METHODS

Antibodies

Rat anti-mouse OX40L mAb (RM134L, rat IgG_{2b}) was isolated from ascitic fluid (QED Bioscience, San Diego, Calif) by using ammonium sulfate precipitation and desalted by means of gel filtration (GE Healthcare, Pittsburgh, Pa). The hybridoma cell line was kindly provided by Dr Hideo Yagita (Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan). Anti-TSLP mAb (M702, rat IgG^{2a}) was prepared and used as previously described.[E1] Isotype control antibody was obtained from BD Biosciences (San Diego, Calif).

Animals

BALB/c mice were obtained from the Jackson Laboratory (Bar Harbor, Me).

Virus preparation

Human RSV A2 Strain (catalog no. VR-1302) and HEp-2 cells (catalog no. CCL-23) were obtained from American Type Culture Collection (Rockville, Md), stocks of purified RSV were prepared, and viral titers were determined.[E2]

RSV infection and treatments

Mice were inoculated with 10^6 plaque-forming units of purified RSV at the indicated age. Anti-OX40L or control antibody was administered intraperitoneally at 15 mg/kg 1 day before primary or secondary RSV infection and on days 1 and 2 after infection. Secondary RSV infection was initiated 5 weeks after primary infection. Airway function and inflammation were assessed on day 7 after either primary or secondary RSV infection. Anti-TSLP (50 μg per pup) or control antibody was administered intraperitoneally 1 day before primary RSV infection in neonates.

Assessment of airway function

Airway function was assessed in anesthetized, tracheostomized, and mechanically ventilated animals by measuring changes in airway resistance in response to increasing doses of inhaled MCh (Sigma-Aldrich, St Louis, Mo), as previously described.[E3] Data are expressed as the percentage of baseline airway resistance obtained after saline challenge.

Measurement of cytokine levels

IFN-γ, IL-4, IL-5, and IL-6 levels were measured in BAL fluid by using commercial ELISA kits, according to the manufacturer's instructions (eBioscience), as was IL-13 (R&D Systems).

Flow cytometry

Lung single-cell suspensions were prepared as previously described.[E4] Briefly, mice were perfused with 10 mL of PBS before lung extraction. The lungs were minced and then digested with 2.5 mg/mL collagenase D (Roche, Carlsbad, Calif) for 30 minutes at 37°C. EDTA was added to stop the digestion, and then the cells were collected with a glass Pasteur pipette and pressed through a 100-μm nylon strainer to provide a single-cell suspension. After digestion, erythrocytes were removed by means of hypotonic lysis with ACK lysis buffer. Single-cell suspensions from the lung were treated with Fc block (CD16/CD32; BD Biosciences) before labeling with fluorochrome-conjugated antibodies. Antibodies against the surface markers CD11c, CD11b, B220, CD103, Siglec-F, and MHCII were purchased from BD Biosciences, and anti-OX40L was obtained from BioLegend (San Diego, Calif). Appropriate isotype-matched control mAbs were obtained from eBioscience or BD. Flow cytometry was carried out with the LSR-II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ashland, Ore).

Intracellular IL-12 staining

Lung single-cell suspensions were obtained 12 hours after RSV infection, and the intracellular expression of IL-12 was evaluated as described previously.[E5] After surface marker staining, cells were fixed and permeabilized with 1% saponin in staining buffer, treated with Fc block, and labeled with phycoerythrin-conjugated anti–IL-12 antibody or control phycoerythrin-conjugated rat IgG_1 (BD Biosciences).

Quantitative RT-PCR analysis

Primers for mouse TSLP were purchased from Applied Biosystems. The probes were conjugated to FAM and TAMRA dyes. RT-PCR was conducted on an ABI7500 RT-PCR system (Applied Biosystems). Cycle threshold values were normalized to glyceraldehyde-3-phosphate dehydrogenase, and the data were expressed as fold change.

In vitro cytokine production by PBLN cells after restimulation with RSV

Seven days after secondary RSV infection, the single-cell suspensions from PBLNs were prepared. Cells (2×10^5) were cultured in 96-well plates in the presence of UV-inactivated RSV (1 multiplicity of infection) for 72 hours. IL-4, IL-5, IL-6, IL-13, and IFN-γ concentrations in the supernatants were measured by using ELISA.

Statistical analysis

All results were expressed as means ± SEMs. Data were analyzed by means of ANOVA with the StatView 4.5 statistical analysis software package (Abacus Concepts). Student *t* tests and 1-way ANOVA were used to determine the level of differences, where appropriate. Nonparametric analysis with the Mann-Whitney *U* test was used to confirm that the statistical differences remained significant, even if the underlying distribution was uncertain. The *P* values for significance were set to .05 for all tests.

REFERENCES

E1. Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. Nat Immunol 2005;6:1047-53.

E2. Dakhama A, Park JW, Taube C, Joetham A, Balhorn A, Miyahara N, et al. The enhancement or prevention of airway hyperresponsiveness during reinfection with respiratory syncytial virus is critically dependent on the age at first infection and IL-13 production. J Immunol 2005;175:1876-83.

E3. Takeda K, Hamelmann E, Joetham A, Shultz LD, Larsen GL, Irvin CG, et al. Development of eosinophilic airway inflammation and airway hyperresponsiveness in mast cell-deficient mice. J Exp Med 1997;186:449-54.

E4. Jakubzick C, Randolph GJ. Methods to study pulmonary dendritic cell migration. Methods Mol Biol 2010;595:371-82.

E5. Dalod M, Salazar-Mather TP, Malmgaard L, Lewis C, Asselin-Paturel C, Brière F, et al. Interferon alpha/beta and interleukin 12 responses to viral infections: pathways regulating dendritic cell cytokine expression in vivo. J Exp Med 2002;195:517-28.

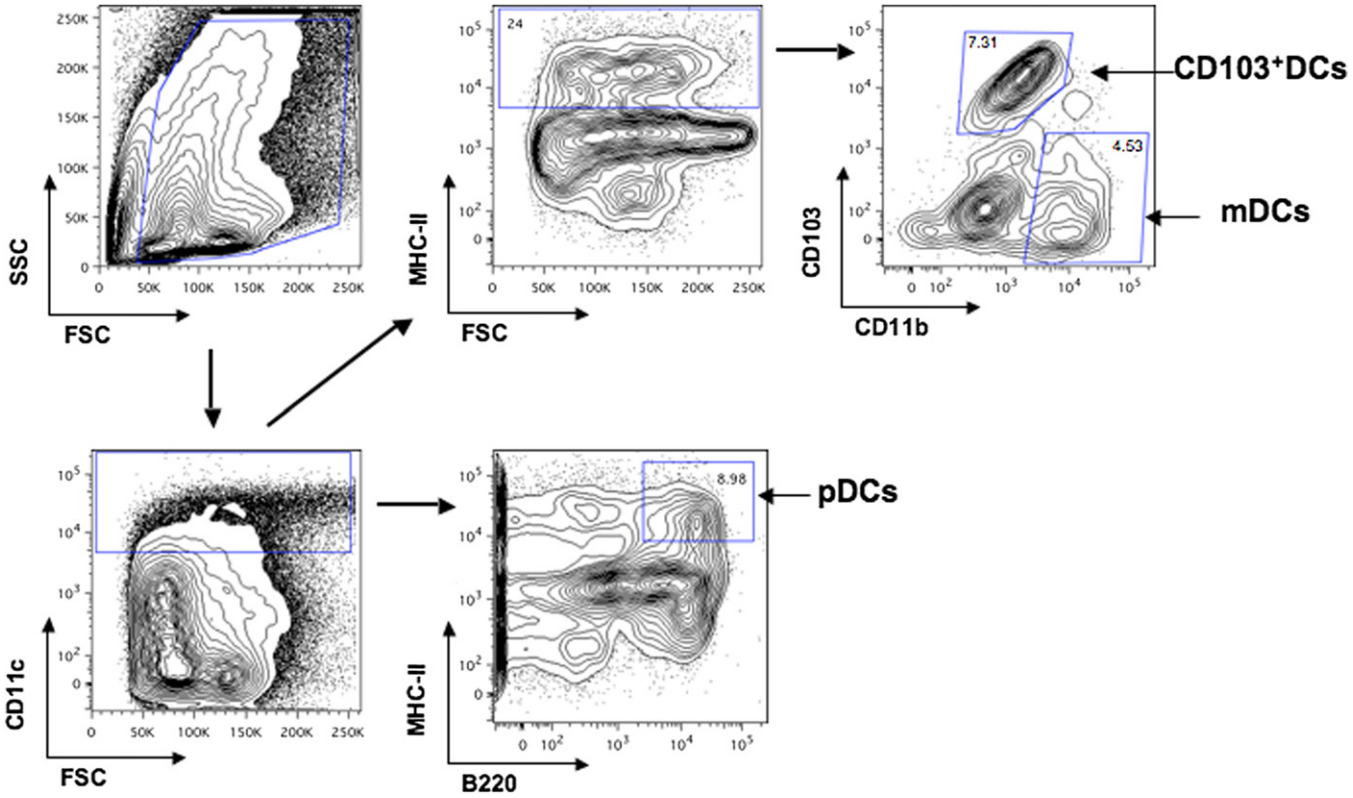

FIG E1. Gating strategy to identify lung DC subsets. Lung single-cell suspensions from RSV-infected or control (PBS-inoculated) mice were evaluated for expression of CD11c, MHCII, CD11b, B220, and CD103. Gated live cells were first plotted as CD11c versus forward scatter *(FSC)* to identify lung DC subsets. The $CD11c^+$ cells were then plotted as MHCII versus FSC or MHCII versus B220. The $CD11c^+MHCII^+$ cells were then plotted as CD103 versus CD11b. As shown in the representative flow plots, $CD103^+$ DCs were identified as $CD11c^+MHCII^+CD103^+CD11b^{lo}$ cells, mDCs were identified as $CD11c^+MHCII^+CD11b^+CD103^-$ cells, and pDCs were identified as $CD11c^+MHCII^+B220^+$ cells. Numbers indicate the percentages of each gated DC subset among total $CD11c^+$ cells. Representative plots from 3 independent experiments with 4 mice per group are shown. *SSC*, Side scatter.

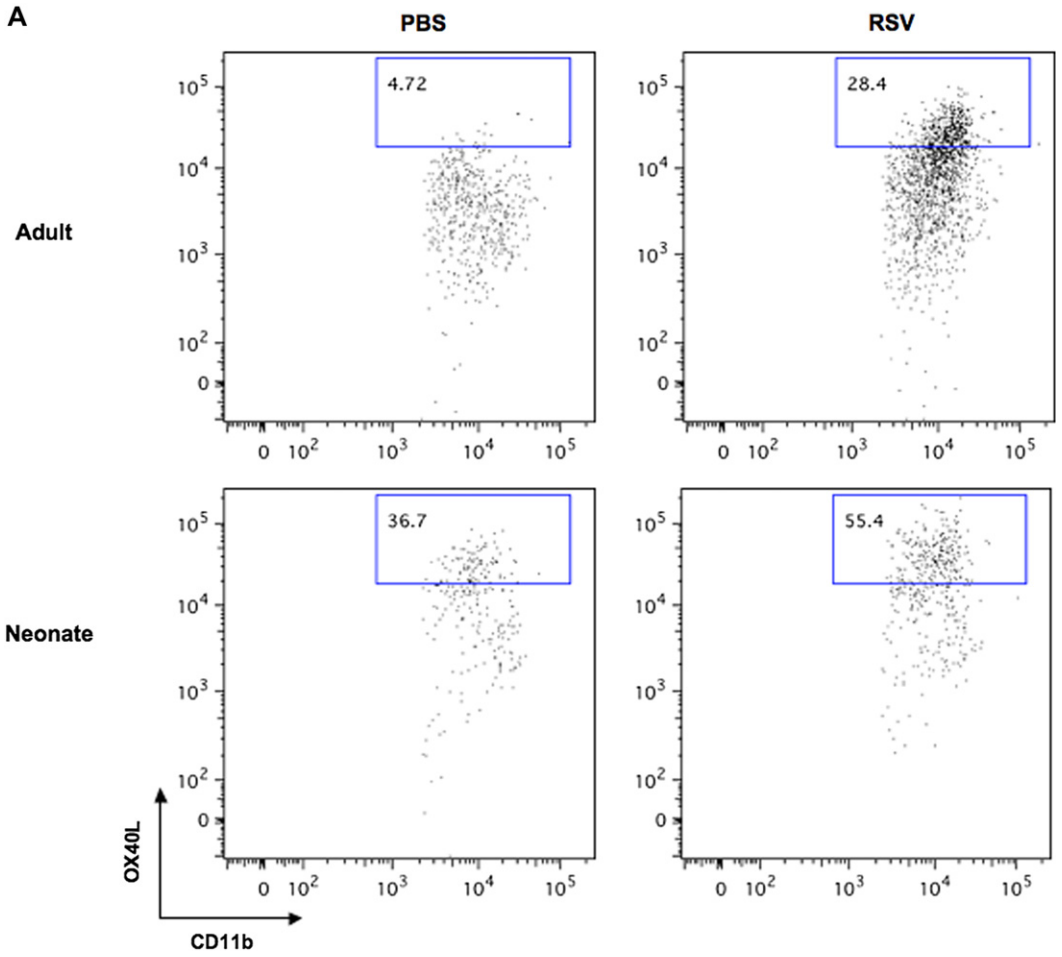

FIG E2. OX40L expression on mDCs and CD103$^+$ DCs. Mice were infected as neonates or at 5 weeks of age. Age-matched control mice were inoculated with PBS. One day after RSV infection, lung single-cell suspensions were prepared, and CD103$^+$ DCs and mDCs were identified as shown in Fig E1. The percentages of OX40L$^+$ cells among mDCs **(A)** and CD103$^+$ DCs **(B)** are shown. The gate for OX40L expression was set based on isotype staining. Representative plots from 3 independent experiments with 4 mice per group are shown.

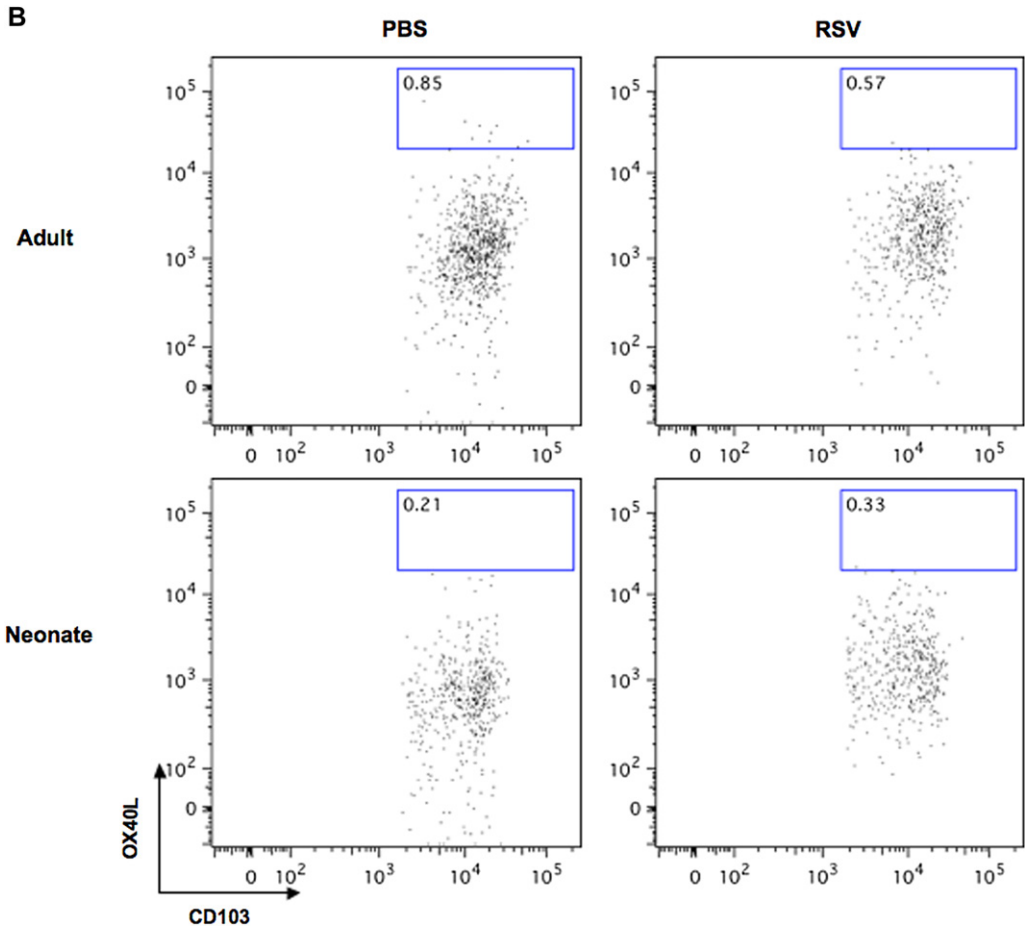

FIG E2. *(Continued)*

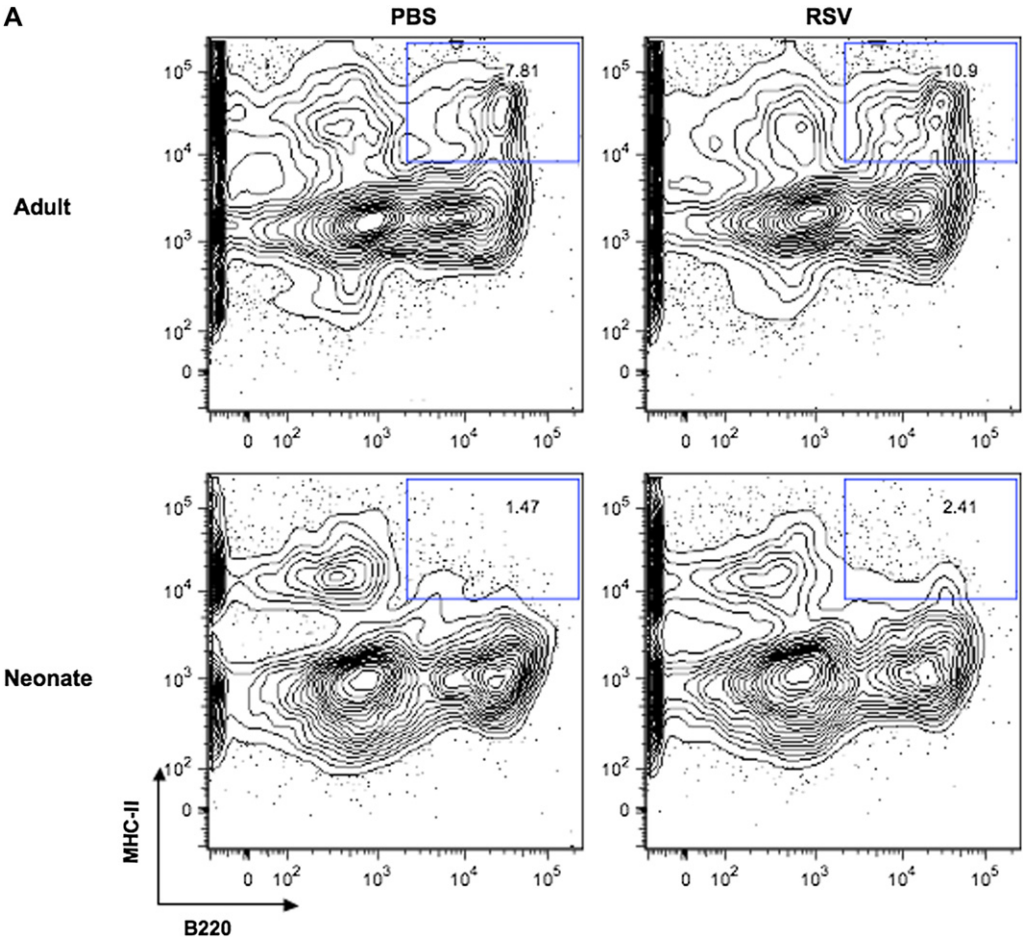

FIG E3. OX40L expression on pDCs. Mice were infected as neonates or at 5 weeks of age. Age-matched control mice were inoculated with PBS. One day after RSV infection, lung single-cell suspensions were prepared and evaluated for expression of OX40L on pDCs. **A,** The lung $CD11c^+$ cells were plotted as MHCII versus B220. Numbers indicate the percentages of pDCs among $CD11c^+$ cells. **B,** Percentage of OX40L-expressing pDCs. Representative plots from 3 independent experiments with 4 mice per group are shown.

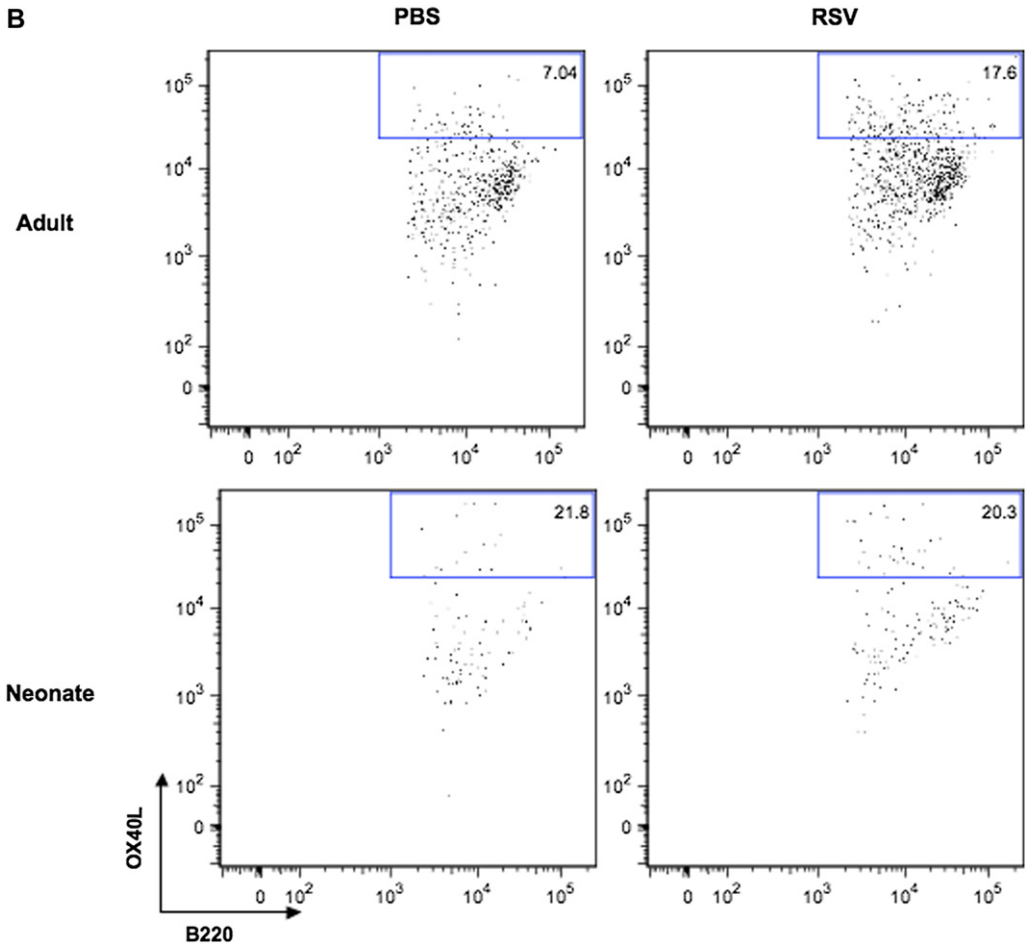

FIG E3. *(Continued)*

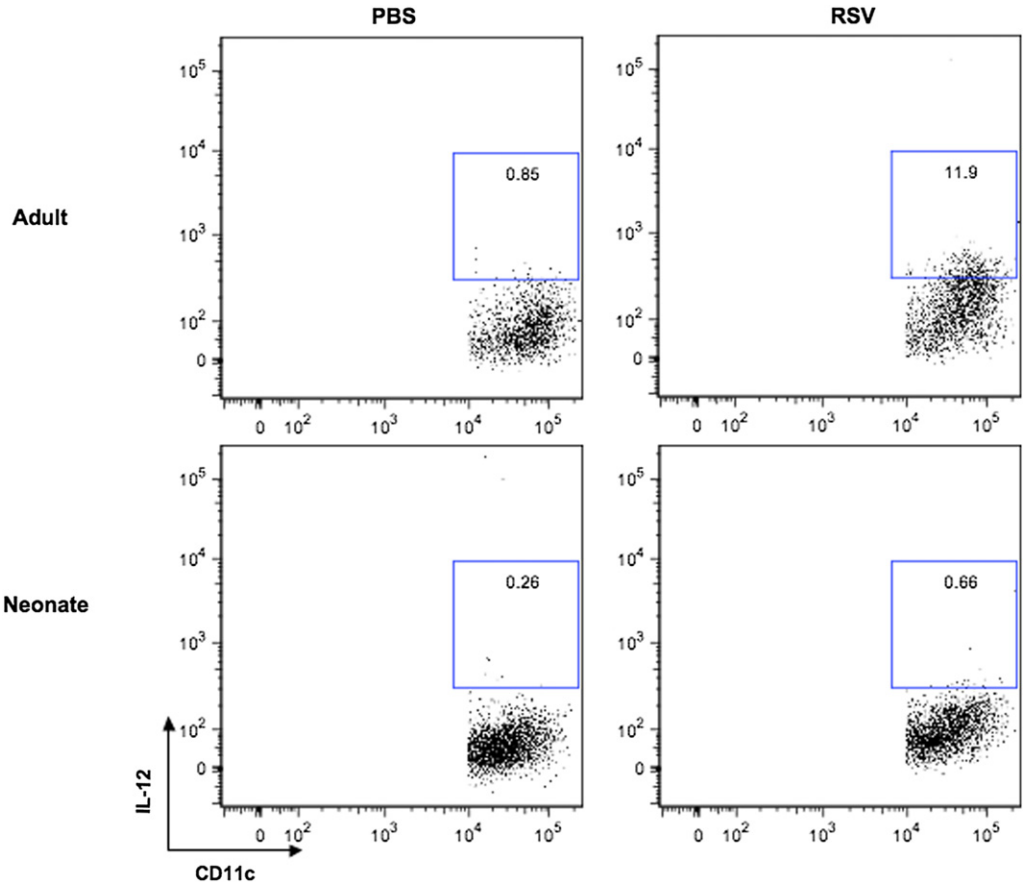

FIG E4. IL-12 expression on CD11c$^+$ cells. Mice were infected as neonates or at 5 weeks of age. Age-matched control mice were inoculated with PBS. Twelve hours after RSV infection, lung single-cell suspensions were prepared and evaluated for intracellular expression of IL-12 on CD11c$^+$ cells. Numbers indicate the percentages of IL-12$^+$ cells among CD11c$^+$ cells. The gate for IL-12 expression was set based on isotype staining. Representative plots from one of 3 independent experiments with 4 mice per group are shown.

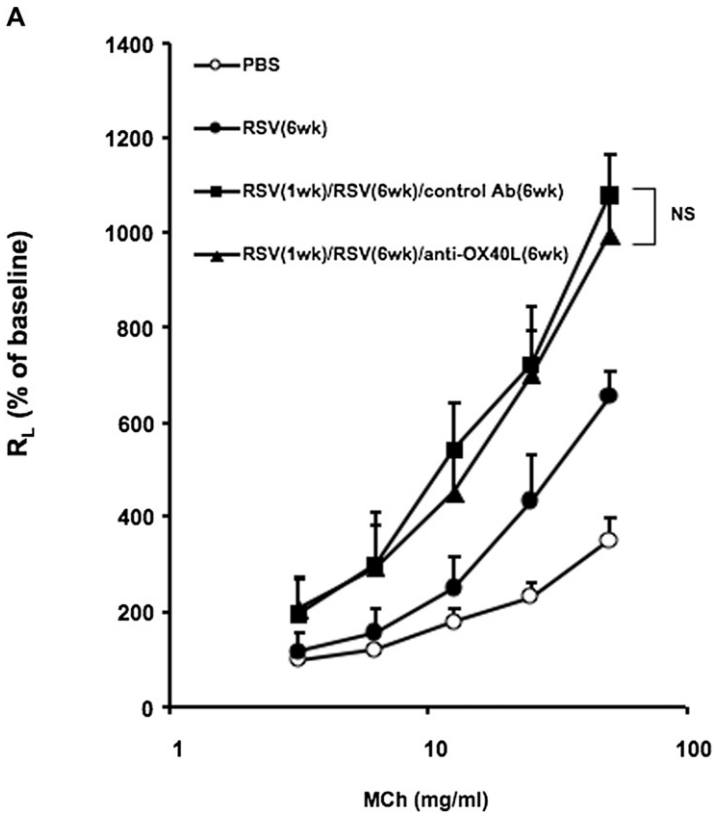

FIG E5. Effect of anti-OX40L during secondary infection on the response to reinfection in newborn mice. Newborn mice were infected with RSV and reinfected with RSV 5 weeks later. Anti-OX40L or control antibody was administered during secondary RSV infection. Airway responsiveness to inhaled MCh **(A)**, BAL cellularity **(B)**, and BAL fluid cytokine levels **(C)** were assessed on day 7 after secondary RSV infection. Results are from 3 independent experiments (n = 12). Data are expressed as means ± SEMs. *Ab*, Antibody; *Eos*, eosinophil; *Lym*, lymphocyte; *Mac*, macrophage; *Neu*, neutrophil; *NS*, nonsignificant; R_L, lung resistance.

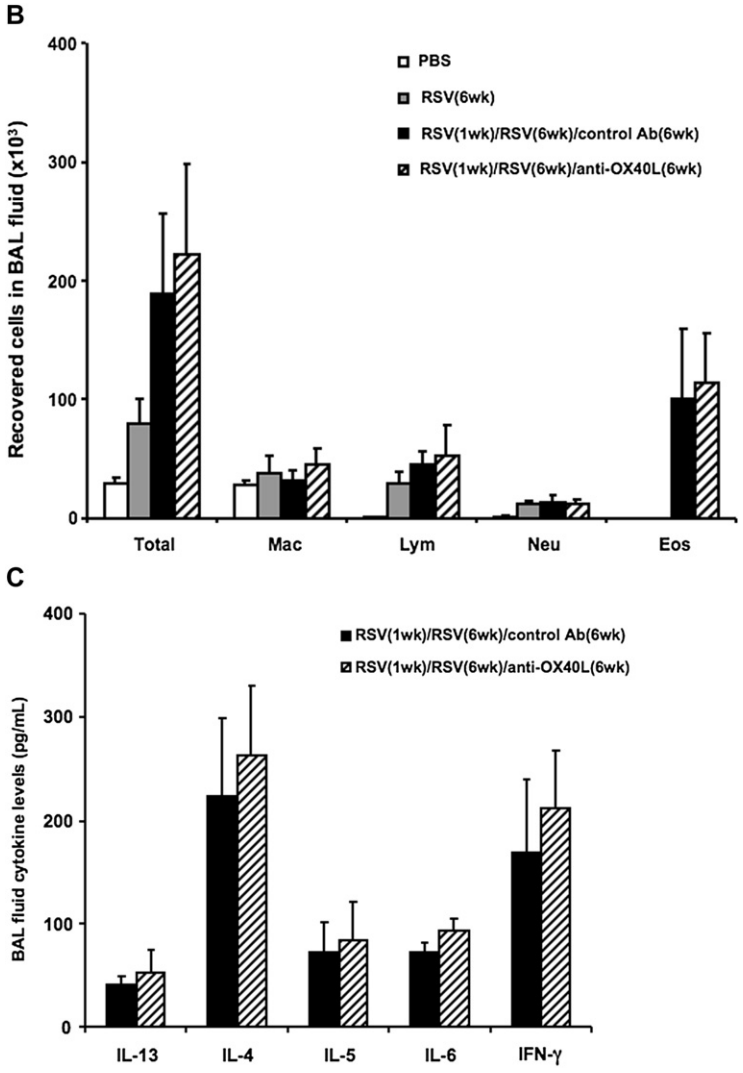

FIG E5. *(Continued)*