H) and spleen (I) in littermate control mice (n=4) and Trpv1^{DTA} mice (n=4) at 24 hpi with hypervirulent *K. pneumoniae* (10^4 CFU/mouse). (**J** to **L**) CFUs recovered in BALF (J), liver (K) and spleen (L) of Trpv1^{DTA} and control mice at 12 hpi of CRKP. (**M**) Weight of the spleen of Trpv1^{DTA} and control mice at 24 hpi of CRKP. Each symbol represents a mouse. Data in (A – E, and G – M) are the means±s.e.m. Statistical analysis was performed by the two-tailed unpaired t-test (A – C, G – H and J – M), Mann-Whitney test (I) and repeated measures two-way ANOVA with Sidak's multiple comparison test (D – E). (*P<0.05, ***P<0.001, ****P<0.0001). ns, not significant.

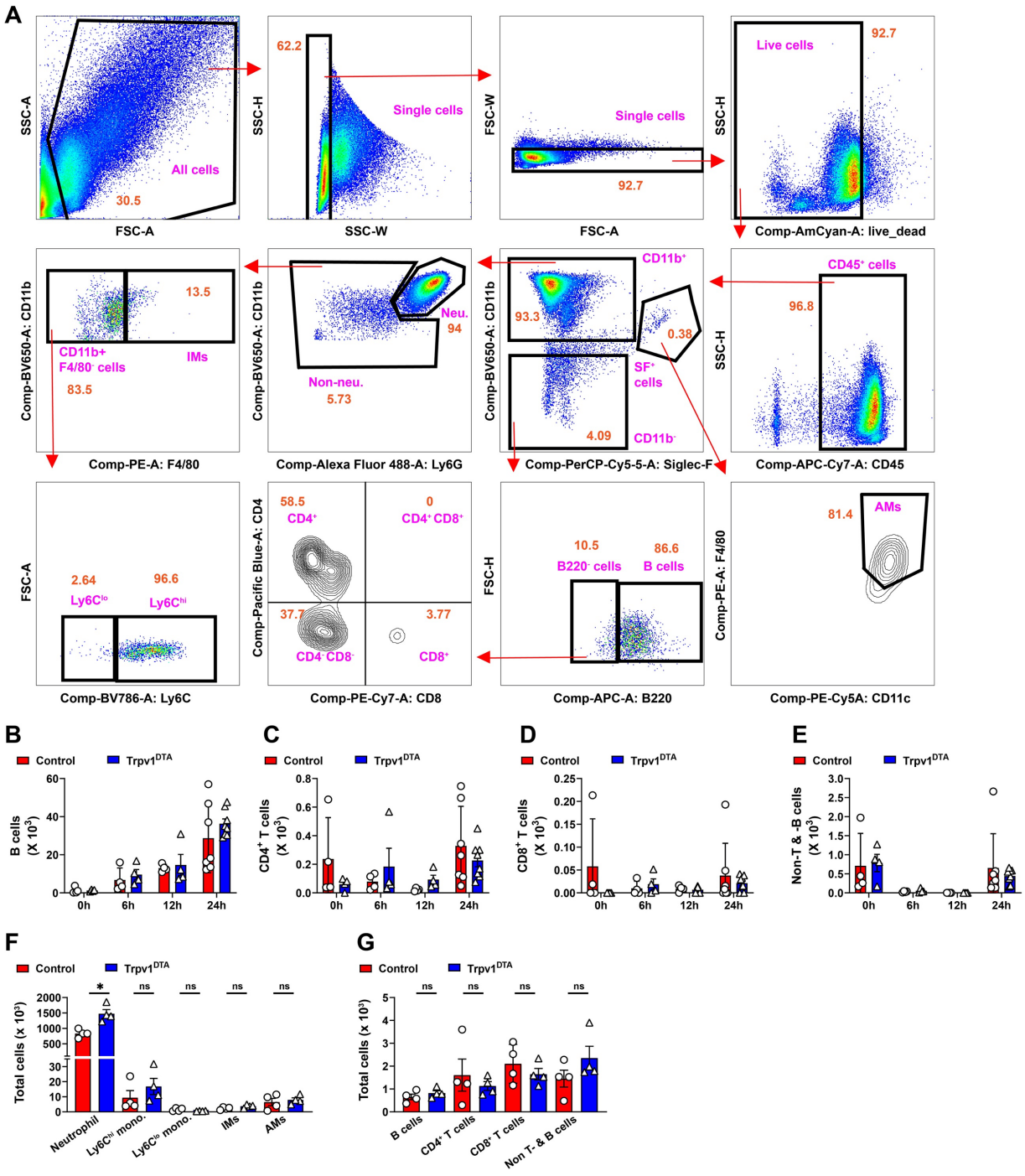

Figure S2: Characterization of BALF immune cells by flow cytometry after infection of Trpv1^{DTA} and control mice with CRKP and hvKp.

(**A**) Gating strategy to characterize immune cells recovered in BALF. BALF cells were gated for live cells by excluding LIVE/DEAD fixable aqua-stained cells. Leukocytes were gated as CD45$^+$ cells, which were further separated into Siglec-F$^+$, CD11b$^+$ and CD11b$^-$ cells. Siglec-F$^+$ (SF$^+$) cells were further gated into F4/80$^+$ and CD11c$^+$; both F4/80$^+$ and CD11c$^+$ cells were identified as alveolar macrophages (AMs). From CD11b$^+$ cells, Ly6G$^+$ cells were gated as neutrophils. CD11b$^+$ Ly6G$^-$ cells were gated for interstitial macrophages (IMs) (CD11b$^+$ F4/80$^+$). Remaining cell

populations were separated into Ly6C^{hi} monocytes (CD11b^{+} Ly6C^{hi}) and Ly6C^{lo} monocytes (CD11b^{+} Ly6C^{lo}). CD11b^{-} cells were gated into B cells (B220^{+} cells) and remaining CD11b^{-} B220^{-} cells were separated into CD4^{+} T cells, CD8^{+} T cells, CD4^{+} CD8^{+} T cells and CD4^{-} CD8^{-} T cells (non-T & -B cells). **(B** to **E)** Quantitative data of B cells (B), CD4^{+} T cells (C), CD8^{+} T cells (D), and non-T & -B cells (E) in BALF of Trpv1^{DTA} and control mice at 24 hpi with CRKP. Data in (B) to (E) involve control mice (n=4/group for 0h, 6h, 12h and n=7/group for 24h) and Trpv1^{DTA} mice (n=4/group for 0h, 6h, 12h and n=8/group for 24h). (**F** and **G**) Recruitment of myeloid subsets (F) and lymphoid subsets (G) in control (n=4) and Trpv1^{DTA} mice (n=4) at 24h post-infection with hvKp (10^4 CFU/mouse). Data in (B to G) are the means±s.e.m. Statistical analysis was done by two-way ANOVA of two-stage liner step-up procedure of Benjamini, Krieger and Yekutieli post-tests (B to E) and Mann-Whitney test (F to G) with the following significance level: (*P<0.05). ns, not significant.

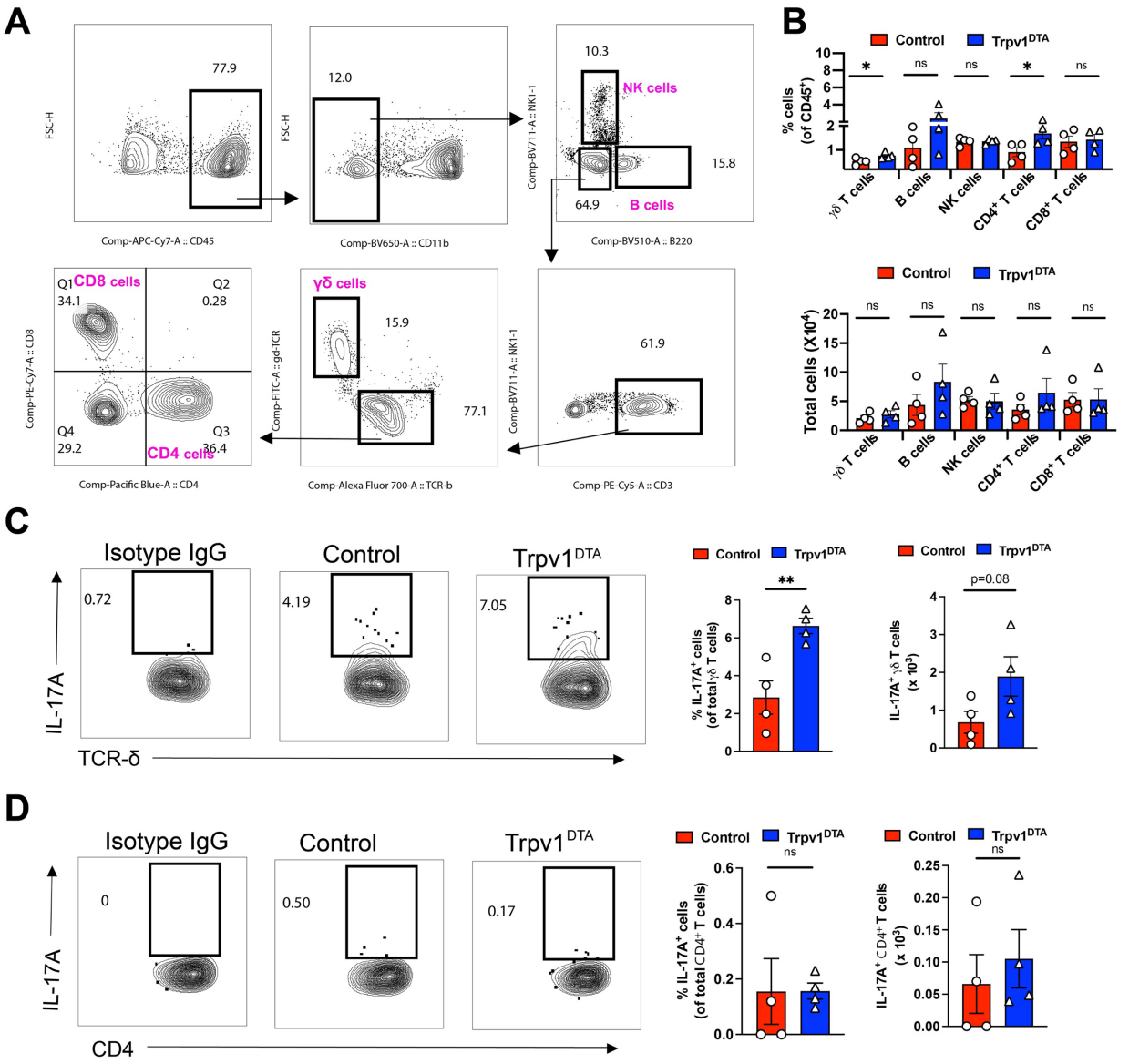

Figure S3. Increased proportion of IL-17A-producing γδ T cells in CRKP-infected nociceptor-ablated mice.

(A) Gating strategy for determining the lymphoid subsets in the whole lungs of CRKP-infected control and Trpv1^{DTA} mice at 24h post-infection. NK cells (CD45$^+$CD11b$^-$B220$^-$NK1.1$^+$), B cells (CD45$^+$CD11b$^-$NK1.1$^-$ B220$^+$ cells), γδ T cells (CD45$^+$CD11b$^-$NK1.1$^-$ B220$^-$ CD3$^+$TCRβ$^-$TCRδ$^+$ cells), CD4$^+$ T cells (CD45$^+$CD11b$^-$NK1.1$^-$B220$^-$CD3$^+$TCRβ$^+$TCRδ$^-$CD8$^-$CD4$^+$cells) and CD8$^+$ T cells (CD45$^+$CD11b$^-$NK1.1$^-$B220$^-$CD3$^+$TCRβ$^+$TCRδ$^-$CD4$^-$CD8$^+$cells). **(B)** Proportions and absolute numbers of lymphoid subsets in the whole lungs of CRKP-infected control (n=4) and Trpv1^{DTA} (n=4) mice at 24h post-infection. Statistical analysis: Unpaired t-tests (B, up) and Mann-Whitney test (B, down) with the following significance level: (*P<0.05). **(C-D)** Flow cytometry plots showing IL-17A$^+$ γδ T cells (C) and IL-17A$^+$ CD4$^+$ T cells (D). Proportions and absolute number of IL-17A$^+$ γδ T cells (C) and IL-17A$^+$ CD4$^+$ T cells (D) in the whole lungs of CRKP-infected control (n=4) and Trpv1^{DTA} (n=4) mice at 24h post-infection. Statistical analysis:

Unpaired t-tests with the following significance levels: (**P<0.01). Isotype IgG was used as negative staining control. ns, not significant.

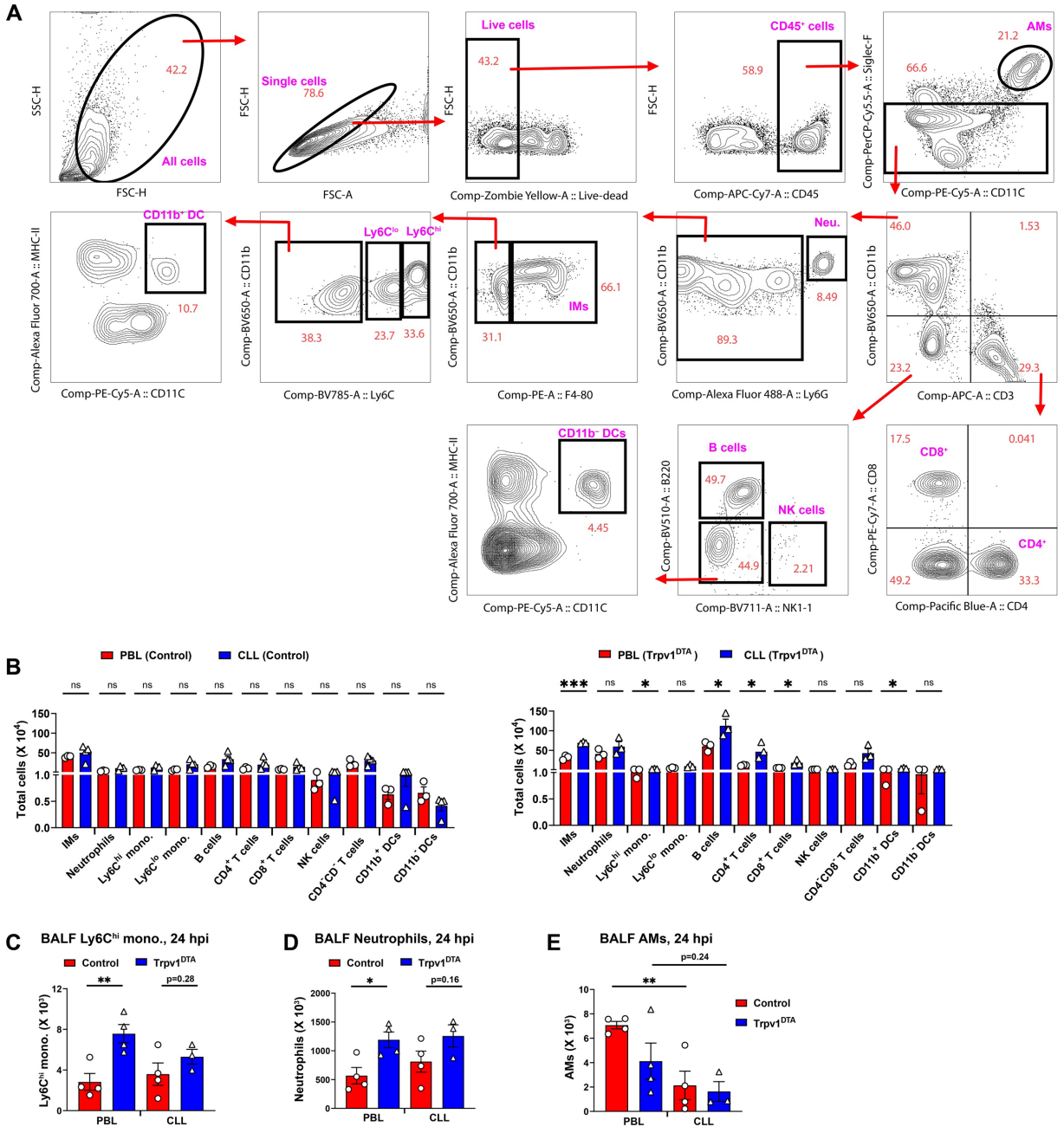

Figure S4: Abundance of myeloid and lymphoid immune cell types after CLL-mediated AM depletion in control and Trpv1^{DTA} mice.

(**A**) Gating strategy for determining the myeloid and lymphoid subsets in the whole lungs of control and Trpv1^{DTA} mice after 48h of clodronate liposome (CLL) administration. PBS-liposome (PBL) was used for control group of mice. CLL or PBL (70 µL/mouse) was inoculated via intratracheal route at 48 hours before the immune cell type analyses. Interstitial macrophages (IMs, CD45^{+}Siglec-F^{-}CD11b^{+}Ly6G^{-} F4/80^{+} cells), neutrophils (CD45^{+}Siglec-F^{-}CD11b^{+}Ly6G^{+} cells), Ly6C^{hi} monocytes (CD45^{+}Siglec-F^{-}CD11b^{+}Ly6G^{-} F4/80^{-} Ly6C^{hi} cells), Ly6C^{lo} monocytes (CD45^{+}Siglec-F^{-}CD11b^{+}Ly6G^{-}F4/80^{-}Ly6C^{lo} cells), CD11b^{+} DC (CD45^{+}Siglec-F^{-}CD11b^{+}Ly6G^{-}

F4/80^{-}Ly6C^{-} CD11c^{+} MHC-II^{+}cells), CD11b^{-} DCs (CD45^{+}Siglec-F^{-}CD11b^{-} CD3^{-} B220^{-} NK1.1^{-} CD11c^{+} MHC-II^{+}cells), B cells (CD45^{+}Siglec-F^{-}CD11b^{-} CD3^{-}NK1.1^{-}B220^{+}cells), CD4^{+} T cells (CD45^{+}Siglec-F^{-}CD11b^{-} CD3^{+} CD8^{-}CD4^{+} cells), CD8^{+} T cells (CD45^{+}Siglec-F^{-}CD11b^{-} CD3^{+} CD4^{-}CD8^{+} cells) and CD4^{-} CD8^{-} T cells (CD45^{+}Siglec-F^{-}CD11b^{-} CD3^{+} CD4^{-}CD8^{-} cells). **(B)** Absolute numbers of myeloid and lymphoid subsets in the whole lungs of CLL- or PBL-treated control (n=3-4) and Trpv1^{DTA} (n=3) mice after 48h of treatment. **(C)** The BALF number of Ly6C^{hi} monocytes, neutrophils, and AMs in CLL- and PBL-treated control (n=4) and Trpv1^{DTA} mice (n=3-4) after 24h of CRKP infection. Statistical analysis: Mann Whitney test (B, left), unpaired t-tests (B, right and C to E) with the following significance levels: (*P<0.05, **P<0.01, ***P<0.001). ns, not significant.

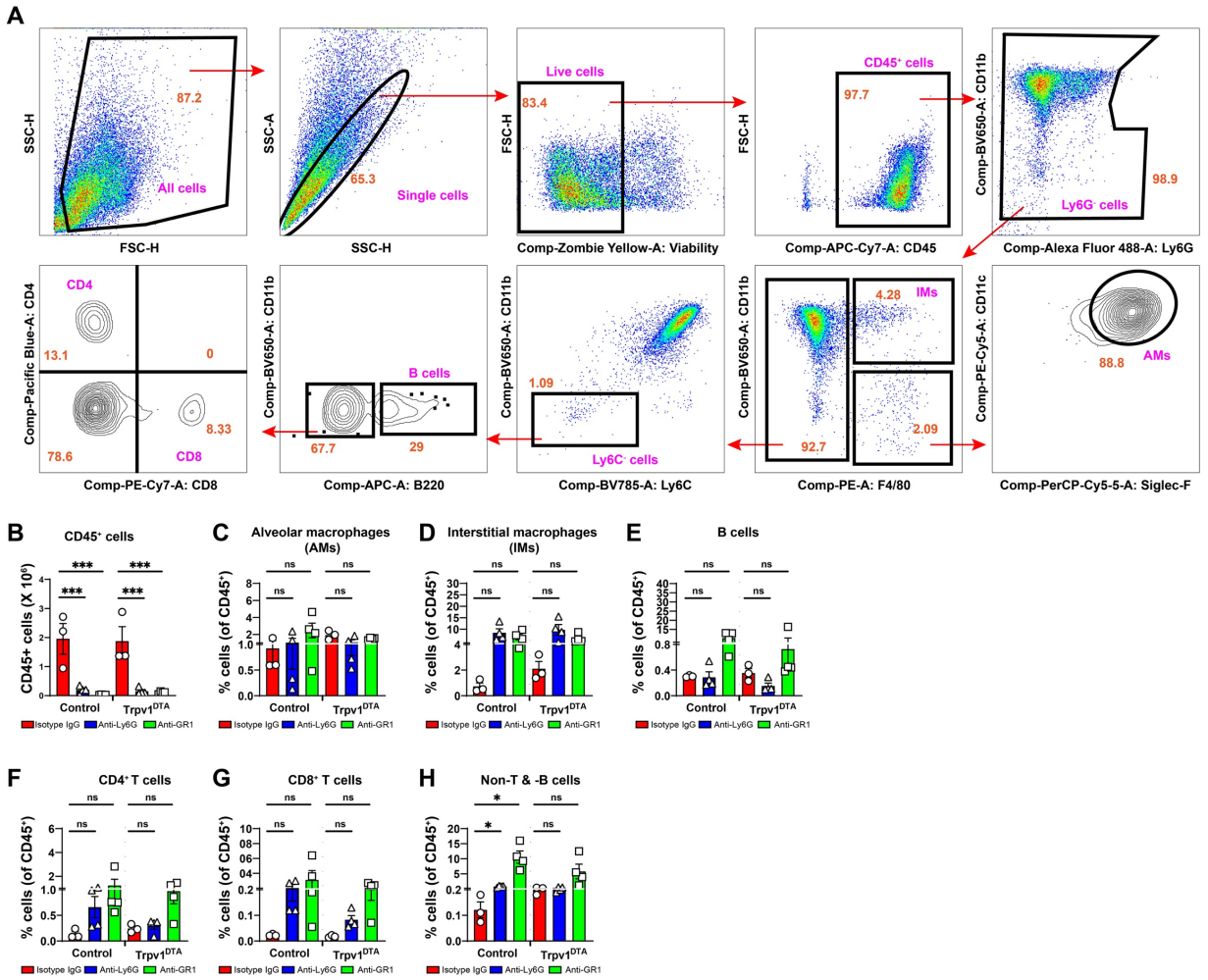

Figure S5: Characterization of BALF immune cells in neutrophil/monocyte-depleted mice with CRKP lung infection

(**A**) Gating strategy to characterize immune cells in BALF collected from neutrophil/monocyte-depleted mice. The shown gating strategy is from a neutrophil-depleted (anti-Ly6G-treated) sample. Zombie Yellow stained cells (dead cells) were excluded to separate live cells. $CD45^+$ cells were gated as leukocytes. From $CD45^+$ cells, $Ly6G^-$ cells were separated into interstitial macrophages (IMs, $CD11b^+$ $F4/80^+$) and $CD11b^-$ $F4/80^+$ cells, latter were further gated as $CD11c^+$ Siglec-F^+ cells (alveolar macrophages, AMs). $F4/80^-$cells were further gated for $Ly6C^-$ cell population for characterizing lymphoid cells. B cells were separated as $CD11b^-$ $B220^+$ cells. $B220^-$ cells were separated into $CD4^+$ T cells, $CD8^+$ T cells, $CD4^+$ $CD8^+$ T cells and $CD4^-$ $CD8^-$ T cells (non-T & -B cells). (**B** to **H**) quantitative data of $CD45^+$ cells (B), alveolar macrophages (C), interstitial macrophages (D), B cells (E), $CD4^+$ T cells (F), $CD8^+$ T cells (G) and non-T and -B cells (H). Each symbol represents a mouse. Data are the means±s.e.m of one experiment. Data were analyzed by one-way ANOVA with Holm-Sidak's multiple comparisons test (B) and Brown Forsythe and Welch ANOVA with Dunnett's T3 multiple comparison post-test (C to H) with the following significance level: (*P < 0.05, ***P < 0.001). ns, not significant.

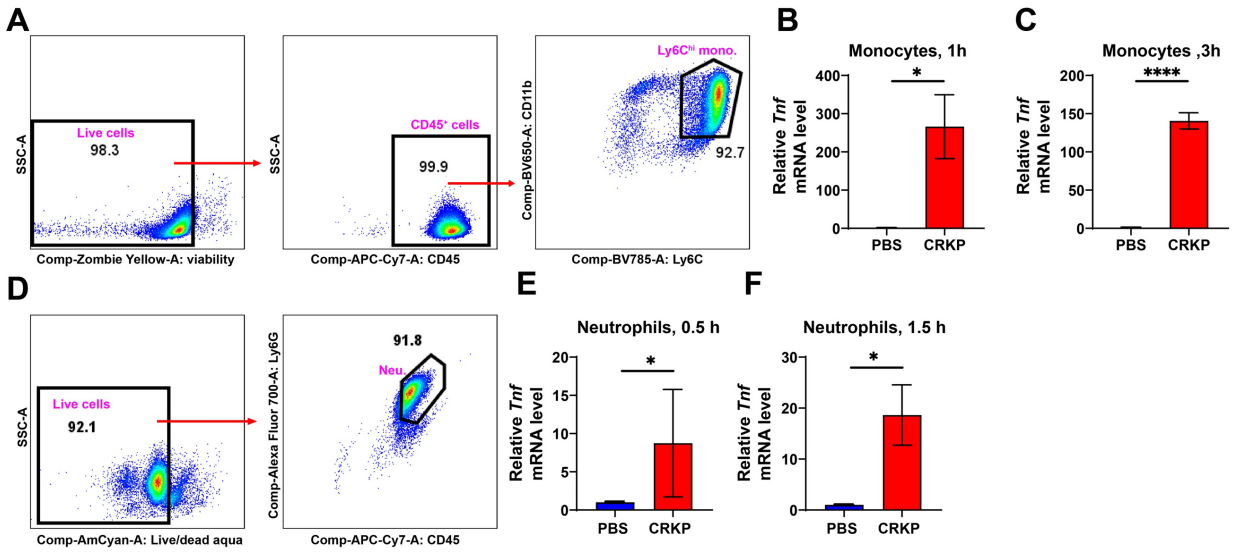

Figure S6: Quantitative polymerase chain reaction (qPCR) for *Tnf* gene expression in purified Ly6C^{hi} monocytes and neutrophils infected with CRKP

(**A**) Flow cytometry plots showing purity of Ly6C^{hi} monocytes isolated from bone marrow (BM) of the C57BL/6 mouse. Zombie Yellow-stained dead cells were separated from live cells, which were further gated for CD45^{+} cells. Ly6C^{hi} monocytes were represented as CD11b^{+} Ly6C^{+} cells. (**B** to **C**) Levels of *Tnf* mRNA in PBS-treated and CRKP-infected monocytes for 1h (B) and 3h (C). (**D**) Flow cytometry plots showing purity of neutrophils isolated from BM from C57BL/6 mouse. Live/dead aqua stain was used to exclude dead cells. Live cells were gated for neutrophils (CD45^{+} Ly6G^{+}). (**E** to **F**) Expression levels of *Tnf* mRNA in PBS-treated and CRKP-infected neutrophils for 0.5 and 1.5h. Data in (B to C) and (E to F) are the means±s.e.m. of 4 samples in each group. Statistical analysis was performed by two-tailed unpaired t-test (B, C and F) and Mann-Whitney test (E) with the following significance levels: (*$P < 0.05$, ****$P < 0.0001$).

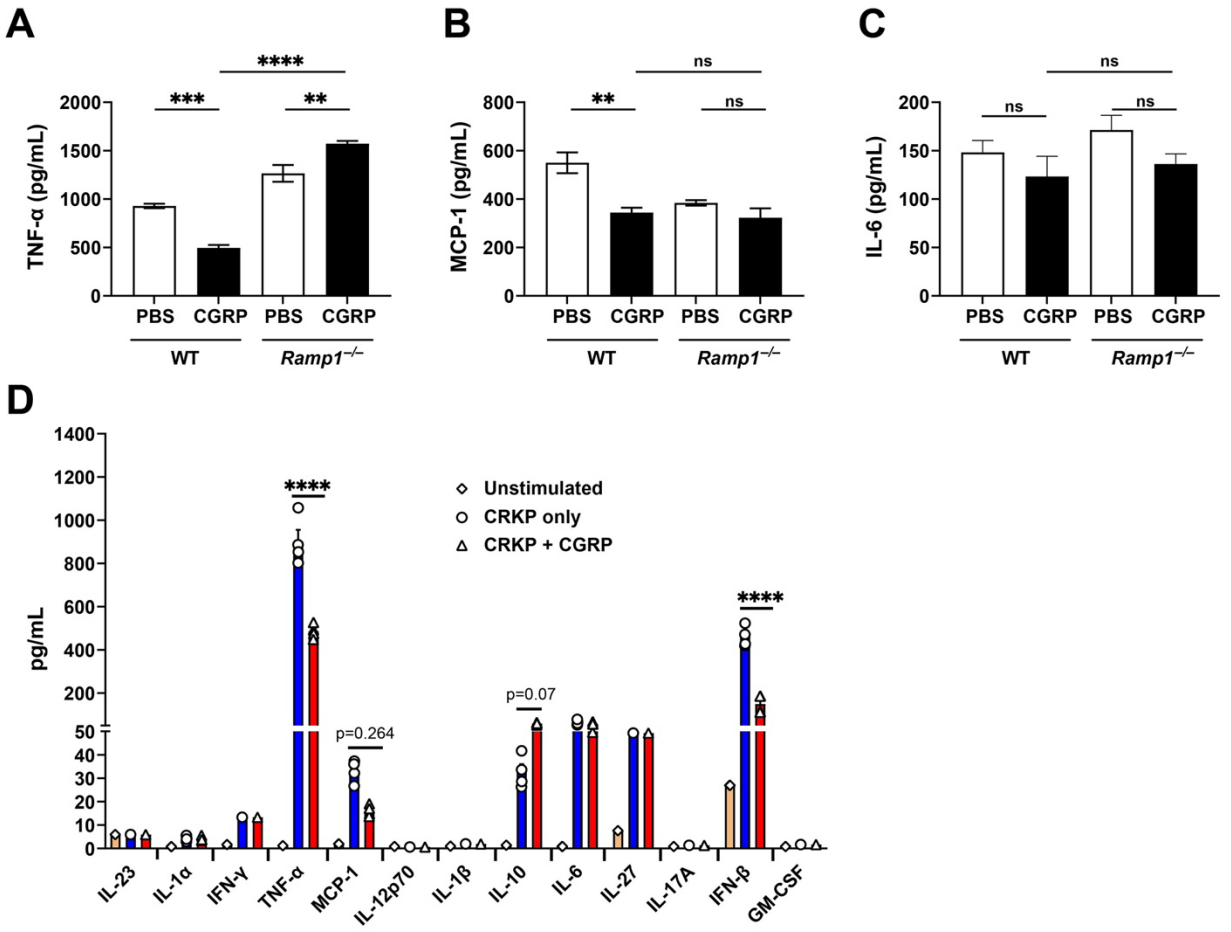

Figure S7: CGRP-RAMP1 signaling suppresses cytokine production by CRKP-infected BMDMs

(A-C) Levels of TNF-α (A), MCP-1 (B) and IL-6 (C) in wild type (WT) and *Ramp1*$^{-/-}$ BMDMs infected with CRKP alone or with CRKP and CGRP. BMDMs were co-cultured with CRKP of MOI of 10 in the presence and absence of CGRP (100 nM) for 6 hours. ELISA was performed to measure the cytokines in cell free supernatant. (**D**) Inflammatory panel of cytokines and chemokine were simultaneously measured by LEGENDplex assay in the supernatant of BMDM-infected culture with CRKP alone or CRKP and CGRP. Data in (A to D) are the means±s.e.m. of four samples in each group. Data were analyzed by one-way ANOVA with Sidak's multiple comparisons test (A to C) and two-way ANOVA of two-stage liner step-up procedure of Benjamini, Krieger and Yekutieli post-tests (D) with the following significance level: (**$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$). ns, not significant.

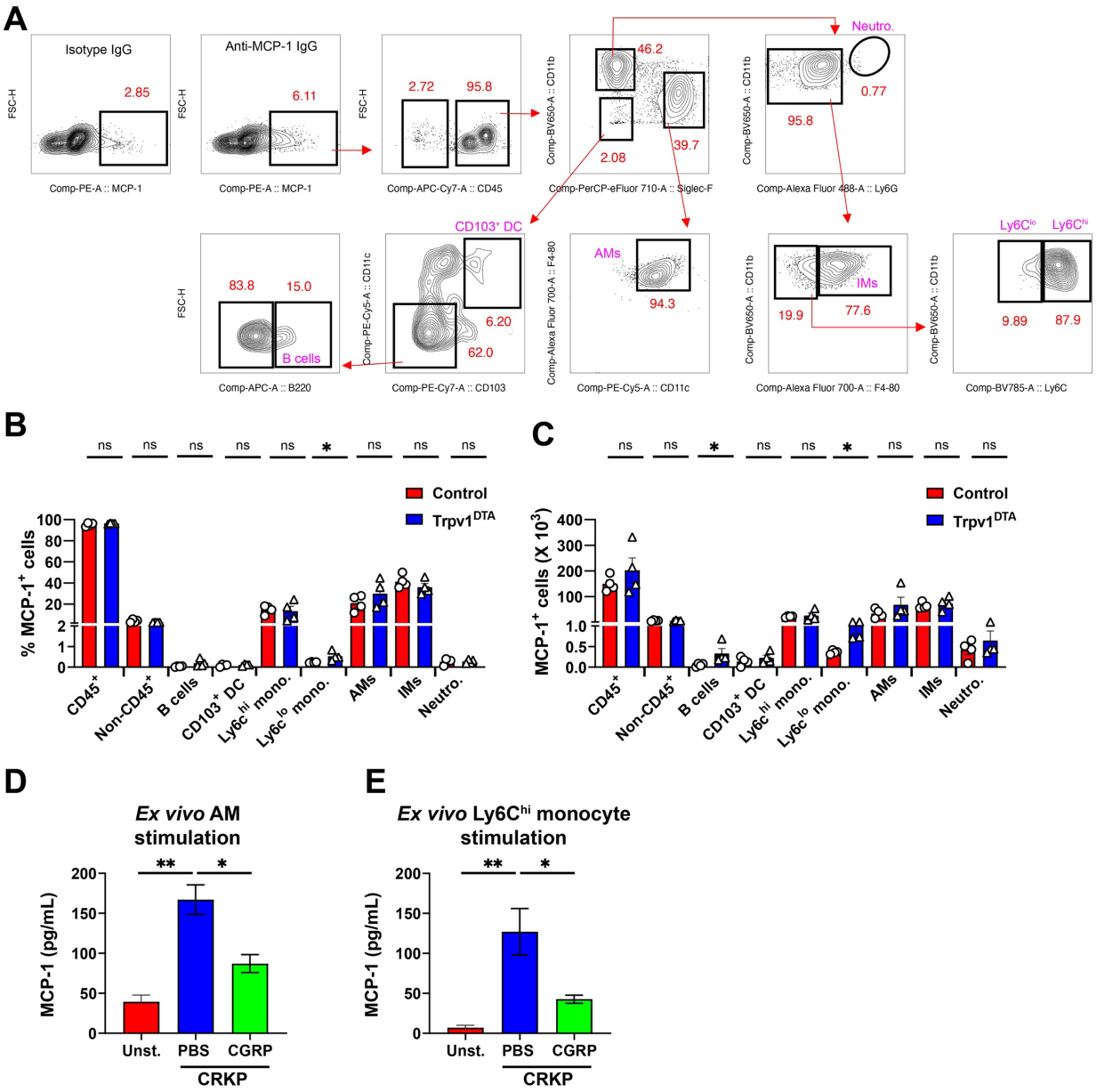

Figure S8. Cellular source of MCP-1 in CRKP infection and CGRP treatment suppresses the production of MCP-1 by CRKP-infected AM and Ly6C^{hi} monocyte cultures

A) Gating strategy for determining the MCP-1^{+} cell types in the whole lungs of CRKP-infected control and Trpv1^{DTA} mice at 24h post-infection. Non-CD45^{+} cells (CD45^{-}cells), Neutrophils (CD45^{+}Siglec-F^{-}CD11b^{+}Ly6G^{+} cells), Alveolar macrophages (AMs, CD45^{+}Siglec-F^{+}CD11b^{-} CD11c^{+}F4/80^{+}cells), Interstitial macrophages (IMs, CD45^{+}Siglec-F^{-}CD11b^{+}Ly6G^{-} F4/80^{+}cells), Ly6C^{hi} monocytes (CD45^{+}Siglec-F^{-}CD11b^{+}Ly6G^{-} F4/80^{-} Ly6C^{hi} cells), Ly6C^{lo} monocytes (CD45^{+}Siglec-F^{-}CD11b^{+}Ly6G^{-}F4/80^{-}Ly6C^{lo} cells), CD103^{+} DCs (CD45^{+}Siglec-F^{-}CD11b^{-} CD11c^{+} CD103^{+}cells) and B cells (CD45^{+}Siglec-F^{-}CD11b^{-}CD11c^{-} CD103^{-} B220^{+}cells). **(B-C)** Proportions (B) and absolute number (C) of MCP-1^{+} cell types in the whole lungs of CRKP-infected control (n=4) and Trpv1^{DTA} (n=4) mice at 24h post-infection. **(D)** Alveolar macrophages (AMs) from BALF, and **(E)** Ly6C^{hi} monocytes from bone marrow cells, were isolated from C57BL/6J mice, seeded in 96 well plates, and stimulated *ex vivo* with CRKP bacteria of MOI of 5

in the presence and absence of CGRP (100 nM) to determine the levels of MCP-1 in culture supernatant by ELISA. Statistical analysis: Unpaired t-tests (B, left), Mann Whitney test (B, right) and one-way ANOVA with Sidak's multiple comparisons post-test (D and E) Levels of significance: (*P<0.05, **P<0.01). ns, not significant.

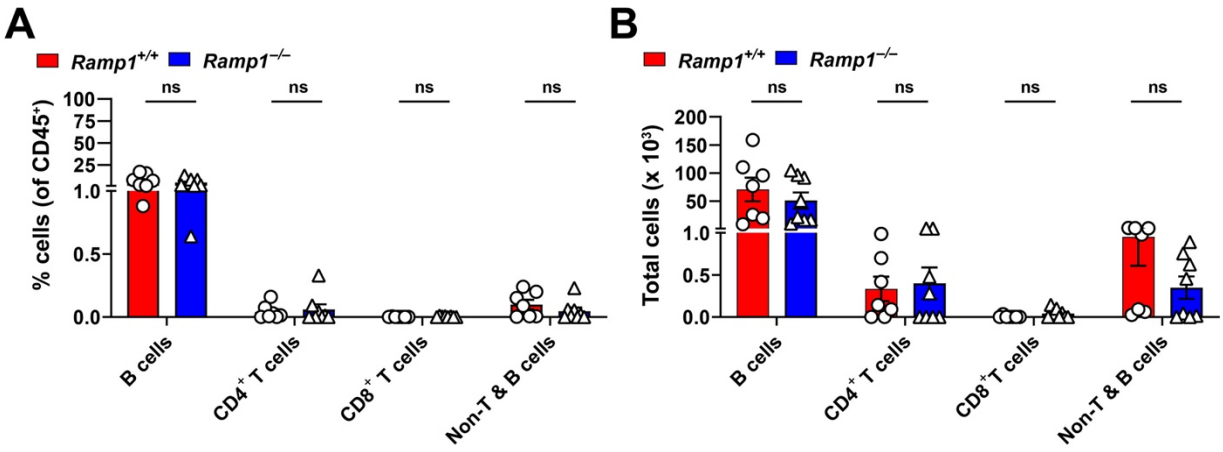

Figure S9: Characterization of lymphoid subsets in BALF of *Ramp1*$^{+/+}$ and *Ramp1*$^{-/-}$ mice
(**A** to **B**) Proportions (A) and total numbers (B) of B cells, CD4^{+} T cells, CD8^{+} T cells and non-T & -B cells recovered in BALF cells of *Ramp1*$^{+/+}$ and *Ramp1*$^{-/-}$ mice after 24 hpi with CRKP. Data in (A) and (B) are the means±s.e.m of *Ramp1*$^{+/+}$ mice (n=7) and *Ramp1*$^{-/-}$ mice (n=8), and of two independent experiments. Data were analyzed by two-way ANOVA of two-stage liner step-up procedure of Benjamini, Krieger and Yekutieli post-tests. ns, not significant.

Table S1. List of primers used for the analysis of neuropeptide receptor gene expression

Genes	Forward (5'- 3')	Reverse (5'- 3')
Ramp1	GGGGCTCTGCTTGCCAT	GGATGAGAGTCCCATAGTCAGG
Calcrl	GCAGCAGCTACCTAGCTTGAA	TTCACGCCTTCTTCCGACTC
Vipr1	ACCATCATCAACTCCTCACT	CAGGATGAAGTTCACCAAGAT
Tacr1	GATGAAGGAGCTGTCCAAGC	TCACGAAACAGGAAACATGC
Tacr2	GCCATGCTGTTTGCGGCTG	CCTTGCTCAGCACTTTCAGC
Oprm1	GGATCGAACTAACCACCAGCCAAC	GGTTCCTCATTCCTCTGTCCATGC
Npy2r	CACCAAATCGGACCTGCT	AGAACCAGTTCACTCTCACTTGG
Htr2b	GCTGACAAAGGACCGCTTTG	ATCAGAGGATTCACCCCCGA
Gapdh	TCGTGGATCTGACGTGCCGCCTG	CACCACCCTGTTGCTGTAGCCGTA
Tnf	CATCTTCTCAAAATTCGAGTGACAA	TGGGAGTAGACAAGGTACAACCC