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Critical role of OX40/OX40L in ILC2-mediated activation of CD4$^+$T cells during respiratory syncytial virus infection in mice

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ABSTRACT

CD4$^+$T cells are crucial cellular source of type 2 cytokines and responsible for RSV-induced asthma-like symptoms and asthma exacerbations. However, the mechanism for regulating the activation of CD4$^+$T cells during RSV infection is not clear completely. We show in this study that infection with RSV may induce an expansion and activation of CD4$^+$T cells in the lungs of BALB/c mice. RSV-induced CD4$^+$T cell expansion and activation seems to depend upon the pulmonary group 2 innate lymphoid cells (ILC2s), since adoptive transfer of lung ILC2s can enhance not only the numbers of CD4$^+$T cells but also the cytokine production by CD4$^+$T cells. Interestingly, blockade of the contact between ILC2s and CD4$^+$T cells, may significantly diminish the CD4$^+$T cell expansion and cytokine production, suggesting that membrane molecules may be involved in ILC2-regulated CD4$^+$T cell activation. In fact, infection with RSV resulted in an increase in the numbers of OX40$^+$CD4$^+$T cells as well as OX40L$^+$ILC2s in the lungs of mice. Moreover, the mRNA expressions of OX40 and OX40L as well as the levels of OX40 and OX40L proteins in the lung CD4$^+$T cells and ILC2s were elevated respectively. When co-culture of CD4$^+$T cells with ILC2s in the presence of anti-OX40L antibody, the cytokine productions by CD4$^+$T cells were reduced markedly, suggesting that lung ILC2s may regulate RSV-induced CD4$^+$T cell expansion and activation perhaps via OX40/OX40L interaction.

1. Introduction

Respiratory syncytial virus (RSV) is a major cause of respiratory disease worldwide. In addition to causing acute respiratory failure, RSV infection is notably associated with a greater risk of asthma and recurrent wheezing later in life [1,2]. Although the precise mechanisms involved in RSV-induced pathogenesis are not known quite well, a relative predominance of Th2 over Th1 cytokines has been demonstrated to be critical for the development of asthma and asthma exacerbations by RSV infection [3,4].

Type 2 cytokines, especially interleukin (IL)-4, IL-5 and IL-13, play key roles in virus-induced airway inflammation and airway hyperresponsiveness (AHR) [5]. IL-4, for instance, is important for IgE synthesis, while IL-13 is required for induction of AHR, mucus hyperproduction and airway remodeling. IL-5, however, recruits and activates eosinophils [6–8]. It is undeniable that the activated CD4$^+$T cells are the major source of type 2 cytokines and contribute to RSV-induced asthma-like symptoms and asthma exacerbations. However, up to now, the potential mechanisms for regulating CD4$^+$T cell activation during RSV infection are not full defined.

It has been reported that group 2 innate lymphoid cells (ILC2s), a novel population of non-B/non-T innate immune cells characterized by the absence of lineage markers (Lin-) and by expression of Sca-1, c-Kit and the IL-33 receptor ST2 [9], can initiate adaptive T cell responses in allergic inflammation [10]. Deficiency of ILC2s results in impairment in allergen-induced expansion and differentiation of Th2 cells [11]. Further analysis showed that the impaired differentiation of naive T cells into Th2 cells in ILC2-deficient mice was rescued by ILC2s transplantation [12], suggesting that ILC2s are a key factor for generation of Th2 cell expansion and activation. However, whether ILC2s regulate CD4$^+$T cell activation during RSV infection and which molecules involved in ILC2-regulated CD4$^+$T cell activation remain unclear.

CD4$^+$T cells express OX40 (also known as CD134) after T cell receptor (TCR)-mediated activation, while ILC2s can express its binding partner OX40 ligand (OX40L; also known as CD252) [13,14]. Ligation of OX40 by OX40L provides a critical signal for the expansion and

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survival of Th2 cells in multiple animal models of autoimmune and inflammatory disease [15–18]. In fact, the numbers of $CD4^+$ effector T cells were elevated significantly in OX40L transgenic mice [19,20]. Meanwhile, blockade of OX40L results in a deficiency in antigen-induced $CD4^+$T cell responses as well as reduced severity of Th2 diseases [21]. An in vitro study found that ILC2s can co-stimulate naïve T cell activation via OX40L molecules [14]. Indeed, treatment of ILC2s with anti-OX40L antibody significantly inhibits IL-4, IL-5, and IL-13 production by CD3/CD28-activated $CD4^+$T cells [14], suggesting that OX40/OX40L interaction may contribute to ILC2-regulated $CD4^+$T cell activation.

However, whether ILC2s as well as OX40L expressed on ILC2s are contribute to the expansion and activation of $CD4^+$T cells during RSV infection are still unknown. Thus, in the present study, by using BALB/c mice that were infected intranasally with respiratory syncytial virus, the regulatory role of ILC2s as well as OX40/OX40L interaction in ILC2-regulated $CD4^+$T cell proliferation and activation was investigated.

2. Materials and methods

2.1. Mice and virus stocks

Female specific-pathogen-free (SPF) BALB/c mice, 8–10 weeks of age, were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Mice had fresh water and autoclaved food and maintained in individual filter cages at the Laboratory Animal Center, China Medical University. The animal protocol was approved by the Institutional Animal Care and Use Committee of China Medical University, China. Human RSV type A2 (RSV A2) strain was grown and assayed for infectivity (expressed as a 50% tissue culture infectious dose, TCID50) in HEp2 cells (ATCC) as previously described [22].

2.2. Infection and adoptive transfer experiment

Mice were inoculated intranasally with RSV at an inoculum dose of 2×10^6 TCID50 per mouse in 20 μl of sterile phosphate-buffered saline (PBS). As a control group (expressed as day 0 in figures), mice were inoculated intranasally with sterile PBS. For adoptive transfer experiment, lung cells from ten mice that were infected with RSV at day 3, were pooled, and $CD45^+$ Lin^- $ST2^+$ cells, which were identified as ILC2s in this study, were isolated by using a MoFlo high-speed cell sorter (Miltenyi). 5×10^5 of ILC2s (purity was > 86%) in 200 μl of PBS were injected via the tail vein into mouse 2 h before RSV infection. At intervals, samples were collected.

2.3. Lung cell preparation

Mice were anesthetized and flushed the pulmonary circulation with 20 ml of PBS to remove intravascular blood pool. Then, lung was minced and incubated with 200 μg/ml collagenase D and 40 μg/ml DNase I (Roche Molecular Biochemicals) at 37 °C for 1 h on a rocker. The digested lung tissue was passed through a stainless steel mesh, and lung cells were collected by density-gradient centrifugation with lymphocyte separation solution.

2.4. Antibodies and flow cytometry

Lung cells were blocked with anti-mouse CD16/32 and then stained with the following specific antibodies to CD45, ST2, CD3, CD4, OX40, OX40L (eBioscience). Lineage cocktail included antibodies to CD3, CD4, CD5, CD8, CD11b, Gr-1, CD19, B220, DX5 (or NK1.1) and TCRδ (eBioscience). The Cytofix/Cytoperm Buffer Set (BD Biosciences) was used for staining of intracellular cytokines, accordance with the manufacturer's instructions.

2.5. Real-time RT-PCR

$CD4^+$T cells and ILC2s were sorted from the lungs of RSV-infected mice. Total RNA was isolated from the purified cell populations using TRIzol reagent (Life Technologies) and converted to cDNA with a SuperScript III Reverse Transcriptase using Oligo (dT) primers (Life Technologies). Quantitative real-time PCR was performed using SYBR Green Master Mix (Life Technologies). Primers used for the detection of expression of mRNAs for IL-2, IFN-γ, IL-4, IL-5, IL-13, OX40 and OX40L were design as IL-2-F, 5′-CTCCCATGACAAATCGAGAAAGC-3′, IL-2-R, 5′-ACTCTGTCCTTCCACGAAATGAT-3′; IFN-γ-F, 5′-TATCTGGAGGAACTGGCAAA-3′,

IFN-γ-R, 5′-GGTGTGATTCAATGACGCTT-3′; IL-4-F, 5′-TGTACCAGGAGCCATATCCA-3′, IL-4-R, 5′-TTCTTCGTTGCTGTGAGGAC-3′; IL-5-F, 5′-GGCTTCCTGTCCCTACTCAT-3′, IL-5-R, 5′-TCCTCGCCACACTTCTCTTT-3′; IL-13-F, 5′-AGCATGGTATGGAGTGTGGA-3′,

IL-13-R, 5′-TTGCAATTGGAGATGTTGGT-3′; OX40-F, 5′-GGCCCTGCATTTGCTGTTCT-3′, OX40-R, 5′- AGGATATGGGTTGTCCGTGC-3′; OX40L-F, 5′-TGCTCCTGATACTCTCTGCG-3′, OX40L-R, 5′-AGAAAGAACCTGTGTCCCGT-3′; β-actin-F, 5′-CAACGAGCGGTTCCGATG-3′, β-actin-R, 5′-GCCACAGGATTCCATACCCA-3′. Real-time RT-PCR was run in a LightCycler®480 (Roche Molecular Biochemicals) under identical amplification conditions. Results are normalized to β-actin expression and presented as fold change (fold change = $2^{-\triangle\triangle Ct}$).

2.6. Co-culture experiment

1×10^6 of lung ILC2s and equal numbers of splenic $CD4^+$T cells, both from the mice on day 3 after RSV infection, were co-cultured in vitro in the presence or absence of 0.5 μg/ml anti-OX40L mAbs (BD Pharmingen). In other experiments, 0.4 μm Transwell porous 24-well plates (Corning Life Sciences) were used for blocking cell-cell contact. 24 h after incubation, the expression of mRNAs for type 2 cytokines in the cultured $CD4^+$T cells was performed by Real-time RT-PCR method.

2.7. Western blot analysis

Lung cells were lysed by using RIPA buffer (Cell Signaling, Boston, MA) containing a cocktail of protease inhibitor (Roche, USA). After heated, the samples were loaded into a 10% acrylamide gel, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were then transferred onto polyvinylidene difluoride (PVDF, Millipore, Billerica, MA, USA) membranes. After blocking the membranes with 5% bovine serum albumin, anti-OX40 or OX40L mAbs (Cell Signaling, Boston, MA, USA) were added. Then, the membranes were incubated with corresponding secondary antibody. Protein bands were determined by chemiluminescent imaging systems (Amersham, Freiburg, Germany). The expression of a target protein was normalized to that of GAPDH.

2.8. Statistical analysis

Data are presented as the mean ± SD of six to ten mice in each group. Statistical analyses were performed using Prism 6 (GraphPad Software). One-way ANOVA with a Tukey post-test was used to compare differences between groups. P values < 0.05 were considered significant.

3. Results

3.1. Infection with RSV induces the expansion and activation of $CD4^+$T cells in the lungs

Mice were infected with RSV at an inoculum dose of 2×10^6 TCID50 per mouse. The proliferation and activation of $CD4^+$T cells in the lungs of tested mice were assayed. It is clear that infection with RSV

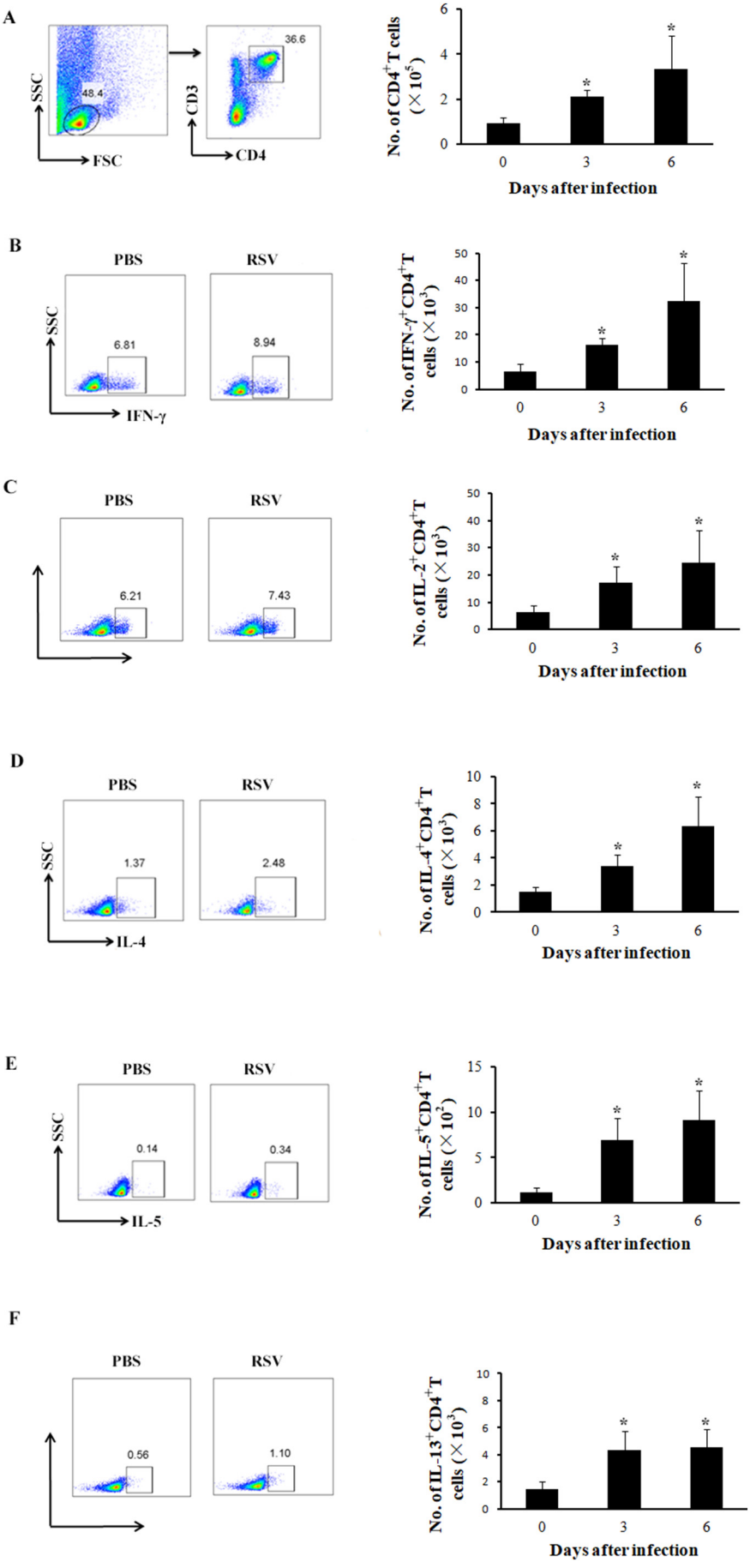

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Fig. 1. The expansion of CD4$^+$T cells in the lungs of RSV-infected mice. BALB/c mice were infected with RSV at an inoculum dose of 2×10^6 TCID50 per mouse. The numbers of total CD4$^+$T cells (A), IFN-γ-producing CD4$^+$T cells (B), IL-2-producing CD4$^+$T cells (C), IL-4-producing CD4$^+$T cells (D), IL-5-producing CD4$^+$T cells (E) and IL-13-producing CD4$^+$T cells (F) in the lungs of tested mice were detected by flow cytometry. Data are mean ± SD of the results for each group of five mice tested.
Significant difference (*$P < 0.05$ by ANOVA), compared with PBS-inoculated mice.

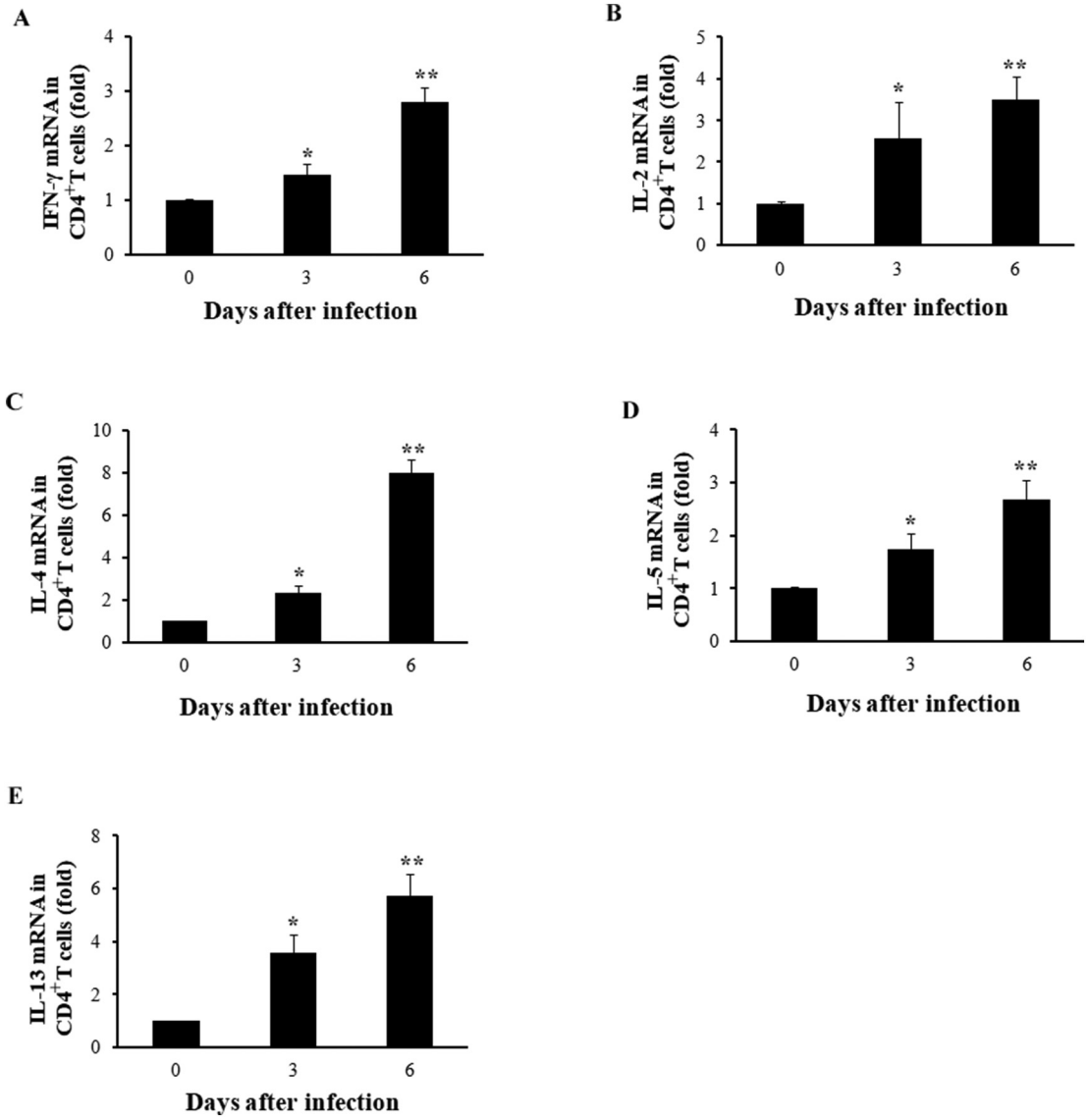

Fig. 2. Infection with RSV enhances the expression of cytokine mRNAs in lung CD4$^+$T cells. Mice were infected with RSV. At intervals, CD4$^+$T cells were isolated from the lungs of RSV-infected mice. The relative expression of mRNAs for IFN-γ (A), IL-2 (B), IL-4 (C), IL-5 (D) and IL-13 (E) in CD4$^+$T cells were determined by Real-time RT-PCR method. Data are mean ± SD of the results for each group of five mice tested.
Significant difference (*$P < 0.05$, **$P < 0.01$ by ANOVA), compared with PBS-inoculated mice.

resulted in an increase in the numbers of total CD4$^+$T cells as well as Th1/Th2-producing CD4$^+$T cells in the lungs (Fig. 1). The relative expression of mRNAs for IFN-γ, IL-2, IL-4, IL-5 and IL-13 in CD4$^+$T cells was also enhanced significantly after RSV infection (Fig. 2), demonstrating that RSV infection can activate CD4$^+$T cells.

3.2. Adoptive transfer of pulmonary ILC2s augments the expansion and activation of CD4$^+$T cells in the lungs

To investigate whether ILC2s contribute to RSV-induced CD4$^+$T cell activation, CD45$^+$Lin$^-$ ST2$^+$ ILC2s were isolated from the lungs of mice on day 3 after infection, and transferred into the corresponding normal mice 2 h before RSV infection. The changes in the numbers of CD4$^+$Tcells as well as the ability to produce Th1/Th2 cytokines by CD4$^+$T cells in the lungs of mice were investigated. As shown in Fig. 3, adoptive transfer of pulmonary ILC2s enhanced the absolute numbers of lung CD4$^+$T cells, in parallel with an augmented expression of Th1/Th2 cytokines, particularly IL-4, IL-5 and IL-13 in the lung CD4$^+$T cells, suggesting that pulmonary ILC2s may act as a promoter for RSV-induced CD4$^+$T cell activation.

3.3. The effect of ILC2s on CD4$^+$T cell activation is dependent upon cell-cell contact

To further confirm the possible ways in which ILC2s stimulate CD4$^+$T cell activation, lung ILC2s and splenic CD4$^+$T cells were

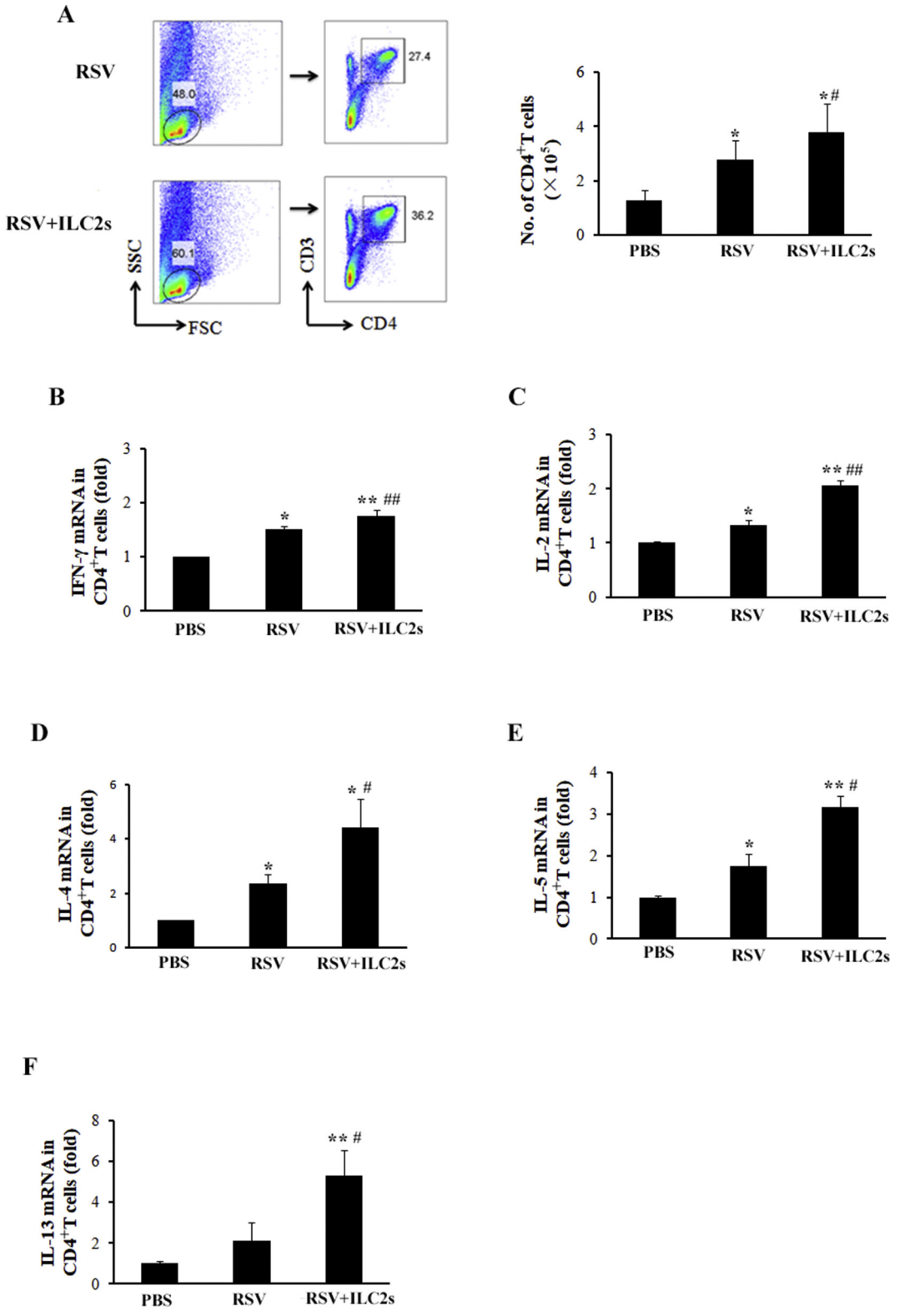

Fig. 3. Adoptive transfer of pulmonary ILC2s promotes the expansion and activation of $CD4^+$T cells in the lungs. 5×10^5 of $CD45^+Lin^-ST2^+$ILC2s were pooled from the lungs of RSV-infected mice and injected via the tail vein into normal mouse 2 h before RSV infection. At day 3 after infection, the absolute numbers of total $CD4^+$T cells (A) as well as the expressions of mRNAs for IFN-γ (B), IL-2 (C), IL-4 (D), IL-5 (E) and IL-13 (F) in the lung $CD4^+$T cells were analyzed. Data are mean ± SD of the results for each group of five mice tested.

Significant difference ($^*P < 0.05$, $^{**}P < 0.01$ by ANOVA), compared with PBS-inoculated mice.

Significant difference ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$ by ANOVA), compared with corresponding non-transferred mice.

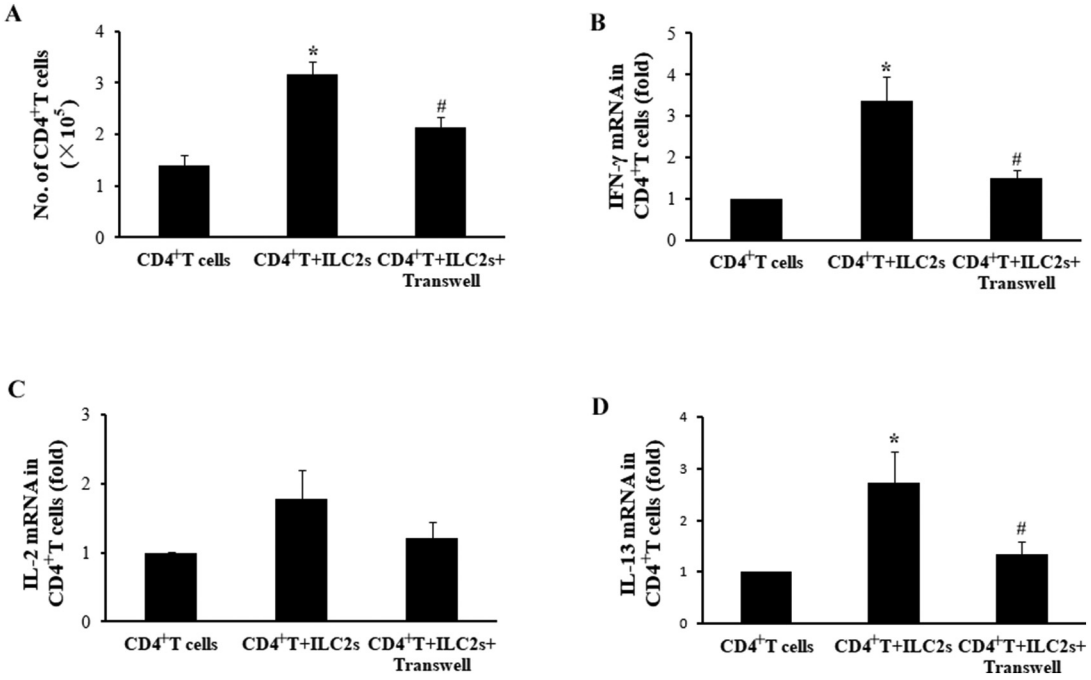

Fig. 4. Blockade of cell-cell contact between CD4$^+$T cells and ILC2s diminishes the activation of CD4$^+$T cells in the lungs of RSV-infected mice. Pulmonary ILC2s and splenic CD4$^+$T cells were collected from BALB/c mice at day 3 after RSV infection, and co-cultured in vitro by using Transwell system. 24 h after co-incubation, the number of CD4$^+$T cells (A) as well as the expression of mRNAs for IFN-γ (B), IL-2 (C) and IL-13 (D) in the cultured CD4$^+$T cells was performed by Real-time RT-PCR method. Data are expressed as mean ± SD.
Significant difference (*$P < 0.05$ by ANOVA), compared with the group of CD4$^+$T cells.
Significant difference ($^{\#}$P < 0.05 by ANOVA), compared with corresponding CD4$^+$T + ILC2s group.

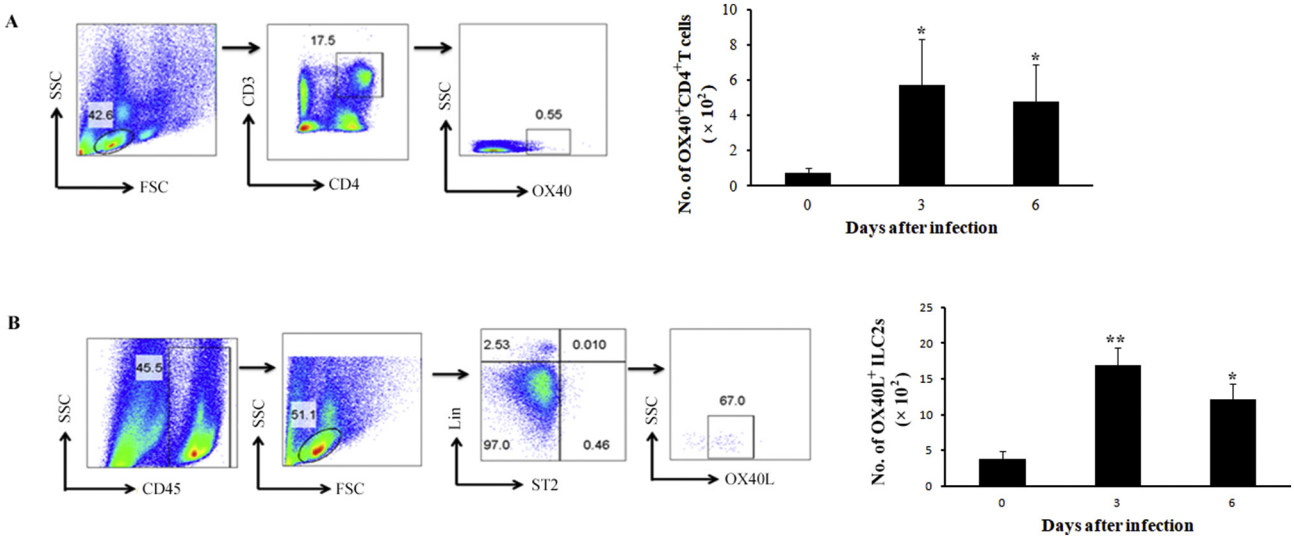

Fig. 5. Infection with RSV increases the numbers of OX40-expressed CD4$^+$T cells and OX40L-expressed ILC2s in the lungs. Mice were infected with RSV and at intervals the numbers of OX40$^+$CD4$^+$T cells (A) as well as OX40L$^+$ILC2s (B) in the lungs of BALB/c mice were detected. Data are mean ± SD of the results for each group of five mice tested.
Significant difference (*P < 0.05, **$P < 0.01$ by ANOVA), compared with PBS-inoculated mice.

collected from the mice on day 3 after RSV infection, and co-cultured in vitro by using Transwell culture system. We found that when blocking cell-cell contact, not only the numbers of CD4$^+$T cells but also the mRNA expressions of IFN-γ and IL-13 in CD4$^+$T cells were reduced significantly (Fig. 4), suggesting that the molecules in the membrane of these cells may contribute to ILC2-regulated CD4$^+$T cell activation during RSV infection. It should be noted that whether co-culture of CD4$^+$T cells with ILC2s or blocking the cell-cell contact, the expression of mRNAs for IL-4 and IL-5 in the cultured CD4$^+$T cells was not altered (data not shown), suggesting that ILC2s might be inefficiency in regulating the production of IL-4 and IL-5 by CD4$^+$T cells in ex vivo culture system.

3.4. OX40/OX40L interaction plays a key role in ILC2-promoted CD4$^+$T cell activation during RSV infection

It has been reported that OX40-OX40L interaction may contribute to CD4$^+$T cell survival, proliferation, and memory cell generation

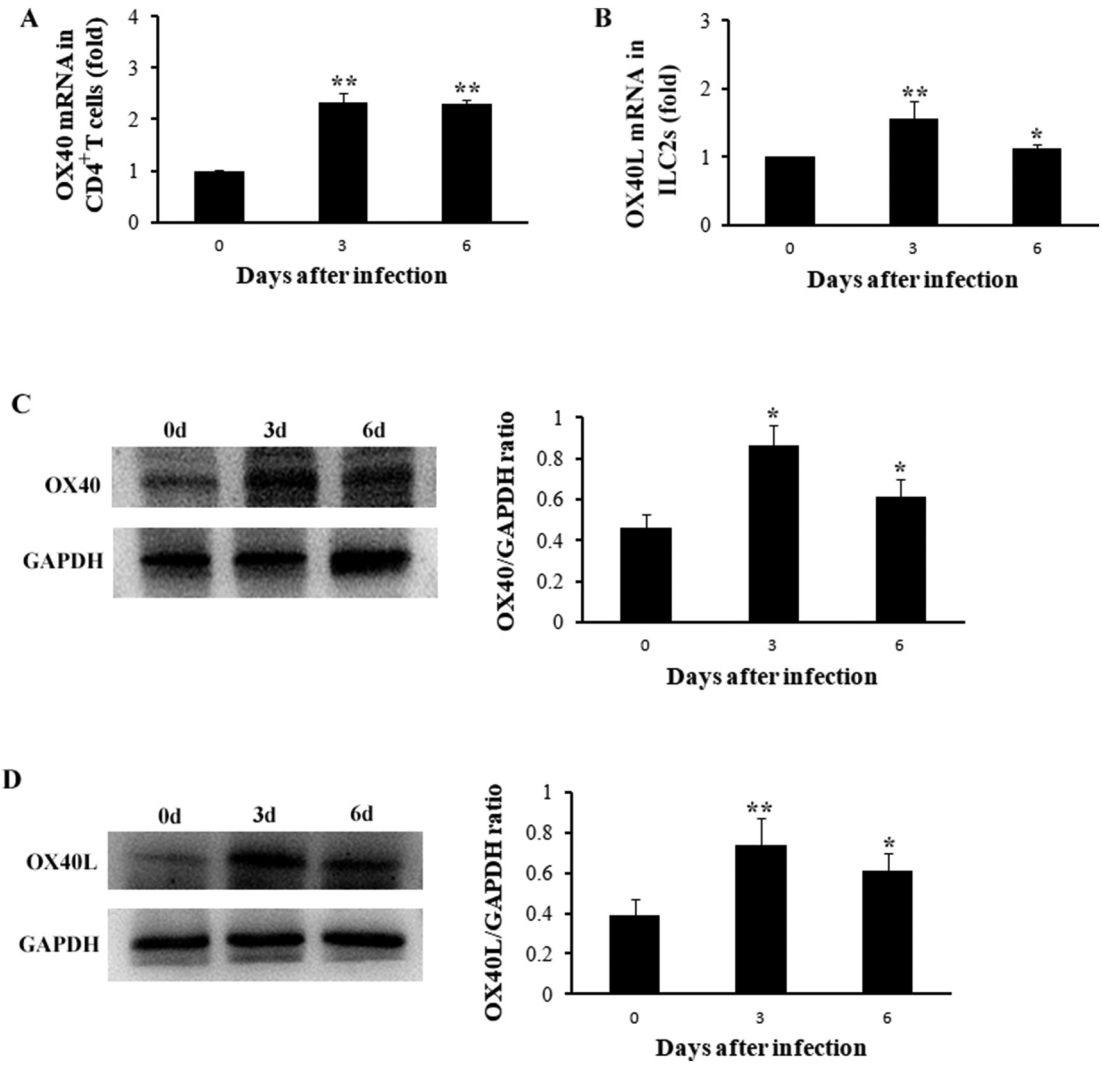

Fig. 6. RSV infection promotes the expressions of OX40/OX40L in $CD4^+$T cells/ILC2s in the lungs of mice. On day 3 and 6 after RSV infection, the $CD3^+CD4^+$T cells and $CD45^+Lin^-ST2^+$ILC2s were sorted from the lungs of RSV-infected mice. The relative expression of OX40 mRNA (A) and OX40 proteins (C) in $CD4^+$T cells as well as OX40L mRNA (B) and OX40L proteins (D) in ILC2s were determined. Data are mean ± SD of the results for each group of five mice tested. Significant difference (*$P < 0.05$, **$P < 0.01$ by ANOVA), compared with PBS-inoculated mice.

[15–17]. Thus, in the present study, the possible roles of OX40 and OX40L in ILC2-regulated $CD4^+$ T cell activation during RSV infection were investigated. It became clear that infection with RSV resulted in an increase in the numbers of $OX40^+CD4^+$T cells and $OX40L^+$ILC2s in the lungs of BALB/c mice (Fig. 5). Moreover, the levels of OX40 mRNA and proteins in the lung $CD4^+$T cells as well as OX40L mRNA and proteins in the lung ILC2s were augmented significantly (Fig. 6), suggesting that OX40/OX40L may be involved in ILC2-regulated $CD4^+$ T cell activation during RSV infection.

To further define the effect of OX40-OX40L interaction on ILC2-regulated $CD4^+$T cell activation, lung ILC2s and splenic $CD4^+$T cells that were isolated from the tested mice on day 3 after RSV infection, were co-cultured in vitro in the presence or absence of anti-OX40L mAbs. As expected, anti-OX40L mAbs treatment significantly decreased the expression of IFN-γ, IL-2 and IL-13 mRNAs in $CD4^+$T cells (Fig. 7), demonstrating that ILC2s may promote $CD4^+$T cell activation in an OX40/OX40L-dependent manner.

4. Discussion

$CD4^+$T cells are crucial cellular source of type 2 cytokines and mainly responsible for RSV-induced asthma-like symptoms and asthma exacerbations [23]. In the present study, by using RSV-infected BALB/c mice, we found that RSV infection can promote $CD4^+$T cell expansion and cytokine expression, particularly type 2 cytokine expression in the lungs of the tested mice (Figs. 1, 2), demonstrating that $CD4^+$T cells may contribute to RSV-induced airway pathogenesis by secreting large amounts of illness-related cytokines.

To acquire a unique cytokine-producing profile, naive $CD4^+$ T cells need to go through an activation and differentiation process. There is no doubt that dendritic cells (DC) and cytokines are critical for inducing activation and differentiation of naïve $CD4^+$T cells [23]. However, other immune cells, for example, group 2 innate lymphoid cells and some molecules may also be involved in $CD4^+$T cell activation. Indeed, RSV-induced $CD4^+$T cell expansion and activation seems likely to rely on lung ILC2s, since adoptive transfer of lung ILC2s can enhance not only the numbers of $CD4^+$T cells but also the ability of type 2 cytokine production by $CD4^+$T cells (Fig. 3), suggesting that ILC2s may play an important role in activation of $CD4^+$T cells during RSV infection. In fact, ILC2s can prime the adaptive type 2 immune responses to inhaled allergens, including the recruitment of eosinophils, Th2 cytokine production and serum IgE levels [12]. When lung ILC2s were co-cultured with naïve splenic $CD4^+$T cells, the proliferation of $CD4^+$T cells as well as type 2 cytokine production by $CD4^+$T cells was clearly enhanced in

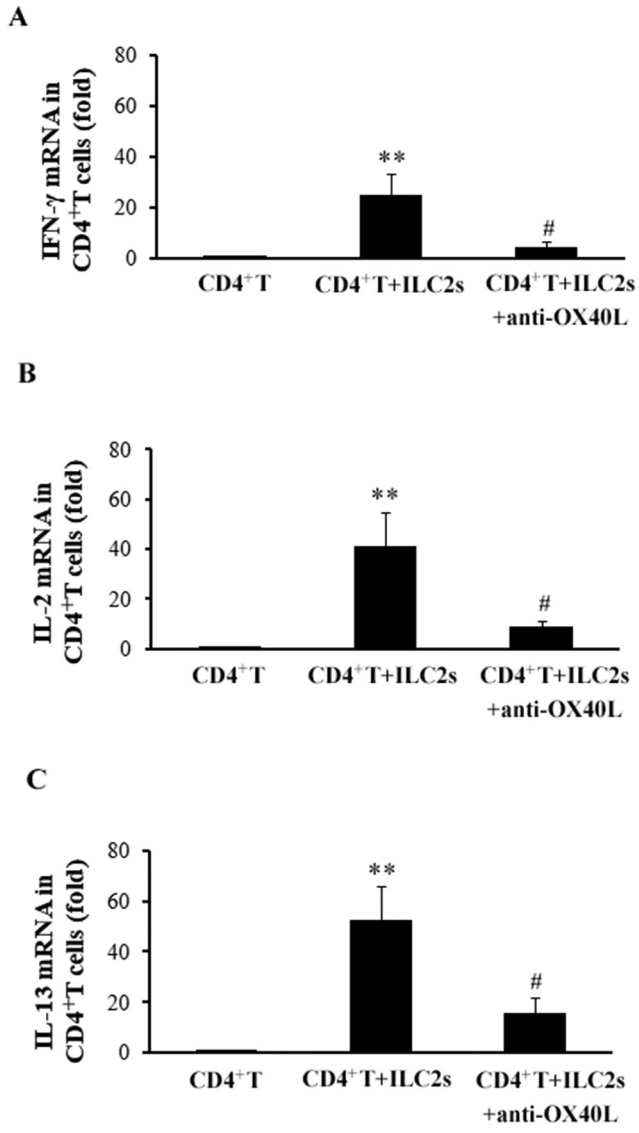

Fig. 7. OX40-OX40L interaction contributes to ILC2-regulated $CD4^+$T cell activation. Pulmonary ILC2s and splenic $CD4^+$T cells were sorted from the lungs of RSV-infected mice at day 3 after infection. Then, the ILC2s and $CD4^+$T cells were co-cultured in vitro in the presence or absence of anti-OX40L mAbs. The expression of IFN-γ (A), IL-2 (B) and IL-13 (C) mRNAs in the $CD4^+$T cells was detected. Data are expressed as mean ± SD.
Significant difference (**P < 0.01 by ANOVA), compared with the group of $CD4^+$T cells.
Significant difference ($^{\#}$P < 0.05 by ANOVA), compared with corresponding $CD4^+$T + ILC2s group.

an allergic animal model [14]. Really, ILC2s can produce large amounts of IL-13 to recruit DC into lymph node and present antigen to protease-specific $CD4^+$T cells [11]. In addition, some ILC2s can express major histocompatibility complex class II molecule and act as antigen-present cells to activate $CD4^+$T cells [24,25]. These reports together with our data confirm that ILC2s may be important for regulating the activation and differentiation of $CD4^+$T cells during RSV infection.

Interestingly, ILC2-regulated $CD4^+$T cell activation during RSV infection seems to depend upon cell-cell contact. When blocking cell-cell contact between ILC2s and $CD4^+$T cells by using Transwell system, the proliferation and activation of $CD4^+$T cells, which were isolated from the lungs of RSV-infected mice on day 3 after RSV infection, were reduced markedly (Fig. 4), suggesting that membrane molecules may contribute to ILC2-promoted $CD4^+$T cell activation after RSV infection.

It has been reported that OX40-OX40L interaction plays a key role in regulating the division and survival of conventional T cells, which has led to the often-used description of OX40 as a costimulatory receptor for T cells [26]. Certainly, the inhibition of OX40L suppressed TSLP-mediated Th2 inflammation in murine and nonhuman primate models of asthma [27]. Moreover, the neutralizing anti-OX40L antibody can diminish the activity of $CD4^+$T cells to secrete cytokines [26], suggesting that OX40/OX40L interaction may be important for $CD4^+$T cell activation. It is clear that ILC2s express high levels of OX40L and ILC2-targeted deletion of OX40L significantly impairs Th2 expansion following allergen exposure and Nippostrongylus brasiliensis helminth infection [28]. In the present study, infection with RSV not only increased the numbers of $OX40^+CD4^+$T cells and $OX40L^+$ILC2s in the lungs (Fig. 5), but also enhanced the expressions of OX40/OX40L mRNAs as well as OX40/OX40L proteins in the lung $CD4^+$T cells/ILC2s, respectively (Fig. 6), suggesting that OX40/OX40L molecules may be involved in ILC2-regulated $CD4^+$T cell activation during RSV infection. Indeed, when co-culture of $CD4^+$T cells with lung ILC2s in the presence of anti-OX40L antibody, the cytokine production by $CD4^+$T cells was reduced significantly (Fig. 7), further demonstrating that ILC2s may promote $CD4^+$T cell activation in an OX40/OX40L-dependent manner.

In summary, in this study, the major observation is that lung ILC2s may regulate RSV-induced $CD4^+$T cell expansion and activation via OX40/OX40L interaction. Our results might provide a novel understanding of the underlying mechanisms of respiratory tract diseases mediated by type 2 immunity and be useful to improve the insights and therapies for RSV-induced or RSV-aggravated asthma.

Author contributions

BL designed the experiments. YC, SB, NZ, WZ and JW performed the experiments. YC analyzed the data. JW and BL contributed to the writing and editing of the manuscript.

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Declaration of Competing Interest

The authors declare no financial or commercial conflict interest.

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