

ICOS ligand expression is essential for allergic airway hyperresponsiveness

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Abstract

Inducible co-stimulator ligand (ICOSL) is a rather newly defined co-stimulatory molecule, which, through interaction with ICOS expressed on T cells, plays an important role in T-cell activation, differentiation and function. T_H2-type immune responses are critical for the development and maintenance of allergic responses including asthma. Using knockout (KO) mice, we have assessed the role of ICOSL in allergic airway inflammation and responsiveness using a standard mouse asthma model induced by ovalbumin (OVA) sensitization and challenge. Our data show that OVA-treated ICOSL KO mice exhibit significantly less lung eosinophilic infiltration, histopathology, mucus production and virtually no airway hyperresponsiveness in contrast to wild-type (Wt) counterparts. Serum antibody analysis showed that antigen-specific IgG1, IgG2a and IgE titers in ICOSL KO mice were significantly lower than those of Wt controls. Also, CD4⁺ T cells isolated from ICOSL KO mice produced less T_H2 cytokines (IL-4, IL-5, IL-10 and IL-13) but more T_H1 (IFN- γ) and IL-17 than their Wt controls. Taken together, we conclude that ICOSL plays an important role in predisposing individuals to allergic airway hyperresponsiveness by enhancing IgE antibody class switching and T_H2 cytokine production and diminishing the T_H17 response and airway eosinophilia.

Keywords: airway hyperreactivity, ICOSL, ovalbumin, T_H17

Introduction

In order to be activated, T cells need to receive three signals: TCR engagement, co-stimulatory molecule stimulation and cytokine stimulation. In the case of the major CD28-B7 co-stimulatory pathway, naive T cells that constitutively express classical CD28 molecules receive the second signal via B7-1 (CD80) and B7-2 (CD86) molecules present on antigen-presenting cells (APCs) (1). Inducible co-stimulator (ICOS), a relatively newly defined member of the CD28 family, is constitutively expressed in low levels by naive T_H cells but is significantly increased after T-cell activation (2, 3). The only endogenous ligand for this molecule is inducible co-stimulator ligand (ICOSL). ICOSL, also named B7RP-1, is a member of the B7 family of co-stimulatory ligands (4, 5) and shares nearly 20% sequence homology with B7.1 and B7.2. ICOSL is constitutively expressed in B-cell areas of secondary lymphoid organs, on most of APCs, including dendritic cells (DCs), B cells and monocyte/macrophages (6). Under inflammatory conditions, some non-professional APCs, such as endothelial cells, also express ICOSL on their surface (7).

Allergic asthma is a significant global public health concern especially in industrialized countries. Allergy involves a state of immediate or delayed hypersensitivity reaction to the common environmental allergens that normally do not pose a threat to humans. Airway eosinophilic inflammation, mucus overproduction, enhanced IgE responses and more importantly airway hyperresponsiveness represent the hallmark pathological changes in allergic asthmatic reactions. Although altered T_H2/T_H1 balance has been shown to be important in the development of allergy and asthma, the mechanism underlying the initiation, development and maintenance of allergy/asthma remains unclear.

Recently, an important role for a novel subset of T_H cells, named T_H17 cells, in the pathogenesis of some inflammatory and autoimmune disorders has been reported (8–10). IL-17 is crucial for the battle against extracellular pathogens (11–13), and *in vivo* expression of IL-17 leads to expansion of neutrophils (14) that, if uncontrolled, may lead to chronic and autoimmune inflammation such as encephalomyelitis, type II collagen-induced arthritis and chronic enterocolitis

(9, 15–20). T_H17 cells probably represent a distinct subset of effector T cells, induced as a consequence of IL-23 production by DCs (21). In addition, transforming growth factor (TGF)- β 1 and IL-6 have been found to be crucial for the development of T_H17 responses (21–23). It has also been reported that IL-17 has an inhibitory effect on established ovalbumin (OVA)-induced allergic hyperreactivity (18, 19). Indeed, mucosal IL-17 administration inhibited the asthma-like reaction by reducing the pulmonary production of IL-5 and the chemokines thymus and activation-regulated chemokine (TARC) and eotaxin, which mediate eosinophilic inflammation in asthma. *In vivo* neutralization of IL-17 by antibodies abolished the asthma-suppressing effect of exogenous IL-17, demonstrating an inhibitory role of IL-17 in established allergic reactions (24, 25).

A strong impact for the ICOS–ICOSL interaction on T-cell-mediated immune responses *in vivo* has been suggested by studies using mice with disrupted *ICOS* gene and antibody-blocking experiments. ICOS-deficient mice show impaired germinal center formation, profound defects in antibody isotype class switching and defects in IL-4 and IL-13 production (26–28). Blockade of ICOS–ICOSL interaction in animal models of experimental allergic encephalomyelitis (29) and of cardiac allograft rejection (30) results in altered local inflammation. In an allergic airway inflammation model, ICOSL-deficient mice show less eosinophilic inflammation and T_H2 cytokine production (31). However, the involvement of ICOS–ICOSL interaction in functional responses to airway inflammation that underpin the development of airway hyperresponsiveness has not been tested.

In the present study, we used ICOSL knockout (KO) mice to investigate the role of ICOSL in OVA-induced changes in airway resistance and responsiveness to inhaled methacholine (MCh). Our results showed that in contrast to wild-type (Wt) counterparts, ICOSL KO mice do not develop airway or peripheral lung tissue hyperresponsiveness after OVA sensitization and challenge. This difference was associated with reduced T_H2 cytokine release, IgE response, airway mucus production and pulmonary eosinophilic inflammation, while T_H17 and T_H1 responses were augmented. This suggests that ICOSL plays an important deterministic role in the development of allergic airway hyperresponsiveness and in controlling T_H17 responses. Notably, this is the first report showing an inhibitory role for ICOS–ICOSL interaction in the development of T_H17 inflammation.

Methods

Animals

Female C57BL/6 Wt mice (7–10 weeks old) were bred at the University of Manitoba (Winnipeg, Manitoba, Canada) breeding facility. ICOSL KO female C57BL/6 littermates were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Animals were used in accordance with the guidelines issued by the Canadian Council on Animal Care.

Reagents

Complete RPMI-16 was prepared according to the manufacturer's instructions. Fluorescence-conjugated anti-CD4⁺, anti-IL-4, anti-IFN- γ and anti-IL-17F antibodies and matched

isotype controls and Fc blocker antibodies against CD16/32 were purchased from eBioscience. Paired antibodies for ELISA measurement of IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-12p40/23, IFN- γ , TGF- β 1 and eotaxin were purchased from BD Pharmingen or eBioscience. CD4⁺ microbeads for isolating CD4⁺ T cells were purchased from Miltenyi Biotec, Germany. Biotinylated anti-IgG1, anti-IgG2a and anti-IgE antibodies were commercially available from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). OVA and alum adjuvant were purchased from Sigma.

Mouse asthma model and lung mechanics measurement

Mice were sensitized and re-challenged with OVA as previously described (32). Briefly, mice were intra-peritoneally immunized with OVA (2 μ g) in 0.5-ml alum adjuvant. After 2 weeks, mice were intra-nasally re-challenged with OVA (50 μ g in 40 μ l PBS).

To determine whether OVA challenge has different effects on respiratory mechanics in ICOSL KO and Wt (C57BL/6) mice, we performed aerosol MCh challenges using a flexiVent small animal ventilator (Scireq, Montreal, Quebec). Twenty-four hours after OVA re-challenge, mice were anesthetized intra-peritoneally (pentobarbital 0.1 ml per 10 gram body weight). After establishing anesthesia, mid-cervical tracheotomy was performed by inserting a polyethylene catheter (1.1 $\times$ 25 mm), which was kept in place by ligating with surgical silk. The catheter was coupled to the small animal ventilator and positive end expiratory pressure was maintained at 3 cmH₂O. The ventilator delivered a tidal volume of 10 ml air per kilogram body weight at a rate of 150 breaths min⁻¹. Mice were subjected to serial aerosol MCh challenge (30 μ l, 3–50 mg ml⁻¹ MCh in saline), and baseline mechanics were determined using saline-only challenge; before each challenge with saline or MCh, lung loading history was normalized by inflation to total lung capacity. Respiratory mechanics were assessed using a preset flexiVent Prime-8 low-frequency forced oscillation protocol to derive respiratory mechanical input impedance (Z_{rs}). Airway resistance (R_{aw}), tissue resistance (G) and lung elastance (H) were derived by fitting Z_{rs} to the constant phase model (33).

Bronchoalveolar lavage and histopathological analysis

The mice sensitized and challenged with OVA were killed and analyzed for airway eosinophilic inflammation and mucus production at 1 week after intra-nasal challenge as described (32). For bronchoalveolar lavage (BAL), the mice tracheas were cannulated, and the lungs were washed three times with 1 ml of PBS. The BAL fluids were centrifuged immediately. The cells were re-suspended for counting and BAL smears preparation. In order to determine the eosinophil count, the BAL smear slides were air-dried, fixed and stained with a Hema-3 stain set (Fisher Scientific). The numbers of eosinophils per 200 cells were counted based on cellular morphology and staining characteristics. For histological analysis, lungs were collected and fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin. Five-micrometer sections were cut, stained with hematoxylin and eosin (H&E) and examined by light microscopy. For analysis of mucus production, typical periodic acid Schiff

(PAS) staining of lung tissues were done as described previously (32, 34) and histological mucus index (HMI) was calculated to represent the proportion of mucus-producing epithelium occupying to total airway epithelium. The examination was blinded as to which samples were derived from ICOSL KO and Wt C57BL/6 mice.

Determination of OVA-specific antibody levels

OVA-specific murine IgG1, IgG2a and IgE were measured by ELISA as described previously (34) using antibodies purchased from Southern Biotechnology Associates, Inc.. Briefly, to determine OVA-specific serum antibodies, ELISA plates (Corning 25805; Corning Science Products, Corning, NY, USA) were coated overnight with 50 $\mu\text{g ml}^{-1}$ OVA (Sigma) in bicarbonate coating buffer (0.1 M, pH 9.6). After blocking for 90 min with a 1% BSA, 0.05% Tween 20 solution and extensive washing, serially diluted sera were incubated for 2 h at 37°C. The plates were washed, and biotinylated rat anti-mouse antibody was added and incubated for overnight at 4°C. Alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Bio/Can Scientific) was added and the plates were kept for incubation at 37°C for 45 min. After extensive washing of the plates, *p*-nitrophenyl phosphate (in 0.5 mM MgCl_2 , 10% diethanolamine, pH 9.8) was added, and the reaction was allowed to proceed for 60 min. The plates were read with a microplate reader (Versamax™; Molecular Devices) at 405 nm. Results are expressed as ELISA titers using the end-point (cutoff at

OD 0.5) of the titration curves compared with a constant internal standard run in each assay. Each serum sample was measured at least three times.

Splenic cell culture

Wt and ICOSL KO mice were intra-peritoneally immunized with OVA in alum adjuvant (five mice per group). After 2 weeks, mice were intra-nasally re-challenged with OVA alone. One week after re-challenge, spleens were collected. Single-cell suspensions of spleen cells were homogenized using a cell grinder and filtered by passing them through 40- μm cell strainer (BD Falcon). RBCs were lysed using NH_4Cl solution and then spleen cells were cultured at $7.5 \times 10^6 \text{ ml}^{-1}$ (2 ml per well) with OVA (50 $\mu\text{g ml}^{-1}$) or alone in 24-well plates at 37°C in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine. Culture supernatants were harvested at 48 and 72 h and stored at -80°C until they were assayed for cytokines.

Cytokine measurements

Murine IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-12p40/IL-23, IFN- γ , TGF- β 1 and eotaxin were measured by a sandwich ELISA purchased from eBioscience (San Diego, CA, USA) as described (32, 35–37). Briefly, 96-well ELISA plates (Corning 25805; Corning Science Products) were coated with capture mAb at 1 $\mu\text{g ml}^{-1}$ in bicarbonate coating buffer (0.1 M NaHCO_3 , pH 9.6). After overnight incubation at 4°C, the plates were blocked with 1% BSA, 0.05% Tween 20 solution

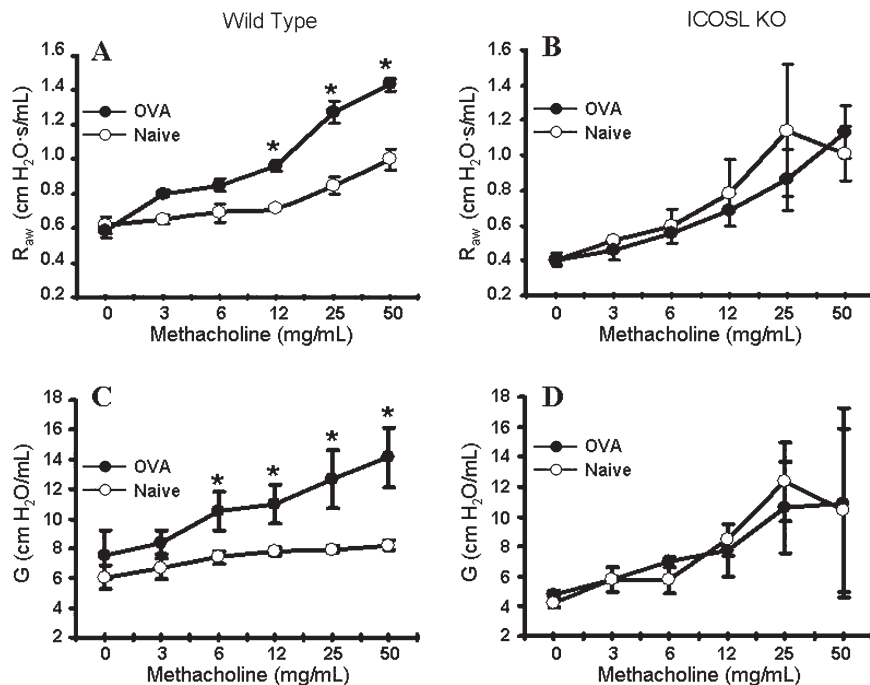


Fig. 1. Development of airway hyperresponsiveness is abrogated in ICOSL KO mice. Wt (A and C) and ICOSL KO (B and D) mice were intra-peritoneally sensitized with OVA (2 μg) in alum adjuvant. After 2 weeks, mice were intra-nasally re-challenged with OVA (50 μg) alone. Twenty-four hours after re-challenge, respiratory mechanics and the concentration-dependent effects of inhaled MCh were measured by low-frequency forced oscillation maneuvers using a small animal ventilator. For all experiments, age- and sex-matched naive mice were also studied. Respiratory parameters measured included central airway resistance (R_{aw}) (A and B), peripheral lung resistance (G) (C and D) and lung elastance (data not shown). Data are shown as the mean $\pm$ SEM for three independent experiments in each group of mice ($N = 3$); $*P < 0.05$; OVA-exposed versus naive mice in each panel. OVA = ovalbumin sensitized and challenged mice.

for 2 h at room temperature and washed four times. Culture supernatants and serially diluted recombinant cytokine standards were added to the plates. The plates were incubated at 37°C for 3 h and then washed four times. Biotinylated relevant anti-cytokine-detecting mAb ($1 \mu\text{g ml}^{-1}$) was added overnight at 4°C. The plates were washed next day and incubated with streptavidin-peroxidase conjugate at 37°C for 45 min. The plates were extensively washed and *p*-nitrophenyl phosphate (Sigma) was added as a substrate. The plates were read at 30, 60 and 90 min with a microplate reader (VersamaxTM; Molecular Devices) at 405 nm.

Intracellular cytokine staining

Spleens were removed aseptically and homogenized by a homogenizer in order to make a homogeneous cell suspension. Splenocytes were obtained by pressing the cell suspension through a 70- μm cell strainer. Erythrocytes were lysed with ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) followed by two washes in RPMI 1640 with 10% FBS and re-suspended in complete RPMI 1640 medium. Cells were incubated with CD4⁺ microbeads (Miltenyi Biotec, Auburn, NY, USA) for 15 min at 4°C. Magnetically labeled cells were isolated using a MACS LS column (Miltenyi Biotec) according to the manufacturer's instructions. For intracellular cytokine staining, splenocytes were stimulated with phorbol myristate acetate (50 ng ml^{-1} ; Sigma), ionomycin ($1 \mu\text{g ml}^{-1}$; Sigma) and anti-CD3 ϵ ($10 \mu\text{g ml}^{-1}$; BD Biosciences) and incubated for 5 h in complete RPMI 1640 medium at 37°C. For the last 4-h incubation, brefeldin A (for

IL-4 and IFN- γ) or monensin (for IL-17F) (eBioscience) was added to accumulate cytokines intracellularly. In brief, cultured cells (2×10^6) were washed twice and incubated with FcR block antibodies (anti-CD16/32; eBioscience) for 20 min at 4°C to block non-specific staining. Cell surface staining was performed first with FITC-conjugated anti-CD4⁺ (L3T4). The cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen) as per the manufacturer's instructions and stained intracellularly with APC-conjugated anti-IFN- γ (XMG 1.2) and FITC-conjugated anti-IL-4 (BVD6-24G2) mAbs (eBioscience) or with corresponding isotope control antibodies in permeabilization buffer (eBioscience) for 30 min at 4°C. Finally, the cells were washed and re-suspended in Dulbecco-PBS containing 2% FBS and acquired using a FACS Calibur flowcytometer (Becton Dickinson) and analyzed using CellQuest ProTM software.

In vitro lymphocyte proliferation assay

Spleens were removed aseptically and homogenized by a homogenizer in order to make a homogeneous cell suspension. Splenocytes were obtained by pressing the cell suspension through a 70- μm cell strainer. Erythrocytes were lysed with lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) followed by two washes in RPMI 1640 with 10% FBS and re-suspended in complete RPMI 1640 medium. In a 96-well plate, $50 \mu\text{l}$ of $0.5\text{--}1 \mu\text{g ml}^{-1}$ (sub-optimal concentration) and/or $10 \mu\text{g ml}^{-1}$ (sledgehammer dosage) of anti-CD3 ϵ antibody (Clone#145-2C11; BD Pharmingen) in PBS were coated at 4°C overnight. PBS only was added to

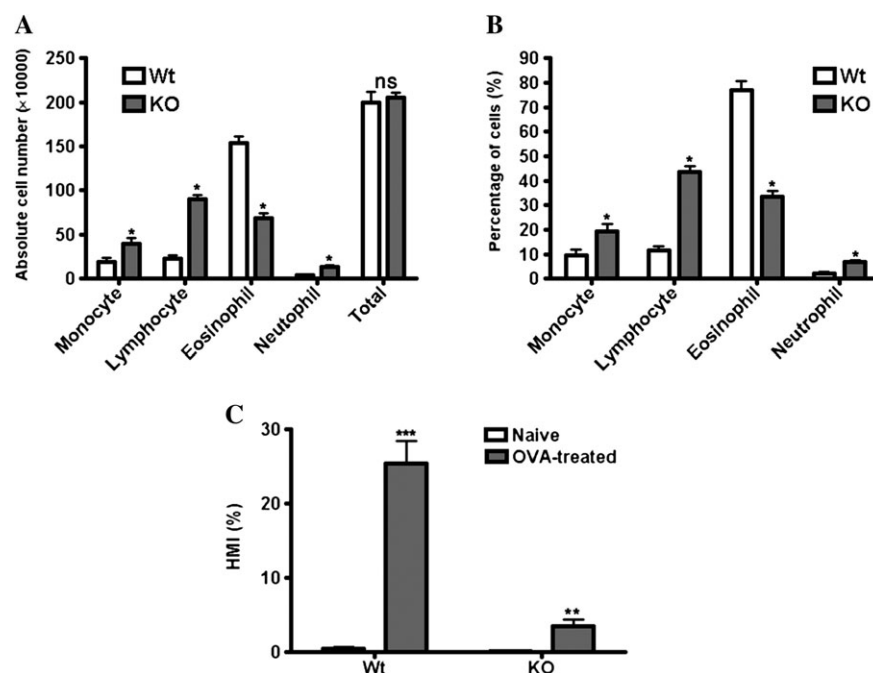


Fig. 2. ICOSL KO mice show reduced pulmonary eosinophilic infiltration into the BAL fluid. Mice were intra-peritoneally sensitized and intra-nasally re-challenged with OVA as described in the legend to Fig. 1. One week after intra-nasal re-challenge, mice were euthanized for analysis. (A and B) BAL fluid was collected and smears of cells were stained and cell differentials were determined. (A) Absolute number of total and different cell types. (B) The percentage of different cell types. (C) Lung tissues were fixed by formalin, sectioned and stained by PAS staining. HMI, as an index of mucus production, was calculated based on the area of mucus-producing epithelium composed of the total area of the airway epithelium. Data are shown as the mean $\pm$ SEM in each group of mice. * $P < 0.05$; ICOSL KO versus Wt C57BL/6 mice. ns, statistically not significant. nd, not detectable.

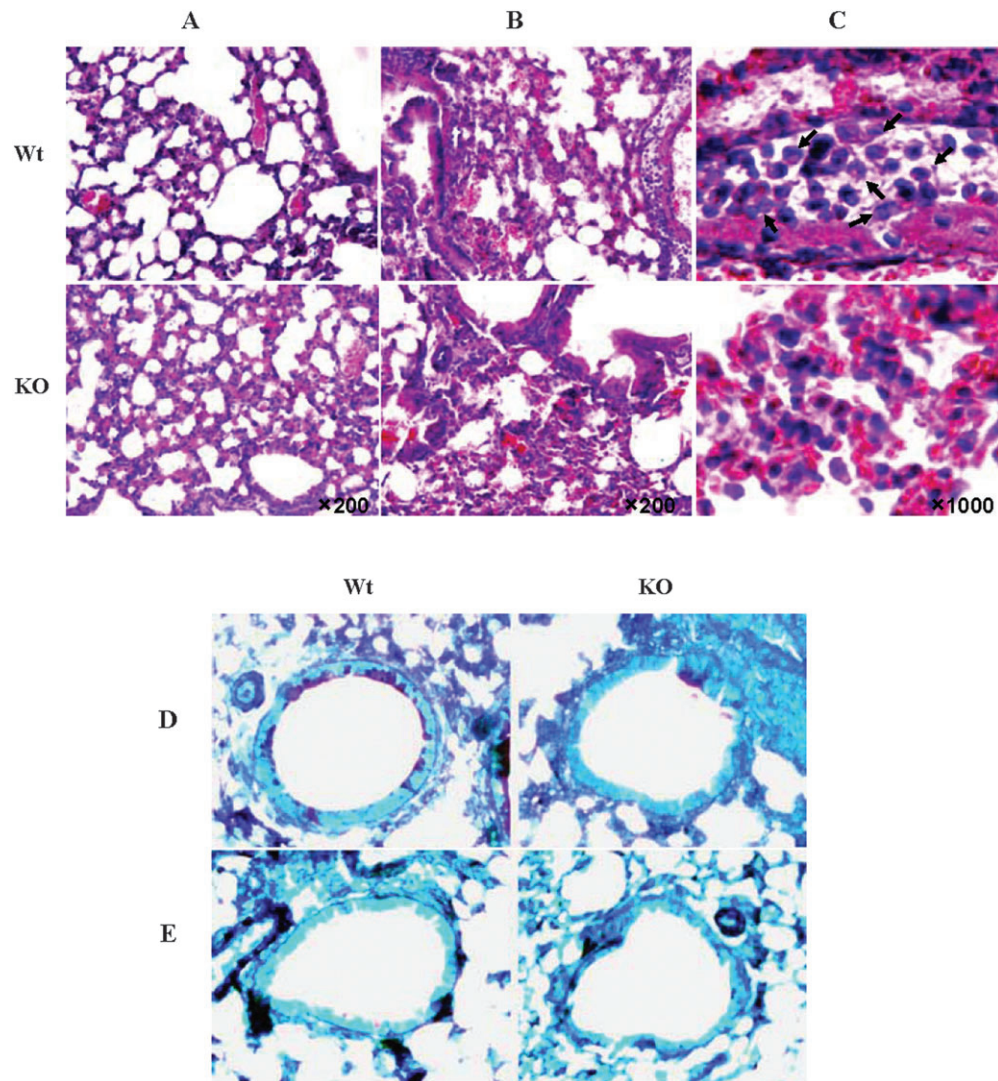


Fig. 3. Histopathological analysis of mice lungs. Mice were intra-peritoneally sensitized and intra-nasally re-challenged with OVA as described in the legend to Fig. 1. One week after intra-nasal re-challenge, lungs were collected. Photomicrographs of H&E-stained sections show the histopathology of hyperreactive lungs in ICOSL KO and Wt mice. The arrows show the abundant number of eosinophils. One of five similar samples is shown. (A) Naive Wt and KO samples as indicated on the figure show a normal lung structure in both the groups of mice. (B and C) OVA-treated Wt and KO samples, respectively, showing higher eosinophil (arrows) infiltration into the lung tissues of Wt mice. OVA-treated (D) and naive mice (E) show typical PAS staining in both Wt and KO mice lung tissue samples. As it is shown, Wt mice show more mucus production (pink) in comparison with that of KO mice.

the control unstimulated wells. After incubation, wells were washed twice with PBS. Spleen cells were cultured at 10^6 ml^{-1} for 2–4 days at 37°C and 5% CO_2 . After the incubation time, 20 μl of 5 mg ml^{-1} 3-(4,5 Dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide (MTT) (Sigma) solution was added to each well and incubated in a humidified cell culture incubator for 4 h. Afterward, 200 μl of MTT lysing solution (20% SDS, 50% DMF; Sigma) was added to each well and incubated in room temperature overnight. The plates were read with a tunable microplate reader (VersamaxTM; Molecular Devices) at 570 nm. All cell samples were used in triplicates.

Statistical analysis

Results are shown as mean $\pm$ SEM were analyzed using unpaired two-tailed Student's *t*-test (GraphPad PRISMTM 4.00).

Comparisons of respiratory mechanics data were performed using Kruskal–Wallis test using Dunn's *post hoc* analyses. Unless otherwise stated each experiment contained five mice in each group, and one of at least three independent experiments with similar results is shown. A *P*-value of <0.05 was considered significant in all quantitative experiments.

Results

Lack of ICOSL prevents OVA-induced airway hyperresponsiveness

In response to inhaled bronchospastic agents such as MCh, elevated central airway resistance (R_{aw}) and peripheral lung resistance, which is manifest in part from airflow resistance in small airways, are cardinal features of asthma that can be

routinely simulated in mice repeatedly exposed to allergen (38–40). To examine the importance of the ICOSL molecule in OVA-induced airway hyperresponsiveness, we compared changes in respiratory mechanics of naive and OVA-sensitized/challenged ICOSL KO and Wt control mice in response to inhaled MCh. For our model of acute allergic airway inflammation, we first sensitized mice by intra-peritoneal immunization with OVA (in alum) and then performed intra-nasal challenge with the same protein 2-weeks post sensitization. Twenty-four hours after challenge, respiratory mechanics were measured using a small animal ventilator. The OVA exposure protocol we used induced marked airway hyperresponsiveness in C57BL/6 mice: R_{aw} induced by MCh concentrations $>6 \text{ mg ml}^{-1}$ was significantly elevated in OVA-exposed mice, and tissue resistance (G) was significantly higher at all MCh concentrations $>3 \text{ mg ml}^{-1}$ (Fig. 1A and C). Moreover, OVA-exposed C57BL/6 mice exhibited marked hypersensitivity to inhaled MCh as the concentration required for a 50% increase in baseline R_{aw} ($5.14 \pm 0.82 \text{ mg ml}^{-1}$) was significantly reduced compared with naive mice ($28.37 \pm 4.11 \text{ mg ml}^{-1}$) ($P < 0.01$). In striking contrast, whereas naive ICOSL KO mice exhibited responsiveness to MCh that mimicked naive C57BL/6 animals, OVA sensitization and challenge had no impact on MCh responses in ICOSL mice (Fig. 1B and D). In addition, we did not detect any increase in sensitivity of R_{aw} to inhaled MCh in ICOSL KO mice as the concentration of MCh required to increase baseline values by 50% was not different for naive and sensitized mice (17.08 ± 6.63 versus 23.66 ± 8.36 , respectively) ($P = 0.72$). As part of our protocol, we also derived total lung elastance, which approximately doubled with maximum MCh challenge in naive and OVA-exposed mice; we found no difference in this effect between ICOSL and C57BL/6 mice (data not shown), suggesting that there were no underlying lung structural abnormalities or intrinsic differences in surfactant function in the two mouse strains. Collectively, these data indicate that ICOSL is essential for the development of airway hyperresponsiveness in OVA-exposed mice.

ICOSL KO mice show decreased pulmonary pathology, eosinophilic inflammation and mucus production

Pulmonary inflammation induced by OVA treatment was examined 7 days after intra-nasal challenge with OVA using BAL and histological analysis as described previously (38). Although BAL analysis of both Wt and KO mice revealed significant airway cellular infiltration, the cellular pattern in each mouse strain was dramatically different. BAL infiltrates in Wt mice were composed mainly of eosinophils, while ICOSL KO mice showed more neutrophils and mononuclear cells (Fig. 2A and B). The cellular infiltration in BAL fluids of naive mice was minimal (<4000 cells). H&E staining also confirmed that there was significantly much more eosinophilic infiltration into the lung tissues of Wt mice compared with KO mice (Fig. 3B and C). HMI, a quantitation of mucus-producing airway epithelial cell revealed by PAS staining, was significantly higher in Wt mice than that of ICOSL KO mice (Figs 2C and 3D and E). These results demonstrate that ICOSL plays an important role in the airway eosinophilic

inflammation in allergic asthma and acute mucosal responses related to mucous production.

Lower OVA-specific antibody production in ICOSL KO mice compared with Wt controls

One week after intra-nasal re-challenge with OVA, sera were collected from OVA-treated mice and analyzed for OVA-specific serum antibodies using ELISA. As shown in Fig. 4, although both groups exhibited an increase in OVA-specific antibody responses following OVA treatment, the titers for specific IgG1, IgG2a and IgE in ICOSL KO mice were significantly lower than those of Wt controls (Fig. 4). The actual antibody titers for OVA-specific IgG1, IgG2a and IgE in ICOSL KO mice were 6.8, 13.4 and 22.7 times lower than those of Wt controls, respectively. The results indicate that ICOSL is a significant determinant of OVA-induced antibody responses, with IgE being the subclass most affected.

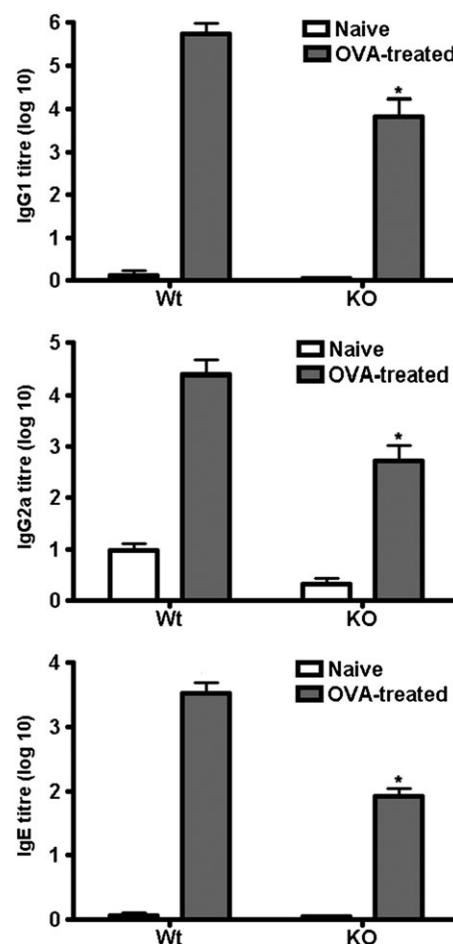


Fig. 4. Lower serum levels of OVA-specific IgG1, IgG2a and IgE antibody production in ICOSL KO mice compared with their Wt controls. Mice were intra-peritoneally sensitized and intra-nasally re-challenged with OVA as described in the legend to Fig. 1. The sera were collected from mice 1 week after intra-nasal re-challenge and analyzed for OVA-specific IgG1, IgG2a and IgE using an ELISA as described in Methods. Data are shown as mean $\pm$ SEM in each group of mice. * $P < 0.05$; OVA-treated KO mice versus OVA-treated Wt C57BL/6 mice.

Reduced T_H2 response and enhanced T_H1 and T_H17 responses in ICOSL KO mice

As T-cell cytokine responses are critical in the regulation of inflammatory reactions including allergic responses, we further examined antigen-driven cytokine production in OVA-treated mice using splenic cell bulk culture and intracellular cytokine staining. The results showed that T_H1 cytokine (IFN- γ) production by splenocytes from OVA-treated ICOSL KO mice was significantly higher than those of Wt controls either with or without OVA re-stimulation *in vitro* (Fig. 5E). In contrast, the production of T_H2 cytokines (IL-4, IL-5, IL-10 and IL-13) in ICOSL KO mice was significantly lower than those of Wt controls (Fig. 5A–D). Similarly, the KO mice showed significantly lower T_H2 cytokine levels but higher T_H1 (IFN- γ) level in the BAL fluids than Wt mice (Fig. 6A–F). To confirm

the cytokine pattern of CD4⁺ T cells in the KO and Wt mice induced by the OVA, we further analyzed the production of T_H2 (IL-4) and T_H1 (IFN- γ) cytokines in the OVA-treated mice using an intracellular cytokine staining assay. Wt mice showed 2.41 times (10.99% versus 4.55%) higher IL-4 production but 4.13 times (2.61% versus 11.07%) lower IFN- γ production by CD4⁺ T cells than those of ICOSL KO mice (Fig. 7). These results demonstrate that ICOSL plays an important role in enhancing antigen-driven T_H2 response but is inhibitory for T_H1 responses. Interestingly, the KO mice also showed 4.06 times higher (10.5% versus 2.5%) intracellular IL-17F production than their Wt counterparts (Fig. 7A and B). Since TGF- β 1, IL-6 and IL-23 are important cytokines in the development of T_H17 population, we further examined the production of these cytokines in the OVA-treated mice.

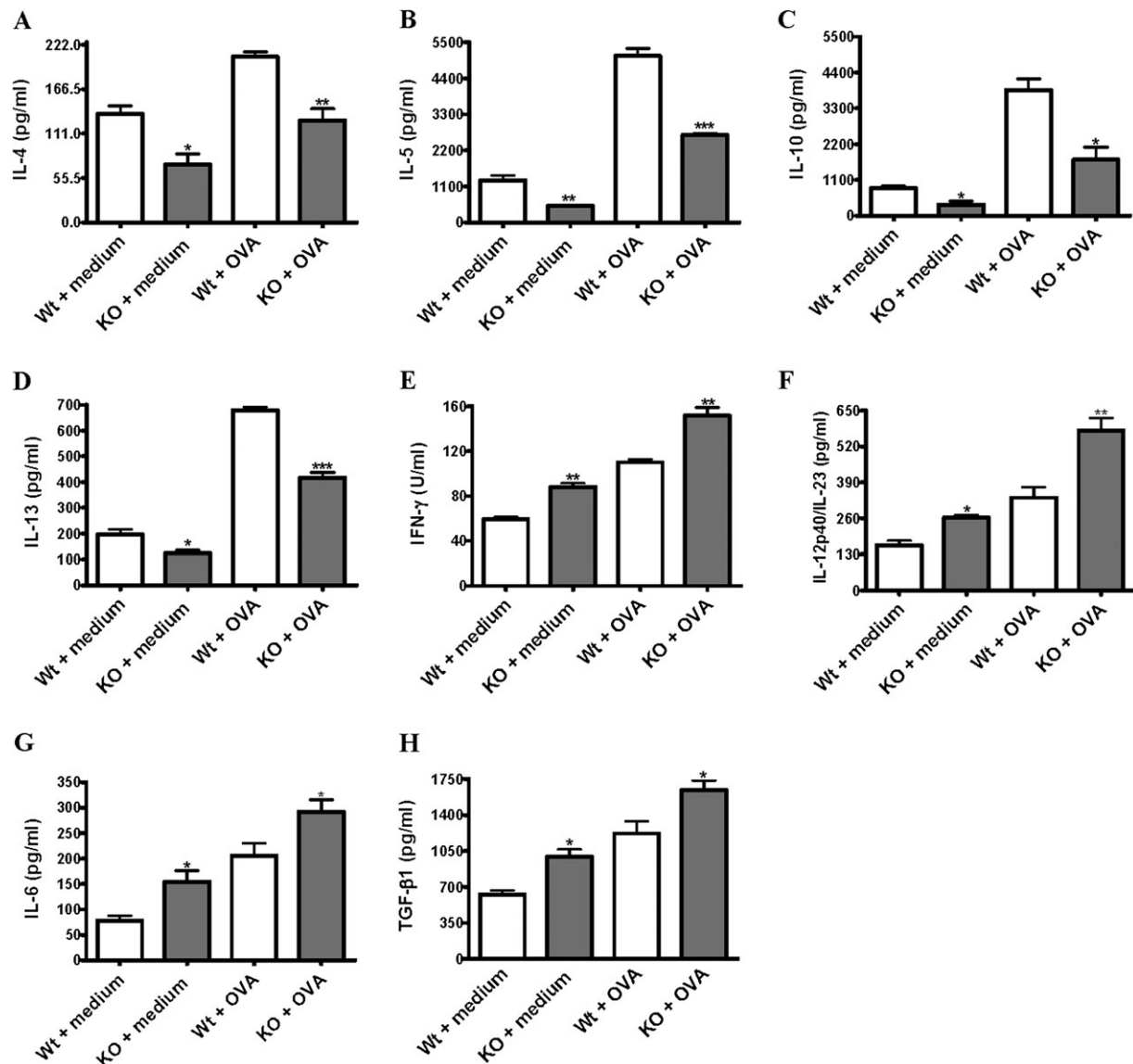


Fig. 5. Reduced T_H2 and enhanced T_H1 and T_H17 promoting cytokine production by spleen cells from ICOSL KO mice. Mice were intra-peritoneally sensitized and intra-nasally re-challenged with OVA as described in the legend to Fig. 1. One week after re-challenge, spleen cells were cultured with or without OVA. Cytokines production (72-h supernatants) was measured by a sandwich ELISA. Data are shown as the mean $\pm$ SEM in each group of mice. * P < 0.05; ** P < 0.01; KO mice versus Wt mice in the same culture condition (with or without OVA re-stimulation).

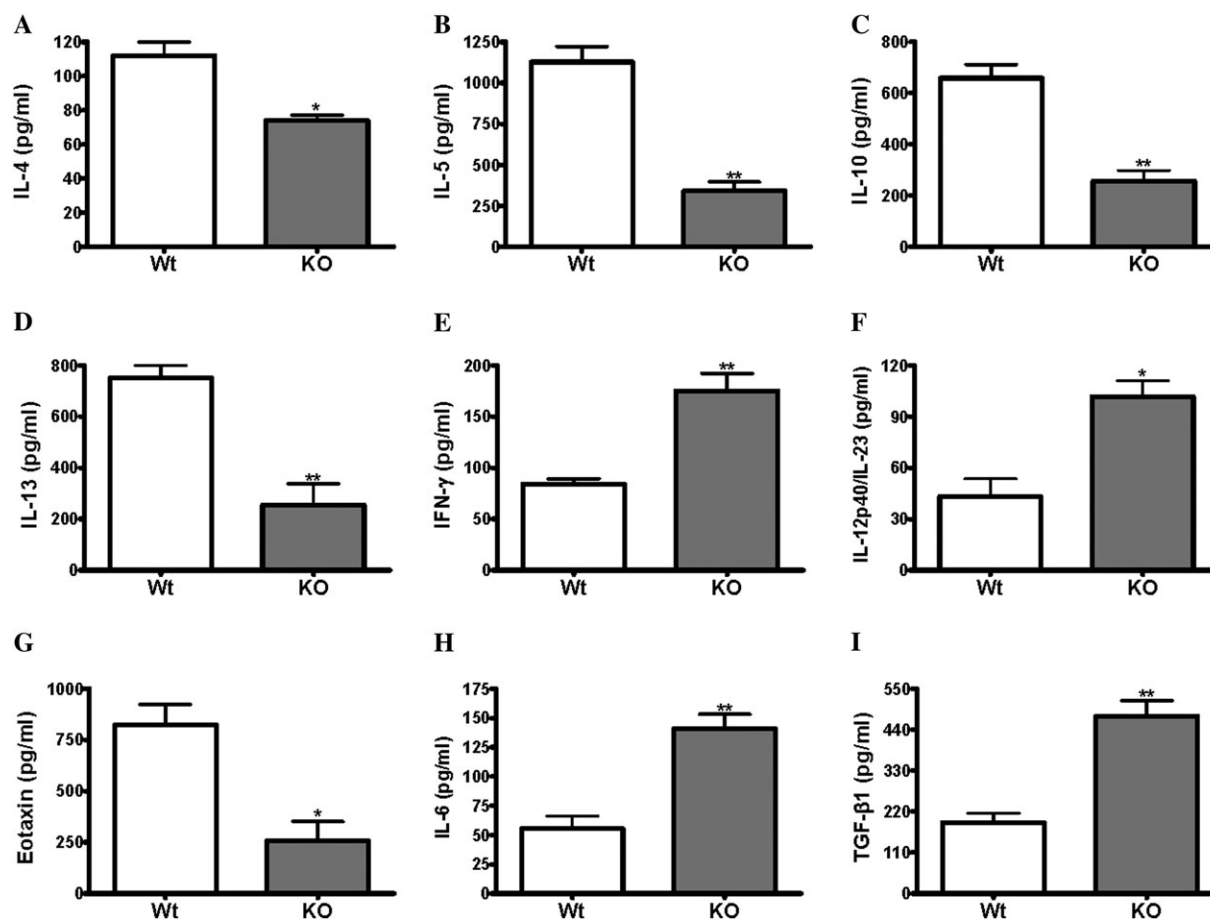


Fig. 6. Reduced T_H2 - and enhanced T_H1 - and T_H17 -promoting cytokines levels in the BAL fluids in ICOSL KO mice. Mice were intra-peritoneally sensitized and intra-nasally re-challenged with OVA as described in the legend to Fig. 1. One week after re-challenge, BAL fluids were collected from both groups of mice and the cytokine/chemokine production was measured by sandwich ELISA. Data are shown as mean $\pm$ SEM in each group of mice. * $P < 0.05$.

The results showed that ICOSL KO mice produced significantly higher TGF- β 1 and IL-6 than those of Wt mice in both splenic cell culture and BAL fluid (Fig. 5G and H and Fig. 6H and I). In addition, IL-12 p40, the α -chain of IL-23, was also significantly higher in the lung of KO mice (Fig. 6F). The results suggest that ICOSL is inhibitory for T_H17 cell development in allergic reactions, which is probably related to its inhibitory effect on T_H17 -promoting cytokines. Last, consistent with our observations that eosinophil infiltration was reduced in ICOSL KO mice, we also showed that KO mice produced significantly less eotaxin (an eosinophil mobilizing chemokine) in BAL fluid compared with Wt mice (Fig. 6G).

ICOSL KO mice show reduced lymphocyte proliferation capability

Lymphocyte proliferation *in vitro* upon antigen-specific (OVA) and polyclonal (coated anti-CD3 mAb) re-stimulation was measured using splenocytes isolated from Wt and ICOSL KO mice that were sensitized and re-challenged with OVA were compared. As shown in Fig. 8(A), upon both antigen-specific and polyclonal T-cell stimulation (anti-CD3 ϵ antibody), splenic cells from ICOSL KO mice showed significantly lower proliferation capability (i.e. less MTT uptake)

than those of Wt mice. Similar differences were observed when the cells were stimulated by the combination of OVA and coated anti-CD3 ϵ (Fig. 8A). Morphologically, the cultured cells with antigen-specific or polyclonal T-cell stimulation showed much more larger size lymphoblasts and cellular clusters in cultures from Wt mice than from KO mice. Interestingly, the production of IL-2, an important cytokine for T-cell proliferation, was not significantly different (Fig. 8B) between the KO and Wt mice, suggesting a critical intrinsic role for ICOS-ICOSL interaction in T-cell proliferation.

Discussion

One of the most significant findings in this study is that airway hyperresponsiveness induced by OVA sensitization and challenge is virtually absent in ICOSL KO mice. Since airway hyperresponsiveness is the hallmark of asthma, the results demonstrate potential for a critical role of ICOS-ICOSL interaction in the axes that underpin the pathogenesis and maintenance of allergic asthma. The suppression of OVA-induced airway hyperresponsiveness in ICOSL KO mice was associated with reduced T_H2 cytokine (IL-4, IL-5, IL-10 and IL-13) production and specific IgE response. Logically, lower

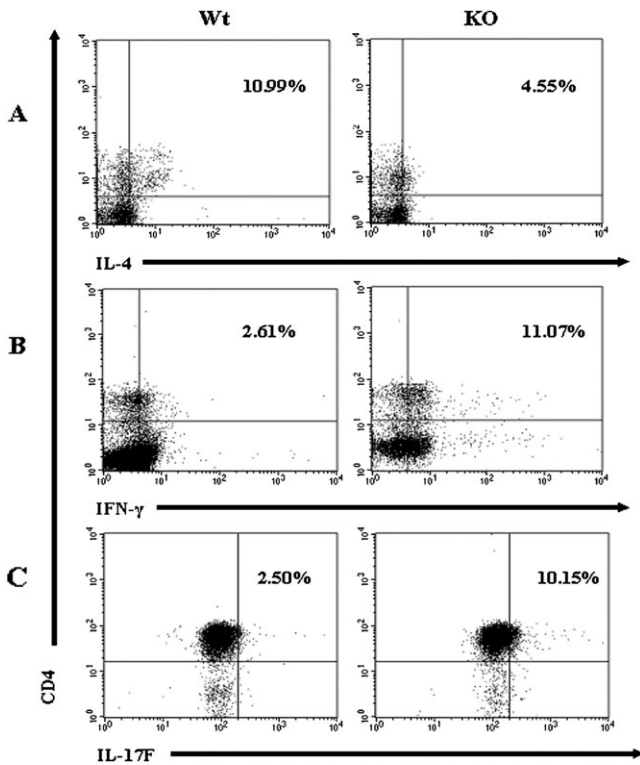


Fig. 7. The intracellular cytokine of CD4⁺ T cells. Mice were intra-peritoneally sensitized and intra-nasally re-challenged with OVA as described in the legend to Fig. 1. One week after re-challenge, CD4⁺ T cells were isolated from spleens and stained for CD4⁺ marker and intracellular IL-4, IFN- γ and IL-17F as indicated in Methods. All density plot charts were based on 10⁴ cells satisfying a gate set on a forward versus side light scatter that defined spleen lymphocytes as well as a second gate that defined CD4⁺ cells. The percentages of cytokine-producing cells (upper right) are indicated on each chart. The flowcytometric data are representative of three independent experiments with similar results.

specific IgE production after intra-nasal re-challenge likely results in less IgE cross-linking on the surface of mast cells and eosinophils, and as a consequence, histamine release is reduced, which could be one mechanism for reduced development of airway hyperresponsiveness. In addition, the lungs of KO mice showed remarkably fewer eosinophils (Fig. 2A and B) and mucus overproduction (goblet cell hyperplasia) after OVA exposure compared with Wt counterparts (Figs 2C and 3D and E). Moreover, these altered responses occur concomitantly with reduced T-cell activation and development, especially T_H2 cells, suggesting an important role for ICOS-ICOSL interaction in the profile of inflammation induced by OVA exposure. As a newly defined costimulatory molecule, ICOS is expressed on the surface of T cells especially CD4⁺ T cells and is elevated during the late phases of the immune response (1–3). Consistent with our study, there are other reports that confirm the involvement of the ICOS-ICOSL interaction in the polarization of cytokine responses toward a T_H2 profile and preferential expression of ICOS on the surface of T_H2 cells (2, 3). Collectively, our study reveals a central role for the ICOS-ICOSL axis in determining the nature of allergic inflammation and immunity that can develop and drive changes in lung function.

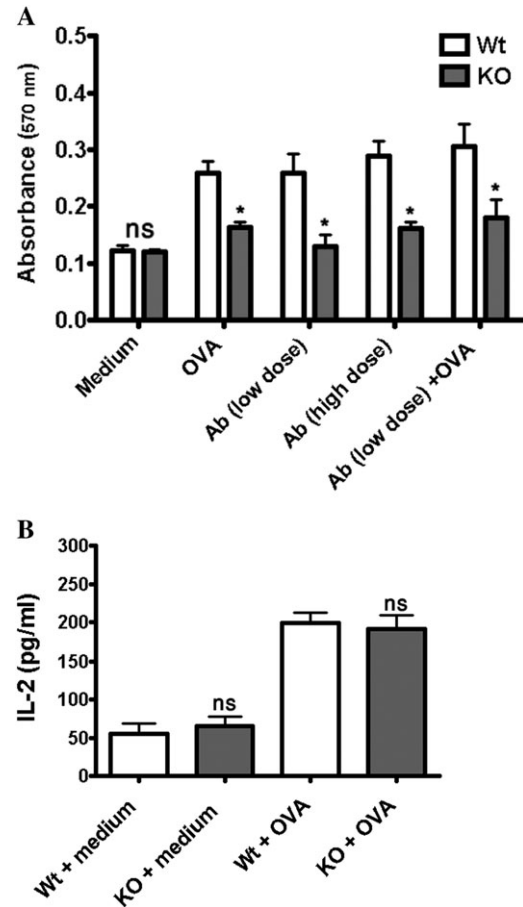


Fig. 8. Reduced lymphocyte proliferation in KO mice. Mice (five mice per group) were intra-peritoneally sensitized and intra-nasally re-challenged with OVA as described in the legend to Fig. 1. (A) One week after re-challenge, spleen cells were stimulated for 3 days with OVA (50 μ g ml⁻¹) or low (1 μ g ml⁻¹) or high doses (10 μ g ml⁻¹) of coated anti-CD3 ϵ antibody or OVA plus low dose of coated anti-CD3 ϵ antibody and assayed using an MTT-based lymphocyte proliferation assay as described in Methods. (B) For IL-2 production, spleen cells were cultured with or without OVA. IL-2 levels in the culture supernatants (72 h) were measured by a sandwich ELISA. Data are shown as mean $\pm$ SEM in each group of mice. **P* < 0.05; ns, not significant.

Another novel finding in the present study is the significant increase of T_H17 response we saw in ICOSL KO mice. To our knowledge, this is the first report showing an important role for ICOS-ICOSL interaction in regulation of T_H17. The higher T_H17 response in ICOSL KO mice was well correlated with the higher production of the three most important cytokines for T_H17 development, i.e. TGF- β 1, IL-6 and IL-23 (IL-12p40 is a shared subunit of IL-23 and IL-12), than that of their Wt controls. Therefore, the increased T_H17 development in ICOSL KO mice may reflect a promoting effect of these cytokines on T_H17 development. Notably, T_H1 response, similar to T_H17 response, was also increased in ICOSL KO mice. The increase of T_H17 and reduction of allergic asthma observed in ICOSL KO mice were consistent with a recent finding by Schnyder *et al.* (18, 19). that showed an inhibitory role of IL-17 on established OVA-induced allergic hyperreactivity. It was found that mucosal IL-17 administration inhibited the

asthma-like reaction by reducing the pulmonary production of IL-5 and the chemokines TARC and eotaxin, while neutralization of IL-17 using antibody exacerbated the established allergic responses.

An additional interesting finding is the significant reduction of T-cell proliferation in KO mice without significant change in IL-2 production. We observed that spleen cells from OVA-treated KO mice, in response to antigen-specific (OVA) and anti-CD3 ϵ antibody stimulation, showed significantly less proliferative activity than those of Wt controls (Fig. 8A). Notably, antigen-driven IL-2 production by spleen cells from the two groups of mice were not statistically significant (Fig. 8B), which is in agreement with the previous finding that CD28 but not ICOS signaling is important for IL-2 production (41–46). The data demonstrate that, although IL-2 is an important cytokine for T-cell proliferation, without ICOS–ICOSL interaction, cell proliferation is not markedly induced even in the presence of IL-2. The reduction in T-cell proliferation fits well with the dramatic decrease in T_H2 response we observed for ICOSL KO mice but is apparently contradictory with the higher T_H1 and T_H17 responses in KO mice. It may be because, due to the nature of the model (allergic T_H2 response), most of the proliferating cells in Wt mice are T_H2 cells, thus a small proportion of proliferating T_H1 and T_H17 cells having no significant impact in total proliferation levels observed in the assay. Moreover, since T_H2 cells express higher ICOS than T_H1 cell (3), it is logical to assume that ICOS–ICOSL interaction is particularly important for T_H2 cell proliferation.

Collectively, our study reveals that the ICOSL molecule plays an important role during the development of allergic inflammatory responses and airway hyperresponsiveness; thus, it can be considered as a molecule that carries a predisposing potential for the initiation and maintenance of the allergic asthma. Therefore, ICOSL might be considered as a potentially useful target in the immunotherapy of allergic diseases. Furthermore, considering the overlapping and synergistic roles of CD28 and ICOS molecule, expressed on T cells (47–50), combinatorial manipulation of the expression of these molecules and T_H17 response would open a new avenue for future immunotherapy of allergy and asthma.

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Disclosure

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