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. Th2-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 Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) but more Th1 (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 Th17 cytokine production and diminishing the Th1 response and airway eosinophilia.

Keywords: airway hyperreactivity, ICOSL, ovalbumin, Th17

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 Tn 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 Th2/Th1 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 Thn cells, named Th17 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.
ICOSL is essential for airway hyperresponsiveness

and Th2 cytokine production (31). However, the involvement in local inflammation. In an allergic airway inflammation model, (29) and of cardiac allograft rejection (30) results in altered production (26–28). Blockade of ICOS–ICOSL interaction in body isotype class switching and defects in IL-4 and IL-13 mediated immune responses 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 Th1,2 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 Th1,2 cytokine release, IgE response, airway mucus production and pulmonary eosinophilic inflammation, while Th1,7 and Th1,1 responses were augmented. This suggests that ICOSL plays an important deterministic role in the development of allergic airway hyperresponsiveness and in controlling Th1,7 responses. Notably, this is the first report showing an inhibitory role for ICOS–ICOSL interaction in the development of Th1,7 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-β1 and etoxacin 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 alun 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 μg) in 0.5-ml alun adjuvant. After 2 weeks, mice were intra-nasally re-challenged with OVA (50 μg in 40 μl 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 × 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 μl, 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 (Zrs). Airway resistance (Raw), tissue resistance (G) and lung elastance (H) were derived by fitting Zrs 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 shift
(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 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; Comin Science Products, Comin, NY, USA) were coated overnight with 50 μg 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 MgCl₂, 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 NH₄Cl solution and then spleen cells were cultured at 7.5 × 10⁶ ml⁻¹ (2 ml per well) with OVA (50 μg 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-β1 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; Coming Science Products) were coated with capture mAb at 1 μg ml⁻¹ in bicarbonate coating buffer (0.1 M NaHCO₃, pH 9.6). After overnight incubation at 4°C, the plates were blocked with 1% BSA, 0.05% Tween 20 solution

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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 (Raw) (A and B), peripheral lung resistance (G) (C and D) and lung elastance (data not shown). Data are shown as the mean ± 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 µg 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 (Versamax™; 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 µg ml⁻¹; Sigma) and anti-CD3ε (10 µg 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 × 10⁶) 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-17F (BD6-24G2) mAbs (eBioscience) or with corresponding isotype 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 Pro™ 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 µl of 0.5–1 µl gm l⁻¹ (sub-optimal concentration) and/or 10 µl gm l⁻¹ (sledgehammer dosage) of anti-CD3ε antibody (Clone#145-2C11; BD Pharmingen) in PBS were coated at 4°C overnight. PBS only was added to

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**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 ± SEM in each group of mice. *P < 0.05; ICOSL KO versus Wt C57BL/6 mice. ns, statistically not significant. nd, not detectable.
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 (Versamax™; Molecular Devices) at 570 nm. All cell samples were used in triplicates.

**Statistical analysis**

Results are shown as mean ± SEM were analyzed using unpaired two-tailed Student’s $t$-test (GraphPad PRISM™ 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: $R_{sw}$ induced by MCh concentrations > 6 mg ml$^{-1}$ was significantly elevated in OVA-exposed mice, and tissue resistance ($G$) was significantly higher at all MCh concentrations > 3 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 $R_{sw}$ (5.14 ± 0.82 mg ml$^{-1}$) was significantly reduced compared with naive mice (28.37 ± 4.11 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 KO mice (Fig. 1B and D). In addition, we did not detect any increase in sensitivity of $R_{sw}$ 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 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 mucus 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.
Reduced Th2 response and enhanced Th1 and Th17 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 Th1 cytokine (IFN-\(\gamma\)) 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 Th2 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 Th2 cytokine levels but higher Th1 (IFN-\(\gamma\)) 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 Th2 (IL-4) and Th1 (IFN-\(\gamma\)) 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-\(\gamma\) 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 Th2 response but is inhibitory for Th1 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-\(\beta\)1, IL-6 and IL-23 are important cytokines in the development of Th17 population, we further examined the production of these cytokines in the OVA-treated mice.
The results showed that ICOSL KO mice produced significantly higher TGF-β1 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 Th17 cell development in allergic reactions, which is probably related to its inhibitory effect on Th17-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 and that were infected with ICOSL KO mice. As shown in Fig. 8(A), upon both antigen-specific and polyclonal T-cell stimulation (anti-CD3: 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: (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 Th2 cytokine (IL-4, IL-5, IL-10 and IL-13) production and specific IgE response. Logically, lower
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 Th2 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 Th2 profile and preferential expression of ICOS on the surface of Th2 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.

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-\(\beta\)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-CD3ε 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 Th2 response we observed for ICOSL KO mice but is apparently contradictory with the higher Th1 response we observed in the assay. Moreover, since Th2 cells express no ICOSL, it can be considered as a molecule that carries a pre-dominant role in the immunotherapy of allergic asthma.

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 ICOSL molecule, expressed on T cells (47–50), combinatorial manipulation of the expression of these molecules and Th17 response would open a new avenue for future immunotherapy of allergy and asthma.

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References
34 Ou, H., Yang, J., Bai, H. et al. 2004. Less inhibition of interferon-gamma to organism growth in host cells may contribute to the high susceptibility of C3H mice to Chlamydia trachomatis lung infection. Immunology 111:453.