

Crucial role of OX40/OX40L signaling in a murine model of asthma

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Abstract. The aim of the present study was to explore the roles of OX40/OX40 ligand (OX40L) signaling and OX40⁺ T cells in ovalbumin (OVA)-induced mouse asthma model. Asthma was induced by OVA exposure and subsequent co-treatment with OX40L protein, neutralizing anti-OX40L blocking antibody, OX40⁺ T cells or PBS. The protein expression levels of interleukin (IL)-4, IL-6, IL-13, IL-17, tumor necrosis factor (TNF)- α and interferon (IFN)- γ in bronchoalveolar lavage fluid (BALF) were examined using murine cytokine-specific ELISA. Eosinophil accumulation as well as proliferation and apoptosis of T cells in BALF were detected by Cell Counting kit-8 and flow cytometric assays. Expression of the apoptosis-related protein cleaved caspase-3 was examined in OX40⁺ T cells using western blot assay. Flow cytometric analysis revealed that OVA-treated mice that were co-treated with OX40L or OX40⁺ T cells exhibited higher eosinophil infiltration compared with control mice treated only with OVA, whereas neutralizing anti-OX40L blocking antibody inhibited eosinophil infiltration. ELISA assays demonstrated that the expression of IL-4, IL-6, IL-13, IL-17, TNF- α and IFN- γ in BALF in OX40L-treated and OX40⁺ T cell-treated mice was increased compared with expression levels in control mice. Treatment with OX40L protein effectively reduced apoptosis of T cells and the expression of cleaved caspase-3 in T cells. OX40L-treated and OX40⁺ T cell-treated mice exhibited increased asthma through OX40/OX40L signaling, which probably promoted inflammatory factor expression, eosinophil infiltration and T cell proliferation.

Introduction

Asthma is a complex disease that involves a number of genetic and environmental influences (1). The chronic inflammation in asthma is characterized by eosinophilic recruitment, airway hyperresponsiveness (AHR), goblet cell hyperplasia/metaplasia, epithelial hypertrophy/hyperplasia, mucus hypersecretion, collagen deposition, smooth muscle cell hypertrophy/hyperplasia and subepithelial fibrosis (2,3). Worldwide ~300 million people suffer from asthma, and this number is predicted to rise over the next 10-15 years to >400 million (4). It has been reported that chronic airway inflammatory processes lead to the recruitment of activated eosinophils and T helper 2 (Th2) lymphocytes to the site of injury and an improper immune response to common allergens (5). Recurrent inflammation and subsequent abnormalities in the tissue repair mechanisms may lead to structural alterations in the airway wall that may develop the clinically detectable features of epithelial injury, goblet cell hyperplasia, subepithelial thickening, airway hyperplasia and angiogenesis (6). Inflammatory infiltrates in the airways that are characteristic of asthma may affect the structural cells and lead to AHR (7). There are a number of different immune cells in the infiltrates, including T lymphocytes that produce cytokines, such as interleukin (IL)-4, IL-5 and IL-13, which serve important roles in the pathogenesis of asthma (8-10). Therefore, targeting these T lymphocytes may have the potential to effectively treat asthma.

OX40 (also known as CD134) and its binding partner OX40 ligand (OX40L; also known as CD252) are members of the tumor necrosis factor (TNF)/TNF receptor superfamily and are expressed on activated CD4 and CD8 T cells, and on a number of lymphoid and non-lymphoid cells (11). OX40L is mainly expressed by antigen-presenting cells (APCs), such as dendritic cells, but is also expressed by B cells, macrophages and Langerhans cells (12). Dendritic cells in the airway express OX40L in response to epithelial cell-derived thymic stromal lymphopoietin stimulation (TSLP) (13). *In vivo* studies using murine and nonhuman primate models of asthma have reported that the inhibition of OX40L suppressed TSLP-mediated Th2 inflammation and reduced the number of OX40L⁺ dendritic cells in the lungs (14). OX40/OX40L interactions have been demonstrated to serve a central role in numerous inflammatory and autoimmune disease development, which

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suggested that they may be suitable candidates for clinical intervention (15); however, the effects and precise mechanisms of OX40/OX40L signaling in the development of asthma remains unclear. Clarification of the underlying mechanisms of the OX40/OX40L signaling in mediating inflammation, immunoreactions or other cell functions in asthma may lead to improved clinical treatment on asthma.

The present study examined the effects of OX40/OX40L signaling on inflammation and T cell functions in a mouse asthma model and investigated the possible underlying mechanisms. The aim was to provide a new perspective and deeper understanding of the etiology of asthma and to provide additional evidence for the potential involvement of OX40/OX40L signaling in the development of asthma.

Materials and methods

Reagents and antibodies. Murine interleukin (IL-) 4 (catalog no. BMS613), IL-6 (catalog no. BMS603-2), IL-13 (catalog no. KMC2221), IL-17 (catalog no. BMS6001), tumor necrosis factor (TNF)- α (catalog no. BMS607-3) and interferon (IFN)- γ (catalog no. 88-8314-77) ELISA kits were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Ovalbumin (OVA) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Neutralizing rat anti-OX40L monoclonal antibody was purchased from Bio X Cell (West Lebanon, NH, USA; catalog no. BE0033-1-25MG). Mouse recombinant OX40L protein was purchased from R&D Systems, Inc. (Minneapolis, MN, USA; catalog no. 1236-OX-025). Rabbit anti-cleaved caspase 3 (Asp175), polyclonal antibody was purchased from AbbeXa, Ltd. (Cambridge, UK; catalog no. abx015533). Rabbit anti-NF- κ B polyclonal antibody (Aviva Systems Biology, San Diego, CA, USA; catalog no. OAAI00072; phosphorylated (p-)Ser337). Anti-GAPDH antibody was purchased from Beyotime Institute of Biotechnology (Shanghai, China; catalog no. AF0006). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Dead Cell Apoptosis kit with Annexin V Alexa Fluor 488 & propidium iodide (PI) was purchased from Thermo Fisher Scientific, Inc. (catalog no. V13241). Fluorescein isothiocyanate-conjugated rat anti-CD4 monoclonal antibody was purchased from LifeSpan BioSciences, Inc. (Seattle, WA, USA; catalog no. LS-C62734-300). Phycoerythrin (PE)-conjugated goat anti-OX40 polyclonal antibody was purchased from R&D Systems, Inc. (catalog no. FAB1256P).

Experimental animals. Specific-pathogen-free female BALB/c mice (n=156; age, 6-8 weeks; weight, 20-25 g) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd. (Shanghai, China), and were kept at 19-22°C and 40-75% relative humidity at all times in the animal facility under specific-pathogen-free conditions. A 12-h light/dark cycle was maintained during the course of the present study. Animals were kept in groups of five and fed regular lab chow and water *ad libitum*. All animal experiments performed in this study conformed to the Guide for the Care and Use of Laboratory Animals (16) and were approved by the Institutional Animal Care and Use Committee of Soochow University (Suzhou, China).

OX40⁺ T cell sorting protocol. Murine CD4⁺ T cells were obtained from mononuclear cells prepared from the bronchoalveolar lavage fluid (BALF) of 18 OVA-challenged mice or the spleen of 6 BALB/c mice and T cells were isolated using a Pan T Cell Isolation Kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; catalog no. 130-095-130) according to the manufacturer's protocol (17) and collected in sterile PBS containing 50% fetal calf serum (FCS). The purified T cells (10⁶ cells/ml) were then cultured in DMEM containing 10% FCS in a 6-well plate with plate-bound anti-CD3 antibody (3 μ g/ml; R&D Systems, Inc.; catalog no. MAB4841-SP) and soluble anti-CD28 antibody (10 μ g/ml; R&D Systems, Inc.; catalog no. MAB4832-SP), as well as IL-2 (5 ng/ml; R&D Systems, Inc.; catalog no. P04351), IL-4 (20 ng/ml; R&D Systems, Inc.; catalog no. P07750), anti-IFN- γ antibody (10 μ g/ml; R&D Systems, Inc.; catalog no. MAB485-SP) and anti-IL-12 antibody (10 μ g/ml; R&D Systems, Inc.; catalog no. AF-419-SP) for 3 days at 37°C. Cells were subsequently removed, washed and cultured with DMEM containing 10% FCS for a further 3-6 days at 37°C without further stimulation (18). T cells were subsequently processed for the sorting assay with phycoerythrin-conjugated OX40 antibodies (10 μ l/10⁶ cells; R&D Systems, Inc.; catalog no. FAB1256P) using flow cytometry to isolate OX40⁺ T cells for further *in vivo* experiments. Cells were analyzed with FlowJo software (version 7.6; FlowJo LLC, Ashland, OR, USA).

Immunization and intervention. Protocols for immunization and intervention were as previously described (19). Briefly, 120 mice were immunized with an intraperitoneal injection of OVA (100 μ g; Sigma-Aldrich; Merck KGaA) and aluminum hydroxide (2 mg; Pierce; Thermo Fisher Scientific, Inc.) in sterile saline on days 1 and 8. On days 9-14 following the initial sensitization, mice were challenged intranasally with 20 μ g of 2% OVA in sterile saline. In other experiments for intervention, 100 μ g/kg of recombinant murine OX40L protein, 12 mg/kg of neutralizing anti-OX40L antibody or 5 \times 10⁶ isolated OX40⁺ T cells or PBS (control) were injected intravenously through the tail vein following anesthesia (20,21).

Cytokine and protein measurements in bronchoalveolar lavage fluid (BALF). BALF was collected by flushing 1 ml ice-cold PBS back and forth three times through a tracheal cannula followed by centrifugation at 2,000 \times g at 4°C for 10 min, as previously described (22). Protein concentrations of IL-4, IL-6, IL-13, IL-17, TNF- α and IFN- γ in the supernatant were measured using murine cytokine-specific ELISA kits (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The concentration of IL-4, IL-6, IL-13, IL-17, TNF- α and IFN- γ in the BALF were measured as markers of inflammatory reaction using a microplate reader at 450 nm (Thermo Fisher Scientific, Inc.; catalog no. 51119000).

Determination of eosinophils. Eosinophils were detected and counted as previously described (23). Briefly, BALF was performed by instilling 0.9% NaCl containing 0.6 mmol/l EDTA in two separate 0.5 ml aliquots. The fluid was recovered by gentle suction and placed on ice for immediate processing. An aliquot of ~0.5 ml BALF was processed immediately for eosinophil count. BALF cell suspensions were stained with

PE-conjugated rat anti-mouse CCR3 at 4°C for 30 min (1:500; R&D Systems, Inc.; catalog no. FAB1551T-100UG), followed by staining with fluorescein isothiocyanate-conjugated anti-mouse CD16 (1:500; Antigenix America Inc., Huntington Station, NY, USA; catalog no. RM160323) for 30 min at 4°C in the dark. Samples stained with non-immunized rat IgG mAb (1:100; R&D Systems, Inc.; catalog no. IC005P) for 30 min at 4°C were used as an isotype control. The percentage of eosinophils (CCR3⁺CD16⁺) was calculated as the number of eosinophils divided by the total number of cells in the BALF sample, as determined by flow cytometry and were analyzed with FlowJo software (version 7.6). All analyses were performed in a blinded fashion. The remainder of the lavage fluid was centrifuged at 1,000 x g for 10 min at 4°C and the supernatant was removed aseptically and stored in individual aliquots at -80°C.

Western blot analysis. Cell pellets (1x10⁷) from fresh BALF collected from lung tissues was homogenized in 600 µl radio-immunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology; catalog no. P0013B) in the presence of protease inhibitors for 30 min at 4°C. Protein concentration was determined with the Bradford assay. Lysates were centrifuged at 9,000 x g for 15 min at 4°C. Samples (20 µg/lane) were boiled for 5 min at 100°C and separated by 12% SDS-PAGE under denaturing conditions and electroblotted to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA). Membranes were blocked with PBS and 5% non-fat dry milk for 12 h at 4°C and incubated with the following antibodies for 1 h at room temperature: Anti-NF-κB (1:500; Aviva Systems Biology; catalog no. OAAI00072; p-Ser337) and anti-cleaved caspase 3 (1:500; Abxbexa Ltd., Cambridge, UK; catalog no. abx015533). Immunoblot assays were then washed and incubated with a horseradish peroxidase-labeled secondary antibody (1:5,000 dilution; GE Healthcare Life Sciences, Shanghai, China; catalog nos. RPN4301 and RPN4201) for 1 h at room temperature. Protein bands were visualized using ECL Plus Enhanced Chemiluminescence Reagent (GE Healthcare Life Sciences), according to the manufacturer's protocol. Protein band intensity was determined relative to GAPDH using ImageJ software (version 2.1.4.7; National Institutes of Health, Bethesda, MD, USA).

Cell proliferation assay in vitro. The CCK-8 assay was used to evaluate proliferation of isolated T cells. T cells were exposed to increasing concentrations of OX40L protein (10, 50, 100 or 200 ng/ml) or 200 ng/ml of OX40L protein combined with 200 ng/ml of neutralizing anti-OX40L antibody. Briefly, T cells (3x10³ cells/well) were seeded in each well of a 96-well plate and stimulated with 0.10 mg/ml phytohemagglutinin (Thermo Fisher Scientific, Inc.; catalog no. 00-4977-93) for 24 h. Subsequently, the cells were cultured in serum-free DMEM for 24 h starvation at 37°C. Cells were treated with different concentrations of OX40L protein (10-200 ng/ml) or 200 ng/ml OX40L protein and 200 ng/ml neutralizing anti-OX40L antibody (200 ng/ml) for 24 h at 37°C, and the proliferative activity was determined by CCK-8 assay, according to the manufacturer's protocol. CCK-8 (10 µl) was added to each well followed by incubation for an additional 2-4 h at 37°C. When the media changed from red to yellow, the absorbance value

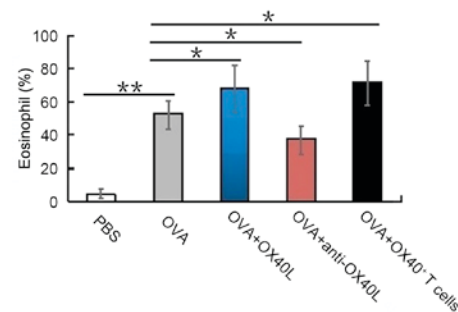


Figure 1. Effects of OX40/OX40L signaling on recruitment of allergen-induced BALF eosinophils in response to OVA treatment. The eosinophil count in the BALF of OVA-treated mice was significantly increased. Furthermore, the number of eosinophils in BALF significantly increased in OX40L protein- and OX40⁺ T cell-treated mice sensitized and challenged with OVA compared with OVA-treated mice. Data are presented as percent of total cells ± SEM; n=6-8 mice/group; *P<0.05, **P<0.01. BALF, bronchoalveolar lavage fluid; OVA, ovalbumin; OX40L, OX40 ligand.

was detected at a wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.; catalog no. 51119000). The experiment was performed at least three times.

Flow cytometric analysis of cell apoptosis. Mononuclear T cells were isolated from BALF according to a previously described procedure with some modifications (24). Briefly, T cells (1x10⁵ cells/test) were washed twice with PBS and centrifuged at 1,000 x g for 5 min at room temperature. Each pellet (~1x10⁵ cells) of the T cells was then re-suspended in 400 µl PBS followed by incubation with 5 µl Annexin V and 1 µl propidium iodide (PI; 1 mg/ml) for 15 min at room temperature. Cells were subsequently analyzed using a flow cytometer without washing the cells. Cells that were PI and Annexin V negative were considered healthy cells, PI negative and Annexin V positive were considered apoptotic and cells that were PI and Annexin V positive were considered necrotic.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical analyses were performed using SPSS 18.0 software package (SPSS Inc., Chicago, IL, USA). Comparisons among multiple groups were performed using one-way analysis of variance followed by the Bonferroni post-hoc test if the data were normally distributed. P<0.05 was considered to indicate a statistically significant difference.

Results

OX40/OX40L signaling effects on eosinophil recruitment in lungs following sensitization and challenge with OVA. The presence of eosinophils in the BALF and lungs is an important indicator of airway inflammation (25). OVA challenged mice had a significantly higher eosinophil count (56±6.8%) compared with PBS-treated control mice (4.4±3.6%; P<0.01); OX40L protein-treated mice and OX40⁺ T cell-treated mice also had a high eosinophil count (65±5.5 and 67±7.2%, respectively) in the BALF following sensitization and challenge with OVA (Fig. 1), compared with those treated with OVA alone. By contrast, eosinophils accounted for only 41±4.5% of all cells in the BALF of mice treated with neutralizing anti-OX40L

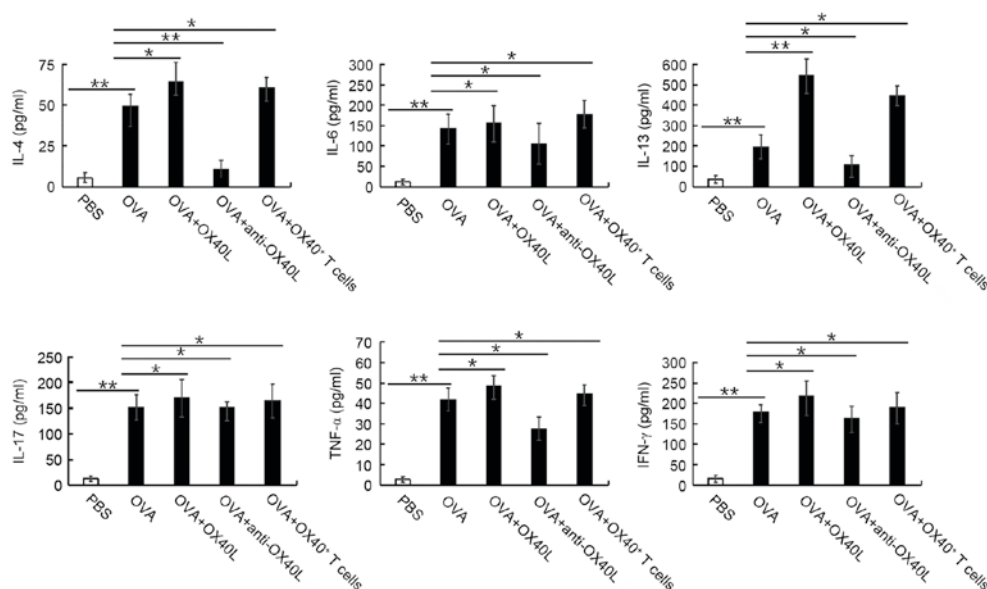


Figure 2. Effects of OX40/OX40L signaling on cytokine secretion in BALF in response to OVA treatment. Expression levels of the cytokines IL-4, IL-6, IL-13, IL-17, TNF- α and IFN- γ were measured by ELISA in the BALF of PBS treated mice and in mice treated with OVA, OVA + OX40L protein, OVA + neutralizing anti-OX40L antibody, and OVA + OX40⁺ T cells. Data are shown as pg/ml \pm SEM, n=6-8 mice/group; *P<0.05, **P<0.01. BALF, bronchoalveolar lavage fluid; IFN, interferon; IL, interleukin; OVA, ovalbumin; OX40L, OX40 ligand; TNF, tumor necrosis factor.

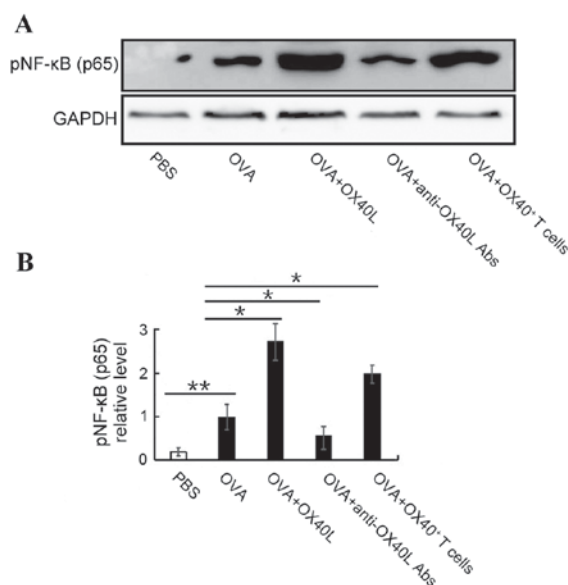


Figure 3. Effects of OX40/OX40L signaling effect on pNF- κ B expression in BALF in response to OVA treatment. (A) Representative western blotting results from three independent experiments. (B) Ratios of pNF- κ B to GAPDH protein bands of PBS treated mice and mice treated with OVA, OVA + OX40L protein, OVA + neutralizing anti-OX40L antibody, and OVA + OX40⁺ T cells. All values represent mean \pm SEM; n=6-8 mice/group; *P<0.05. BALF, bronchoalveolar lavage fluid; NF, nuclear factor OVA, ovalbumin; OX40L, OX40 ligand.

antibody (Fig. 1). These results suggested that OX40/OX40L signaling may serve an important role in eosinophil recruitment into the lungs.

Effects of OX40/OX40L signaling on cytokine expression levels *in vivo*. Mice challenged with OVA exhibited a significant increase in IL-4, IL-6, IL-13, IL-17, TNF- α and

IFN- γ expression levels in BALF, compared with the levels in untreated mice (P<0.05; Fig. 2). In mice sensitized and challenged with OVA that were co-treated with neutralizing anti-mouse OX40L antibody, this increase in IL-4, IL-6, IL-13, IL-17, TNF- α and IFN- γ cytokine levels was significantly suppressed (P<0.05 or P<0.01 vs. OVA; Fig. 2); whereas mice co-treated with OX40L protein or OX40⁺ T cells exhibited a significant increase in these cytokine levels in the BALF (P<0.05 or P<0.01; Fig. 2), compared with the levels the OVA group. These results suggested that OX40/OX40L signaling may significantly increase the IL-4, IL-6, IL-13, IL-17, TNF- α and IFN- γ cytokine levels in the BALF.

NF- κ B activation promotes inflammatory cell migration and proliferation, and mediates inflammatory factor secretion (26). To determine whether OX40/OX40L signaling is able to effect NF- κ B activation, the expression of activated NF- κ B was detected in inflammatory cells from the BALF. Activated NF- κ B expression was significantly increased in the OVA-challenged group compared with the PBS group (P<0.01). Furthermore, pNF- κ B expression was significantly increased in OX40L protein or OX40⁺ T cell-treated mice and significantly reduced in anti-OX40L antibody-treated mice compared with mice in the OVA group (Fig. 3). These results indicated that OX40/OX40L signaling may induce NF- κ B activation and suggests that OX40/OX40L signaling had a pro-inflammatory effect by regulating the expression of IL-4, IL-6, IL-13, IL-17, TNF- α and IFN- γ through NF- κ B activation.

Effects of OX40/OX40L signaling on proliferation and polarization of CD4⁺ T cells *in vitro*. It has been previously reported that OX40L promoted the number of OX40⁺ CD4 T cells in an asthma model (27), which indicated that OX40/OX40L signaling may serve an important role in the process of CD4⁺ T cell polarization, which may eventually affect the

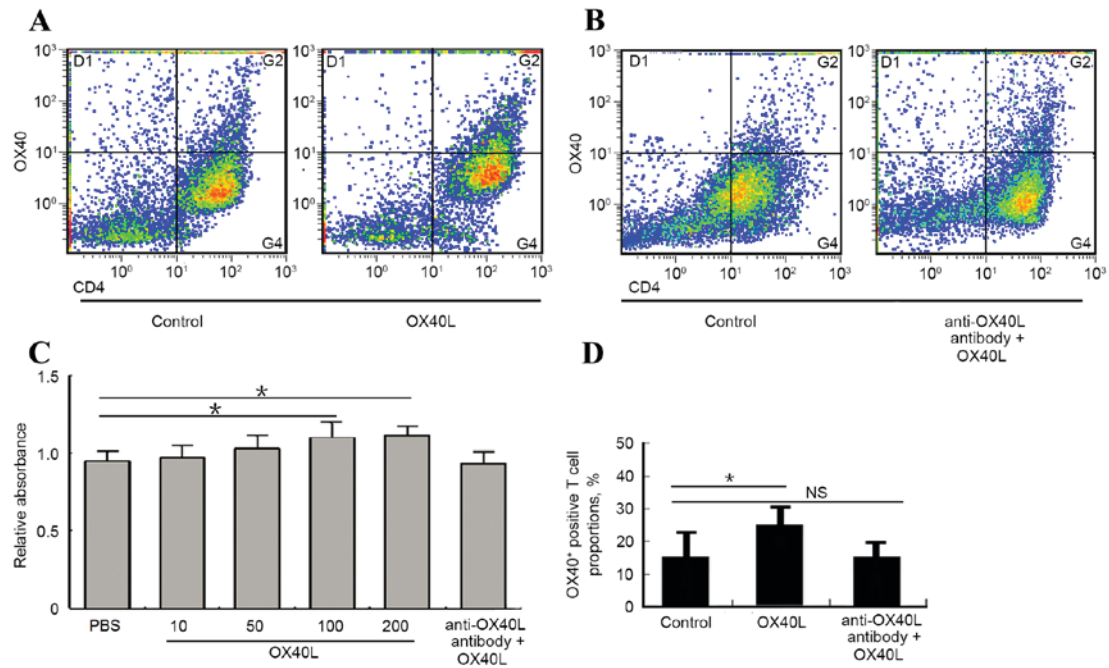


Figure 4. Effects of OX40/OX40L signaling on T cell proliferation and polarization. (A and B) Flow cytometric analysis of OX40⁺ T cell polarization *in vitro*. Representative results from 3-4 tests of CD4⁺/OX40⁺ T cells from control PBS-treated, OX40L protein-treated or OX40L protein + neutralizing anti-OX40L antibody-treated groups. Absorbance was detected using a microplate reader at a wavelength of 450 nm in cells treated with (C) different concentrations of OX40L protein (ng/ml) or 200 ng/ml OX40L protein + 200 ng/ml neutralizing anti-OX40L antibody; All values represent mean \pm SEM; n=3; *P<0.05 vs. PBS group. (D) Proportion of OX40⁺/OX40⁺ T cells from (A and B), respectively. All values are presented as the mean \pm SEM; n=5; *P<0.05. NS, not significant; OX40L, OX40 ligand.

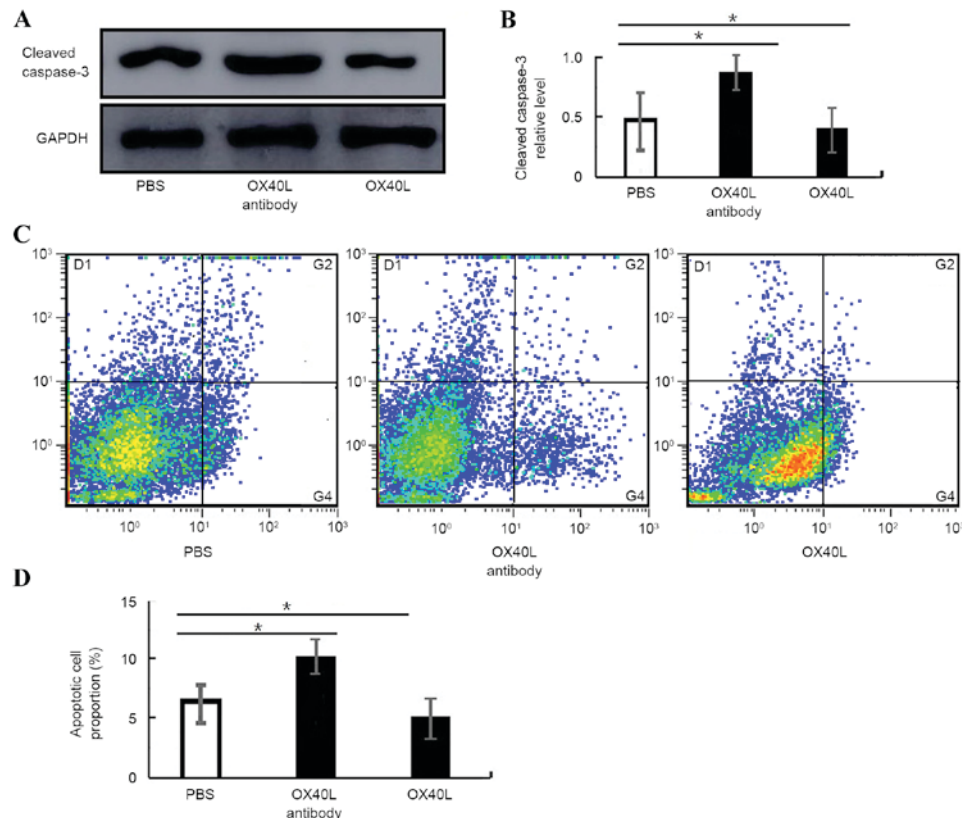


Figure 5. Effects of OX40/OX40L signaling on protein expression of cleaved caspase-3 and cell apoptosis. (A) Protein extracts were obtained and subjected to western blotting for cleaved caspase-3 expression analysis. Representative results from three independent experiments are presented. (B) Ratios of cleaved caspase-3 to GAPDH protein bands of PBS-, OVA + OX40L protein-, and OVA + neutralizing anti-OX40L antibody-treated group. n=6-8; *P<0.05. (C) Representative results from 3-4 tests of Annexin V/propidium iodide-positive cells from either PBS-treated, OVA + OX40L-treated and OVA + neutralizing anti-OX40L antibody-treated mice. (D) The proportion of apoptotic cells from PBS, OVA + OX40L and OVA + neutralizing anti-OX40L antibody-treated mice was determined. n=6-8; All values are presented as the mean \pm SEM; *P<0.05. OVA, ovalbumin; OX40L, OX40 ligand.

progression of asthma. The present study examined the effects of OX40/OX40L signaling on CD4⁺ T cell proliferation and polarization *in vitro*. CD4⁺ T cells isolated from mouse spleen cultured with 100 or 200 ng/ml OX40L protein exhibited a significant increase in proliferation, whereas no change in proliferation was detected between the OX40L protein and neutralizing anti-OX40L antibody co-culture group and the PBS-treated control group (Fig. 4A). Furthermore, OX40L treatment significantly promoted the number of OX40⁺ T cells, whereas co-treatment with neutralizing anti-OX40L antibody suppresses OX40⁺ T cell number (Fig. 4B-D). These data indicated that OX40/OX40L signaling may be involved in CD4⁺ T cell proliferation and polarization, and thus influences T cell bio-function in asthma pathology.

OX40/OX40L signaling effects on T cell apoptosis in lungs following sensitization and challenge with OVA. To further explore the mechanism underlying OX40/OX40L signaling involved in asthma process, the expression levels of apoptosis-related protein cleaved caspase-3 were examined in isolated T cells from PBS-treated, OX40L protein-treated or neutralizing anti-OX40L antibody-treated mice. Western blot analysis demonstrated that the relative protein expression level of cleaved-caspase-3 was significantly increased in anti-OX40L antibody-treated mice (0.87 ± 0.11) and decreased in OX40L protein-treated mice (0.36 ± 0.18) compared with PBS-treated mice (0.51 ± 0.17 ; $P < 0.05$ Fig. 5A and B). Similar results were obtained by flow cytometric analyses for apoptosis (Fig. 5C and D).

Discussion

OX40/OX40L signaling serves a key role in the development, differentiation and physiological functions of T cells and other immunological cells (28). The expression of OX40 is upregulated on activated T cells (29), whereas it is constitutively expressed on T regulatory cells (30,31). This difference in expression is consistent with the previously hypothesized role of OX40/OX40L interactions in the propagation of the immune response and in initial T cell priming (32). OX40L is predominantly expressed in APCs, such as B cells, macrophages, microglia, dendritic cells and endothelial cells (33-39). Signaling through OX40/OX40L interactions during effector T cell responses has been previously reported to enhance T cell survival (40,41), cytokine production (42) and increase the number of memory CD4⁺ T cells (43). Experimental models of autoimmunity and inflammation have indicated the potential role for OX40/OX40L, as inhibiting the interaction between OX40 and OX40L attenuates disease progression or severity (44,45). Owing to this important role in the pathological process of numerous diseases, the present study examined the effects and the mechanisms of OX40/OX40L signaling in experimentally induced asthma.

A number of previous *in vivo* studies (15,46-48) have demonstrated a pathogenic role for OX40/OX40L signaling in autoimmune diseases, and the disruption of this axis was reported to be beneficial for prevention and treatment (49). However, whether OX40/OX40L serves pathogenic roles in human asthma requires further exploration. Our previous study demonstrated that OX40L was overexpressed by

myeloid APCs in peripheral blood and BALF in a mouse asthma model (27). Another previous study reported that the number of OX40L-expressing myeloid APCs was positively correlated with disease activity, as assessed by intrafluid expression of inflammatory factors (50). In the present study, the results indicated that OX40/OX40L signaling promoted CD4⁺ T cell proliferation and polarization into CD4⁺OX40⁺ T cells, as evidenced by the significantly increased proportion of CD4⁺OX40⁺ T cells present when OX40L was administered to isolated T cells from experimental mice *in vitro*. Conversely, CD4⁺ T cell proliferation was suppressed in cells co-treated with the neutralizing anti-mouse OX40L antibody. These observations suggest that OX40/OX40L interactions may have activated CD4⁺OX40⁺ T cells and this event may represent a crucial component for affecting the progression of experimental asthma (51).

Although OX40/OX40L signaling inhibition was demonstrated to reduce inflammation, as determined by its effect on IL-4, IL-6, IL-13, IL-17, TNF- α and IFN- γ expression in peripheral blood and bronchoalveolar lavage fluid, there are still many signal transduction processes that may be involved in the pathogenesis of asthma progression, which makes these types of investigations very complex. Previous studies have focused on NF- κ B signaling, which is necessary for the proliferation and migration of cells, the expression of multiple cytokines and the inflammatory response (52). The present study examined activated NF- κ B expression in asthma lung tissues by western blot analysis and demonstrated that activated NF- κ B expression was elevated in OX40L protein- and CD4⁺OX40⁺ T cell-treated groups, and it was decreased in anti-OX40L antibody treated group, suggesting that OX40/OX40L interactions may affect asthma progression through the NF- κ B pathway. Song *et al* (53) reported that the activation of NF- κ B1 by OX40 contributes to antigen-driven T cell expansion and survival. Burrows *et al* (54) demonstrated that OX40 blockade inhibits house dust mite-driven allergic lung inflammation in mice and allergic responses in humans *in vitro*. The effects of OX40/OX40L on T cell function and the inflammatory response through the NF- κ B pathway demonstrated in these previous reports is consistent with the results of the present study.

Inflammatory responses are important factors for asthma-induced lung injury (55-57). During the acute pathological process, inflammatory cells (including neutrophils and macrophages) are recruited into lung lesions, where they are induced to secrete pro-inflammatory cytokines to further accelerate inflammatory responses and promote asthma (58). Therefore, effective blocking or inhibiting of the inflammatory responses may be a fundamental treatment strategy for asthma. OX40/OX40L interaction is a promoting factor in pathological processes of asthma (59). Consistently, OX40/OX40L interactions in the present study were also demonstrated to promote inflammatory cell infiltration and facilitated pro-inflammatory cytokine expression. A clear association has been demonstrated between an experimental model of asthma and the regulation of the inflammatory response by OX40/OX40L (60,61).

In conclusion, although it requires further exploration, OX40/OX40L signaling may be a prospective intervention target for inhibiting CD4⁺OX40⁺ T cell activation, inflammatory factor secretion and inflammatory cell infiltration into BALF

in clinical settings of asthma therapy. Results from the present study revealed that the neutralizing anti-mouse OX40L antibody was an efficient inhibitor of CD4⁺OX40⁺ T cell production in OVA-induced asthma, and this inhibiting effect may further impact IL-4, IL-6, IL-13, IL-17, TNF- α and IFN- γ expression, reduce inflammatory responses and thus alleviate asthma progression. Targeting of the OX40/OX40L axis may provide therapeutic effects in the treatment of asthma.

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