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Supplemental Information

Programming Multifaceted Pulmonary T Cell Immunity by Combination Adjuvants

Chandranaik B. Marinaik, Brock Kingstad-Bakke, Woojong Lee, Masato Hatta, Michelle Sonsalla, Autumn Larsen, Brandon Neldner, David J. Gasper, Ross M. Kedl, Yoshihiro Kawaoka, and M. Suresh

SUPPLEMENTAL INFORMATION

SUPPLEMENTARY FIGURES

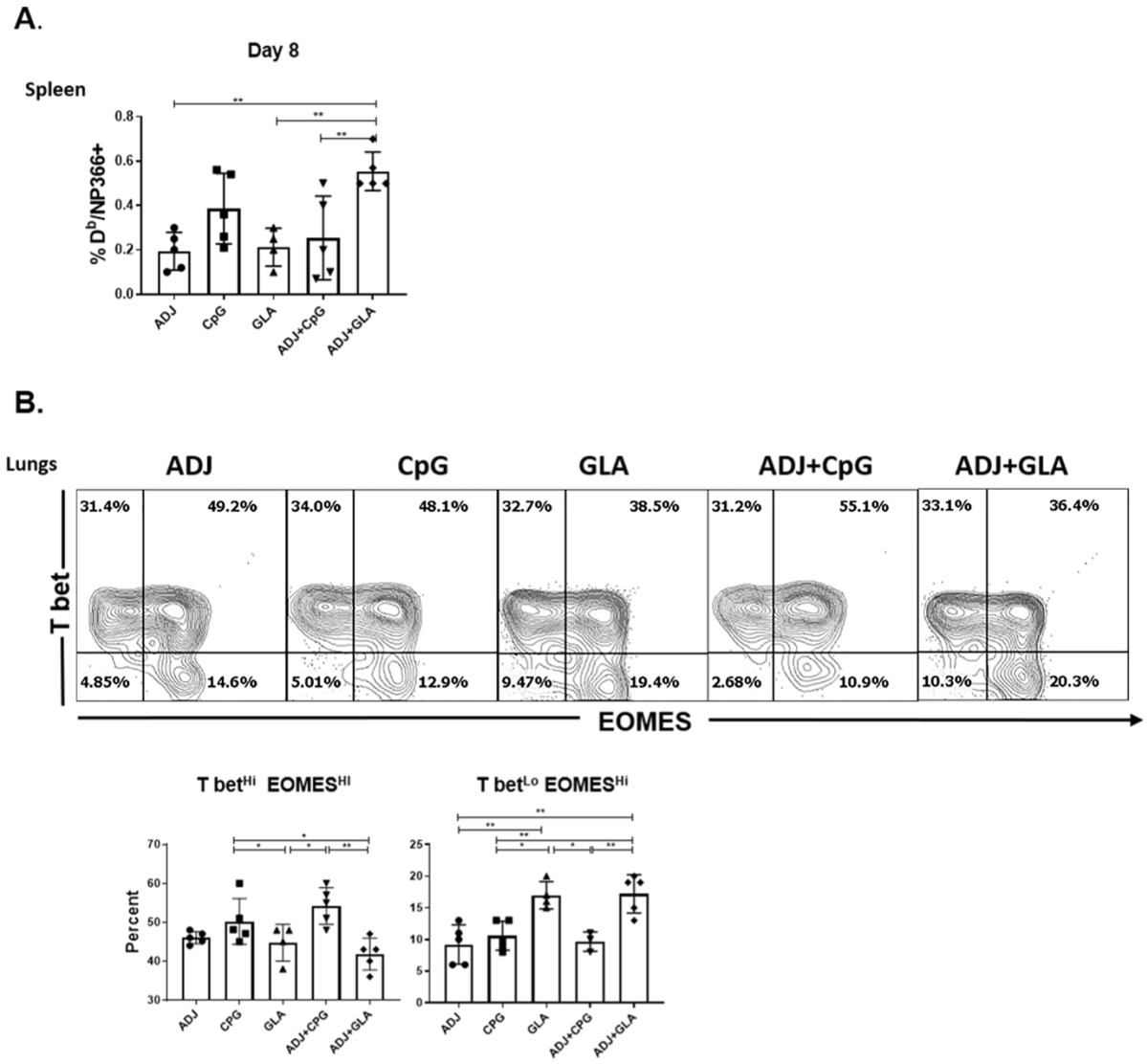

Figure S1. Systemic and mucosal CD8 T cell responses to adjuvanted vaccines. Related to Figure 1. C57BL/6 mice were vaccinated intranasally twice (at 3-week intervals) with NP formulated with the indicated adjuvants. (A-B) At days 8 and 100 after booster vaccination, splenocytes were stained with anti-CD8, anti-CD44 and D^b/NP366 tetramers. (A) Percentages of NP366-specific CD8 T cells in spleen at day 8 after vaccination. (B) On the 8^{th} day after vaccination, lung cells were stained with D^b/NP366 tetramers, anti-CD8, anti-T-bet and anti-

EOMES antibodies. FACS plots in B are gated on D^b/NP366 tetramer-binding CD8 T cells and numbers in each quadrant are the percentages among the gated cells. Adjoining bar graphs in B show percentages of T-bet^{HI}EOMES^{HI} or T-bet^{LO}EOMES^{HI} cells among D^b/NP366-specific CD8 T cells. Data are representative of two independent experiments. One-way ANOVA test with Tukey corrected multiple comparisons or Students t test; *, **, and *** indicate significance at $P<0.05$, 0.01 and 0.001 respectively.

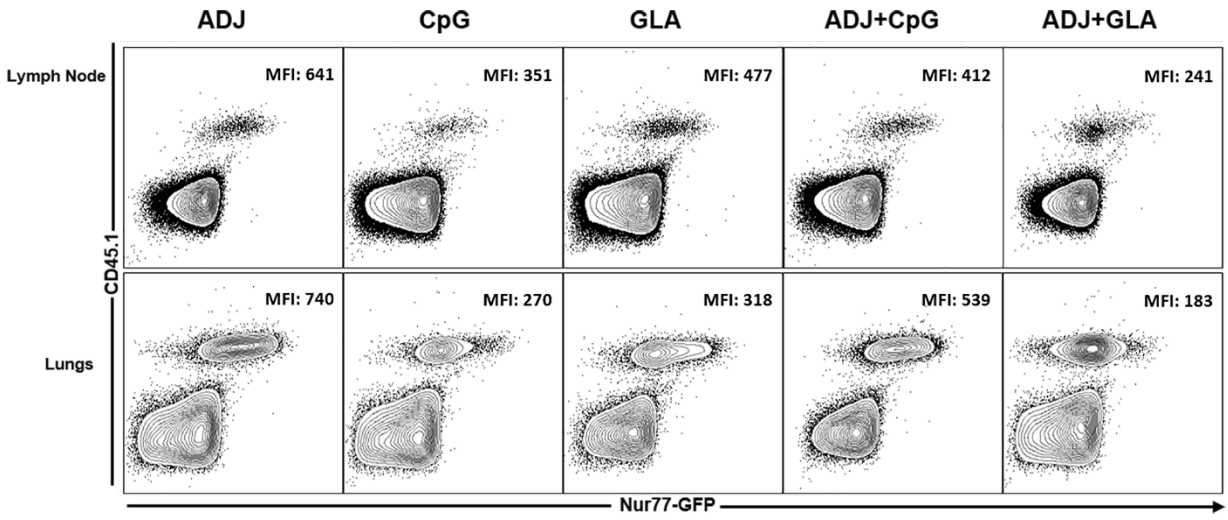

Figure S2. Quantification of TCR signaling in vaccinated mice using Nur77-GFP OT-I T cells. Related to Figure 4**.** One thousand Ly5.1 Nur77-GFP OT-I CD8 T cells were adoptively transferred into congenic Ly5.2 B6 mice and vaccinated a day later with OVA protein formulated with the indicated adjuvants. On the 8th day after vaccination, cells from lymph nodes and lungs were stained with K^{b}/SIINFEKL tetramers, anti-Ly5.1, anti-Ly5.2, anti-CD8 and anti-CD44 antibodies. The median fluorescence intensities (MFI) for GFP in Ly5.1^{+ve} OT-I CD8 T cells were quantified by flow cytometry. FACS plots are gated on total CD8+ T cells. Data are representative of 4 mice/group.

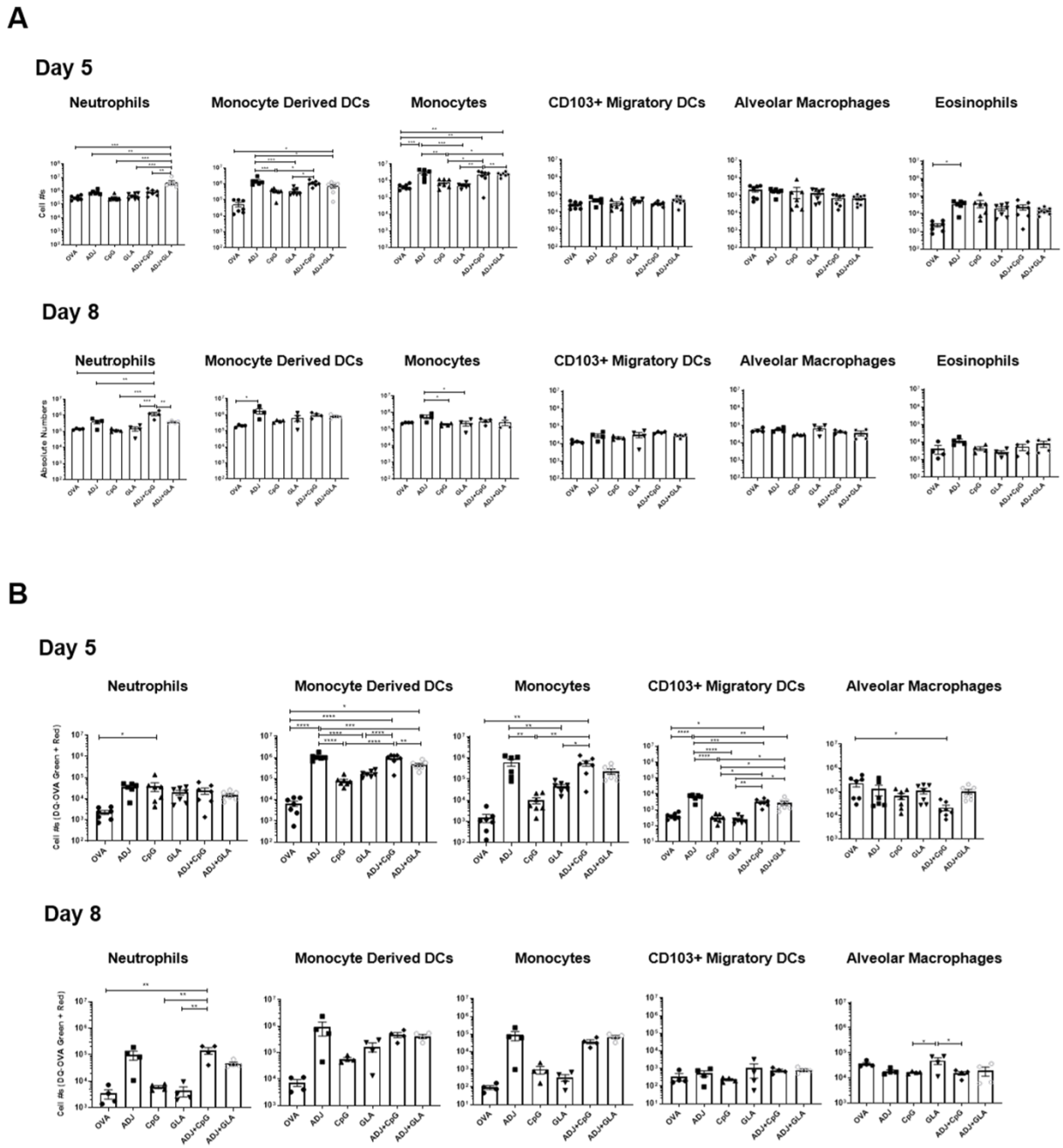

Figure S3. Innate immune cell subsets and antigen-processing cells in lungs of vaccinated mice. Related to Figure 4. Groups of C57BL/6 mice were vaccinated with DQ-OVA protein formulated in various adjuvants. At day 5 and 8 after vaccination, lung cells were stained with anti-CD11b, anti-Siglec-F, anti-CD11c, anti-CD64, anti-Ly6G, anti-Ly6C, anti-CD103, and anti-I-A/I-E. The numbers of neutrophils (Ly-6G^{HI}/Siglec-F^{LO}/CD64^{LO}), alveolar

macrophages Ly6G^{LO}/Siglec-F^{HI}/CD64^{HI}CD103^{LO}), monocytes (Ly6G^{LO}/Siglec-F^{LO}/MHC-II^{Lo}/CD11c^{LO}/CD64^{LO}/CD103^{LO}CD11b^{H}/Ly6C^{HI}), monocyte-derived DCs (Ly6G^{LO}/SiglecF^{LO}MHC-II^{HI}/CD11c^{HI}/CD64^{HI}/CD103^{LO}/CD11b^{HI}/Ly6C$^{LO\text{-}INT}$, CD103^{+ve} migratory DCs (Ly6G^{LO}/Siglec-F^{LO}/CD64^{LO}/MHC-II^{HI}/CD11c^{HI}/CD103^{HI}/CD11b^{LO}) and eosinophils (Ly-6G^{LO}/Siglec-F^{HI}/CD64^{LO}/CD103^{LO}) were enumerated by flow cytometry. Cells that contained processed DQ-OVA were visualized by green and red fluorescence (Green^{+ve}/Red^{+ve}). (A) Numbers of innate immune cell subsets in lungs of vaccinated mice. (B) Numbers of innate immune subsets containing processed DQ-OVA. Data are pooled from two experiments. One-way ANOVA test with Tukey corrected multiple comparisons or Students t test. *, **, and *** indicate significance at P<0.1, 0.01 and 0.001 respectively.

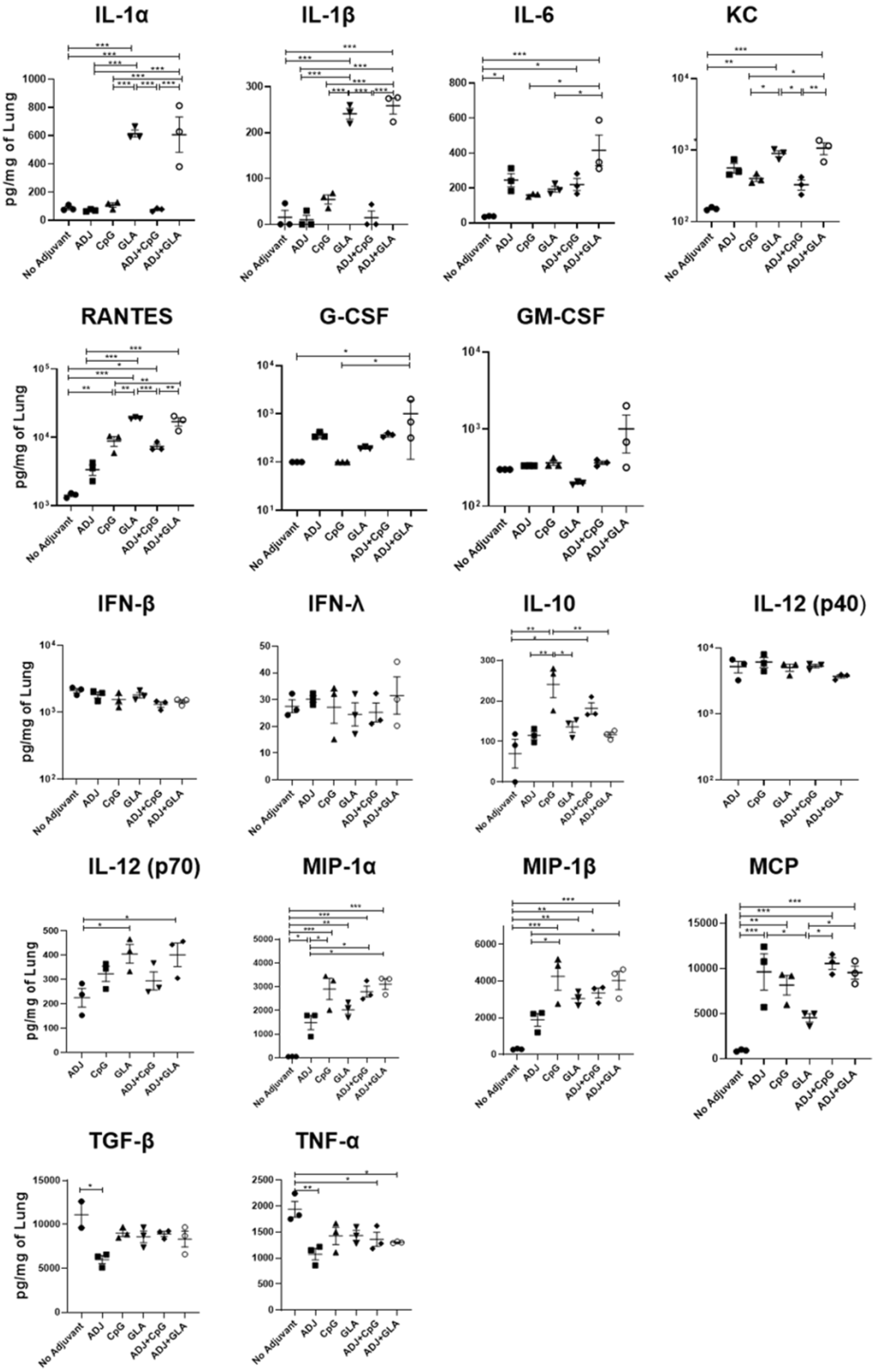
IL-1α
IL-1β
IL-6
KC
RANTES
G-CSF
GM-CSF
IFN-β
IFN-λ
IL-10
IL-12 (p40)
IL-12 (p70)
MIP-1α
MIP-1β
MCP
TGF-β
TNF-α
pg/mg of Lung
No Adjuvant
ADJ
CpG
GLA
ADJ+CpG
ADJ+GLA

Figure S4. Early cytokine production in lungs of mice vaccinated with combination adjuvants. Related to Figures 3 and 4. C57BL/6 mice were vaccinated intranasally with OVA protein formulated with various adjuvants. Cytokine/chemokine levels in the lungs were quantified at 24 hours after vaccination. Data are representative of two independent experiments and analyzed by One-way ANOVA test with Tukey corrected multiple comparisons or Students t test. *, **, and *** indicate significance at $P<0.1$, 0.01 and 0.001 respectively.

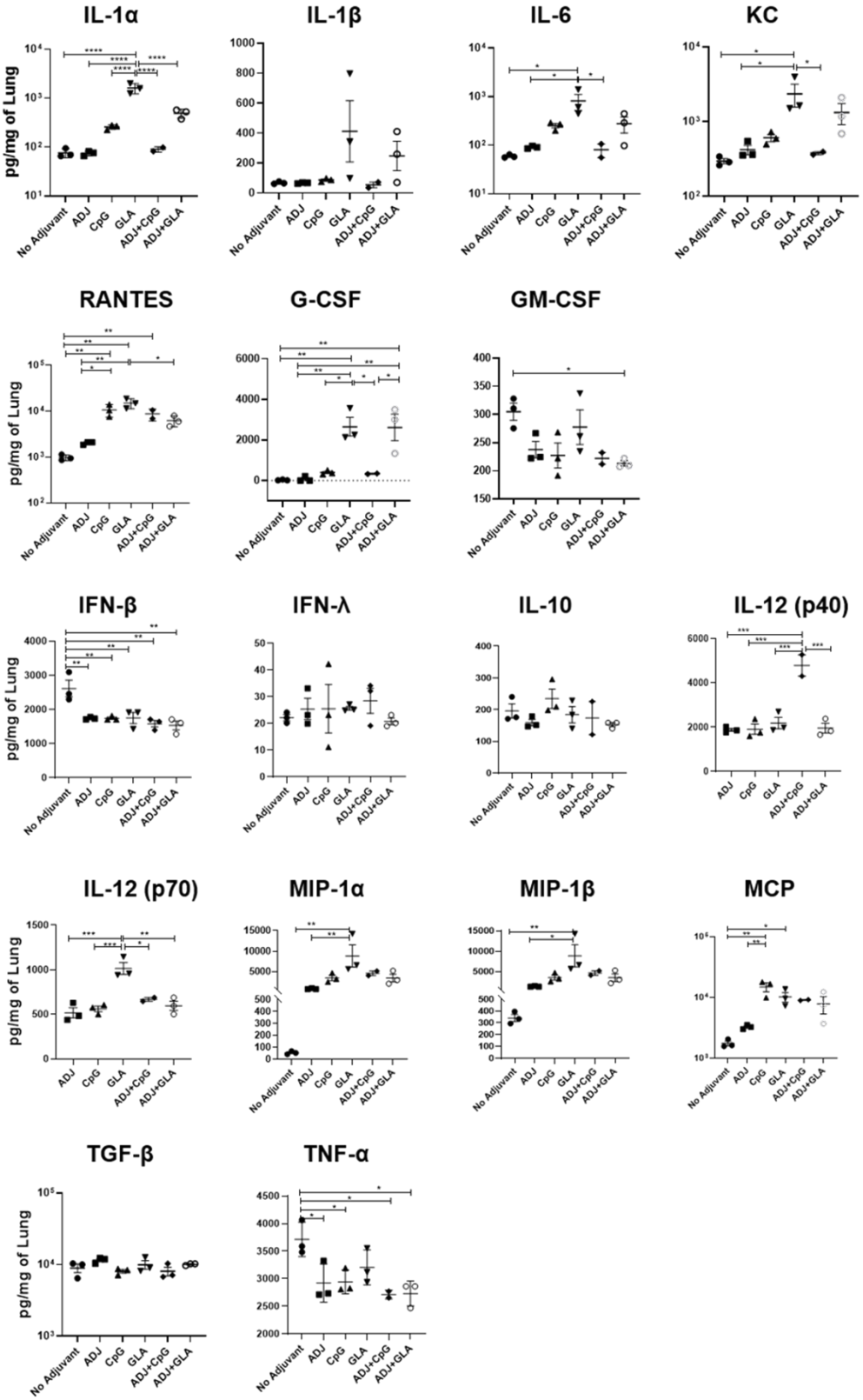
IL-1α
IL-1β
IL-6
KC
RANTES
G-CSF
GM-CSF
IFN-β
IFN-λ
IL-10
IL-12 (p40)
IL-12 (p70)
MIP-1α
MIP-1β
MCP
TGF-β
TNF-α
pg/mg of Lung
No Adjuvant
ADJ
CpG
GLA
ADJ+CpG
ADJ+GLA

Figure S5. Early cytokine production in lungs of mice vaccinated with combination adjuvants. Related to Figures 3 and 4. C57BL/6 mice were vaccinated intranasally with OVA protein formulated with various adjuvants. Cytokine/chemokine levels in the lungs were quantified at 48 hours after vaccination. Data are representative of two independent experiments and analyzed by One-way ANOVA test with Tukey corrected multiple comparisons or Students t test. *, **, and *** indicate significance at $P<0.1$, 0.01 and 0.001 respectively.

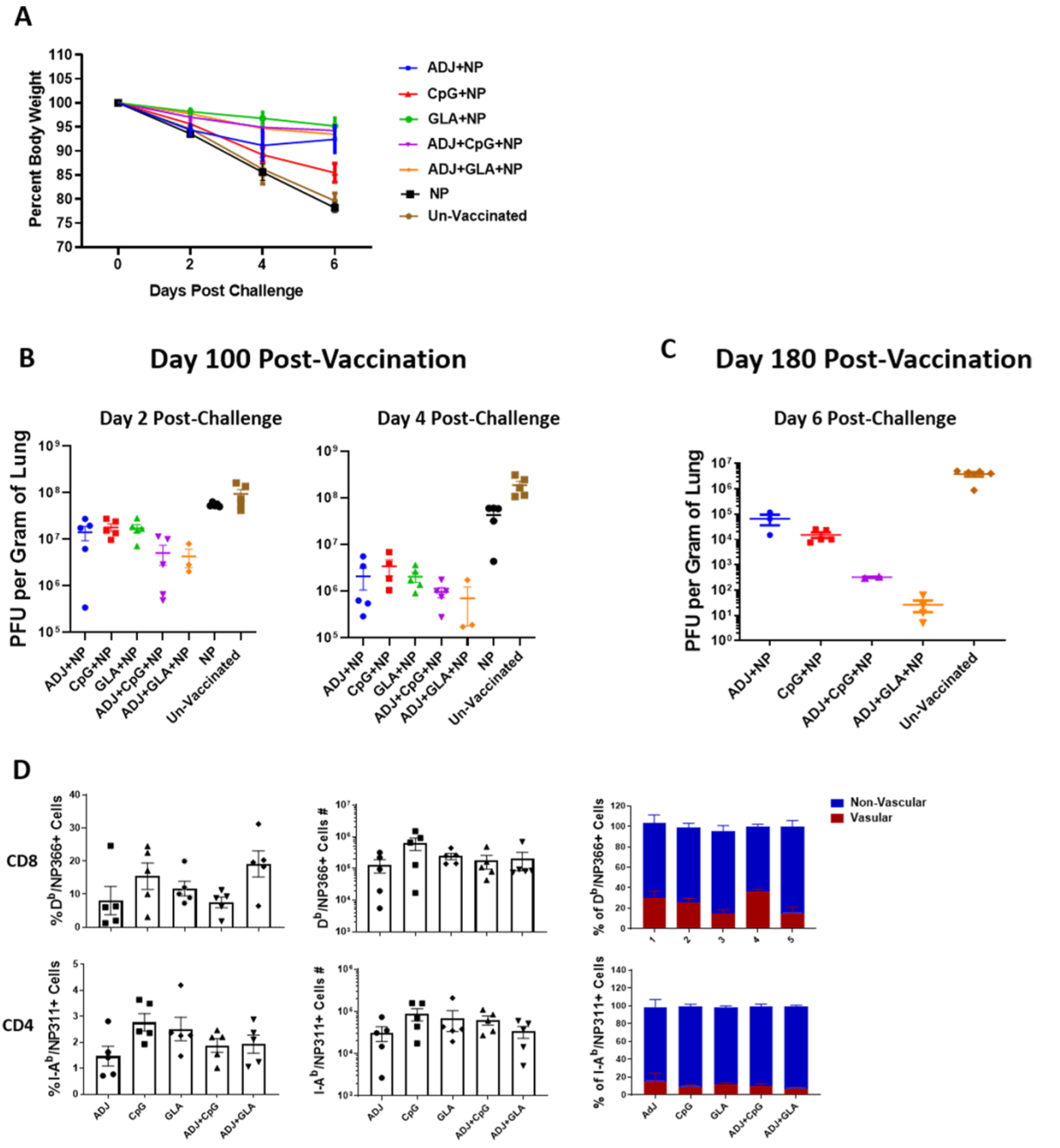

Figure S6. Kinetics and durability of influenza viral control in vaccinated mice. Related to Figure 6. C57BL/6 mice were vaccinated twice (at 3 weeks intervals) intranasally with NP protein formulated with the indicated adjuvants. Unvaccinated mice and mice vaccinated with NP only (without adjuvants) served as controls. At 100 and 180 days after booster vaccination, mice were

challenged intranasally with PR8/H1N1 influenza virus. (A) Body weight loss was assessed by calculating bodyweight at different days after challenge, relative to bodyweight before challenge at 100 days after vaccination. (B) Vaccinated mice were challenged with PR8/H1N1influenza virus at 100 days after vaccination and viral titers in lungs were quantified at day 2 and 4 after challenge, using a plaque assay. (C) At day 180 after booster vaccination, mice were challenged with PR8/H1N1 influenza virus, and viral titers in lungs were assessed at day 6 after challenge. (D) Percentages and numbers of NP366-specific CD8 T cells and NP311-specific CD4 T cells in lungs and percentage of these cells in the vascular and non-vascular compartment at day 6 after challenge (challenged at 100 days after vaccination). Comparisons were made using one-way ANOVA test with Tukey corrected multiple comparisons or two-way ANOVA test with multiple comparisons and Student's T test; $p<0.05$ = *, $p<0.01$ = **, $p<0.001$ = *** were considered significantly different among groups. Viral titers were log transformed prior to analysis. Nonlinear regression analysis was used for percent body weight loss graph. No data or outliers were excluded from analyses.

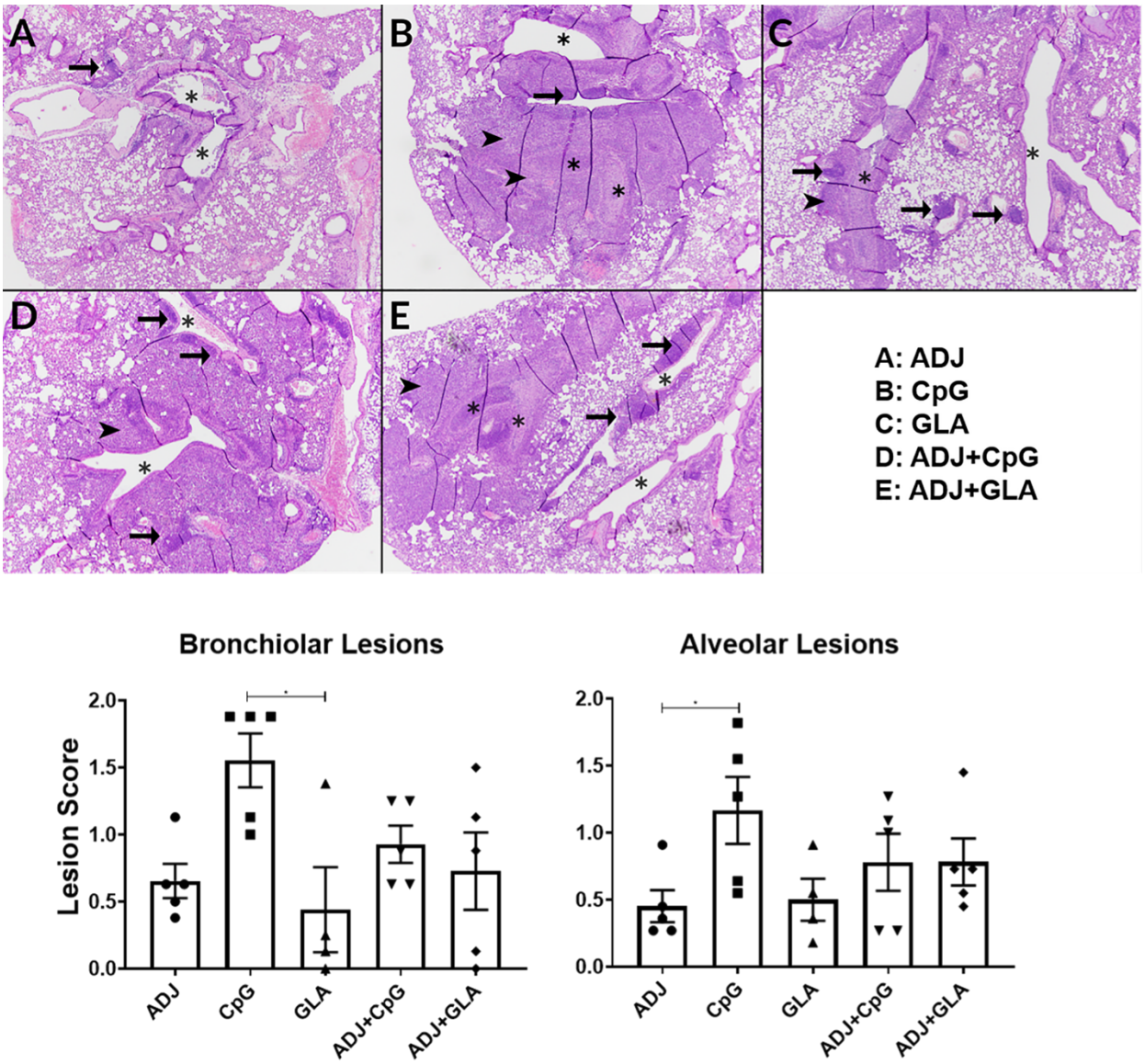

Figure S7. Histopathological analysis of lungs following viral challenge of vaccinated mice. Related to Figure 6. Groups of C57BL/6 mice were vaccinated twice (at 3 weeks interval) with NP protein formulated in various adjuvants. At 100 days after booster vaccination, vaccinated mice were challenged intranasally with H1N1/PR8 strain of influenza A virus. On the 6th day after viral challenge, lungs were collected in neutral-buffered formalin, and tissue sections were stained with Hematoxylin and Eosin (H&E). Lung sections were evaluated by a board-certified pathologist (Dr. Gasper); he was blinded to the identity of sections. In each image (40X magnification), asterisks

indicate similarly sized large bronchioles, arrow heads indicate regions in which bronchial lesions extend in to the adjacent alveoli, and arrows indicate perivascular lymphoid nodules. A. Adjuplex-vaccinated mouse: there is mild necrotizing bronchitis asterisks). B. CpG-vaccinated mouse: there is obliteration of two bronchioles by inflammation that extends far into the surrounding alveoli (arrowheads). C. GLA-vaccinated mouse: there is bronchiolitis affecting 1 of the larger bronchioles, with minimal extension into the adjacent alveoli. D. ADJ+CpG-vaccinated mouse. Bronchiolitis is similar to that in A, but alveolar regions around the affected bronchiole (center) are infiltrated by inflammatory cells. E. ADJ+GLA vaccinated mouse: bronchiolitis is of intermediate severity between B and C, and regionally extends into the adjacent alveolar tissue (arrowhead). Each lung section was scored individually, and lesion scores from 0-3 were assigned for bronchial lesions, alveolar lesions, and specific disease patterns, with 0 = absent, 1 = mild, 2 = moderate, 3 = severe.

Bronchiolar Lesions: Epithelial degeneration/necrosis; Intraepithelial neutrophils; Intraepithelial eosinophils; Intraepithelial lymphocytes; Luminal dislodged epithelial cells/debris; Luminal cellular exudate; Peribronchiolar neutrophils; Pavementing/Subendothelial leukocytes.

Alveolar Lesions: Alveolar wall thickening; Interstitial macrophages; Interstitial lymphocytes; Interstitial granulocytes; Epithelial necrosis; Luminal edema; Luminal hemorrhage; Luminal cellular exudate; Luminal alveolar macrophages; Luminal neutrophils; Luminal sloughed epithelial cells. One-way ANOVA test with Tukey corrected multiple comparisons or Students t test. * significance at $P<0.1$.

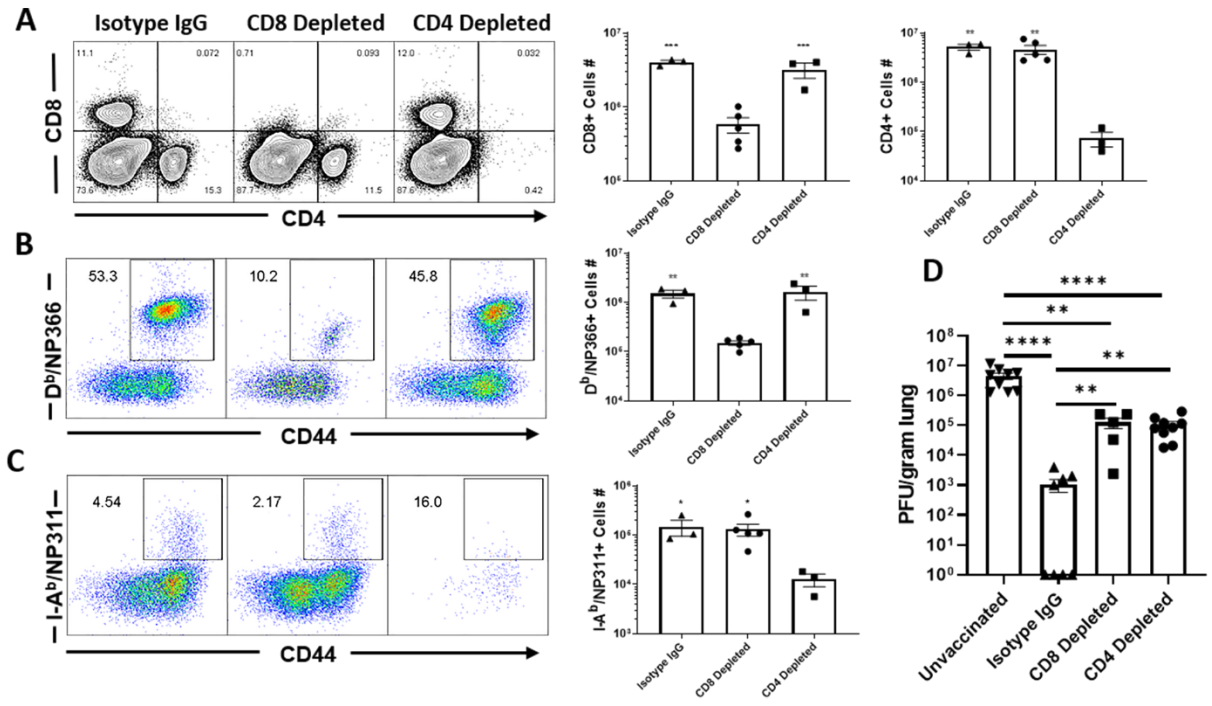

Figure S8. CD4 and CD8 T cells are required for protective immunity to influenza A virus. Related to Figure 7. C57BL/6 mice were vaccinated twice (at 3 weeks interval) with NP protein formulated in ADJ+GLA. At 70 days after booster vaccination, mice were challenged intranasally with H1N1/PR8 strain of influenza A virus; unvaccinated mice were challenged as controls. Cohorts of vaccinated mice were treated (intravenously and intranasally) with isotype control IgG, anti-CD4 or anti-CD8 antibodies at days -5, -3, -1 and 1, 3 and 5, relative to viral challenge. On the 6th day after viral challenge, virus-specific T cells and viral titers were quantified in lungs. (A) FACS plots are gated on live lymphocytes and numbers are percentages among gated cells. (B) FACS plots are gated on CD8 T cells and numbers are percentages of D^{b}/NP366 tetramer-binding CD8 T cells among gated cells. (C) FACS plots are gated on CD4 T cells and numbers are percentages of I-A^{b}/NP311 tetramer-binding CD4 T cells among gated cells. (D) Viral titers in lungs were quantified by a plaque assay. Data are from two independent experiments. One-

way ANOVA test with Tukey corrected multiple comparisons or Students t test. *, **, and *** indicate significance at $P<0.1$, 0.01 and 0.001 respectively.