MATERIALS AND METHODS

Various adjuvants tested for immunization

TLR2/6/9-agonist Pam2-ODN was provided by Dr. Scott Evans and TLR1/2-agonist diprovocim was provided by Dale Boger, Scripps Research Institute. Mice were immunized with 40 μg heat-killed *K. pneumoniae*-43816 or 1 μg OmpX formulated with 10 μg LTA1, 4 μM Pam2+1 μM ODN (TLR2/6/9-agonist) or 10 μg Diprovocim (TLR1/2-agonist). All immunization was performed using the oropharyngeal aspiration-tongue pull technique (referred as intratracheal, i.t.) and boosted with the same vaccine 3 weeks later based on prior work with influenza (*11*).

Antibodies for flow cytometry

Fluorophore conjugated antibodies used for mouse cell analysis include: PE-Cy5 anti-TCRβ Chain (Cat #553173, BD Bioscience), Alexa Fluor 700 anti-CD4 (Cat #100536, Biolegend), APC anti-CD3 (Cat #100236, Biolegend), PerCP anti-CD44 (Cat #103036, Biolegend), PE-Cy7 anti-CD69 (Cat #25-0691-82, eBioscience), eFluor 450 anti-CD19 (Cat #48-0193-82, eBioscience), PE anti-CD45R/B220 (Cat #103207, Biolegend), PE-Cy7 anti-IFNγ (Cat #505825, Biolegend), FITC anti-IL-17A (Cat #506907, Biolegend), APC-eFluor 780 anti-Thy1.1 (Cat #47-0900-82, eBioscience), APC anti-Ly-6G/Ly-6C (Cat #108412, Gr-1) (Biolegend), PerCP anti-CD11b (Cat #101230, Biolegend), PE anti-F4/80 (Cat #123110, Biolegend), PE anti-CD45.1 (Cat #110707, Biolegend), FITC anti-IgG (Cat #406001, Biolegend), FITC anti-IgA (Cat #559354, BD Bioscience), FITC anti-IgG (Cat #406001, Biolegend), FITC anti-rat IgG1 isotype control (Cat #554684, BD

Bioscience), and eFluor 450 anti-CD45.2 (Cat #48-0454-82, eBioscience). LIVE/DEAD™ BacLight™ Bacterial Viability and Counting Kit (Cat #L34856, ThermoFisher) was used for detecting live bacteria. CellTrace™ Violet Cell Proliferation Kit (Cat #34571, ThermoFisher) was used for cell proliferation analysis.

Intravascular labeling using anti-CD45.2 mAb

Three minutes before mice were sacrificed and perfused with 10ml cold PBS via right ventricular of the heart, the mice were injected with anti-CD45.2 mAb intravenously (*14*). The percentage of contaminated blood $CD4^+$ T cells in single cell suspensions from the lung digestion was identified by flow cytometry.

Th17 differentiation and plasticity

Naïve splenic $CD4^+$ T cells were isolated from spleens of IL-17F-Thy1.1 reporter mice with a CD4+CD62L+ T-cell isolation kit (Cat #130-106-643, Miltenyi Biotec). Cells were cultured in RPMI supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in 5% CO2. 2 x 10^5 cells per well were seeded in a 96 well plate, precoated with 2.5 μg/ml anti-CD3 and 2.0 μg/ml anti-CD28 at 4°C overnight, and cultured in the presence of 5 ng/ml TGF-β, 40 ng/ml IL-6, 10 ng/ml IL-23, 5 μg/ml anti-IL-4, and 5 μg/ml anti-IFNγ for 5 days (*22*). Thy1.1+ cells were isolated with a kit (Cat #130-121-273, Miltenyi Biotec) and incubated with IL-12 or vehicle. Some Thy1.1+ cells were collected 30 minutes post incubation with IL-12 to confirm STAT4

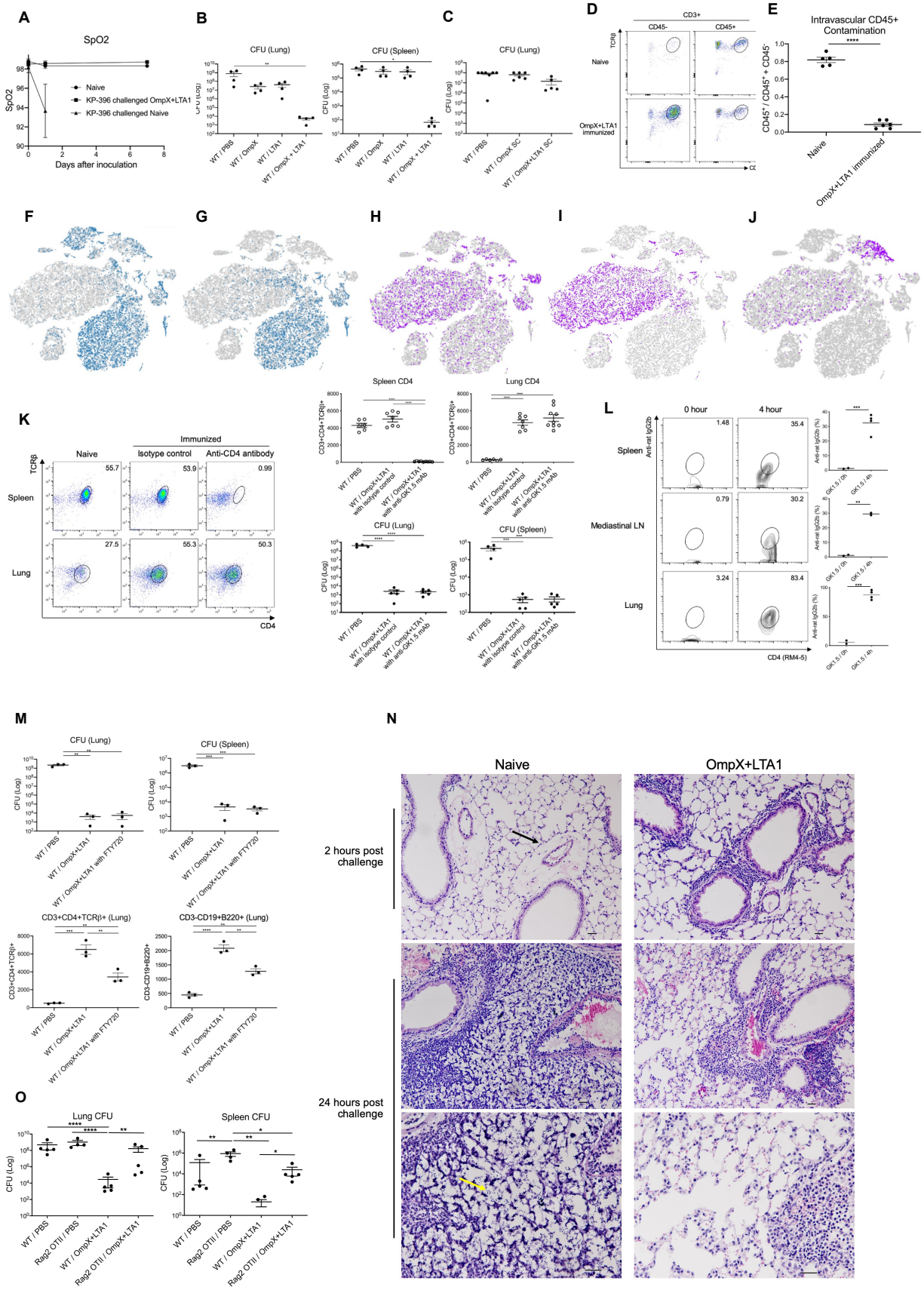
phosphorylation by FACS. After 5 days of incubation, the cells were stained with antibodies specific for intracellular cytokines.

TCRVβ repertoire analysis

Lungs of naive mice or OmpX+LTA1 immunized mice were removed and processed to obtain single cell suspensions. Those cells were stained with anti-Mouse TCR Vβ Screening Panel (BD Bioscience) and $CD4^+$ T cell marker. Frequency of %TCRVβ+ cells in $CD4^+$ T cells was calculated by flow cytometry analysis.

Bulk RNA sequencing

Lung tissue RNA was used to perform RNA sequencing. RNA concentration was determined with a Qubit 3.0 Fluorometer (ThermoFisher Scientific). RNA integrity number (RIN) and were obtained with either the Agilent 2100 Bioanalyzer or the Agilent 4150TapeStation. Illumina TruSeq Stranded mRNA sample prep kit was used for library preparation. The cDNA libraries were pooled at a final concentration 1.8 pM. Cluster generation and 75 bp paired read single-indexed sequencing was performed on Illumina NextSeq 550 Raw reads were processed and mapped, then gene expression and nucleotide variation were evaluated as previously described (47, 48). Raw read counts were normalized across all samples and then used for differential expression analysis using DESeq, EdgeR and Cuffdiff (Slug Genomics, UV Santa Cruz). The EdgeR output was used to generate volcano plots in R. Data was deposited in the Gene Expression Omnibus and the accession number is GSE174849.


A
SpO2
Naive
KP-396 challenged OmpX+LTA1
KP-396 challenged Naive
Days after inoculation
B
CFU (Lung)
CFU (Spleen)
WT / PBS
WT / OmpX
WT / LTA1
WT / OmpX + LTA1
C
CFU (Lung)
WT / OmpX SC
WT / OmpX+LTA1 SC
D
CD3+
CD45-
CD45+
TCRβ
Naive
OmpX+LTA1 immunized
E
Intravascular CD45+ Contamination
CD45+ / CD45+ + CD45-
Naive
OmpX+LTA1 immunized
F
G
H
I
J
K
Immunized
Naive
Isotype control
Anti-CD4 antibody
Spleen
Lung
55.7
53.9
0.99
27.5
55.3
50.3
CD4
Spleen CD4
Lung CD4
CD3+CD4+TCRβ+
WT / OmpX+LTA1 with isotype control
WT / OmpX+LTA1 with anti-GK1.5 mAb
CFU (Lung)
CFU (Spleen)
L
0 hour
4 hour
Spleen
Mediastinal LN
Lung
1.48
35.4
0.79
30.2
3.24
83.4
Anti-rat IgG2b
CD4 (RM4-5)
GK1.5 /0h
GK1.5 /4h
M
CFU (Lung)
CFU (Spleen)
WT / PBS
WT / OmpX+LTA1
WT / OmpX+LTA1 with FTY720
CD3+CD4+TCRβ+ (Lung)
CD3-CD19+B220+ (Lung)
N
Naive
OmpX+LTA1
2 hours post challenge
24 hours post challenge
O
Lung CFU
Spleen CFU
WT / PBS
Rag2 OTII / PBS
WT / OmpX+LTA1
Rag2 OTII / OmpX+LTA1

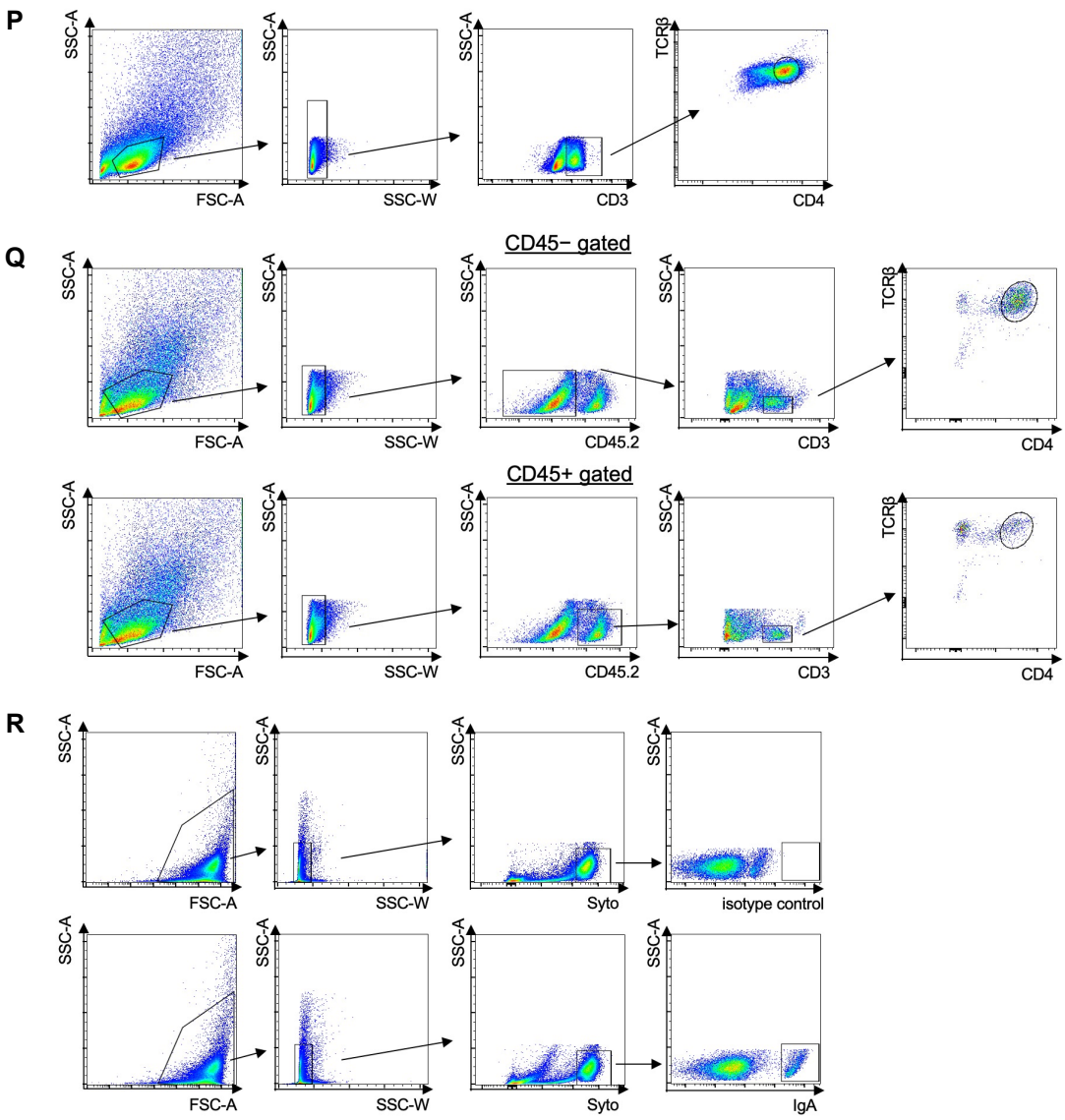


Fig. S1. Subunit immunized lung CD4⁺ T cells are compatible to tissue resident phenotypes. (**A**) Blood oxygen concentration (SpO2) was assessed between naïve mice, KP-396 challenged OmpX+LTA1 vaccinated mice, and KP-396 challenged naïve mice. Any desaturation was not observed in naïve and KP-396 challenged vaccinated mice in contrast to KP-396 challenged naïve mice (*n* = 3-4, representative from 2 independent experiments) (**B**) Mice immunized with unadjuvanted OmpX or LTA1 alone and 10^4 CFU KP-396 challenged, as in Fig.1A, were sacrificed and bacterial burden (CFU) 24 hours post infection in lungs were determined (*n* = 4, representative from 2 independent experiments) (**C**) Similarly, naïve, OmpX only, or OmpX+LTA1 subcutaneously vaccinated 6-10 week old male mice were challenged with 10^4 CFU KP-396 strain. Lung CFU 24 hours post challenge is shown (*n* = 5-7,

representative from 2 independent experiments) (**D**) Lung tissue $CD4^+$ T cells after in vivo CD45.2 labeling were compared between naïve and OmpX+LTA1 immunized mice CD3+CD4+TCRβ+ were gated followed by analysis of the CD45.2 negative or positive population. (**E**) 80% of the cells in perfused lung tissue from naïve mice were comprised of intravascular CD45+ cells, however only approximately 10% from OmpX+LTA1 immunized mice were from the vascular space (*n* = 5-6, representative from 2 independent experiments). Using TSNE plots from single cell RNAseq, some egress markers and lung tissue resident markers were compared between naïve $CD4^+$ T cells and OmpX+LTA1 immunized $CD4^+$ T cells, such as (**F**) *Klf2*, (**G**) *S1pr1*, (**H**) *Cd69*, (**I**) *Cxcr6* and (**J**) *Itgae*. (**K**) Representative dot plots show lung or spleen $CD4^+$ T cells in naïve WT C57BL/6 mice and OmpX+LTA1 immunized mice treated with 300 µg anti-CD4 antibody (GK1.5) or isotype control intraperitoneally 2 days before sacrifice, and the lung and spleen CFU are shown. (*n* = 4-7, representative from 2 independent experiments) (**L**) Secondary antibody (anti-rat IgG2b) depicted the penetration of anti-CD4 antibody (GK1.5) into spleen, mediastinal LN, and lung tissues. (*n* = 4, representative from two separate experiments) (**M**) Lung and spleen bacterial burdens, 24 hours post infection with KP-396 strain in naïve C57BL/6 mice and OmpX+LTA1 immunized mice with or without FTY720 treatment. FTY720 (1 µg/g mouse) was administered intraperitoneally on Day 28-Day31 and infected on Day 32. Also, lung $CD4^+$ T cells and B cells in OmpX+LTA1 immunized mice with or without FTY720 treatment are shown. (*n* = 3, representative from two separate experiments). (**N**) Representative H and E stains in naïve and OmpX+LTA1 immunized lung 2 or 24 hours post KP-396 challenge is shown (scale bar = 100 µm). OmpX immunized mice showed early influx of lymphoid cells aligning the bronchus. In contrast, naïve mice 2 hours post KP-396 challenge showed edema around the blood vessels (black arrow). At 24 hours post challenge, naïve mice showed marked edema and lung inflammation with severe peri-vascular and peri-bronchial edema and recruitment of myeloid cells as well as bacteria in this compartment (yellow arrow) in contrast to OmpX immunized mice. (**O**) Rag2 OT-II mice and WT mice were immunized with the OmpX+LTA1, as is in Fig. 1A, and challenged with 10^4 CFU KP-396. Bacterial burden (CFU) 24 hours post challenge were enumerated (*n* = 4). Data are presented as mean ± SEM. Significant differences are designated by using one-way ANOVA followed by Tukey's multiple comparisons test (**K**, **M**, **O**), Kruskal-Wallis followed by Dunn's multiple comparisons test (**B**, **C**) and unpaired t-test (E, L). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. (**P**) Representative gating strategy for $CD4^+$ T cells detection. (**Q**) Representative gating strategy for intravascular $CD45^+$ or $CD45^-$ $CD4^+$ T cells. (**R**) The gating strategy for IgA bound bacteria is shown.

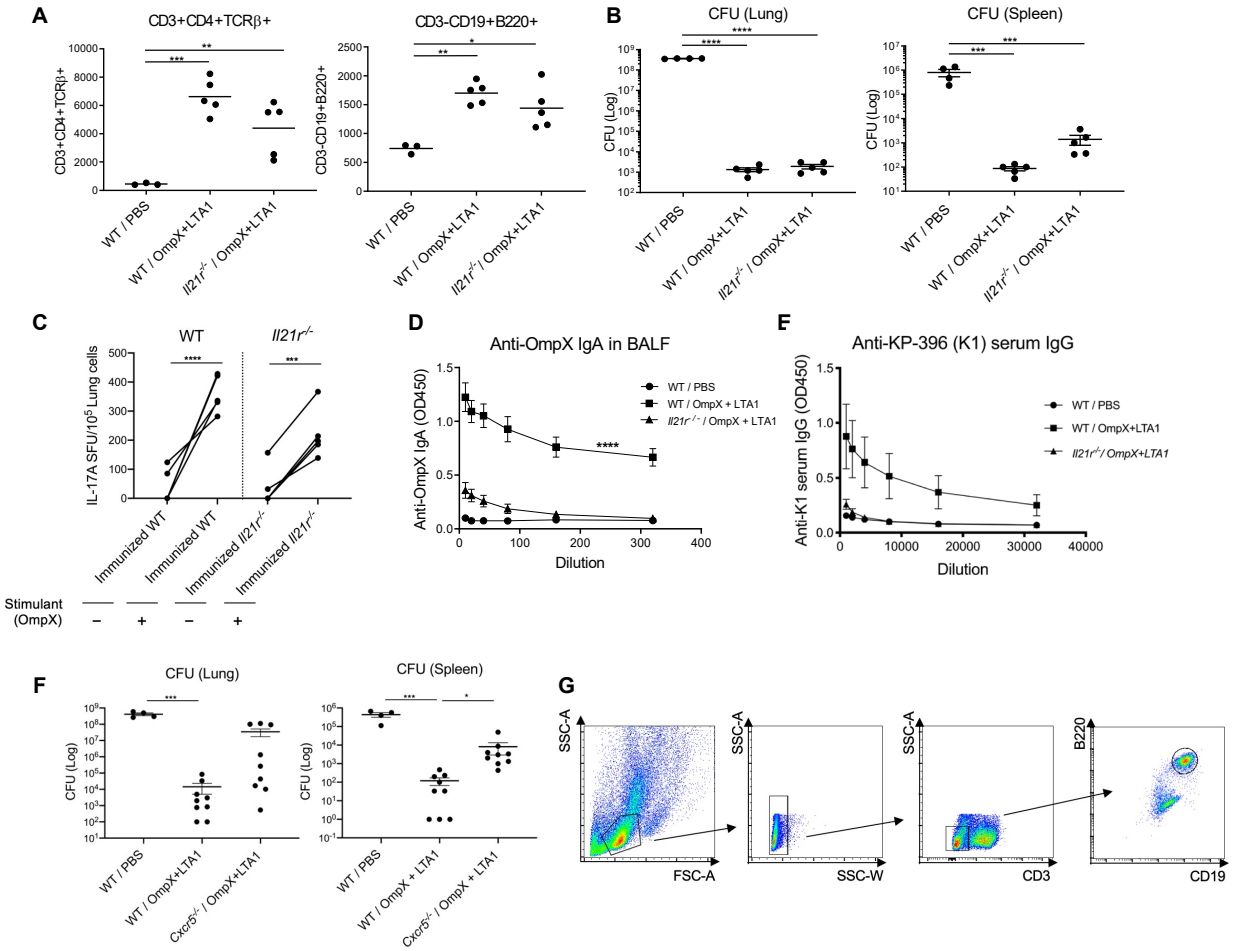


Fig. S2. Vaccine efficacy is independent of IL-21R signaling. (**A**) Total lung CD4$^+$ T cells and B cells in naïve WT C57BL/6 mice and OmpX+LTA1 immunized WT C57BL/6 mice and *Il21r*$^{-/-}$ mice. (**B**) Lung and spleen bacterial burden, 24 hours post infection in naïve WT C57BL/6 mice and OmpX+LTA1 immunized WT C57BL/6 mice and *Il21r*$^{-/-}$ mice are shown (n = 4-5, data shown representative of two separate experiments). (**C**) ELISpot analysis of OmpX-specific IL-17A-producing CD4$^+$ T cells from the immunized lung in WT C57BL/6 mice and *Il21r*$^{-/-}$ mice. Significant difference was designated by paired t-test. (**D**) OmpX specific IgA in BALF and (**E**) KP-396 (K1 strain) specific IgG in serum are compared among naïve WT C57BL/6 mice and OmpX+LTA1 immunized WT C57BL/6 mice and *Il21r*$^{-/-}$ mice. Data are presented as mean ± SEM. (**F**) Naïve, OmpX+LTA1 vaccinated 6-10-week-old male WT or *Cxcr5*$^{-/-}$ mice were challenged with 10^4 CFU KP-396 strain. Lung and spleen CFU 24 hours post challenge were assessed. Representative data are pooled from 2 independent experiment (n = 4-9 per group). (**G**) The gating strategy for B cells detection is shown. Significant differences were designated by one-way ANOVA followed by Tukey's multiple comparisons test (A, B, D, E) and Kruskal-Wallis followed by Dunn's multiple comparisons test (F). *, P < 0.05, ***, P < 0.001.

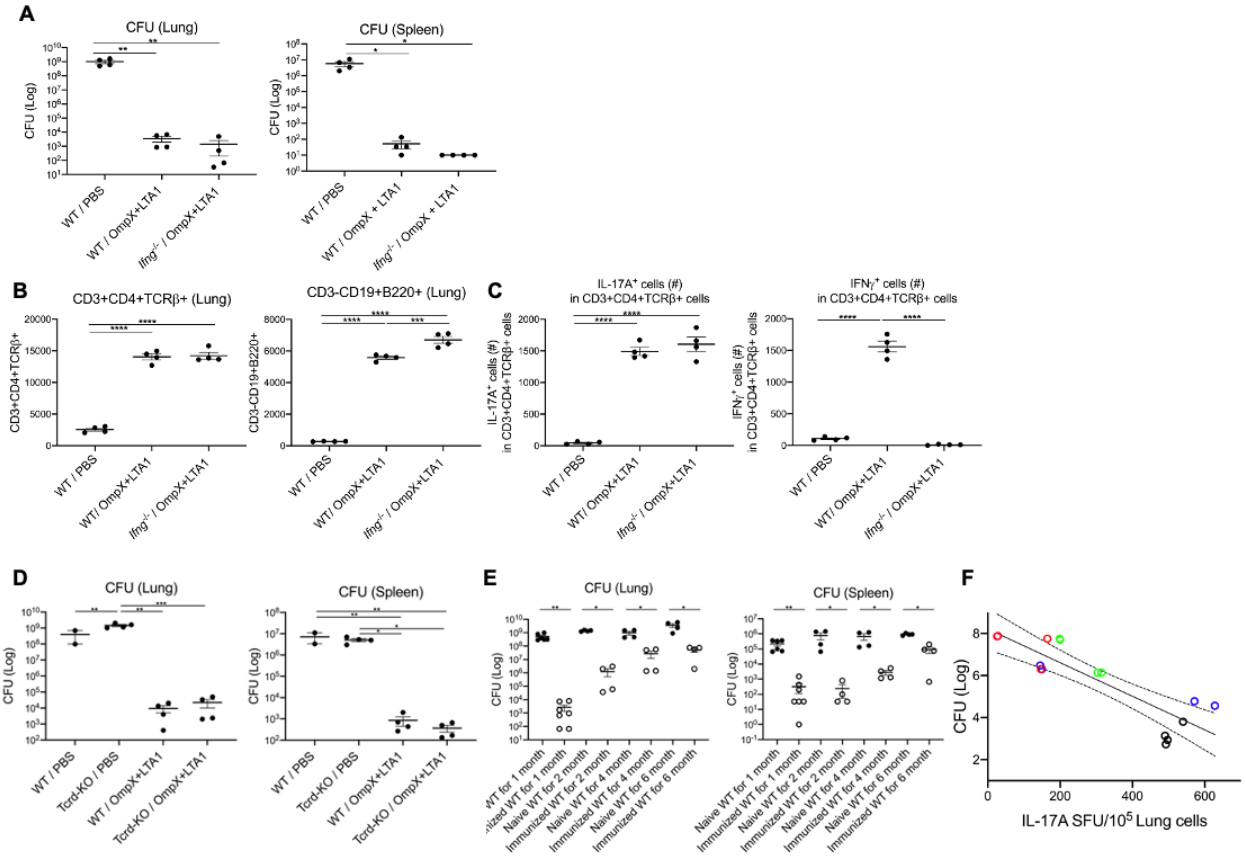


Fig. S3. IFNγ and γδ cells are dispensable for OmpX+LTA1 vaccine efficacy. (**A**) Lung and spleen bacterial burden 24 hours post infection in naïve WT C57BL/6 mice and OmpX+LTA1 immunized WT mice or *Ifng*$^{-/-}$ mice (n = 4, representative data are shown from two separate experiments). (**B**) Representative dot plots of lung CD4$^+$ T cells (left), gated with CD3+, and B cells (right), gated with CD3-, in naïve WT C57BL/6 mice and OmpX+LTA1 immunized WT mice or *Ifng*$^{-/-}$ mice. (**C**) Intracellular cytokine analysis of lung CD4$^+$ T cells for IL-17A and IFNγ production is shown. (**D**) Lung and spleen bacterial burden, 24 hours post infection in naïve WT C57BL/6 mice and *Tcrd*$^{-/-}$ mice with or without OmpX+LTA1 immunization are shown (n = 4, representative data are shown from two separate experiments). (**E**) Lung and spleen CFU 1, 2, 4, or 6 months post last immunization were assessed. Mice were challenged with 10^4 CFU of the KP-396 strain and euthanized 24 hours post challenge. Data are presented as mean ± SEM. Significant differences are designated by using one-way ANOVA followed by Tukey's multiple comparisons test (A, B, C, D) or Mann-Whitney test (E). *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001. (**F**) Scatter plot of IL-17A ELISpot and lung CFU were enumerated (black; 1 month, blue; 2 months, green; 4 months, red; 6 months post last immunization) and shows a significance by Pearson test. R^2 = 0.7413, ****, P < 0.0001.

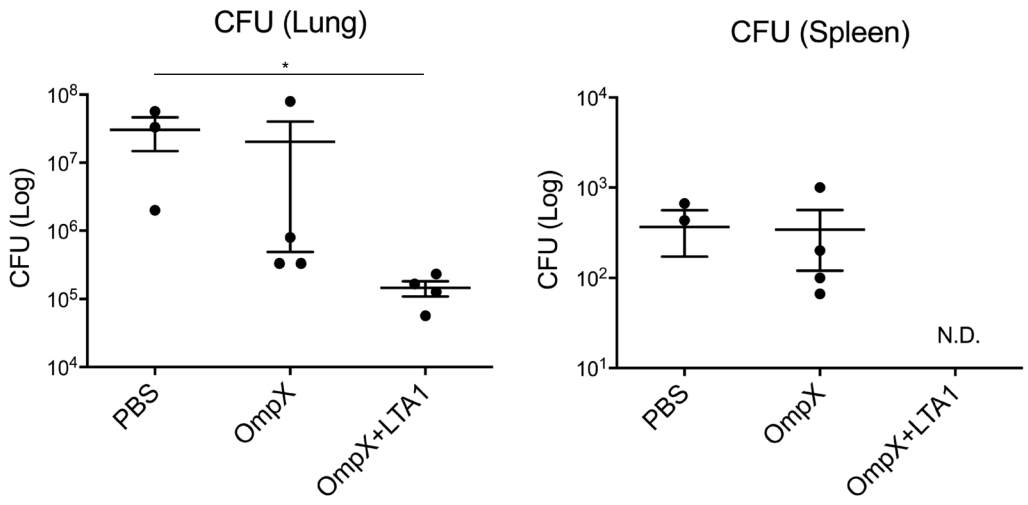


Fig. S4. OmpX+LTA1 immunization potentially ameliorate the Serratia pulmonary infection. Wild type C57BL/6 mice were immunized with OmpX+LTA1, OmpX alone, or PBS following the dosing and immunization schedule as described in Figure 1A (*n* = 3-4, representative data are shown from two separate experiments). Three weeks after the final immunization mice were challenged with 2 x 10^6 CFU *S. marcescens* in 100 µL via oropharyngeal aspiration. At 9 hours post infection, all mice were euthanized and their lungs and spleens were collected and homogenized for bacterial enumeration. When comparing CFUs between immunization groups, mice immunized with OmpX+LTA1 had significantly less lung burdens than the PBS immunized mice. Additionally, there was no detectable bacteria in the spleens of mice vaccinated with OmpX+LTA1, while the other groups had similar bacterial burdens. Data presented as mean ± SEM. N.D.; not detected. Significant differences were calculated using a Kruskal-Wallis test followed by Dunn's multiple comparisons test. *, $P < 0.05$.

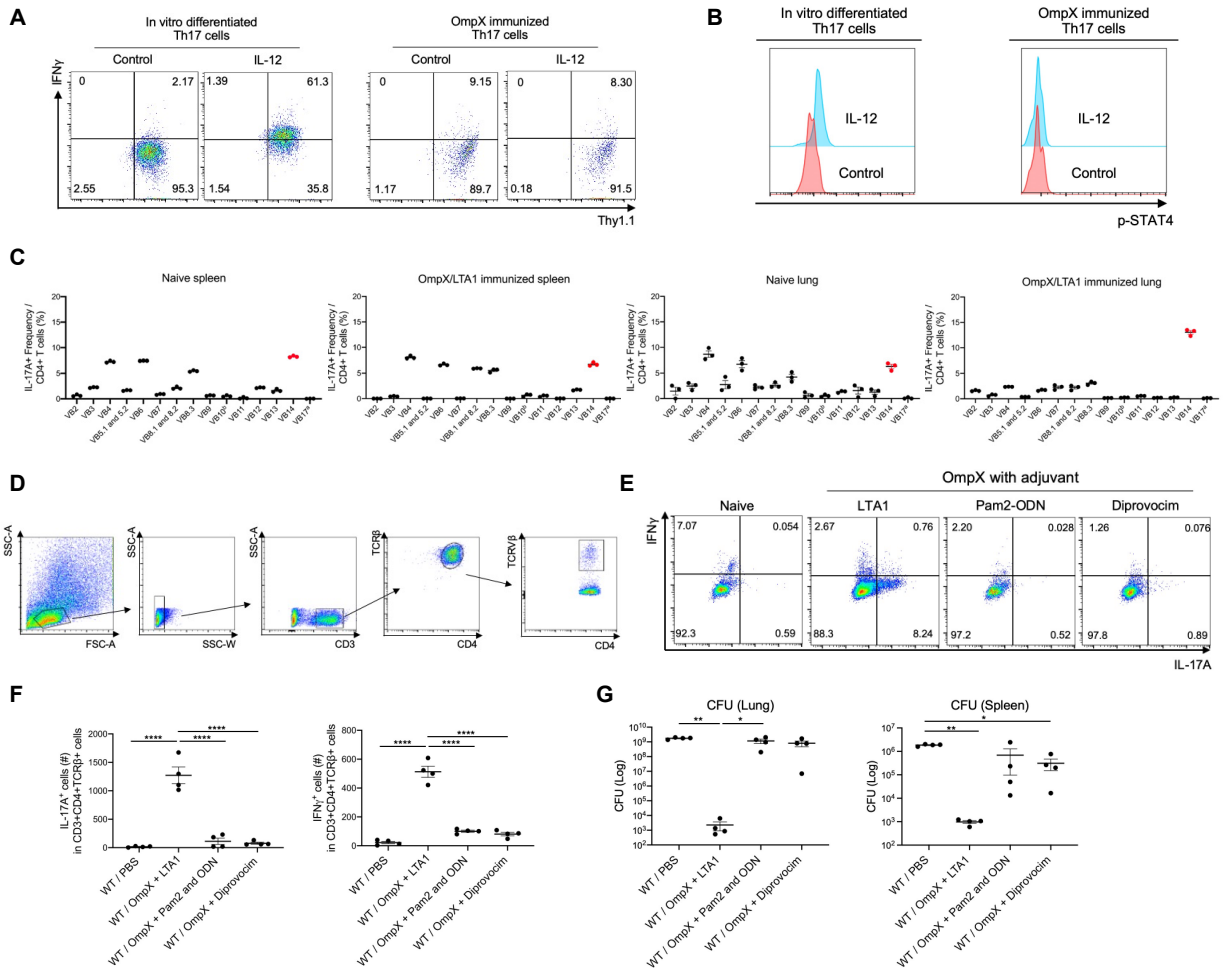


Fig. S5. LTA1 but not TLR2- or TLR9- based adjuvants, prime lung Th17 cells. (**A**) Representative dot plots of Th1 and Th17 cells 5 days post incubation of thy1.1+ cells with IL-12p70, which are gated with CD3+CD4+TCRβ+, with in vitro differentiation compared to OmpX+LTA1 immunization in IL-17F Thy1.1 reporter mice. (**B**) Overlays depicting STAT4 phosphorylation (p-STAT4) 30 min after IL-12 stimulation of in vitro differentiated Th17 cells and OmpX immunized lung Th17 cells from IL-17F Thy1.1 reporter mice. (The representative from 2 independent experiment) (**C**) TCR-Vβ repertoire of naïve spleen or lung $CD4^+$ T cells and OmpX+LTA1 immunized spleen or lung $CD4^+$ T cells. (*n* = 3, representative from 2 independent experiment). (**D**) A gating strategy for TCRVβ detection in $CD4^+$ T cells is shown. (**E**) Representative dot plots showing IL-17A and IFNγ positive lung $CD4^+$ T cells from mice immunized with OmpX plus the different adjuvants. (**F**) Summary of (E) from individual animals after immunization. (**G**) Bacterial burden in lungs of immunized mice 24 hours post challenge with KP-396. Data are presented as mean ± SEM (*n* = 4, representative from 2 independent experiment). Significant differences are designated using one-way ANOVA followed by Tukey's multiple comparisons test. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

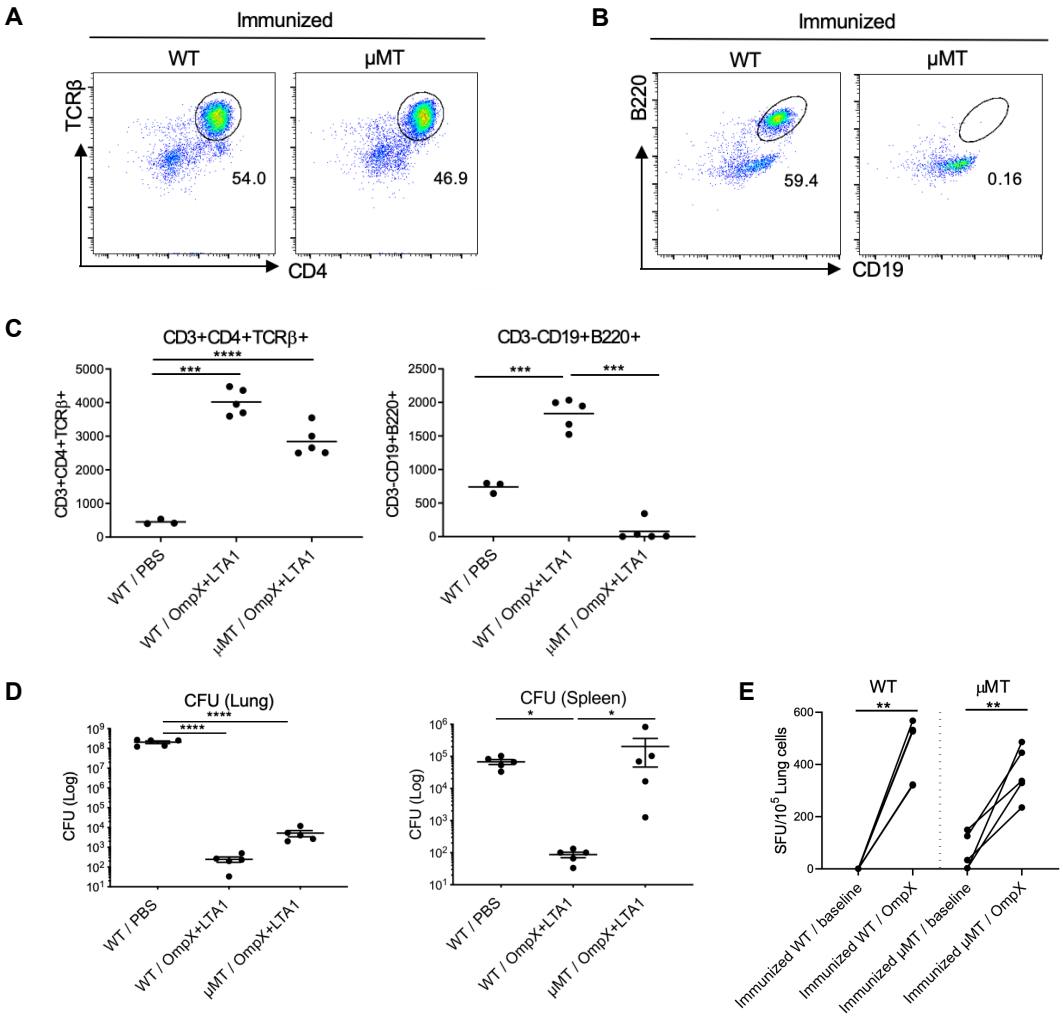


Fig. S6. B cells are dispensable for lung immunity in OmpX+LTA1 immunization but required to prevent systemic bacterial dissemination. Representative dot plots of lung (**A**) $CD4^+$ T cells, gated on CD3+ cells, and (**B**) B cells, gated one CD3- cells, in OmpX+LTA1 immunized WT C57BL/6 mice and μMT mice, and (**C**) the summary of individual mice is shown. Data are presented as mean ± SEM (*n* = 3-5, shown representative of two separate experiments). (**D**) Lung and spleen bacterial burdens 24 hours post infection in naïve WT C57BL/6 mice and OmpX+LTA1 immunized WT C57BL/6 mice and μMT mice. (**E**) IL-17A ELISpot was performed to detect OmpX-specific $CD4^+$ T cells from the immunized lung in WT C57BL/6 mice and μMT mice. SFU; spot forming units. Data are presented as mean ± SEM (*n* = 5, data are representative of two separate experiments). Significant differences are designated using one-way ANOVA followed by Tukey's multiple comparisons test (**C**), Kruskal-Wallis followed by Dunn's multiple comparisons test (**D**) or paired t-test (**E**). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$.

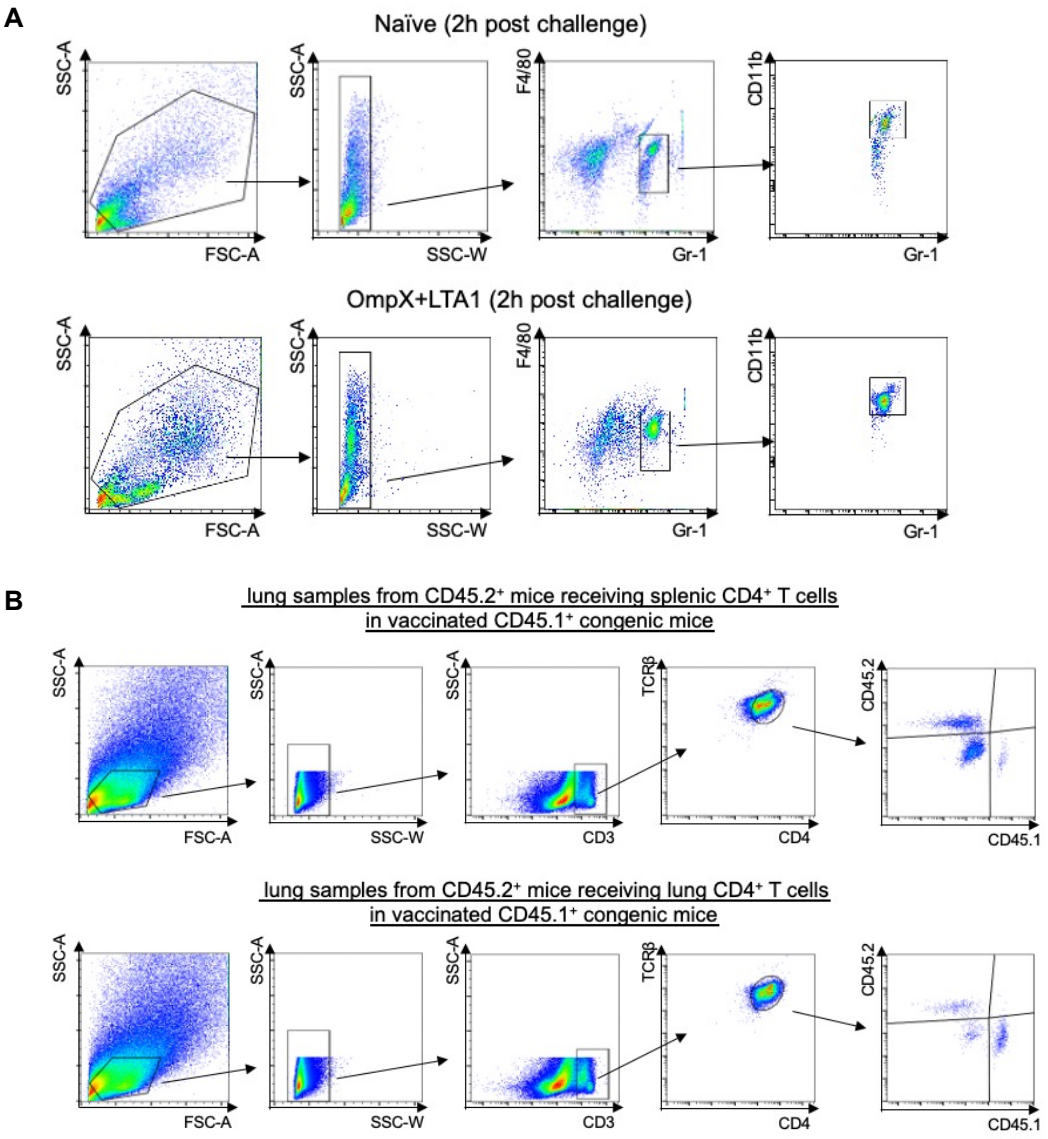


Fig. S7. Representative gating strategies for neutrophils and transferred CD4⁺ T cells from CD45.1 mice.

(**A**) Gating strategies for Gr-1⁺F4/80⁻ cells and Gr-1⁺CD11b⁺ cells in BALF 2 hours post challenge are shown. (**B**) More lung CD4+ T cells in the OmpX+LTA1 immunized CD45.1 mice homed to the lung in a transferred mice.

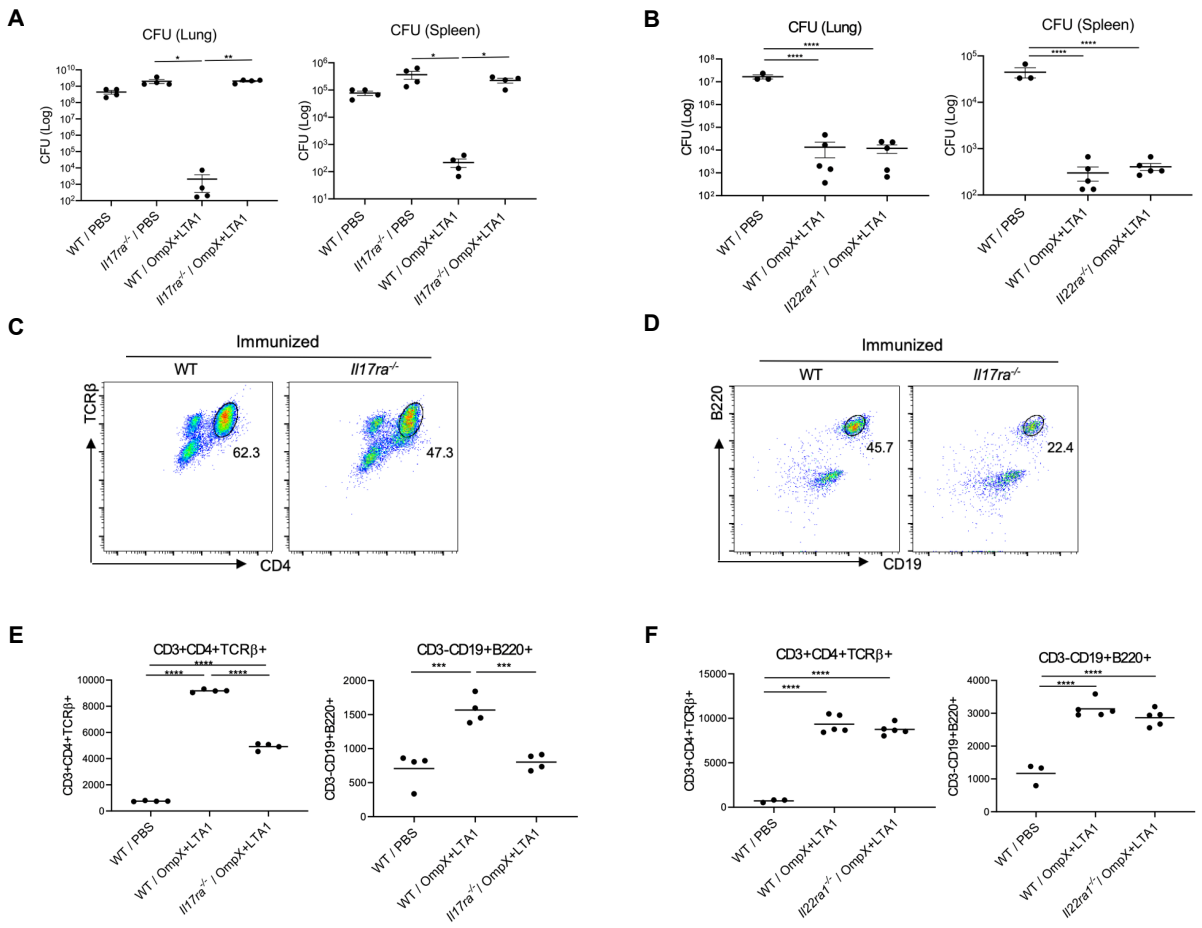


Fig. S8. Efficacy of OmpX+LTA1 immunization requires IL-17R signaling, but not IL-22R signaling. (**A**) Lung and spleen bacterial burdens in naïve C57BL/6, OmpX vaccinated C57BL/6 and *Il17ra*$^{-/-}$ mice were estimated at 24 hours post infection. (**B**) Bacterial burdens in lung and spleen in naïve C57BL/6, OmpX vaccinated C57BL/6 and *Il22ra1*$^{fl/fl}$ *E2a-Cre*$^{+}$ mice *(Il22ra1*$^{-/-}$ mice*)*. Representative dot plots of lung (**C**) CD3^{+}CD4^{+} T cells, and (**D**) CD19^{+} B cells of OmpX+LTA1 vaccinated C57BL/6 and *Il17ra*$^{fl/fl}$ *E2a-Cre*+ mice *(Il17ra*$^{-/-}$ *mice)*, and (**E**) total numbers of CD3^{+}CD4^{+} T cells and B cells in lung. (**F**) Total lung CD3^{+}CD4^{+} T cells and CD19^{+} B cells in the lungs of unchallenged C57BL/6, vaccinated C57BL/6 and *Il22ra1*$^{-/-}$ mice. Data are presented as mean ± SEM (*n* = 3-5, the representative data are from two separate experiments). Significant differences were calculated with Kruskal-Wallis followed by Dunn's multiple comparisons test (**A**) or one-way ANOVA followed by Tukey's multiple comparisons test (**B**, **E**, **F**). **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

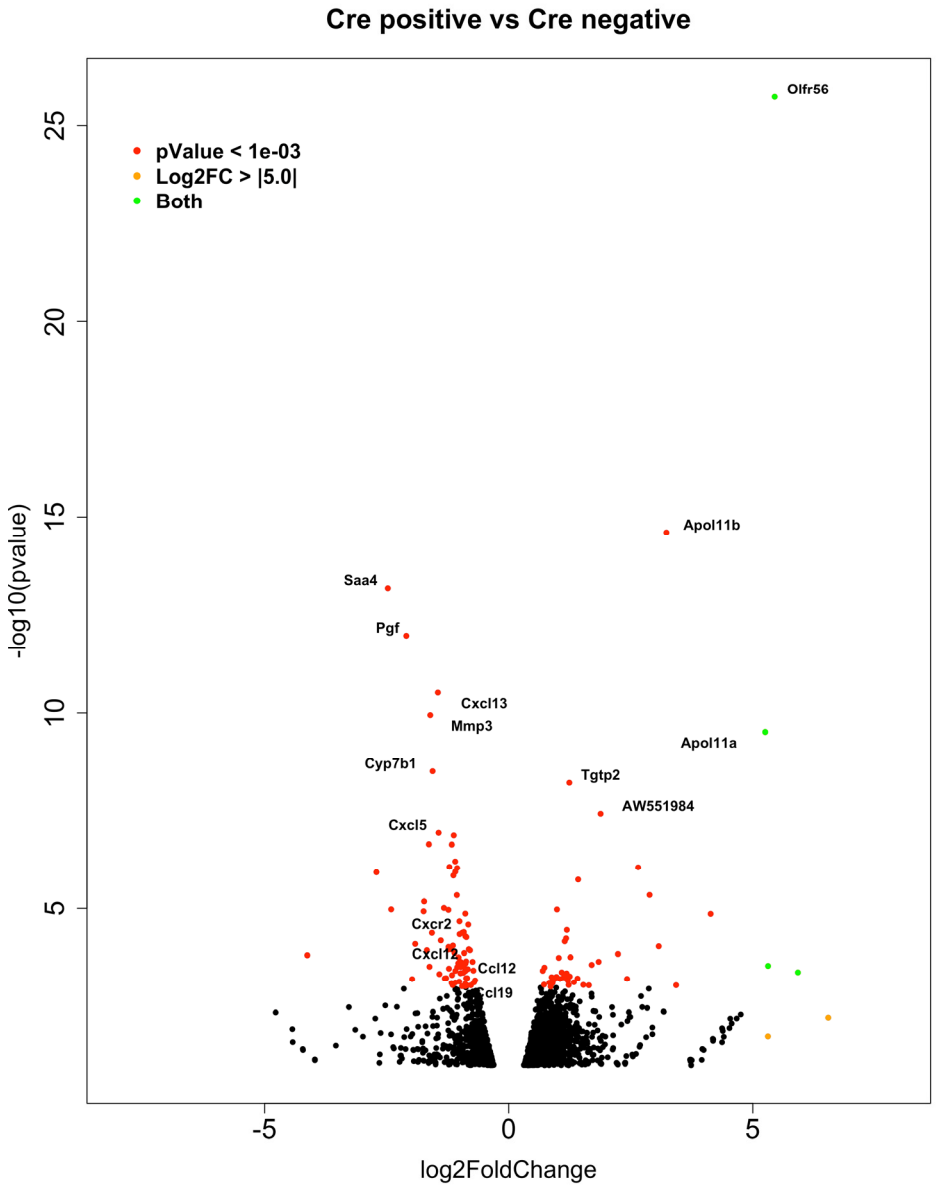


Fig. S9. Differential gene expression associated with OmpX immunization. Volcano plot of differentially expressed genes in lungs between infected OmpX+LTA1 immunized *Il17ra*$^{fl/fl}$ mice versus *Il17ra*$^{fl/fl}$*DermoCre*$^{+}$ mice 4 hours post infection (*n* = 3).