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Natural Killer and CD8 T Cells Contribute to Protection by Formalin Inactivated Respiratory Syncytial Virus Vaccination under a CD4-Deficient Condition

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ABSTRACT

Respiratory syncytial virus (RSV) causes severe pulmonary disease in infants, young children, and the elderly. Formalin inactivated RSV (FI-RSV) vaccine trials failed due to vaccine enhanced respiratory disease, but the underlying immune mechanisms remain not fully understood. In this study, we have used wild type C57BL/6 and CD4 knockout (CD4KO) mouse models to better understand the roles of the CD4 T cells and cellular mechanisms responsible for enhanced respiratory disease after FI-RSV vaccination and RSV infection. Less eosinophil infiltration and lower pro-inflammatory cytokine production were observed in FI-RSV vaccinated CD4KO mice after RSV infection compared to FI-RSV vaccinated C57BL/6 mice. NK cells and cytokine-producing CD8 T cells were recruited at high levels in the airways of CD4KO mice, correlating with reduced respiratory disease. Depletion studies provided evidence that virus control was primarily mediated by NK cells whereas CD8 T cells contributed to IFN-γ production and less eosinophilic lung inflammation. This study demonstrated the differential roles of effector CD4 and CD8 T cells as well as NK cells, in networking with other inflammatory infiltrates in RSV disease in immune competent and CD4-deficient condition.

Keywords: Respiratory syncytial virus; FI-RSV; Vaccine enhanced disease; CD8-positive T-lymphocytes; NK cell

INTRODUCTION

Human respiratory syncytial virus (RSV) is an enveloped RNA virus and causes severe respiratory disease leading to bronchiolitis, pneumonia, and mortality in infants and young children less than 5 years of age. As the name of the virus refers, RSV induces formation of cell fusion syncytia in respiratory epithelium. In 1960s, an attempt to develop prophylactic RSV vaccine trials failed due to enhanced respiratory disease. The children who received alum adjuvanted formalin-inactivated RSV (FI-RSV) resulted in 80% of hospitalizations and

Abbreviations

BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluids; CD4KO, CD4 knockout; cDC, conventional dendritic cell; DC, dendritic cell; FI-RSV, formalin inactivated respiratory syncytial virus; GSU, Georgia State University; H&CR, hematoxylin and Congo red; pDC, plasmacytoid dendritic cell; PenH, enhanced pause; RSV, respiratory syncytial virus; WT, wild type.

Author Contributions

Conceptualization: Ko EJ, Kang SM; Data curation: Ko EJ, Kang SM; Formal analysis: Ko EJ, Kang SM; Funding acquisition: Ko EJ, Kang SM; Investigation: Ko EJ, Lee Y, Lee YT, Hwang HS, Park Y, Kim KH; Methodology: Lee Y, Lee YT, Hwang HS, Park Y, Kim KH; Project administration: Kang SM; Resources: Kang SM; Supervision: Kang SM; Validation: Ko EJ, Kang SM; Visualization: Ko EJ, Kang SM; Writing - original draft: Ko EJ, Kang SM; Writing - review & editing: Ko EJ, Lee Y, Lee YT, Hwang HS, Park Y, Kim KH, Kang SM.

2 deaths during RSV epidemic season (1-3). Many scientists have been developing protective and safe RSV vaccines without causing enhanced respiratory disease, but there is still no licensed RSV vaccine. In preclinical murine animal models, other platforms of RSV vaccines including recombinant vaccinia expressing RSV glycoproteins (G, F), RSV fusion (F) protein and F encoding plasmid DNAs were reported to induce enhanced respiratory disease after vaccination and then RSV infection (4-8).

A mechanism of FI-RSV vaccine enhanced respiratory disease is an imbalance in CD4 Th1 and Th2 immune responses as reported in studies using BALB/c mouse models (9). Generally, Th1 immune responses induce IFN-γ secretion and cytotoxic CD8 T cell activation which are desirable responses for antiviral protection. However, FI-RSV vaccination induced Th2-biased immune responses, which include RSV-specific IgG1 Ab production and IL-4, IL-5, and IL-13 producing T cell responses in BALB/c mouse models (10,11). Moreover, FI-RSV immunization elicited severe eosinophil infiltration and lung inflammation after RSV infection (11-13). Depletion of CD4 and CD8 T cells but not B cells before and during the RSV infection resulted in diminishing RSV pathogenesis in BALB/c mice (14,15). Therefore, vaccination inducing balanced Th1 CD4 T cell and cytotoxic CD8 T cell responses as well as neutralizing Ab production is highly desirable to prevent enhanced respiratory disease after RSV infection (16). However, host immune parameters contributing to enhanced respiratory disease have not been fully understood yet.

BALB/c mouse models have been commonly used in previous studies reporting RSV pathogenesis and vaccine efficacy probably due to being relatively high susceptible to RSV infection (17,18). In this study, we have used wild type (WT) C57BL/6 and CD4 knockout (CD4KO) mice to better understand the roles of the CD4 T cells and immune cell networks contributing to enhanced respiratory disease after FI-RSV vaccination and following RSV challenge. In addition, depletion of CD8 T cells and NK cells from the FI-RSV-immunized CD4KO mice demonstrated further cellular mechanisms of RSV protection and enhanced respiratory disease.

MATERIALS AND METHODS

Animals and reagents
C57BL/6 WT and CD4KO (B6.129S6-Cd4^tm1Knw/J) mice were purchased from the Jackson laboratory and maintained in the Georgia State University (GSU) animal facility under the guidelines of a GSU-approved Institutional Animal Care and Use Committee protocol (protocol A18001). Six to eight-wk old female mice were used in this study. FI-RSV was prepared by a method previously described (19). Briefly, RSV was amplified in Hep2 cells and inactivated with formalin for 3 days at 37°C. The formalin-treated RSV was ultra-centrifuged at 30,000 rpm, 4°C for 1 h. The pellet was resuspended in serum-free DMEM and stored at −80°C until use.

Immunization, cell depletion, and virus infection
WT and CD4KO mice (n=5, 6–8 wk old age) were immunized with PBS or FI-RSV (2 μg) with aluminum hydroxide (Alum, 100 μg) intramuscularly at a 3-wk interval. Blood samples were collected at 2 wk after prime and boost immunizations. For specific cell depletion before RSV infection, 150 μg of anti-CD8α monoclonal Ab (clone 53-6.7) for CD8 T cell depletion and 50 μl of anti-asialo GM1 monoclonal Ab for NK cell depletion were injected intraperitoneally 2

times on −2 and 0 days relative to the day of challenge. Naïve control and FI-RSV immunized mice were infected with 5×10⁵ PFU of RSV A2 intranasally under isoflurane anesthesia at 4 wk after boost immunization. Body weight changes were daily monitored for 5 days and airway resistance was measured at day 4 post infection by using plethysmography. For measuring the airway resistance and hyper-responsiveness, mice were treated with aerosolized 100 mg/ml of methacholine (50 µl/mouse) by a nebulizer and the enhanced pause (PenH) was measured before and after methacholine treatment by a mouse whole-body plethysmograph (EMKA technologies). The percent increases above the baseline PenH were calculated and presented. At day 5 post infection, all mice were euthanized and bronchoalveolar lavage fluids (BALF) and lung tissues were obtained for further analysis. The RSV titers in lung samples were determined by using a plaque forming assay on Hep2 cells as previously described (13).

Ab and cytokine ELISA

To measure RSV-specific Ab levels in immunized mice, immune sera were serially diluted and treated to 96-well ELISA plates coated with 200 ng/well of FI-RSV after a blocking process. HRP-conjugated anti-mouse IgG, IgG1, and IgG2c Abs were used for detecting specific Ab subtypes. The levels of IL-1β, IL-6, and IFN-γ cytokines in BALF and lung homogenates were measured by using cytokine ELISA kits (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocol. The OD values were measured at 450 nm by using an ELISA reader (Bio-Rad, Hercules, CA, USA).

Lung histopathology

Lung samples from individual mice were harvested at day 5 post RSV infection and fixed in 10% neutral buffered formalin. The fixed lung samples were processed and then embedded paraffin blocks for tissue sections. Lung tissue sections with a thickness of 5 µm were stained with H&E or hematoxylin and Congo red (H&CR) to evaluate lung histopathologic changes and eosinophil infiltration, respectively as previously described (13,20).

Flow cytometry

Cells prepared from BALF and lung tissues collected at day 5 post infection were stained with specific phenotypic marker Abs including CD45 (30-F11), F4/80 (BM8), CD11b (M1/70), CD11c (N418), CD103 (2E7), B220 (RA3-6B2), Ly6c (HK1.4), CD49b (DX5), Siglec F (E50-2440), CD3 (17A2), CD4 (RM4-5), and CD8 (53-6.7) to distinguish the cell populations. To determine the RSV-Specific cytokine producing T cells, bronchoalveolar lavage (BAL) cells were stimulated with 4 µg/ml RSV peptide mixture (F$_{92-106}$; ELQLLMQSTPATNNR + F$_{85-93}$; KYKNAVTEL for CD8 T cells and G$_{183-195}$; WAICKRIPNKK for CD4 T cells) for 5 h. And then the stimulated cells were fixed and permeabilized by using a Cytofix/Cytoperm kit (BD Biosciences, Franklin Lakes, NJ, USA) and stained with the surface markers and intracellular cytokines such as TNF-α, IL-4, and IFN-γ. The stained cells were acquired using a BD LSR-II/Fortessa flow cytometer and the data were analyzed by FlowJo software. The gating strategy of the immune cells and cytokine-producing cells were presented in **Supplementary Figs. 1** and **2**.

Statistical analysis

The data were presented as the mean±SEM. Statistical analysis of data was performed by 2-way ANOVA with Tukey's multiple comparison test or non-parametric Mann-Whitney test by using GraphPad Prism.

RESULTS

FI-RSV immunization in CD4KO mice induces lower levels of IgG Ab responses than those in WT mice

It was known that FI-RSV induced Th2-biased immune responses, contributing to vaccine enhanced disease. In this study, we have examined immune responses to FI-RSV vaccination in WT and CD4KO mice to better understand the roles of CD4 T cells and cellular immune mechanisms in inducing Ag specific Ab production and vaccine-enhanced respiratory disease. WT and CD4KO mice were immunized with FI-RSV intramuscularly 2 times with a 3-wk interval, immune sera were collected for RSV-specific Ab ELISA at 2 wk after each immunization (**Fig. 1**). In CD4KO mice, RSV-specific Ab levels were not detectable after prime immunization, but IgG and IgG1 Abs were increased to significant levels after boost (**Fig. 1A and B**). The levels of RSV-specific Ab in CD4KO mice were lower than those in WT mice, suggesting critical roles of CD4 T cells in inducing IgG responses to FI-RSV vaccination. Higher IgG2c (or IgG2a in BALB/c mice) production reflects Th1-biased immune responses whereas IgG1-dominant Ab response indicates Th2 in mice correlating with the induction of Th1 and Th2 cytokines respectively (21,22). No significant levels of IgG2c Ab were detected in CD4KO mice whereas low but substantial levels of IgG2c in WT mice (**Fig. 1C**). IgG1 subtype was the dominant RSV-specific IgG Ab induced by FI-RSV immunization in both WT and CD4KO mice as evident in the ratio of IgG2c/IgG1 isotype Ab levels (**Fig. 1D**).

CD4 T cells contribute to enhanced respiratory disease by FI-RSV vaccination upon RSV infection
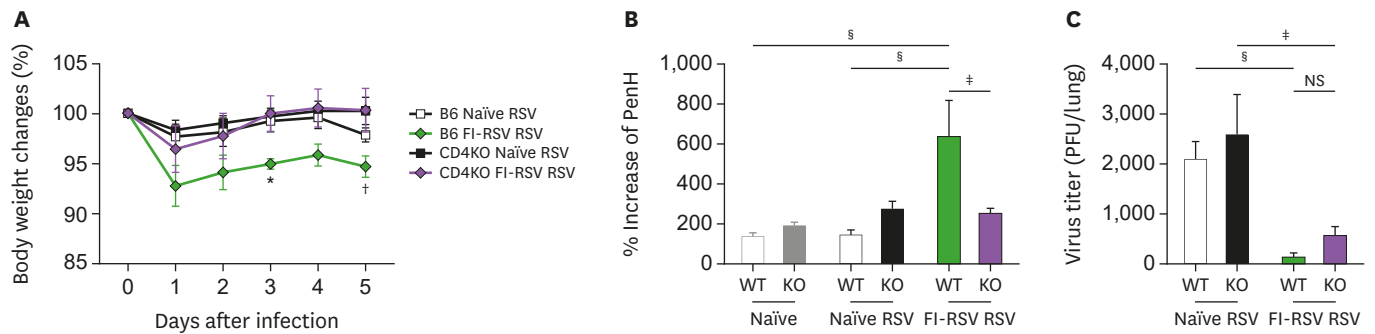
The naïve and FI-RSV immunized WT and CD4KO mice were infected with RSV to determine the roles of CD4 T cells in inducing vaccine enhanced respiratory diseases by FI-RSV after RSV infection. The body weight changes of the infected mice were monitored daily for 5 days and the PenH values were measured at day 4 post infection. In FI-RSV immunized WT mice, a substantial loss (7%) in body weight was observed from day 1 post RSV infection and not recovered until the day 5 post infection (**Fig. 2A**). In the naïve RSV infected WT mice, body weight loss was less but not fully recovered until day 5 post infection. However, the naïve and FI-RSV immunized CD4KO group showed slight weight loss (≤3% or less) at day 1 post infection, and fully recovered at day 3 post infection. Airway resistance of the RSV infected mice at day 4 post infection was measured by plethysmography under methacholine
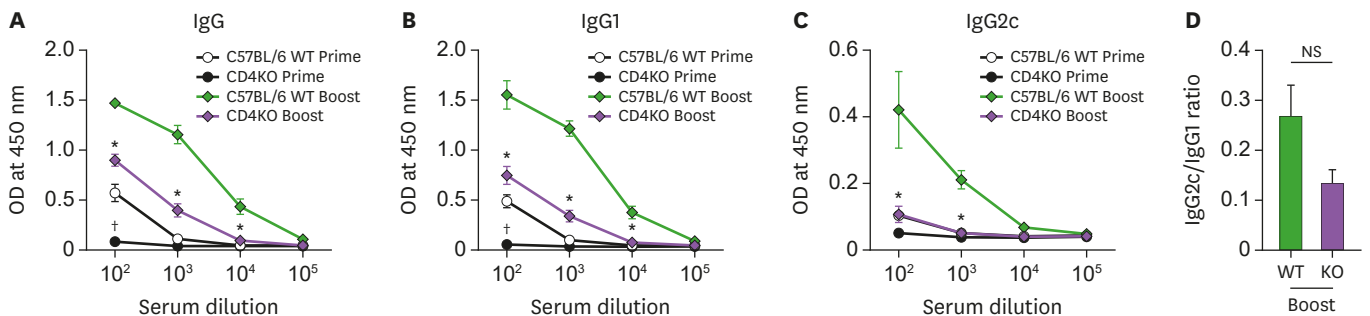
Figure 1. RSV-specific Ab production in immunized WT and CD4KO mice. WT and CD4KO mice (n=5 each) were immunized with FI-RSV intramuscularly 2 times with a 3-wk interval. The immune sera were collected at 2 wk post prime and boost immunization and RSV-specific IgG ELISA was performed. (A-C) RSV-specific IgG, IgG1, and IgG2c levels of diluted sera from each immunization groups were shown. Experimental data were presented in mean±SEM. Two-way ANOVA and Tukey's multiple comparison tests were performed for statistical analysis. (D) IgG2c/IgG1 ratio of 100-times diluted boost sera from FI-RSV immunized WT and CD4KO mice.

NS, not significant.

*p<0.001 compared to C57BL/6 WT boost; †p<0.001 compared to C57BL/6 WT prime.

Figure 2. FI-RSV immunized CD4KO mice are protected against RSV disease and viral replication. The immunized WT and CD4KO mice (n=5 each) were challenged with RSV intranasally and monitored body weight daily. Two-way ANOVA and Tukey's multiple comparison tests were performed between groups and days. (A). At day 5 post infection, PenH were measured by plethysmography (B) and the lung samples were collected for RSV viral titers (C). Experimental data were presented in mean±SEM. Non-parametric Mann-Whitney test was performed between the indicated groups for statistical analysis.
*p<0.05 compared to B6 naïve RSV group; †p<0.05 compared to CD4KO FI-RSV RSV group; ‡p<0.05; §p<0.001.
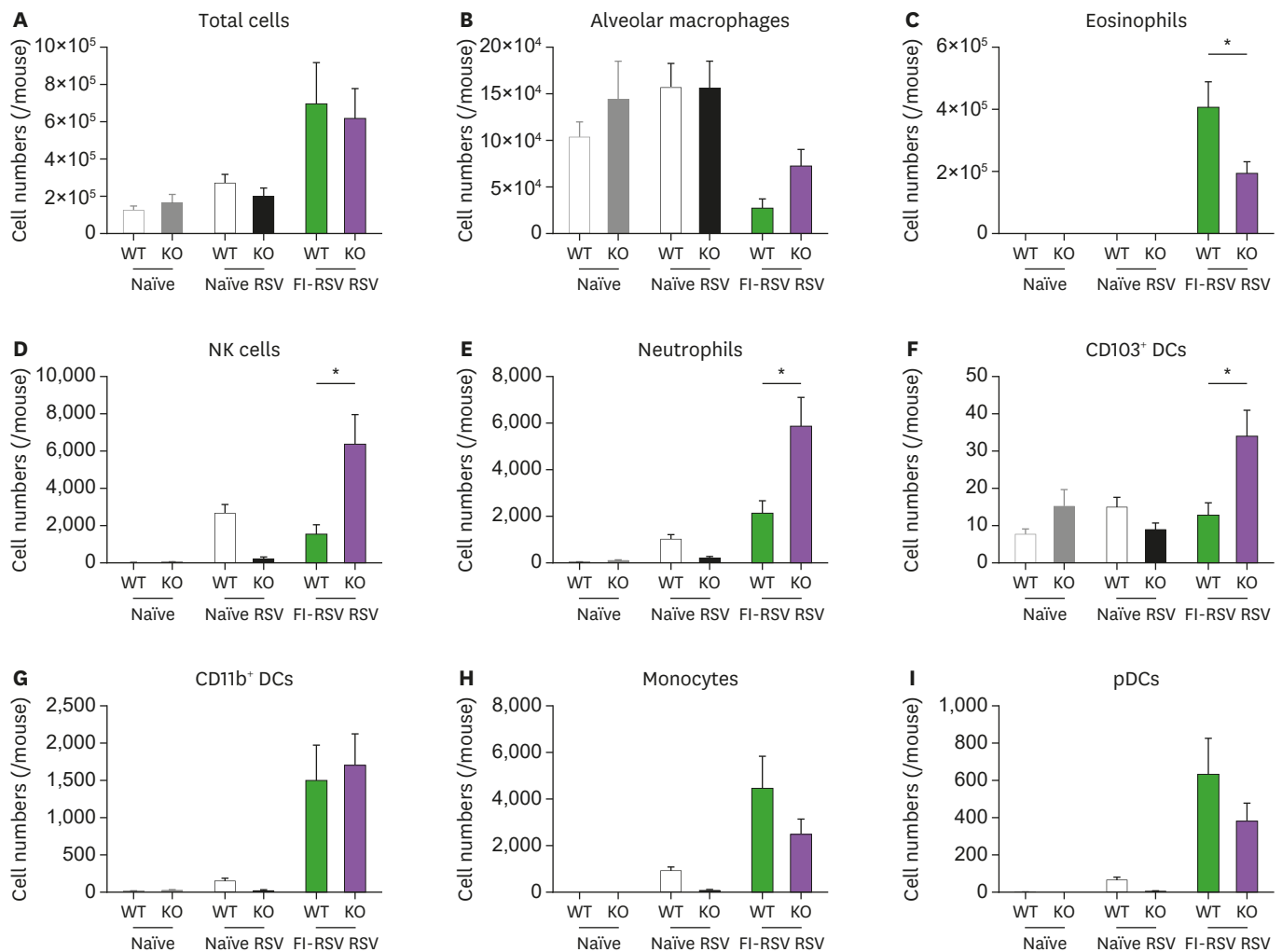
treatment (**Fig. 2B**). FI-RSV immunized WT mice showed significantly enhanced PenH values, but CD4KO mice immunized with FI-RSV displayed significantly less airway obstruction, indicating reduced respiratory disease. Lung virus titers at day 5 post RSV infection were significantly lower in both FI-RSV immunized WT and CD4KO mice than those of the naïve RSV groups (**Fig. 2C**). There were no significant differences in lung virus titers between FI-RSV immunized WT and CD4KO mice.

Lung inflammation and eosinophil infiltration are the representative pathological changes of vaccine enhanced diseases by RSV infection in FI-RSV immunized population. Histopathology appeared to be less severe in the lung from CD4KO mice compared to that from WT mice without FI-RSV vaccination after RSV infection (**Supplementary Fig. 3**). Perivascular inflammation after RSV infection was most severe in both FI-RSV immunized WT and CD4KO mice despite of low viral loads (**Supplementary Fig. 3**). The CD4KO mice immunized with FI-RSV significantly reduced eosinophil infiltration, but not fully, compared with eosinophil infiltration of FI-RSV immunized WT mice in H&CR staining for eosinophils in lungs (**Fig. 3A**). This result suggests that CD4 T cells are partially responsible for eosinophil infiltration in FI-RSV immunized mice after RSV infection. In addition, pro-inflammatory cytokines such as IL-6 and IL-1β were reduced in lung extracts from FI-RSV CD4KO mice compared to WT mice, and anti-viral cytokine IFN-γ was significantly increased in FI-RSV CD4KO mice (**Fig. 3B**). These results provide evidence for reduced lung inflammation in FI-RSV immunized CD4KO mice after RSV infection.

FI-RSV vaccination in CD4KO mice recruits NK cells and effector CD8 T cells into the airway BALF upon RSV infection

Under a CD4 T cell deficient condition, CD4KO mice might display different cellular recruitment in the airways after RSV infection from WT mice. We collected BAL cells at day 5 post infection and carried out flow cytometric analysis of the cellular phenotypes and intracellular cytokine staining (**Figs. 4** and **5**). The gating strategy of the cells were presented in **Supplementary Figs. 1** and **2**. FI-RSV immunization in both WT and CD4KO mice reduced the number of alveolar macrophages in BAL after RSV infection (**Fig. 4B**). As observed in H&CR-stained lung histopathology (**Fig. 3A**), significantly lower levels of eosinophils (CD45$^+$CD11b$^+$CD11c$^-$SiglecF$^+$) were detected in FI-RSV immunized CD4KO mice compared to WT mice. Nonetheless, substantial amounts of eosinophils were recruited in CD4KO mice with FI-RSV vaccination after RSV challenge (**Fig. 4C**). Previous studies have reported

Figure 3. Eosinophil infiltration and inflammatory cytokine levels in lung are reduced in FI-RSV immunized CD4KO mice. The immunized WT and CD4KO mice (n=5 each) were challenged with RSV intranasally and lung samples were harvested at day 5 post challenge. (A) Representative eosinophil infiltrations around the airways were shown after H&CR staining. The number of eosinophils was counted from the H&CR-stained lung specimen. (B) Cytokine levels in lung extracts were measured by ELISA. Experimental data were shown in mean±SEM. Non-parametric Mann-Whitney test was performed between the indicated groups for statistical analysis. *p<0.05; ***p<0.001.

no detection or low levels of eosinophil infiltration in RSV infected naïve animals and in less severe human patients (11,12,20,23). In consistent, we observed no eosinophils or below the detection limit in the lung from WT and CD4KO mice without FI-RSV vaccination after challenge with RSV (**Fig. 4C**). NK cells (CD45+CD3−CD49b+), neutrophils (CD45+CD11b+F4/80− Ly6cint), and CD103+ dendritic cells (DCs; CD45+F4/80−CD11c+MHCII+CD103+) were significantly increased in FI-RSV immunized CD4KO mice compared to WT mice (**Fig. 4D-F**). There were similar levels of CD11b+ DCs (CD45+F4/80−CD11c+MHCII+CD11b+) between FI-RSV immunized WT and CD4KO mice but we observed a trend of reduced levels in monocytes (CD45+CD11b+F4/80+Ly6chi) and plasmacytoid DCs (pDCs) (CD45+F4/80−CD11c+MHCII+B220+) in BAL samples from CD4KO with FI-RSV vaccination and RSV challenge (**Fig. 4G-I**).

In addition to significant increase of NK cell population, FI-RSV immunized CD4KO mice showed significant increases in cytokine-producing effector CD8 T cells (CD45+CD3+CD8+TNF-α+ or CD45+CD3+CD8+IFN-γ+, **Fig. 5A**) in BAL, which is correlating with high levels of airway CD103+ DCs (**Fig. 4F**). In contrast, WT mice recruited high levels of CD4 T cells producing TNF-α, IFN-γ, and IL-4 into the airway BALF (**Fig. 5B**). These results suggest that NK cells and CD8 T cells might play a role in providing better protective outcomes of less weight loss and inflammation in CD4KO mice upon RSV infection whereas high levels of effector CD4 T cells secreting inflammatory cytokines (TNF-α, IL-4) in the airways contribute to severe FI-RSV enhanced inflammation in WT mice.

Figure 4. Cellular phenotypes infiltrated into the BALF upon RSV infection. The immunized WT and CD4KO mice (n=5 each) were challenged with RSV intranasally and BALF samples were harvested at day 5 post challenge. Cell phenotypes were determined by flow cytometry. All data were shown in mean±SEM. Non-parametric Mann-Whitney test was performed between the WT and CD4KO FI-RSV RSV groups for statistical analysis. *p<0.05.

Extensive lung effector CD4 T cells correlate with inflammatory infiltrates in WT mice upon RSV infection

Lung infiltrates were analyzed by flow cytometry to better understand inflammation and pathology in WT mice after RSV infection (**Figs. 6** and **7**). Flow cytometry results of CD4 T cells confirmed CD4 deficiency in CD4KO mice. TNF-α and IL-4 producing total lung cells in FI-RSV WT mice were significantly higher than those in CD4KO mice (**Fig. 6A and C**). IFN-γ+ CD4 T and TNF-α+ CD4 T cells were recruited at high to moderate levels into the lung from naïve WT mice after RSV infection (**Fig. 6D and E**). In addition, higher levels of total infiltrates, alveolar macrophages, monocytes, NK cells as well as diverse DC populations (pDCs, CD103+ DCs, CD11b+ DCs) were infiltrated into the lung from naïve WT mice compared to those in CD4KO naïve mice after RSV infection (**Fig. 7A-G**). These effector CD4 T cells and infiltrates appear to correlate with moderate histopathology in naïve WT C57BL/6 mice compared to CD4KO mice after RSV infection (**Supplementary Fig. 3**).
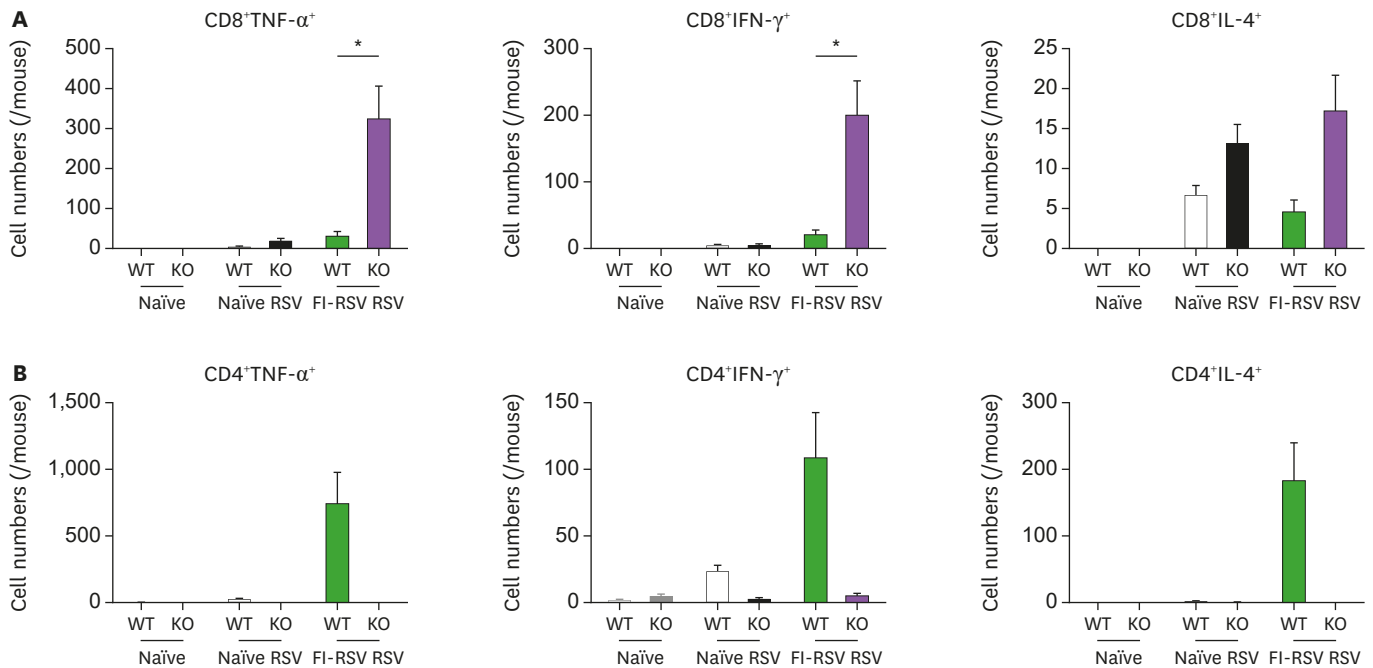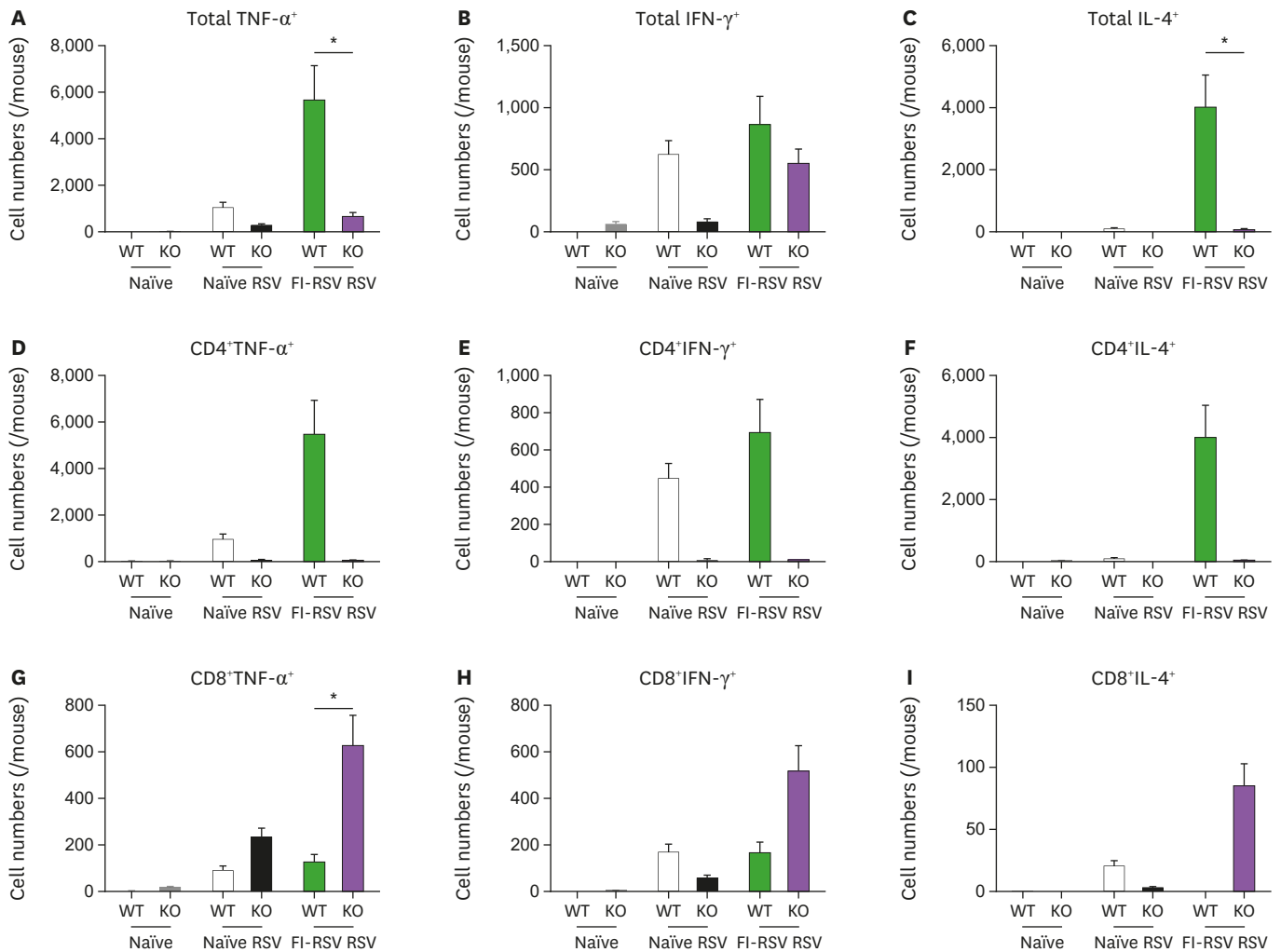
Figure 5. Intracellular cytokine staining of CD4 and CD8 T cells infiltrated into the airway BALF upon RSV infection. BAL cells were harvested as described in **Fig. 4**. Intracellular cytokine staining of cells in BALF was performed to determine cytokine producing cells. Experimental data were shown in mean±SEM. Non-parametric Mann-Whitney test was performed between the indicated groups for statistical analysis.
*p<0.05.

FI-RSV vaccination in WT C57BL/6 mice resulted in extensive induction of lung effector CD4 T cells producing TNF-α and IL-4 cytokines most dominantly and IFN-γ+ at moderate levels upon RSV challenge (**Fig. 6D-F**). The high levels of pro-inflammatory TNF-α+ and Th2 type IL-4+ CD4 T cells contributed to weight loss and might be partially responsible for eosinophil infiltration in mice after FI-RSV vaccination and RSV challenge. In contrast, cytokine-producing CD8 T cells were recruited into the lung at significantly higher levels in FI-RSV immunized CD4KO mice after RSV infection (**Fig. 6G-I**). Lung eosinophils were significantly recruited in WT mice compared to those in CD4KO mice after FI-RSV vaccination and RSV challenge (**Fig. 7H**). There were no significant differences in the levels of other myeloid cells such as monocytes, neutrophils, and NK cells recruited into the lung in WT and CD4KO mice after FI-RSV vaccination and RSV challenge (**Fig. 7C, D, and I**).

NK and CD8 T cell depletion results in increases in weight loss and lung viral loads in FI-RSV immunized CD4KO mice upon RSV infection

To investigate the roles of CD8 T cells and NK cells in protective outcomes in FI-RSV immunized CD4KO mice, we depleted CD8 T cells and NK cells before RSV challenge by treating with cell specific depleting Abs (**Fig. 8**). At day 1 post infection, the CD8 and NK-depleted FI-RSV immunized CD4KO mice showed similar body weight loss (3%) as control (no depletion) FI-RSV immunized CD4KO mice. At day 2 after RSV infection, NK-depleted CD4KO mice showed 8% of weight loss and CD8-depleted CD4KO mice showed 7% of weight loss, a level similar to that observed in FI-RSV immunized WT mice whereas control FI-RSV immunized CD4KO mice displayed 3% weight loss. The control FI-RSV immunized CD4KO mice recovered by day 3 post infection, but the NK-depleted mice recovered after 5 days, and CD8-depleted mice could not fully recover body weight until day 5 post infection (**Fig. 8A**). PenH values were measured at day 4 post RSV infection, but there was no significant difference in FI-RSV immunized CD4KO mice

Figure 6. Intracellular cytokine staining of CD4 and CD8 T cells infiltrated into the lung tissues upon RSV infection. The immunized WT and CD4KO mice (n=5 each) were challenged with RSV intranasally and lungs were harvested at day 5 post challenge. Intracellular cytokine staining of lung cells was performed to determine cytokine producing cells. Experimental data were shown in mean±SEM. Non-parametric Mann-Whitney test was performed between the indicated groups for statistical analysis.
*$p < 0.05$.

with CD8 T cell or NK cell depletion (**Fig. 8B**). However, the control of viral loads in lung was highly affected as a result of depleting NK or CD8 T cells (**Fig. 8C**). Significantly increased viral loads were observed in both CD8 and NK cell depleted CD4KO mice compared with the control FI-RSV immunized CD4KO mice. In particular, NK cell-depleted CD4KO mice with FI-RSV vaccination could not control lung viral loads and showed similar viral loads to naïve CD4KO mice. These results suggest that the effector cells such as CD8 T cells and NK cells also play a role in controlling RSV replication and in alleviating weight loss due to RSV infection.

NK and CD8 T cell depletion has differential impact on lung inflammation in FI-RSV immunized CD4KO mice after RSV infection
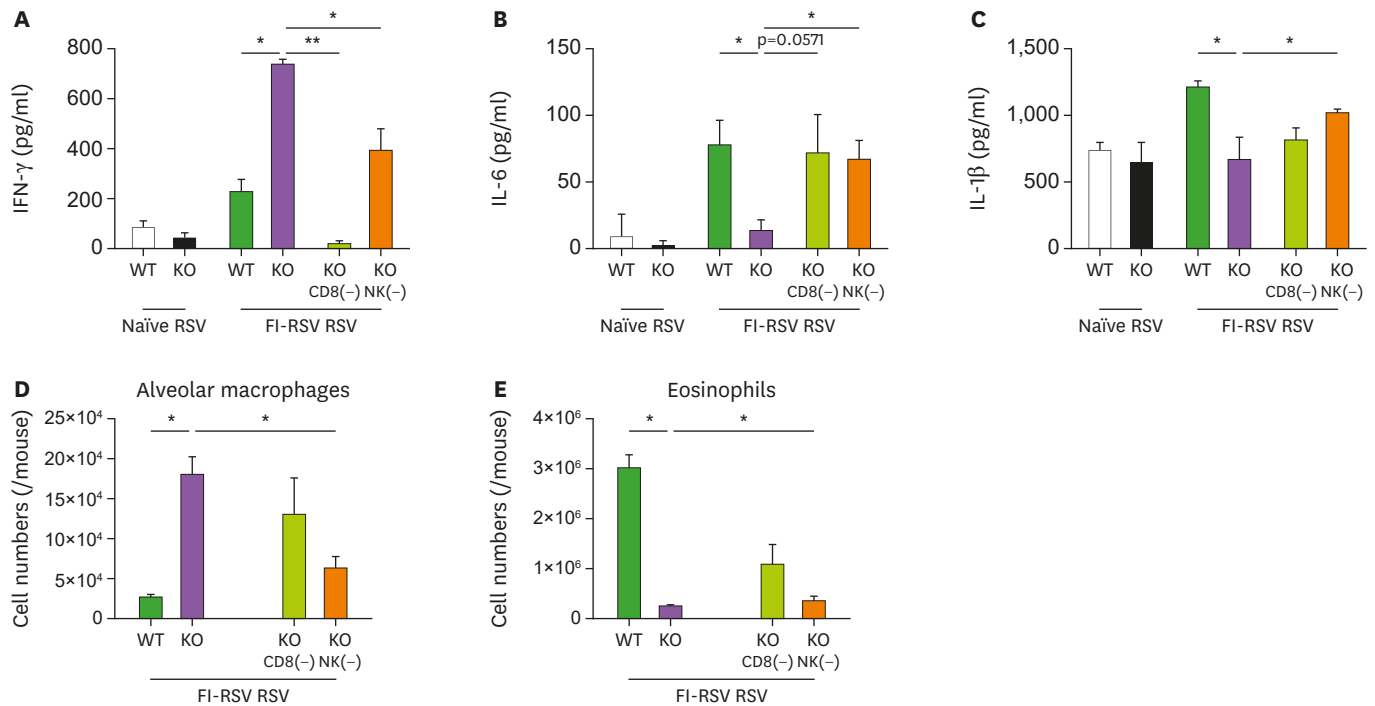
BAL and lung samples were harvested at day 5 after infection in non-depleted control, CD8-depleted and NK-depleted CD4KO mice to assess lung inflammation. IFN-γ production was completely suppressed in CD8-depleted FI-RSV CD4KO mice and significantly reduced in NK-depleted FI-RSV CD4KO mice (**Fig. 9A**). Pro-inflammatory cytokine IL-6 production
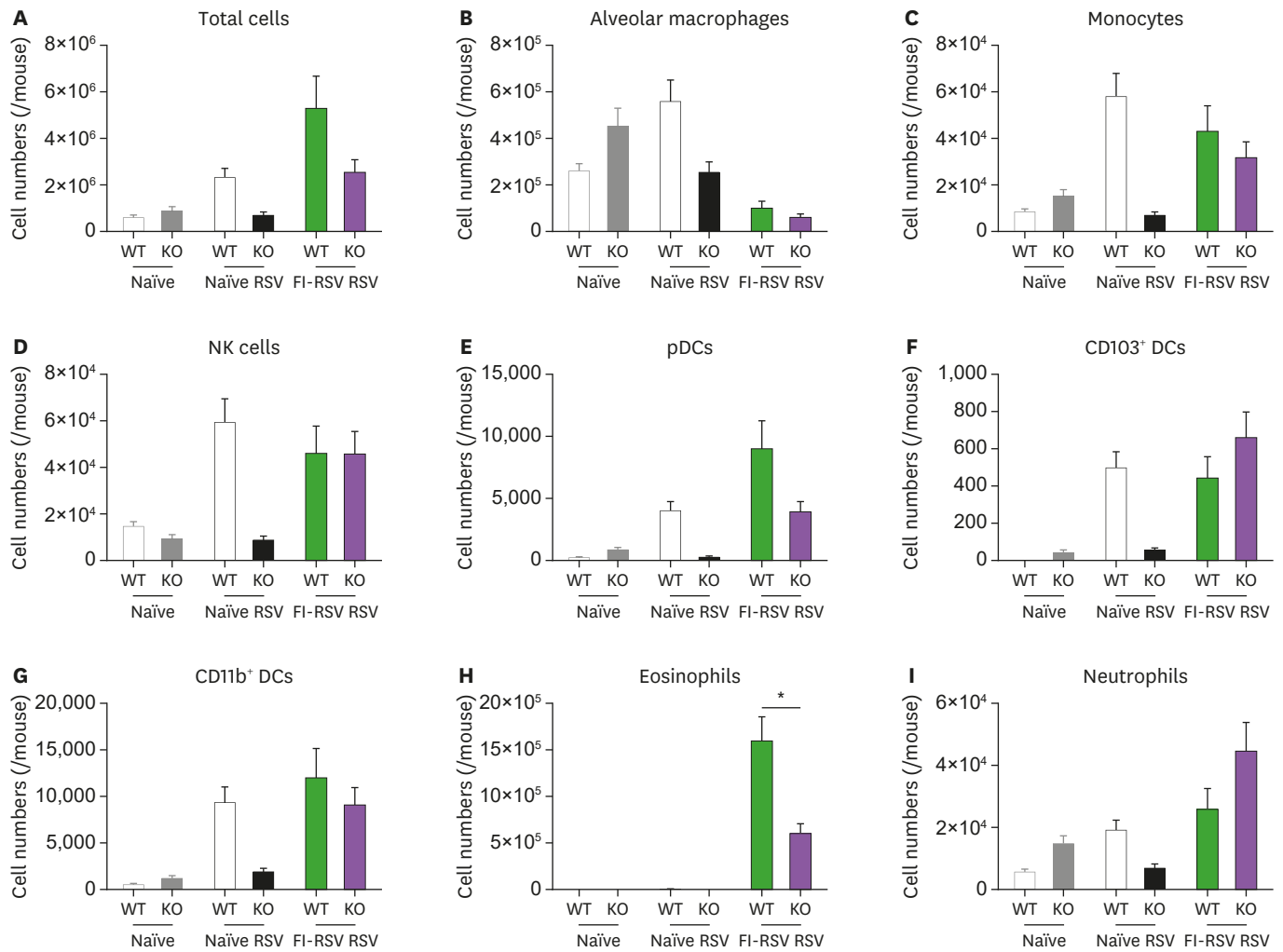
Figure 7. Cellular phenotypes of inflammatory infiltrates ion into the lung upon RSV infection. Lung tissues were harvested at day 5 post challenge and cells prepared for a flow cytometry assay. Cell phenotypes were determined by staining with phenotypic marker Abs. Experimental data were shown in mean±SEM. Non-parametric Mann-Whitney test was performed between the WT and CD4KO FI-RSV RSV groups for statistical analysis. *p<0.05.
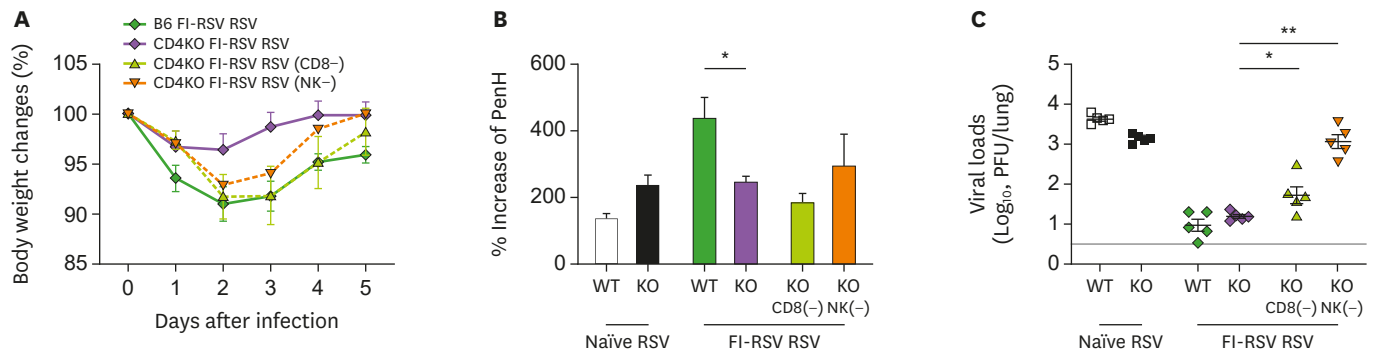
Figure 8. Body weight changes and viral loads in CD8 T or NK cell depleted FI-RSV CD4KO mice after RSV infection. WT (n=5) and CD4KO mice (n=15) were immunized with FI-RSV intramuscularly 2 times at a 3-wk interval. FI-RSV immunized CD4KO mice were injected with anti-CD8 or anti-NK monoclonal Abs (n=5, each) for depletion 2 days in advance and right before RSV infection. (A) Body weight changes were monitored for 5 days after RSV infection. (B) PenH values were measured at day 4 post RSV infection before and after aerozolized methacholine treatment. (C) RSV viral loads were measured from the lung extracts of each group. Experimental data were shown in mean±SEM. Non-parametric Mann-Whitney test was performed between the indicated groups for statistical analysis. *p<0.05; **p<0.01.

Figure 9. Inflammatory responses in FI-RSV CD4KO mice after CD8 T and NK cell depletion. Naïve and FI-RSV immunized WT, CD4KO, CD8 T-depleted CD4KO, NK-depleted CD4KO mice (n=5 each) were challenged with RSV intranasally. BAL and lung samples were harvested at day 5 post infection. (A-C) Cytokine levels in lung extracts. (D, E) Immune cell infiltration in BALF. Experimental data were shown in mean±SEM. Non-parametric Mann-Whitney test was performed between the indicated groups for statistical analysis.
*p<0.05; **p<0.01.

in lung was significantly enhanced in CD8-depleted and NK-depleted FI-RSV CD4KO mice, which is similar to those in FI-RSV WT mice (**Fig. 9B**). A moderate increase in IL-1β cytokine was observed in NK-depleted FI-RSV CD4KO mice (**Fig. 9C**). A significant reduction in alveolar macrophages was observed in the BAL cells from NK-depleted CD4KO mice (**Fig. 9D**). In addition, a significant increase in eosinophil infiltration was detected in CD8-depleted FI-RSV CD4KO mice (**Fig. 9E**). These results indicate that CD8 T cells are responsible for most IFN-γ production in lung and partial inhibition of eosinophil infiltration and that NK cells play a role in reducing lung inflammation by controlling viral loads in CD4KO mice with FI-RSV vaccination upon RSV infection.

DISCUSSION

CD4 T cells orchestrate humoral and cellular immune responses by interacting with Ag presenting cells through a T cell receptor and modulating a pattern of Th1 and Th2 cytokines. CD4 T cells have been considered to be mainly responsible for enhanced respiratory disease after RSV infection of FI-RSV vaccinated BALB/c mice (24,25). Alum adjuvant in FI-RSV vaccination of BALB/c mice induced RSV-specific IL-4⁺ and TNF-α⁺ CD4 T cells rather than IFN-γ⁺ CD8 T cells, which exacerbated pulmonary inflammation after RSV infection (26). We tested whether FI-RSV vaccine enhanced respiratory disease could be induced in WT C57BL/6 and CD4KO mice upon RSV infection. Consistent with those in BALB/c mice (27), FI-RSV vaccine enhanced respiratory disease was induced in WT C57BL/6 mice as determined by Th2 biased IgG responses after vaccination, weight loss, increased airway resistance, enhanced

inflammatory cytokine production, and eosinophil infiltration in lung after RSV infection. Significantly lower IgG levels by 10 folds were induced in CD4KO mice than those in WT mice after FI-RSV vaccination but the efficacy of lung viral clearance was similarly observed in both mice after RSV infection. There was no correlation between lung viral titers and inflammatory disease in naïve and FI-RSV vaccinated WT C57BL/6 and CD4KO mice upon RSV infection, which was also reported in BALB/c mice (28-32), suggesting a limitation in murine models for RSV pathogenesis. Nonetheless, there were significant differences in FI-RSV vaccine enhanced respiratory disease between WT C57BL/6 and CD4KO mice with FI-RSV vaccination or not upon RSV infection, supporting the rationale justification of this study on the roles of CD4 T cells in RSV disease.

In a CD4KO mouse model, the vaccine enhanced disease parameters monitored during RSV infection after FI-RSV immunization were significantly reduced. Lung eosinophil infiltration was diminished in the FI-RSV immunized CD4KO mice as well as body weight loss and airway resistance compared to the FI-RSV immunized WT mice. These results suggest that Th2 biased CD4 T cell responses to FI-RSV vaccination were a parameter deriving immunopathology responsible for vaccine-enhanced respiratory disease. Consistently, previous studies reported that depletion of CD4 T cells but not B cells before and during RSV infection significantly lessened inflammatory RSV disease in BALB/c mice (14,15). Nonetheless, there were still substantial levels of lung eosinophil infiltration even under a CD4-deficient condition after FI-RSV vaccination upon RSV infection.

CD4 T cell deficiency would not generate an imbalanced immune condition of Th2 CD4 T cells which are associated with inducing eosinophil infiltration and suppressing the induction and recruitment of cytotoxic CD8 T and NK cells into the lungs. As a result, protective CD8 T and NK cell responses would be promoted in CD4KO mice, contributing to diminishing FI-RSV vaccine enhanced respiratory disease. In this study, we provide evidence that CD8 T cells and NK cells play a protective role in FI-RSV vaccine enhanced respiratory diseases in a CD4 deficient condition. We observed significant increases in NK cells and cytokine-producing CD8 T cells in the BALF of FI-RSV vaccinated CD4KO mice upon RSV infection, suggesting the possible protective roles of NK and CD8 T cells. There were previous studies reporting that a small subset of conventional DC (cDC) expresses CD4 molecules (33,34). Therefore, it is possible that CD4KO mice not only have no CD4 T cells, but also might have a defect with developing cDC expressing CD4. It should be noted that the phenomenon observed such as the attenuation of eosinophil recruitment and subsequent inflammatory disease in CD4KO mice might be the result from the outcomes by deficiency of both CD4 T cells and a subset of cDCs expressing CD4 molecules. It is also possible that the increase of CD103 DCs in CD4KO mice together with CD8 T cells and NK cells might have contributed to the inhibition of respiratory disease.

CD8 T cell plays a role in antiviral responses by its cytotoxic effector function and cytokine production like IFN-γ. Cytotoxic CD8 T cell transfer to the naïve immune-deficient mice could provide protection against RSV infection by successful RSV clearance (35-37). A recent study reported the roles of resident memory CD8 T cells in providing protection against RSV infection (38). It was also demonstrated that a lack of CD8 T cell induction by FI-RSV vaccination might promote eosinophil infiltration and disease enhancement in lungs (39). In WT mice with intact CD4 T cells, FI-RSV immunization induced strong Th2-biased CD4 T cell activation. IL-4 Th2 unbalanced responses cause relative inhibition of IFN-γ producing CD8 T cell and Th1 CD4 T cell responses. In addition, other Th2 cytokines produced by

CD4 T cells such as IL-5 and IL-13 can promote eosinophil infiltration in lung. In a CD4 deficient condition, there would be no such Th2-CD4 T cell-mediated inhibition of IFN-γ producing effector CD8 T cells, contributing to better protection against RSV infection in FI-RSV immunized CD4KO mice. In this study, we found significant increases in CD8 T cells secreting TNF-α and IFN-γ from FI-RSV immunized CD4KO upon RSV infection. In addition, CD8 T cell depletion from FI-RSV immunized CD4KO mice resulted in lowering IFN-γ levels to a background and increases in lung viral loads, inflammatory IL-6 cytokine, severe weight loss, and eosinophil infiltration. This finding suggests that a deficiency of CD4 T cells during FI-RSV vaccination appears to promote the induction of protective CD8 T cell responses contributing to protection against weight loss upon RSV infection.

NK cells are known to play dual roles in RSV infection. NK cells play a role in providing protection against RSV by inducing cytotoxicity of virus-infected airway epithelial cells, but also can aggravate lung injury by producing excessive cytokines and cytotoxicity, which results in severe injury and immune regulation in the lung (40). NK cell is one of the innate immune cells which produce IFN-γ, TNF-α, and cytotoxic granules and induce cell death of virus-infected cells. It also contributes to regulate the adaptive immune responses including CD8 T cell differentiation into effector cytotoxic lymphocyte as well as CD4 T cell activation (41,42). During RSV infection, NK cells were recruited to the lung and highly activated to induce acute lung injury. The activated NK cells produce IFN-γ which increases lung CD8 T cell recruitment and aggravates lung pathology (43,44). Consistent with these previous studies on NK cells, our preliminary studies showed that inclusion of an adjuvant Addavax (MF59-like oil-in-water emulsion) in FI-RSV split vaccination of BALB/c mice resulted in increased recruitment of activated NK cells and other inflammatory innate and adaptive infiltrates causing severe histopathology despite lung viral clearance upon RSV infection (data not shown). However, the roles of NK cells in FI-RSV vaccination enhanced respiratory disease are not full elucidated yet. NK cells and effector CD8 T cells were not recruited into the airway BALF collected from alum adjuvanted FI-RSV vaccinated WT C57BL/6 mice upon RSV infection. It might be due to the high levels of RSV-specific CD4 T cells in WT mice. The virus infection and replication were blocked by the RSV-specific serum Ab and Th2-biased strong CD4 T cell responses including IL-4 and IL-13 cytokine production, promoting eosinophil infiltration greatly in the airway rather than recruitment of effector CD8 T cells or NK cells. In contrast, FI-RSV vaccination and RSV infection of CD4KO mice recruited NK cells and effector CD8 T cells at higher levels. NK cell depletion from FI-RSV immunized CD4KO mice during RSV infection led to substantial weight loss similar to FI-RSV WT mice and more increases in lung RSV titers compared to those in CD8 depletion, suggesting that NK cells are a critical cell population to clear lung viral loads in FI-RSV vaccinated CD4KO mice. It is interesting to note that CD8 depletion led to a complete loss in IFN-γ production whereas NK depletion to a partial loss in IFN-γ and no control of viral loads, indicating a cross-talk between NK cells and effector CD8 T cells.

This study supports that CD4 T cells are required for inducing high levels of humoral IgG responses to alum adjuvanted FI-RSV vaccination but also contribute to FI-RSV vaccine enhanced respiratory disease. It is also likely that alum adjuvanted FI-RSV vaccination of WT C57BL/6 mice appears to suppress the induction of effector CD8 T cells and NK cells in the airways in contrast to CD4KO mice during RSV infection. RSV vaccination should induce neutralizing Abs while avoiding the induction of Th2 type CD4 T cell responses. Excessive induction of RSV-specific memory CD8 T cells can contribute to severe immunopathology following RSV infection (45). Similarly, NK cell immunity needs to be cautious due to its

potential contribution to immunopathology and eosinophilia upon RSV infection (44). Further studies are required to investigate the molecular mechanisms of CD8 T cells and NK cells in RSV vaccine-induced protection and lung pathology to provide better insight in developing an effective and safe RSV vaccine.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1

Gating strategy for innate immune cells. Alveolar macrophages, CD11b⁻CD11c⁺F4/80⁺; eosinophils, CD11b⁺CD11c⁻SiglecF⁺; neutrophils, CD45⁺CD11b⁺F4/80⁻Ly6c^int; monocytes, CD45⁺CD11b⁺F4/80⁻Ly6c^hi; NK cells, CD45⁺CD3⁻CD49b⁺, pDCs, CD45⁺F4/80⁻CD11c⁺MHCII⁺B220⁺; CD103 DCs, CD45⁺F4/80⁻CD11c⁺MHCII⁺CD103⁺; CD11b DCs, CD45⁺F4/80⁻CD11c⁺MHCII⁺CD11b⁺.

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Supplementary Figure 2

Gating strategy for cytokine-producing lymphocytes. CD4 and CD8 T cells were gated from CD3⁺ cells. Ag-specific cytokine production was evaluated after 5-h RSV F and G peptide treatment.

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Supplementary Figure 3

Histopathological changes in lung. Lung samples were harvested from the naïve mice or the RSV infected mice at day 5 post challenge. Lung sections were stained with H&E.

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REFERENCES

1. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parrott RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am J Epidemiol* 1969;89:422-434.
   PUBMED | CROSSREF
2. Peebles RS Jr, Graham BS. Pathogenesis of respiratory syncytial virus infection in the murine model. *Proc Am Thorac Soc* 2005;2:110-115.
   PUBMED | CROSSREF

3. van Drunen Littel-van den Hurk S, Watkiss ER. Pathogenesis of respiratory syncytial virus. *Curr Opin Virol* 2012;2:300-305.
    PUBMED | **CROSSREF**

4. Openshaw PJ, Clarke SL, Record FM. Pulmonary eosinophilic response to respiratory syncytial virus infection in mice sensitized to the major surface glycoprotein G. *Int Immunol* 1992;4:493-500.
    PUBMED | **CROSSREF**

5. Castilow EM, Varga SM. Overcoming T cell-mediated immunopathology to achieve safe RSV vaccination. *Future Virol* 2008;3:445-454.
    PUBMED | **CROSSREF**

6. Murphy BR, Sotnikov AV, Lawrence LA, Banks SM, Prince GA. Enhanced pulmonary histopathology is observed in cotton rats immunized with formalin-inactivated respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV 3-6 months after immunization. *Vaccine* 1990;8:497-502.
    PUBMED | **CROSSREF**

7. Connors M, Kulkarni AB, Collins PL, Firestone CY, Holmes KL, Morse HC 3rd, Murphy BR. Resistance to respiratory syncytial virus (RSV) challenge induced by infection with a vaccinia virus recombinant expressing the RSV M2 protein (Vac-M2) is mediated by CD8+ T cells, while that induced by Vac-F or Vac-G recombinants is mediated by antibodies. *J Virol* 1992;66:1277-1281.
    PUBMED | **CROSSREF**

8. Kwon YM, Hwang HS, Lee YT, Kim KH, Lee Y, Kim MC, Lee YN, Quan FS, Moore ML, Kang SM. Respiratory syncytial virus fusion protein-encoding DNA vaccine is less effective in conferring protection against inflammatory disease than a virus-like particle platform. *Immune Netw* 2019;19:e18.
    PUBMED | **CROSSREF**

9. Domachowske JB, Rosenberg HF. Respiratory syncytial virus infection: immune response, immunopathogenesis, and treatment. *Clin Microbiol Rev* 1999;12:298-309.
    PUBMED | **CROSSREF**

10. Connors M, Kulkarni AB, Firestone CY, Holmes KL, Morse HC 3rd, Sotnikov AV, Murphy BR. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. *J Virol* 1992;66:7444-7451.
    PUBMED | **CROSSREF**

11. Hwang HS, Lee YT, Kim KH, Ko EJ, Lee Y, Kwon YM, Kang SM. Virus-like particle vaccine primes immune responses preventing inactivated-virus vaccine-enhanced disease against respiratory syncytial virus. *Virology* 2017;511:142-151.
    PUBMED | **CROSSREF**

12. Ko EJ, Kwon YM, Lee JS, Hwang HS, Yoo SE, Lee YN, Lee YT, Kim MC, Cho MK, Lee YR, et al. Virus-like nanoparticle and DNA vaccination confers protection against respiratory syncytial virus by modulating innate and adaptive immune cells. *Nanomedicine (Lond)* 2015;11:99-108.
    PUBMED | **CROSSREF**

13. Hwang HS, Kwon YM, Lee JS, Yoo SE, Lee YN, Ko EJ, Kim MC, Cho MK, Lee YT, Jung YJ, et al. Co-immunization with virus-like particle and DNA vaccines induces protection against respiratory syncytial virus infection and bronchiolitis. *Antiviral Res* 2014;110:115-123.
    PUBMED | **CROSSREF**

14. Graham BS, Bunton LA, Rowland J, Wright PF, Karzon DT. Respiratory syncytial virus infection in anti-mu-treated mice. *J Virol* 1991;65:4936-4942.
    PUBMED | **CROSSREF**

15. Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. *J Clin Invest* 1991;88:1026-1033.
    PUBMED | **CROSSREF**

16. Polack FP, Karron RA. The future of respiratory syncytial virus vaccine development. *Pediatr Infect Dis J* 2004;23:S65-S73.
    PUBMED | **CROSSREF**

17. Prince GA, Horswood RL, Berndt J, Suffin SC, Chanock RM. Respiratory syncytial virus infection in inbred mice. *Infect Immun* 1979;26:764-766.
    PUBMED | **CROSSREF**

18. Altamirano-Lagos MJ, Díaz FE, Mansilla MA, Rivera-Pérez D, Soto D, McGill JL, Vasquez AE, Kalergis AM. Current animal models for understanding the pathology caused by the respiratory syncytial virus. *Front Microbiol* 2019;10:873.
    PUBMED | **CROSSREF**

19. Quan FS, Kim Y, Lee S, Yi H, Kang SM, Bozja J, Moore ML, Compans RW. Viruslike particle vaccine induces protection against respiratory syncytial virus infection in mice. *J Infect Dis* 2011;204:987-995.
    PUBMED | **CROSSREF**

20. Lee Y, Lee YT, Ko EJ, Kim KH, Hwang HS, Park S, Kwon YM, Kang SM. Soluble F proteins exacerbate pulmonary histopathology after vaccination upon respiratory syncytial virus challenge but not when presented on virus-like particles. *Hum Vaccin Immunother* 2017;13:2594-2605.
   PUBMED | **CROSSREF**

21. Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc Natl Acad Sci U S A* 1998;95:15553-15558.
   PUBMED | **CROSSREF**

22. Visciano ML, Tagliamonte M, Tornesello ML, Buonaguro FM, Buonaguro L. Effects of adjuvants on IgG subclasses elicited by virus-like particles. *J Transl Med* 2012;10:4.
   PUBMED | **CROSSREF**

23. Everard ML, Swarbrick A, Wrightham M, McIntyre J, Dunkley C, James PD, Sewell HF, Milner AD. Analysis of cells obtained by bronchial lavage of infants with respiratory syncytial virus infection. *Arch Dis Child* 1994;71:428-432.
   PUBMED | **CROSSREF**

24. Christiaansen AF, Knudson CJ, Weiss KA, Varga SM. The CD4 T cell response to respiratory syncytial virus infection. *Immunol Res* 2014;59:109-117.
   PUBMED | **CROSSREF**

25. Knudson CJ, Hartwig SM, Meyerholz DK, Varga SM. RSV vaccine-enhanced disease is orchestrated by the combined actions of distinct CD4 T cell subsets. *PLoS Pathog* 2015;11:e1004757.
   PUBMED | **CROSSREF**

26. Kim KH, Lee YT, Hwang HS, Kwon YM, Jung YJ, Lee Y, Lee JS, Lee YN, Park S, Kang SM. Alum adjuvant enhances protection against respiratory syncytial virus but exacerbates pulmonary inflammation by modulating multiple innate and adaptive immune cells. *PLoS One* 2015;10:e0139916.
   PUBMED | **CROSSREF**

27. Moghaddam A, Olszewska W, Wang B, Tregoning JS, Helson R, Sattentau QJ, Openshaw PJ. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. *Nat Med* 2006;12:905-907.
   PUBMED | **CROSSREF**

28. Boelen A, Andeweg A, Kwakkel J, Lokhorst W, Bestebroer T, Dormans J, Kimman T. Both immunisation with a formalin-inactivated respiratory syncytial virus (RSV) vaccine and a mock antigen vaccine induce severe lung pathology and a Th2 cytokine profile in RSV-challenged mice. *Vaccine* 2000;19:982-991.
   PUBMED | **CROSSREF**

29. Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. Respiratory synctial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. *J Virol* 1996;70:2852-2860.
   PUBMED | **CROSSREF**

30. Prince GA, Denamur F, Deschamps M, Garçon N, Prieels JP, Slaoui M, Thiriart C, Porter DD. Monophosphoryl lipid A adjuvant reverses a principal histologic parameter of formalin-inactivated respiratory syncytial virus vaccine-induced disease. *Vaccine* 2001;19:2048-2054.
   PUBMED | **CROSSREF**

31. Kamphuis T, Meijerhof T, Stegmann T, Lederhofer J, Wilschut J, de Haan A. Immunogenicity and protective capacity of a virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid A in mice. *PLoS One* 2012;7:e36812.
   PUBMED | **CROSSREF**

32. Johnson TR, Teng MN, Collins PL, Graham BS. Respiratory syncytial virus (RSV) G glycoprotein is not necessary for vaccine-enhanced disease induced by immunization with formalin-inactivated RSV. *J Virol* 2004;78:6024-6032.
   PUBMED | **CROSSREF**

33. Vremec D, Pooley J, Hochrein H, Wu L, Shortman K. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J Immunol* 2000;164:2978-2986.
   PUBMED | **CROSSREF**

34. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 2013;31:563-604.
   PUBMED | **CROSSREF**

35. Cannon MJ, Stott EJ, Taylor G, Askonas BA. Clearance of persistent respiratory syncytial virus infections in immunodeficient mice following transfer of primed T cells. *Immunology* 1987;62:133-138.
   PUBMED

36. Cannon MJ, Openshaw PJ, Askonas BA. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. *J Exp Med* 1988;168:1163-1168.
   PUBMED | **CROSSREF**

37. Ostler T, Davidson W, Ehl S. Virus clearance and immunopathology by CD8(+) T cells during infection with respiratory syncytial virus are mediated by IFN-gamma. *Eur J Immunol* 2002;32:2117-2123.
    PUBMED | **CROSSREF**

38. Retamal-Díaz A, Covián C, Pacheco GA, Castiglione-Matamala AT, Bueno SM, González PA, Kalergis AM. Contribution of resident memory CD8+ T cells to protective immunity against respiratory syncytial virus and their impact on vaccine design. *Pathogens* 2019;8:147.
    PUBMED | **CROSSREF**

39. Olson MR, Varga SM. CD8 T cells inhibit respiratory syncytial virus (RSV) vaccine-enhanced disease. *J Immunol* 2007;179:5415-5424.
    PUBMED | **CROSSREF**

40. Bhat R, Farrag MA, Almajhdi FN. Double-edged role of natural killer cells during RSV infection. *Int Rev Immunol* 2020;39:233-244.
    PUBMED | **CROSSREF**

41. Vankayalapati R, Klucar P, Wizel B, Weis SE, Samten B, Safi H, Shams H, Barnes PF. NK cells regulate CD8+ T cell effector function in response to an intracellular pathogen. *J Immunol* 2004;172:130-137.
    PUBMED | **CROSSREF**

42. Crouse J, Xu HC, Lang PA, Oxenius A. NK cells regulating T cell responses: mechanisms and outcome. *Trends Immunol* 2015;36:49-58.
    PUBMED | **CROSSREF**

43. Hussell T, Openshaw PJ. Intracellular IFN-gamma expression in natural killer cells precedes lung CD8+ T cell recruitment during respiratory syncytial virus infection. *J Gen Virol* 1998;79:2593-2601.
    PUBMED | **CROSSREF**

44. Li F, Zhu H, Sun R, Wei H, Tian Z. Natural killer cells are involved in acute lung immune injury caused by respiratory syncytial virus infection. *J Virol* 2012;86:2251-2258.
    PUBMED | **CROSSREF**

45. Schmidt ME, Knudson CJ, Hartwig SM, Pewe LL, Meyerholz DK, Langlois RA, Harty JT, Varga SM. Memory CD8 T cells mediate severe immunopathology following respiratory syncytial virus infection. *PLoS Pathog* 2018;14:e1006810.
    PUBMED | **CROSSREF**