Detailed Methods

Cells and virus

Spodoptera frugiperda SF9 insect cells (ATCC, CRL-1711) were maintained in SF900-II serum free medium (Invitrogen, Carlsbad, CA) and used for production of RSV F and G VLP vaccines [1]. HEp-2 cells obtained from the ATCC were maintained in Dulbecco modified Eagle medium (DMEM) media (GIBCO-BRL). RSV A2 strain was used for the preparation of FI-RSV, live RSV infection and challenge [1]. An expression plasmid encoding human codon bias-optimized RSV A2 F was previously described [2]. RSV A2 stocks were grown and titrated in HEp-2 cells [1]. RSV, grown in HEp-2 cells, was harvested from infected cell cultures, and inactivated with formalin (1:4000 vol/vol) for 3 days at 37^{o}C, and then purified using ultracentrifugation [1,3]. Inactivation was confirmed by an immune-plaque assay [1]. The FI-RSV vaccine adsorbed to aluminium hydroxide adjuvant (alum, 4 mg/ml) was used for immunization.

Antibody ELISA and RSV neutralizing activity

Naïve or immunized sera were collected 6 months after boost immunization (prior to RSV challenge). Diluted sera (100 fold dilution) from immunized mice were added to the plates after blocking. For antibody determination, ELISA plate wells were treated with horseradish peroxidase labeled goat anti-mouse IgG, IgG1 or IgG2a antibodies. Tetramethylbenzidine was used as a substrate and optical density (O.D.) was measured at 450 nm. The data were presented as mean ± standard error (SEM). Heat-inactivated naïve and immunized mice sera were serially diluted and mixed with 1000 PFU of A2-K-line19F virus. After 1 hour culture at 37°C, the sera and virus mixtures were added to confluent monolayers of HEp-2 cells in a separated 96-well

culture plate. After 2 hours infection, the remaining virus was removed and the infected HEp-2 cells were incubated at 37°C, 5% CO_2 for 22 hours. The plates were washed and fluorescent intensity as a result of replication of A2-K-line19F virus was read (588 nm excitation, 635nm emission) by using an Synergy H1 hybrid Reader (BioTek Instruments,Inc, Winooski, VT). For lung RSV titers, at 26 weeks after boost immunization, the mice were intranasally challenged with RSV (1×10^6 PFU/mouse) and lung tissues were collected day 5 post challenge (n=15). RSV titers were measured by using a plaque assay and presented as numbers of plaques per gram lung tissues.

Flow Cytometry analysis

For cell phenotype analysis, the harvested BAL cells from BALF samples (n=5, pooled) were stained with fluorophore-labeled surface markers. Anti-mouse CD16/32 (clone 93, eBioscience) was used as a Fc receptor blocker and then, an antibody cocktail which contained anti-mouse CD45-PerCP (clone 30-F11, BD Pharmigen), CD11b-APC (clone M1/70, eBioscience), CD11c-PE-Cy7 (clone N418, eBioscience), CD3-Pacific Blue (clone 17A2, BioLegend), CD8-APC eFluor780 (clone 53-6.7, eBioscience) and SiglecF-PE (clone E50-2440, BD Pharmigen) was used to treat the cells. For intracellular interferon-gamma (IFN- γ) staining, Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit with BD GolgiStop™ was used as manufacturer's protocol. The stained cells were washed after incubation, acquired by flow cytometer LSR Fortessa (BD Biosciences) and analyzed by FlowJo program (Tree Star Inc.).

For flow cytometry profiles in Fig. 3, upper oval gates were designated as Region 1, which include granulocytes/myeloid cells including alveolar macrophages as a major population and

lower oval gates (Region 2) include lymphocytes as a major population. The dot plots are representative of three independent experiments. BAL cells were analyzed at day 5 post RSV challenge of immunized mice (n=15, BAL cells from 5 mice were pooled). Vaccination and RSV challenge groups are the same as described in the legend of Fig. 2.In figure 4, upper-left area indicates $CD11b^-$ and $CD11c^+$ population in $CD45^+$ granulocyte/myeloid in BAL. Upper-right area indicates $CD11b^+CD11c^+$ population. Lower-right area indicates $CD11b^+CD11c^-$ population. The number in dot plots indicates cell percentages of the population in each area. The dot plots are representative of three independent experiments. In Figure 5, the indicated cells are $CD11b^+SiglecF^+$ cells (eosinophils) and their percentages marked in the dot plots. The dot plots are representative out of three independent experiments (n=5 mice, pooled in each set of experiment).

In vitro cytokine production in bone marrow-derived dendritic cells and splenocytes

Bone marrow (BM) cells were harvested from femur and tibia of naïve Balb/c mice as described [4] and cultured with 10 ng/ml of mouse granulocytes macrophage-colony stimulating factor (mGM-CSF) to enrich dendritic cells (BMDCs). After 6 to 10 days culture, the enriched BMDCs were collected and used for experiments. Splenocytes were prepared as described [5] and used for experiments. The generated BMDCs were seeded at the concentration of 5×10^4 cells/well in 96-well cell culture plates and treated with 10 µg/ml of FI-RSV, F VLP, G VLP and live RSV. 2×10^5 cells/well of the harvested splenocytes in 96-well cell culture plates were treated with 10 µg/ml of FI-RSV, F VLP and G VLP. After 2 days culture, the cell culture supernatants were harvested and cytokine production were measured. BMDC and splenocyte culture supernatants

were used to determine cytokine levels by mouse interleukin (IL)-12 p70, IL-6, tumor necrosis factor (TNF)-alpha, and IFN-γ ELISA Ready-Set-Go® kits (eBioscience).

Pulmonary histopathology

For histological analysis of lung tissues, the lung tissues were collected after fixation via infusion through the trachea with 4% formalin at day 5 post RSV challenge. Collected lung tissues were immersed in 4% formalin for 24 hours, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) [6,7]. Images were acquired under a microscope (Zeiss Axiovert 100, Carl Zeiss) at a magnification of ×100, using an attached Canon 30D digital camera.

Statistical Analysis

Statistical analysis was performed by One-way ANOVA and Tukey's multiple comparison tests or student t test.

Supplementary Figure Legends

Supplementary Figure S1. RSV-specific serum IgG isotype antibodies after individual vaccine components immunization.

Serum samples were collected 3 weeks after prime and boost immunization from mice (n=15) that were immunized with F DNA+F VLP+G VLP RSV vaccine cocktail (50, 10, 10 µg for prime, 25, 5, 5 µg for boost, respectively), F VLP+G VLP (10, 10 µg for prime and boost, respectively) or F DNA (50 µg for prime and boost) at weeks 0 (prime) and 4 (boost). RSV-specific IgG1 and IgG2a levels were measured by ELISA. (A) RSV F protein specific IgG isotypes. (B) RSV G protein specific IgG isotypes. The results were representative of three independent experiments and presented by concentration (µg/ml) as mean± SEM (standard error of mean). Statistical analysis was by One-way ANOVA and Tukey's multiple comparison test. *** indicates $p<0.001$, between prime and boost immunized sera. $^{\#\#}$ and $^{\#\#\#}$ mean $p<0.01$ and 0.001, respectively, compared to the F DNA groups.

Supplementary Figure S2. Cytokine production from bone marrow-derived dendritic cells and splenocytes after stimulation with RSV vaccines.

Bone marrow-derive dendritic cells (BMDCs) and splenocytes were treated with FI-RSV, F VLP, G VLP, or live RSV. After 2 days culture, cell culture supernatants were collected and used to determine IL-6 (A), TNF-alpha (B) and IL-12p70 (C) production in BMDC and IFN-γ production in splenocytes (D). The concentration of the cytokines were presented as mean ± SEM and the data were representative of three independent experiments. *, ** and *** bars with

comparing groups indicates $p<0.05$, 0.01 and 0.001 by one-way ANOVA and Tukey's multiple comparison test.

Supplementary Figure S3. Comparison of pulmonary histopathology after RSV challenge. (A) Representative pulmonary histopathology of lung tissue sections is shown in each group of mice at day 5 post RSV challenge. Representative images of peribronchiolar regions after hematoxylin and eosin (H&E) staining were acquired at a magnification of ×100. (B) Inflammation scores of airways. (C) Inflammation scores of interstitial spaces. *, ** and *** indicates $p<0.05$, 0.01 and 0.001 by one-way ANOVA and Tukey's multiple comparison test. Naïve+R; naïve mice challenged with RSV. FI-RSV+R; FI-RSV group challenged with RSV. FdFG-VLP+R; FdFG VLP group challenged with RSV. Live RSV+R; Live RSV group challenged with RSV.

Supplementary Figure S4. Cytokine profiles in bronchoalveolar lavage fluid of RSV challenged mice.

Bronchoalveolar lavage fluid (BALF) was collected from the immunized mice day 5 post RSV challenge and used to determine IL-4 (A), IL-5 (B), IL-13 (C), and IFN-γ (D). The concentration of the cytokines were presented as mean ± SEM and the data were representative of three independent experiments. * indicates $p<0.05$ compared to all other groups and # indicates $p<0.05$ compared to FI-RSV+R group by one-way ANOVA and Tukey's multiple comparison test.

References for supplementary text

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Figure S1

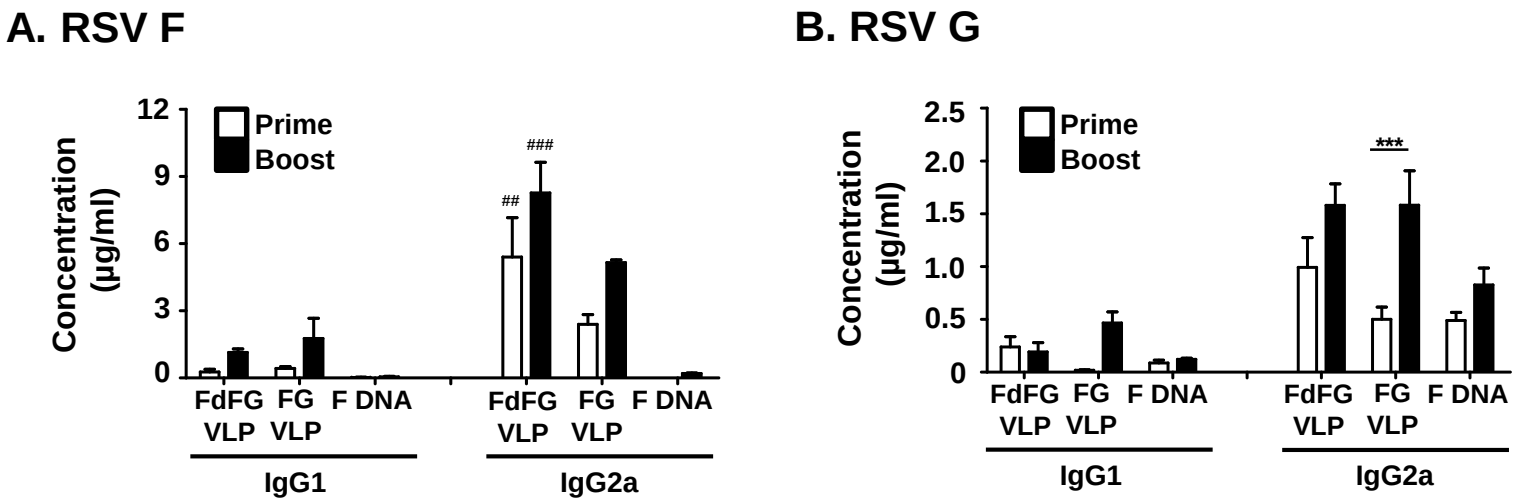

A. RSV F
Concentration (µg/ml)
12
9
6
3
0
Prime
Boost
##
###
FdFG VLP
FG VLP
F DNA
FdFG VLP
FG VLP
F DNA
IgG1
IgG2a
B. RSV G
Concentration (µg/ml)
2.5
2.0
1.5
1.0
0.5
0
Prime
Boost
***
FdFG VLP
FG VLP
F DNA
FdFG VLP
FG VLP
F DNA
IgG1
IgG2a

Figure S2

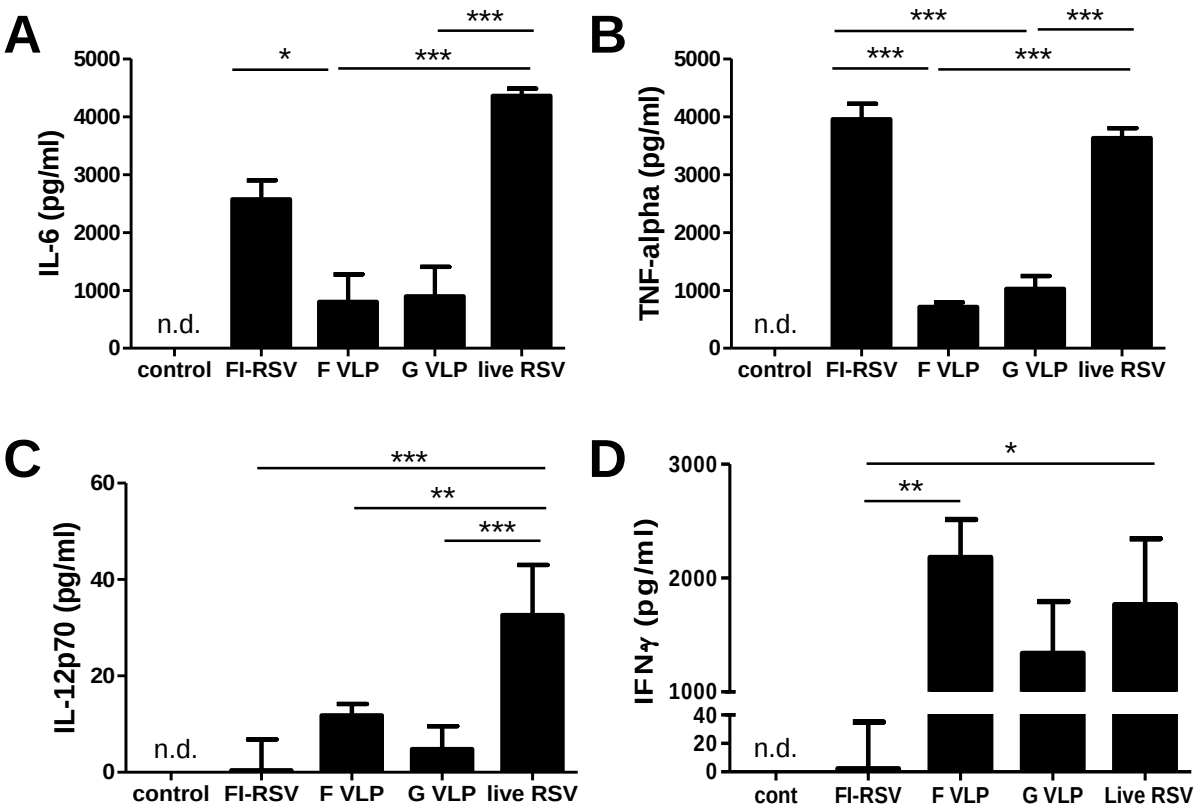

A
IL-6 (pg/ml)
5000
4000
3000
2000
1000
0
n.d.
*
***
***
control
FI-RSV
F VLP
G VLP
live RSV
B
TNF-alpha (pg/ml)
5000
4000
3000
2000
1000
0
n.d.
***
***
***
control
FI-RSV
F VLP
G VLP
live RSV
C
IL-12p70 (pg/ml)
60
40
20
0
n.d.
***
**
***
control
FI-RSV
F VLP
G VLP
live RSV
D
IFNγ (pg/ml)
3000
2000
1000
40
20
0
n.d.
**
*
cont
FI-RSV
F VLP
G VLP
Live RSV

Figure S3

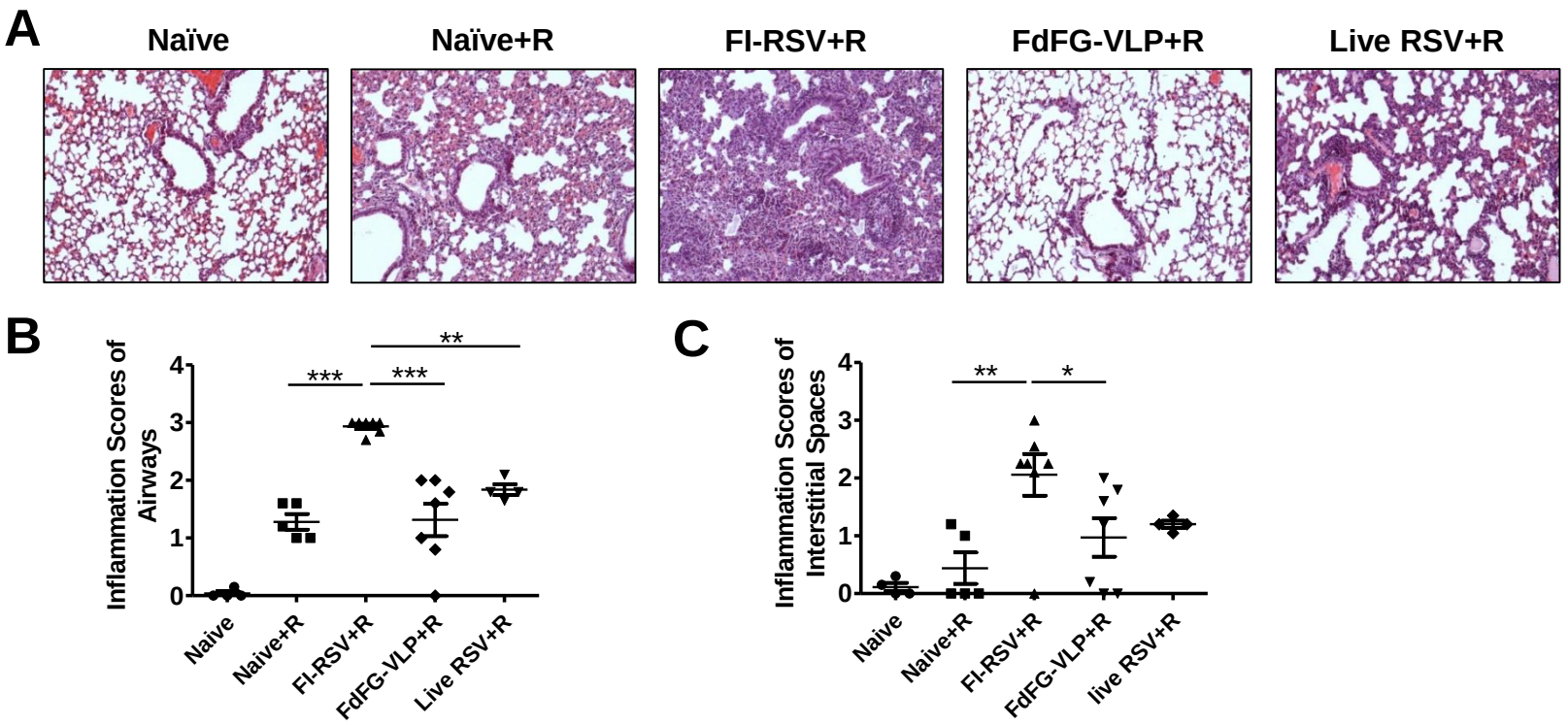
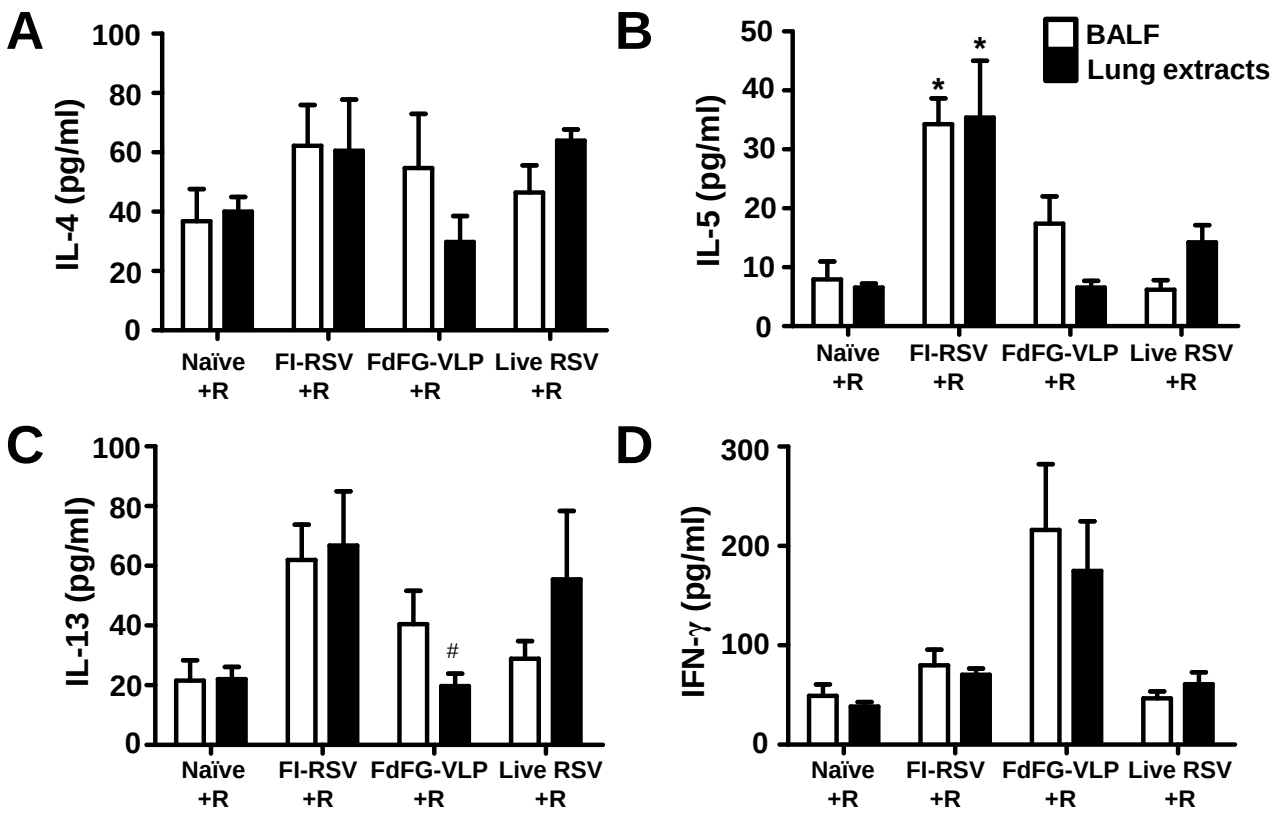

A
Naïve
Naïve+R
FI-RSV+R
FdFG-VLP+R
Live RSV+R
B
Inflammation Scores of Airways
0
1
2
3
4
***
***
**
Naïve
Naïve+R
FI-RSV+R
FdFG-VLP+R
Live RSV+R
C
Inflammation Scores of Interstitial Spaces
0
1
2
3
4
**
*
Naïve
Naïve+R
FI-RSV+R
FdFG-VLP+R
live RSV+R

Figure S4


A
IL-4 (pg/ml)
100
80
60
40
20
0
Naïve +R
FI-RSV +R
FdFG-VLP +R
Live RSV +R
B
IL-5 (pg/ml)
50
40
30
20
10
0
*
*
BALF
Lung extracts
Naïve +R
FI-RSV +R
FdFG-VLP +R
Live RSV +R
C
IL-13 (pg/ml)
100
80
60
40
20
0
#
Naïve +R
FI-RSV +R
FdFG-VLP +R
Live RSV +R
D
IFN-γ (pg/ml)
300
200
100
0
Naïve +R
FI-RSV +R
FdFG-VLP +R
Live RSV +R

Supplementary Table 1

(x10^3 cells/mouse)

	Naïve	Naïve+R	FI-RSV+R	FdFG-VLP+R	Live RSV+R
Leukocytes ($CD45^+$)	117.6±49.1	652.4±225.7	1938.6±296.1***	656.3±94.1##	1609.9±256.8**
Region 1 gated cells	95.0±37.3	298.2±73.0	1126.0±283.3**	323.0±76.0#	582.7±39.8
$CD11b^+CD11c^+$	0.3±0.1	12.5±10.2	60.0±35.4	32.6±9.3	53.1±24.9
$CD11b^-CD11c^+$	87.7±36.7	180.8±120.6	123.5±15.3	164.2±61.1	212.4±98.9
$CD11b^+CD11c^-$	2.1±1.3	29.8±14.4	633.4±219.3**	82.0±11.8#	155.8±52.6#
Eosinophils ($CD11b^+CD11c^-SiglecF^+$)	1.0±0.4	9.1±7.0	590.4±309.0	7.2±3.6	17.1±1.4
Region 2 gated cells	4.8±4.1	259.4±205.8	562.7±112.8	212.5±42.8	698.9±261.4
$CD4^+$	3.5±2.8	183.1±150.2	447.4±82.4	148.4±45.5	493.1±194.5
$CD8^+$	1.2±1.2	76.0±55.8	115.0±30.7	63.7±8.2	204.8±68.2*

The region 1 and 2 gates are indicted in the Fig. 3. Each indicated phenotypic BAL cell populations were calculated with the percentages of each cell population according to flow cytometry analysis and total cell counts by trypan-blue dye staining. The data are expressed as mean ± SEM out of three independent experiments. BAL cells were analyzed at day 5 post RSV challenge (1 x 10^6 PFU/mouse) of immunized mice (n=5). Naïve; unimmunized mice. Naïve+R; naïve mice infected with RSV. FI-RSV+R; FI-RSV group challenged with RSV. FdFG-VLP+R; FdFG VLP group challenged with RSV. Live RSV+R; Live RSV group infected with RSV. *, ** and ***; $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared to the naïve group. # and ##; $p<0.05$ and $p<0.01$ compared to the FI-RSV+R group by One-way ANOVA and Tukey's multiple comparison test.