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ICOS promotes group 2 innate lymphoid cell activation in lungs

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ABSTRACT

Group 2 innate lymphoid cells (ILC2s) are newly identified, potent producers of type 2 cytokines, such as IL-5 and IL-13, and contribute to the development of allergic lung inflammation induced by cysteine proteases. Although it has been shown that inducible costimulator (ICOS), a costimulatory molecule, is expressed on ILC2s, the role of ICOS in ILC2 responses is largely unknown. In the present study, we investigated whether the interaction of ICOS with its ligand B7-related protein-1 (B7RP-1) can promote ILC2 activation. Cytokine production in ILC2s purified from mouse lungs was significantly increased by coculture with B7RP-1-transfected cells, and increased cytokine production was inhibited by monoclonal antibody-mediated blocking of the ICOS/B7RP-1 interaction. ILC2 expansion and eosinophil influx induced by papain, a cysteine protease antigen, in mouse lungs were significantly abrogated by blocking the ICOS/B7RP-1 interaction. Dendritic cells (DCs) in the lungs expressed B7RP-1 and the number of DCs markedly increased with papain administration. B7RP-1 expression on lung DCs was reduced after papain administration. This downregulation of B7RP-1 expression may be an indication of ICOS/B7RP-1 binding. These results indicate that ILC2s might interact with B7RP-1-expressing DCs in allergic inflammatory lung, and ICOS signaling can positively regulate the protease allergen-induced ILC2 activation followed by eosinophil infiltration into the lungs.

1. Introduction

Allergic asthma is characterized by chronic airway inflammation with pronounced eosinophil infiltration, mucus overproduction, and airway hypersensitivity. Although a variety of cell types are involved in allergic inflammation, there is substantial evidence that infiltration of Th2 cells and increased levels of type 2 cytokines, including IL-4, IL-5, and IL-13, are common in the lungs of patients with allergic asthma [1]. Thus, Th2 cells have long been thought to be the primary source of type 2 cytokines [2]. In contrast, subsequent studies revealed that novel innate lymphocytes, called group 2 innate lymphoid cells (ILC2s), potently produce type 2 cytokines in response to epithelial-derived cytokines, such as IL-33 [3–7]. Protease allergens, such as papain, which is a plant-derived cysteine protease, induce the production of these cytokines in epithelial cells [8]. Intranasal administration of papain or IL-33 induced lung eosinophilia and mucus overproduction in recombination activating gene (*Rag*)$^{-/-}$ mice that lack T/B cells [9,10]. Treatment of lung explants with papain increased the production of IL-33 from stromal cells and that of IL-5 and IL-13 by ILC2s [6]. Moreover, papain did not induce eosinophilic lung inflammation in *Il33*$^{-/-}$ or *Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice that lack ILC2s [6,10]. Consequently, it appears that ILC2s are capable of inducing allergic lung inflammation by producing type 2 cytokines.

Optimal T cell activation requires two signals: one produced by the engagement of T cell receptors with antigen/major histocompatibility complexes and the other is the costimulatory signal delivered by the interaction of receptors on T cells and their corresponding ligands on antigen-presenting cells (APCs) [11]. The interaction of B7/CD28 family members plays a major role in providing costimulatory signals for T cells [12]. Inducible costimulator (ICOS), a member of CD28 receptor family, is expressed on activated T cells, and its ligand B7-related protein-1 (B7RP-1; also known as B7h, B7-H2, GL50, and ICOSL) is constitutively

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expressed on APCs, including dendritic cells (DCs) [12]. ICOS ligation by B7RP-1 enhances T cell differentiation and effector function [12]. In addition, anti-ICOS monoclonal antibody (mAb)-treated or ICOS-deficient mice showed the attenuated Th2 cell responses and eosinophil accumulation in ovalbumin-induced allergic lung inflammation [13–15]. Thus, ICOS is thought to play a crucial role in Th2 cell responses during the development of allergic lung inflammation. It has been shown that ICOS is also expressed on ILC2s and is a useful target for identifying ILC2s [16]. However, the role of ICOS in ILC2 responses is largely unknown. In this study, we investigated whether ICOS signaling can regulate ILC2 activation.

2. Materials and methods

2.1. Mice

Male C57BL/6 mice were purchased from Charles River Laboratories (Kanagawa, Japan). C.B-17/Icr-scid/scid Jcl (SCID) mice and C.B-17/Icr-+/+Jcl control mice were obtained from Crea (Tokyo, Japan). All mice were used at 6–10 weeks of age and kept under specific pathogen-free conditions during the experiments. All animal experiments were approved by Juntendo University Animal Experimental Ethics Committee.

2.2. Airway inflammation

Mice were intranasally injected with 30 μg papain (Merck Millipore, Darmstadt, Germany) in 40 μl of PBS or PBS alone on 3 consecutive days. Some groups of mice were intraperitoneally administered with 300 μg of anti-B7RP-1 mAb (HK5.3, rat IgG2a) [17] or control rat IgG2a 4 h before each papain injection. At 24 h after the last papain instillation, lungs were collected for subsequent assays.

2.3. Lung cell preparation

Lungs obtained from mice were minced with scissors and incubated in RPMI1640 medium (supplemented with 10 mM HEPES, 2 mM L-glutamine, 0.1 mg/ml penicillin/streptomycin, 50 μM 2-ME) containing 50 μg/ml Liberase TM (Roche, Basel, Switzerland) and 20 μg/ml DNase I (Roche) for 45 min at 37 °C. Digested tissues were further incubated for 5 min in the presence of 5 mM EDTA (Dojindo Laboratories, Kumamoto, Japan) and passed through a 70-μm cell strainer (BD Biosciences, San Jose, CA, USA). The cells were used for sorting or flow cytometry after lysis of red blood cells.

2.4. Flow cytometry

Cells were pre-incubated with unlabeled anti-CD16/32 mAb (2.4G2) to avoid non-specific binding of antibodies to FcγR. Cells were then incubated with the antibodies listed in Supplemental Table S1. Biotin-B7-H3 (MJ18), biotin-GITR (MIH44), biotin-TIM-1 (RMT1-4), biotin-TIM-2 (RMT2-26), biotin-TIM-3 (RMT3-23), biotin-TIM-4 (RMT4-53), and biotin-4-1BBL (TKS-1) were from our laboratory [18–23]. Cells incubated with biotinylated mAb were stained with PE-labeled streptavidin (eBioscience, San Diego, CA, USA). Stained cells were analyzed by BD LSRFortessa (BD Biosciences), and data were processed by FlowJo Version 7.6.5 software (FlowJo, Ashland, OR, USA). Live cells were gated by forward- and side-scatter profiles and 7-AAD (TONBO Biosciences, San Diego, CA, USA) exclusion.

2.5. ILC2 stimulation in vitro

Lineage$^-$ ($CD3\varepsilon^-CD11b^-CD11c^-CD19^-Gr\text{-}1^-TER\text{-}119^-$) and $CD90.2^+CD25^+CD127^+$ ILC2s were sorted from lung cells by JSAN cell sorter (Bay Bioscience, Kobe, Japan). Purified ILC2s (1×10^3 cells/well) were cultured with mitomycin C (Nacalai Tesque, Kyoto, Japan)-treated B7RP-1/P815 or P815 parent cells (1×10^3 cells/well) in the presence of 50 U/ml recombinant human IL-2 (Shionogi, Osaka, Japan), 10 ng/ml recombinant mouse IL-33 (R and D Systems, Minneapolis, MN, USA), anti-B7RP-1 mAb (HK5.3), anti-ICOS mAb (7E.17G9, rat IgG2b, BioLegend), or control rat IgG (20 μg/ml). To determine cytokine production, supernatants were collected after 7 days and assayed for IL-5 by enzyme-linked immunosorbent assay using OptEIA kit (BD Biosciences) and IL-13 using a Ready-SET-Go! Kit (eBioscience), according to the manufacturer's instructions.

2.6. Statistical analysis

Statistical analyses were performed by unpaired Student's *t*-tests. Results are expressed as mean ± SD or SEM, as described in each figure legend. $p < 0.05$ was considered significant.

3. Results

3.1. Expression of costimulatory molecules on lung ILC2s

ILC2s reside in the lung tissue of naïve mice, and intranasal administration of papain rapidly induced the activation of lung ILC2s [6]. We first obtained lung cells from PBS- or papain-treated mice and investigated ICOS expression on resting and activated lung ILC2s by flow cytometry. As shown in Fig. 1A, lung ILC2s were identified as Lineage$^-$ (CD3ε, CD4, CD8α, CD11b, CD11c, CD19, Gr-1, FcεRI, NK1.1, and TER-119) and c-Kit$^+$Sca-1$^+$CD25$^+$CD127$^+$ cells. Lung ILC2s constitutively expressed ICOS as previously reported, and ICOS expression increased after papain treatment (Fig. 1B). We next analyzed the expression of other costimulatory molecules and found that lung ILC2s from PBS-treated mice marginally expressed CD28, programmed cell death-1 (PD-1), its ligand PD-L1 (also known as B7-H1), and receptor activator of NF-κB ligand (RANKL) (Supplemental Fig. S1). CD28 expression slightly decreased and PD-1 expression increased after papain inhalation (Supplemental Fig. S1). Glucocorticoid-induced TNFR family-related gene (GITR), herpes virus entry mediator (HVEM), and Fas were clearly expressed on resting lung ILC2s, and Fas expression increased with papain injection (Fig. 1C). Although these three molecules were expressed in large populations of Lineage$^+$ cells, ICOS was only expressed in a few Lineage$^+$ cells (Fig. 1D). ICOS was expressed on a portion of CD3$^+$ T cells (Fig. 1E). Thus, the ICOS-expressing cells in the lungs included ILC2s and some T cells. Therefore, we focused on the role of ICOS in lung ILC2 activity because of its substantial and relative limited expression.

3.2. ICOS/B7RP-1 interaction increases cytokine production by ILC2s

To reveal whether ICOS/B7RP-1 interaction leads to ILC2 activation, ILC2s were purified from lungs of naïve C57BL/6 mice and cocultured with B7RP-1-transfected P815 (B7RP-1/P815) cells or P815 parental cells. IL-5 and IL-13 production by ILC2s was not observed from coculture with B7RP-1/P815 or P815 cells (Fig. 2), which indicates that ICOS signaling alone could not elicit cytokine production by ILC2s. We next investigated whether ICOS enhances cytokine production by ILC2s in the presence of stimulants. A combination of IL-33 with IL-2, but not IL-33 alone, induces production of cytokines by ILC2s purified from naïve mouse lungs *in vitro* [6]. As reported, the high amounts of IL-5 and IL-13 were

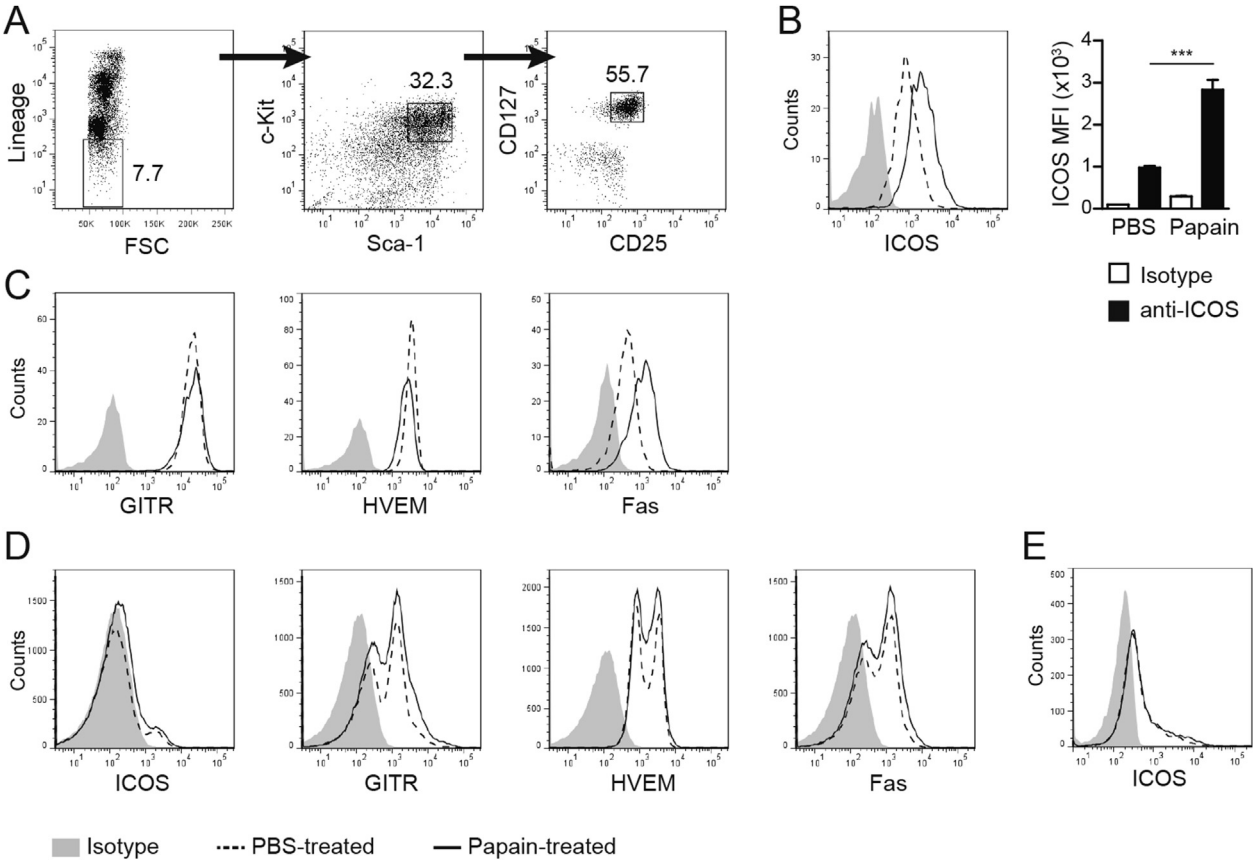

Fig. 1. Expression of costimulatory molecules on lung ILC2s. Mice were treated with papain or PBS for 3 consecutive days. At 24 h after the last instillation, lungs were collected for subsequent analysis. (A) Gating strategy for identifying lung ILC2s as Lineage$^-$c-Kit$^+$Sca-1$^+$CD25$^+$CD127$^+$ in C57BL/6 mice by flow cytometry is illustrated. Lineage; CD3ε, CD4, CD8α, CD11b, CD11c, CD19, Gr-1, FcεRI, NK1.1, and TER-119. The numbers in the plots indicate the percentage of cells in the indicated gates. (B) Surface expression of ICOS on lung ILC2s from PBS-treated mice (dashed line) and papain-treated mice (solid line), shown as the histogram overlay (*left*). Isotype control is shown as the shadowed area. Mean fluorescence intensity (MFI) of ICOS expression on lung ILC2s is expressed as the mean ± SEM of 3 mice in each group (*right*). (C, D) Surface expression of indicated molecules on (C) lung ILC2s and (D) Lineage$^+$ cells from PBS-treated mice (dashed line) and papain-treated mice (solid line), shown as the histogram overlay. Isotype control is shown as the shadowed area. (E) Surface expression of ICOS on CD45.2$^+$CD3$^+$ T cells in lungs from PBS-treated mice (dashed line) and papain-treated mice (solid line), shown as histogram overlay (*left*). Isotype control is shown as the shadowed area. ***$p < 0.001$. Similar results were obtained from 3 independent experiments.

detected in the culture supernatants of IL-2/IL-33-stimulated ILC2s (Fig. 2). Of note, ILC2s cocultured with B7RP-1/P815 cells produced higher amounts of IL-5 and IL-13 compared with those cocultured with P815 cells in the presence of IL-2 and IL-33 (Fig. 2). Enhancement of cytokine production from ILC2s by coculture with B7RP-1/P815 cells was significantly inhibited by both anti-B7RP-1 mAb and anti-ICOS mAb compared with each isotype-matched control (Fig. 2). These results indicate that ICOS engagement by

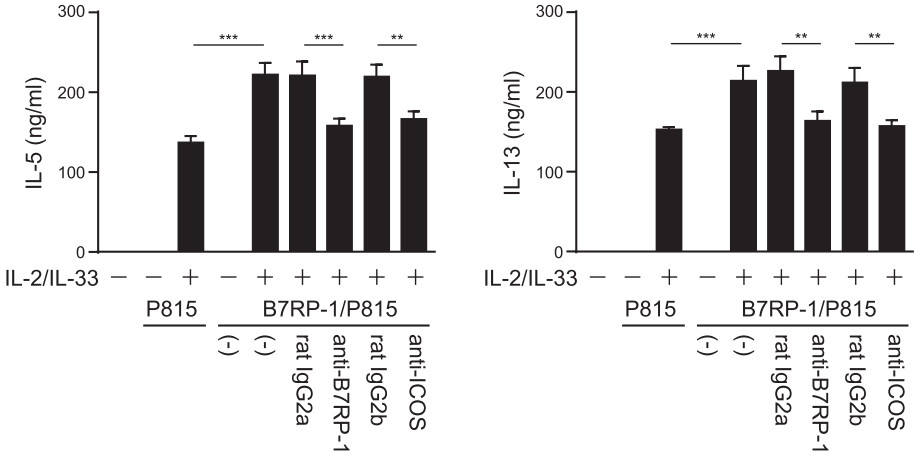

Fig. 2. ICOS/B7RP-1 interaction promotes IL-5 and IL-13 production by lung ILC2s. Purified ILC2s from C57BL/6 mouse lungs were cultured with mitomycin C-treated B7RP-1/P815 cells or P815 parent cells in the presence of recombinant human IL-2 (50 U/ml), recombinant mouse IL-33 (10 ng/ml), anti-B7RP-1 mAb (HK5.3), anti-ICOS mAb (7E.17G9), or corresponding isotype-matched control rat IgG (20 μg/ml). Production of IL-5 and IL-13 in the culture supernatants at 7 days were determined by ELISA. Data are presented as the mean ± SD. **$p < 0.01$ and ***$p < 0.001$. Similar results were obtained from 2 independent experiments.

B7RP-1, which is a natural ligand for ICOS, can promote IL-2/IL-33-induced cytokine production by ILC2s.

3.3. B7RP-1 is expressed on CD11c^{hi} DCs in the lungs

We next investigated which cells express B7RP-1 in lungs by flow cytometry. B7RP-1 was not detectable on CD3^{+}NK1.1^{-} T cells, CD3^{+}NK1.1^{+} T cells, CD3^{-}NK1.1^{+} NK cells, CD11c^{hi}Siglec-F^{+} alveolar macrophages, CD11c$^{-/dim}$Siglec-F^{+} eosinophils, Gr-1^{+} neutrophils, and CD45.2^{-}EpCAM^{+} epithelial cells in neither PBS- nor papain-treated lung (data not shown). CD19^{+} B cells in PBS-treated lungs marginally expressed B7RP-1 (Fig. 3A, B). Papain treatment did not alter B7RP-1 expression on CD19^{+} B cells and the number of B cells compared with those in the PBS-treated group (Fig. 3). Conventional DCs (cDCs), which reside in the lungs, can be subdivided into two distinct subsets based on the expression of cell surface markers: CD11c^{hi}CD11b^{-} cDCs (CD11b^{-} cDCs; also known as CD103^{+} cDCs) and CD11c^{hi}CD11b^{+} cDCs (CD11b^{+} cDCs). In addition, CD11c^{hi}CD11b^{+}CD64^{+} monocyte-derived DCs (moDCs) are recruited to the lungs when the lungs are challenged with foreign antigens [24]. B7RP-1 was present on CD11b^{-} cDCs, CD11b^{+} cDCs, and moDCs (Fig. 3A, B). The numbers of these DC subsets remarkably increased in papain-treated lungs compared with PBS-treated lungs, although B7RP-1 expression was reduced (Fig. 3). These data

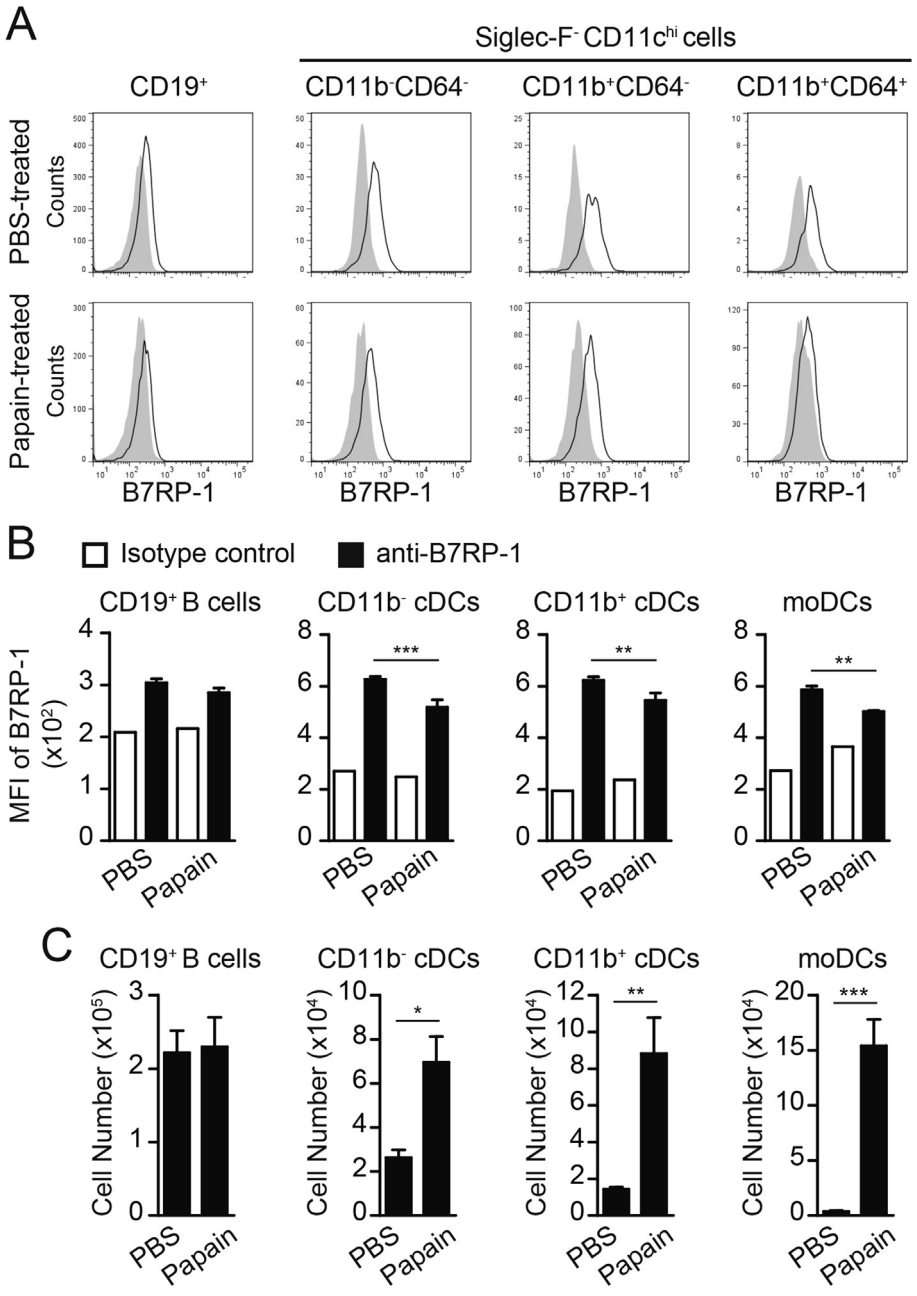

Fig. 3. B7RP-1 expression on lung cells. Mice were treated with papain or PBS for 3 consecutive days. At 24 h after the last instillation, lung cells were collected for flow cytometric analysis. (A) Surface expression of B7RP-1 on CD19^{+} B cells, CD11c^{hi}CD11b^{-}CD64^{-} DCs, CD11c^{hi}CD11b^{+}CD64^{-} DCs, and CD11c^{hi}CD11b^{+}CD64^{+} DCs within Siglec-F^{-} cells. (B) Mean fluorescence intensity (MFI) of B7RP-1 expression on CD19^{+} B cells, CD11c^{hi}CD11b^{-}CD64^{-} DCs (CD11b^{-} cDCs), CD11c^{hi}CD11b^{+}CD64^{-} DCs (CD11b^{+} cDCs), and CD11c^{hi}CD11b^{+}CD64^{+} DCs (moDCs) is expressed as mean ± SEM of four mice in each group. (C) The numbers of CD19^{+} B cells, CD11b^{-} cDCs, CD11b^{+} cDCs, and moDCs in lungs are expressed as mean ± SEM of four mice in each group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Similar results were obtained from 2 independent experiments.

indicate that B7RP-1-expressing DCs are abundant in lungs experiencing allergic inflammation.

3.4. Blocking ICOS/B7RP-1 interaction inhibits papain-induced eosinophil infiltration into the lungs

Finally, we evaluated the contribution of ICOS to ILC2 activation *in vivo*. As ICOS was also detected on $CD3^+$ T cells in the lungs (Fig. 1E), we examined the effect of anti-B7RP-1 mAb on papain-induced lung inflammation not only in wild-type mice but also in T/B cell-deficient SCID mice to eliminate the influence of ICOS expressed on T cells. In SCID mice, ICOS was rarely expressed on $Lineage^+$ cells, and almost all ICOS-expressing cells were $Lineage^-$ in both PBS- and papain-treated lungs (Fig. 4A, B). Thus, ICOS is preferentially expressed on ILC2s of SCID mouse lungs. The numbers of eosinophils ($CD11c^{-/dim}Siglec\text{-}F^+$) and ILC2s ($Lineage^-CD90.2^+CD25^+CD127^+$) in the lungs were significantly increased in papain-injected SCID mice to a similar or slightly

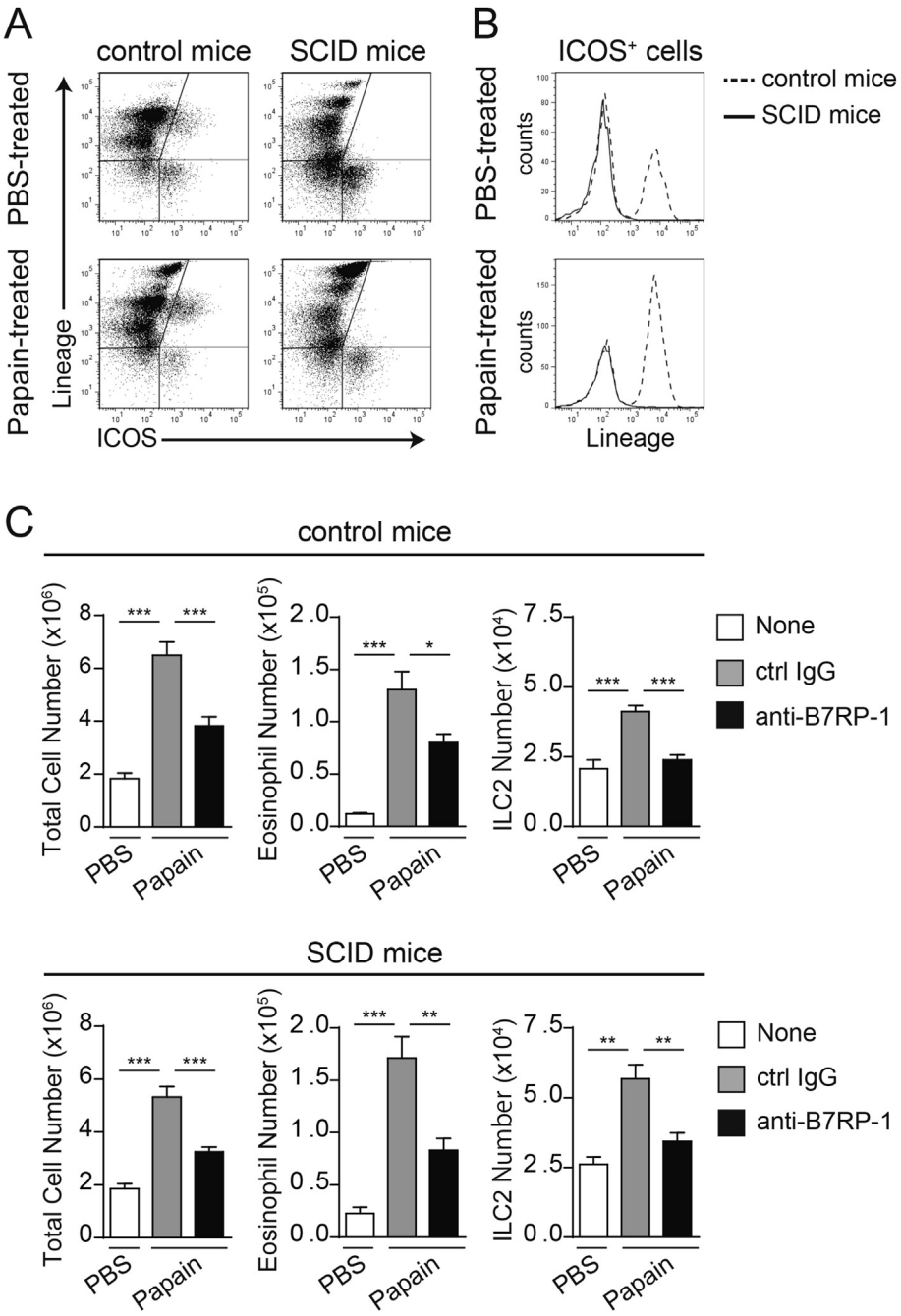

Fig. 4. Inhibitory effect of anti-B7RP-1 mAb on papain-induced lung inflammation in mice. SCID (C.B-17/Icr-scid/scid) and control mice (C.B-17/Icr-+/+) were treated with papain or PBS and administered anti-B7RP-1 mAb or isotype-matched control rat IgG (ctrl IgG) for 3 consecutive days. At 24 h after the last papain instillation, lung cells were collected for flow cytometry. (A) Surface expression of ICOS on $Lineage^+$ and $Lineage^-$ cells in lungs. (B) Surface expression of lineage markers on $ICOS^+$ cells in SCID mouse lungs (solid line) and wild-type control mouse lungs (dashed line). (C) Total cell number was counted, and $CD11c^{-/dim}Siglec\text{-}F^+$ eosinophils and $Lineage^-CD90.2^+CD25^+CD127^+$ ILC2s were analyzed by flow cytometry. Data are presented as mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Similar results were obtained from 2 independent experiments.

greater extent compared to those in wild-type control mouse lungs (Fig. 4C). Anti-B7RP-1 mAb administration significantly reduced the increased number of total lung cells, eosinophils, and ILC2s in SCID as well as control mice (Fig. 4C). Collectively, these data indicate that ICOS signaling can promote lung ILC2 activation *in vivo* during allergic inflammation.

4. Discussion

We elucidated whether ICOS-mediated signaling can promote activation of lung ILC2s. ICOS was expressed on ILC2s and a portion of T cells in the lungs. We found that intranasal papain administration resulted in the pronounced eosinophil infiltration and ILC2 expansion in the lungs of T/B cell-deficient SCID mice as well as T/B cell-sufficient wild-type mice, similar to that reported in a study using *Rag*$^{-/-}$ mice [6,10]. This result indicates that T/B cells are not involved in eosinophilic inflammation that was observed in the present study. We also found that mAb-mediated blocking of ICOS/B7RP-1 interaction significantly abrogated papain-induced eosinophil influx and ILC2 expansion in SCID as well as wild-type mouse lungs. Furthermore, almost all ICOS-expressing cells were Lineage^{-} in SCID mice. Collectively, the inhibitory effects of anti-B7RP-1 in the present study might be explained by inhibiting ICOS signaling in ILC2s but not T cells. In addition, cytokine production by ILC2s purified from lungs was significantly promoted by coculture with B7RP-1/P815 cells, but not P815 parent cells, and the increased cytokine production was inhibited by blocking ICOS/B7RP-1 interaction. Taken together, our findings indicate that ICOS signaling positively regulates the protease allergen-induced ILC2s activation followed by eosinophil infiltration into the lungs.

More recently, Maazi et al. showed that ILC2s expressed both ICOS and B7RP-1, and the ICOS/B7RP-1 interaction promoted cytokine production by ILC2s, although B7RP-1 expression was constitutively downregulated in ILC2s upon binding to ICOS [25]. Consistent with this, B7RP-1 expression was not detected on ILC2s in the current study. We observed that CD11b^{-} cDCs, CD11b^{+} cDCs, and moDCs in the lungs express B7RP-1, and the numbers of these DC subsets were markedly increased by papain administration. Crosstalk between group 3 ILCs and DCs in the gut has been reported [26], and it is possible that lung ILC2s may directly interact with DCs in a cell–cell contact-dependent manner. Watanabe et al. showed that B7RP-1 was downregulated in APCs, including DCs, by interaction with ICOS [27]. We found that B7RP-1 expression levels on lung DCs were significantly reduced after papain administration, which indicates that these DCs interact with ICOS-expressing cells in the lungs. It has been demonstrated that lung DCs localize in the peribronchial and perivascular areas [28–30]. Although the precise localization of ILC2s in lung tissue is unclear, a study using IL-5-reporter mice demonstrated that lung ILC2s accumulated in the perivascular area when mice were intranasally administered with the non-protease allergen chitin, which is a polysaccharide from arthropods, parasites, and fungi [31]. Additionally, ILC2s are detectable in bronchoalveolar fluids from inflamed lungs, but not from healthy lungs [16,32,33]. These observations led us to speculate that ILC2s move from the lung interstitium to peribronchial and perivascular areas under inflammatory conditions and then interact with B7RP-1-expressing DCs as well as ILC2s themselves, although further studies are needed to reveal whether coculture with DCs can promote cytokine production by ILC2s and whether ILC2s colocalize with DCs in the lungs under allergic inflammatory conditions.

In conclusion, the present study demonstrates that ICOS, a costimulatory molecule, promotes protease allergen-induced ILC2 activation, and that blocking the ICOS/B7RP-1 interaction could be useful for asthma therapy.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.06.005.

Transparency document

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