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Group 2 Innate Lymphoid Cells Promote an Early Antibody Response to a Respiratory Antigen in Mice

Li Yin Drake,* Koji Iijima,* Kathleen Bartemes,* and Hirohito Kita*,†

Innate lymphoid cells (ILCs) are a new family of immune cells that play important roles in innate immunity in mucosal tissues, and in the maintenance of tissue and metabolic homeostasis. Recently, group 2 ILCs (ILC2s) were found to promote the development and effector functions of Th2-type CD4⁺ T cells by interacting directly with T cells or by activating dendritic cells, suggesting a role for ILC2s in regulating adaptive immunity. However, our current knowledge on the role of ILCs in humoral immunity is limited. In this study, we found that ILC2s isolated from the lungs of naive BALB/c mice enhanced the proliferation of B1- as well as B2-type B cells and promoted the production of IgM, IgG1, IgA, and IgE by these cells in vitro. Soluble factors secreted by ILC2s were sufficient to enhance B cell Ig production. By using blocking Abs and ILC2s isolated from IL-5-deficient mice, we found that ILC2-derived IL-5 is critically involved in the enhanced production of IgM. Furthermore, when adoptively transferred to *Il7r*^{-/-} mice, which lack ILC2s and mature T cells, lung ILC2s promoted the production of IgM Abs to a polysaccharide Ag, 4-hydroxy-3-nitrophenylacetyl Ficoll, within 7 d of airway exposure in vivo. These findings add to the growing body of literature regarding the regulatory functions of ILCs in adaptive immunity, and suggest that lung ILC2s promote B cell production of early Abs to a respiratory Ag even in the absence of T cells. *The Journal of Immunology*, 2016, 197: 000–000.

Group 2 innate lymphoid cells (ILC2s) belong to a growing family of innate lymphoid cells (ILCs) that play important roles in innate immunity and the maintenance of tissue and metabolic homeostasis (1). Although ILC2s do not express Ag receptors, ILC2s react rapidly to infection and tissue injury by responding to cytokines such as IL-33 secreted by tissue cells. Mirroring the function of their adaptive counterpart Th2 cells, activated ILC2s produce a large quantity of type 2 cytokines, such as IL-5 and IL-13, and initiate innate type 2 immune responses (2). Recent literature now suggests that ILC2s modulate adaptive immunity by regulating the development and effector functions of CD4⁺ T cells (3–7). Indeed, ILC2s enhance CD4⁺ T cell function through direct cellular contact via MHC class II and OX40 ligand (OX40L) molecules and by secreting soluble factors, such as IL-4 (4–6, 8). Alternatively, ILC2s promote migration and chemokine production by dendritic cells (DCs), and thus promote CD4⁺ T cell functions (3, 7). Despite our growing knowledge about the effects of ILC2s on T cell-mediated adaptive immunity, information has been limited regarding the role of ILC2s in humoral immunity.

Ab responses can be generally divided into two arms: T cell-dependent Ab responses that are mediated primarily by B2 cells,

and T cell-independent Ab responses that are mediated by marginal zone B cells and B1 cells (9). The critical roles of T cells in promoting humoral immune responses to protein Ag have been known for many years. In addition, both T cell-dependent and -independent Ab responses can be regulated by innate immune cells, such as invariant NK T cells, DCs, and granulocytes (9). More recently, Magri et al. (10) identified splenic ILC3-like cells that enhance Ab production by marginal zone B cells. Moro et al. (11) showed that adipose tissue-derived ILC2s support self-renewal of B1 cells and promote production of IgA, suggesting the ability of certain ILCs to regulate B cell function and Ig production.

The aim of this study was to better understand the effects of ILC2s on B cells, in particular the regulation of T cell-independent Ab responses. We performed a series of experiments in vitro using isolated ILC2s and B cells, and in vivo using an airway polysaccharide Ag exposure model in mice. Our results indicate that lung ILC2s promote B cell production of early Abs to a respiratory Ag even in the absence of T cells. Soluble factors secreted by ILC2s, such as IL-5, most likely play a key role.

Materials and Methods

Mice and reagents

BALB/cJ, C57BL/6, and C57BL/6 *Il7r*^{-/-} mice were from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 *Il5*^{-/-} mice were provided by K. Takatsu (University of Toyama, Toyama, Japan). Female mice ages 6–12 wk were used in all experiments. All animal experiments and handling procedures were approved by the Mayo Clinic Institutional Animal Care and Use Committee, and were performed according to established guidelines.

Fluorescence-labeled Abs to CD3 (145-2C11), CD25 (PC61; 7D4), CD44 (IM7), CD14 (rmC5-3), CD11b (M1/70), CD16/CD32 (2.4G2), CD45R/B220 (RA3-6B2), CD23 (B3B4), purified anti-CD40 (HM40-3), and purified anti-ICOS (7E.17G9) were purchased from BD Biosciences. Fluorescence-labeled anti-ICOS (7E.17G9) was from Miltenyi Biotec. Anti-IL-5 (TRFK4), anti-IL-13 (eBio1316H), anti-IL-6 (BMS178), anti-IL-9 (16-7093), anti-GM-CSF (MMGM-CSFB2.6), and rIL-33 were from eBioscience. Control Abs were purified goat IgG, rat IgG (both from BD Biosciences), or mouse IgG (eBioscience). Mouse rIL-7 and rIL-25 and blocking polyclonal anti-OX40L Ab were from R&D Systems. Mouse rIL-4 was from PeproTech. LPS (L4516) was from Sigma-Aldrich. Abs to mouse IgG1, IgM, IgA, and IgE were from BD Pharmingen. The 4-hydroxy-3-nitrophenylacetic

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L.Y.D. and H.K. designed the studies and experiments, interpreted the data, and wrote the manuscript, and L.Y.D., K.I., and K.B. performed the experiments.

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Abbreviations used in this article: DC, dendritic cell; ILC, innate lymphoid cell; ILC2, group 2 ILC; i.n., intranasal; Lin, lineage; NP, 4-hydroxy-3-nitrophenylacetic; OX40L, OX40 ligand; WT, wild-type.

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(NP) hapten conjugated to aminoethylcarboxymethyl-Ficoll (NP-Ficoll) and NP (16)-BSA were from Biosearch Technologies.

ILC2 isolation and culture

ILC2s were isolated from the lungs of naive BALB/c or C57BL/6 mice, as described previously (12). Briefly, lungs were minced and digested with a mixture of collagenases (Roche Diagnostics) at 35.7 $\mu\text{g}/\text{ml}$ and DNase I (StemCell Technologies) at 25 $\mu\text{g}/\text{ml}$ at 37°C to obtain single-cell suspensions. RBCs were lysed with ammonium chloride/potassium lysing buffer. Subsequently, lung cells were stained with PE-conjugated Abs to CD3, CD14, CD11b, CD16/CD32, and B220, followed by magnetic depletion of PE⁺ cells with EasySep PE selection kit, as per the manufacturer's instructions (StemCell Technologies). These lineage[−] (Lin[−]) cell-enriched lung cells were then stained with fluorescence-labeled Abs to CD3, CD14, CD11b, CD16/CD32, B220, CD25, and CD44. ILC2s were isolated as the Lin[−]CD25⁺CD44^{hi} cell population by FACS sorting (BD FACSAria). ILC2s were resuspended in RPMI 1640 medium supplemented with 50 μM 2-ME, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% FBS, and then expanded by culturing in a 96-well tissue culture plate at 10^4 cells/well with a mixture of IL-33 (10 ng/ml) and IL-7 (10 ng/ml). Fresh IL-33 and IL-7 were added to the culture every 3 or 4 d, and ILC2s were used for experiments after 1–2 wk in culture. Before use, ILC2s were washed once with PBS to remove residual IL-33 and IL-7. Furthermore, supernatants of ILC2s that were cultured for 3 or 4 d were collected, pooled, and stored at −20°C for culture with B cells (see below).

B cell isolation and culture

Splenic B cells were purified using a Negative Selection EasySep mouse B cell enrichment kit (StemCell Technologies) to >90% cell purity. For cell proliferation assays, B cells were labeled with CFSE using the CellTrace CFSE cell proliferation kit, according to the manufacturer's instructions (Invitrogen). Labeled B cells were cultured in a 96-well plate at 2×10^4 cells/well with or without ILC2s at 10^4 cells/well. The cells were stimulated with indicated concentrations of anti-CD40 Ab or LPS in the presence or absence of 10 ng/ml IL-4 for 4 d. Cells were then harvested, stained with PE-conjugated anti-CD19 or anti-B220, and counted by FACS. For B1 and B2 cell isolation, peritoneal lavage cells were collected by flushing the peritoneal cavity of naive mice with 3–5 ml PBS. The cells were then stained with fluorescence-conjugated anti-CD23 and anti-B220 Abs. B1 cells (B220^{low}CD23[−]) and B2 cells (B220^{high}CD23⁺) were isolated by FACS sorting (Supplemental Fig. 1) (11, 13). Purified B1 cells and B2 cells were cultured as described above. In some experiments, neutralizing anti-IL-5, anti-IL-6, anti-IL-9, anti-IL-13, and anti-GM-CSF Abs were added at a concentration of 2 $\mu\text{g}/\text{ml}$. In separate experiments, the ILC2 culture supernatant was added to the B cell culture at 50 $\mu\text{l}/\text{well}$ in a total volume of 200 $\mu\text{l}/\text{well}$. Cell-free supernatants were harvested from the B cell culture wells and analyzed for Ab concentrations. The remaining cells were harvested and stained with PE-conjugated anti-CD19 or anti-B220 Ab. After washing, cells were resuspended in 400 μl buffer and analyzed by FACS by collecting the cells at a constant time (1 min).

Ab ELISA

To determine the levels of Igs in B cell supernatants, ELISA plates were coated with Abs to mouse IgG1, IgM, IgA, or IgE, blocked with 1% BSA, and incubated with culture supernatants or serum samples. After washing, the IgM and IgG1 plates were incubated with HRP-conjugated anti-mouse IgM or IgG1, followed by peroxidase substrate. The IgA and IgE plates were incubated with biotinylated secondary Abs, followed by streptavidin and peroxidase substrate. In all assays, the absorbance was read in a microplate autoreader after stopping the reaction with hydrochloric acid. To determine the levels of Abs specific for NP-Ficoll, ELISA plates were coated with NP (16)-BSA, blocked with BSA, and incubated with the diluted samples.

Adoptive cell transfer and airway Ag exposure in vivo

Lung ILC2s from C57BL/6 mice were expanded in vivo by i.p. injection with IL-25 and IL-33 (400 ng/mouse) daily for 4 d. Twenty-four hours after the last injection, lungs and mediastinal lymph nodes were collected. The lung single-cell suspensions were prepared as described above, and then pooled with the lymph node cells that were dispersed by using a cell strainer and syringe. Lin[−] lung cells were enriched by staining with a mixture of PE-conjugated lineage Abs, followed by immunomagnetic depletion of PE⁺ cells with EasySep PE selection kit. These cells were then stained with fluorescence-labeled Abs to CD3, CD14, CD11b, CD16/CD32, B220, and ICOS. Lin[−]ICOS⁺ ILC2s were sorted by FACS, as described previously (12). By FACS analysis, we confirmed that these Lin[−]ICOS⁺ ILC2s are ST2⁺ (Supplemental Fig. 2). Sorted ILC2s were resuspended in PBS and

adoptively transferred by i.v. injection (1.5×10^5 cells/mouse) to recipient *IL7r^{−/−}* mice (day 0). On days 1, 3, and 6, mice were exposed intranasally (i.n.) to NP-Ficoll (50 μg) plus bromelain (10 μg). Twenty-four hours after the last exposure, sera were collected and the levels of total Ab and NP-Ficoll-specific Ab were determined by ELISA.

Statistical analysis

Statistical significance was assessed with Student *t* test and a *p* value < 0.05 was considered significant.

Results

Lung ILC2s enhance B cell proliferation

To investigate the effects of ILC2s on B cell proliferation, we first used an in vitro culture system. ILC2s were isolated from the lungs of naive BALB/c mice, expanded, and maintained with IL-33 plus IL-7, and then washed before use. We have previously shown that these in vitro expanded ILC2s are lineage negative, CD44⁺CD25⁺, and ST2⁺ (5). B cells were isolated from the spleens of naive BALB/c mice. To analyze whether ILC2s affect B cell proliferation, we took two different approaches. First, we cultured CFSE-labeled B cells alone or together with ILC2s and then analyzed the B cell division profiles by FACS (Fig. 1A). Second, we cultured unlabeled B cells with or without ILC2s for 4 d and then counted the B cells in the cultures (Fig. 1B). Because the cytokine IL-4 and the costimulatory molecule CD40 play critical roles in B cell proliferation and class switch (14), we examined the effects of ILC2s on B cell proliferation in the presence or absence of IL-4 or anti-CD40 Ab. ILC2s significantly enhanced proliferation of B cells that were cultured without IL-4 or anti-CD40, as judged by CFSE staining and cell numbers (Fig. 1A, 1B, *p* < 0.01). IL-4 alone or anti-CD40 Ab alone induced modest proliferation of B cells, and the response was significantly enhanced in the presence of ILC2s (*p* < 0.01 and *p* < 0.05). In addition, a combination of IL-4 and anti-CD40 induced robust B cell proliferation, which was also further enhanced by ILC2s (*p* < 0.01).

To examine whether ILC2s have similar effects on B1 and B2 subpopulations of B cells, we isolated B1 cells (B220^{low}CD23[−]) and B2 cells (B220^{high}CD23⁺) from the peritoneal cavity of naive BALB/c mice and cultured them with or without ILC2s. Two common B cell agonists, anti-CD40 Ab and LPS, were used together with IL-4 to stimulate these B cells. Both B1 cells and B2 cells proliferated when stimulated with anti-CD40 or LPS in a concentration-dependent manner (Fig. 1C, 1D). Consistent with a previous report (11), ILC2s enhanced the proliferation of B1 cells and shifted the dose-response curves of anti-CD40 or LPS to the left (Fig. 1C); approximately one-third to one-tenth of the concentrations of anti-CD40 or LPS was required to achieve maximum proliferation in the presence of ILC2s as compared with the absence of ILC2s. Furthermore, ILC2s also enhanced the proliferation of B2 cells (Fig. 1D), although the effects were not as pronounced as B1 cells. Additional replicate data are provided in Supplemental Fig. 3. Collectively, these data indicate that ILC2s promote the proliferation of B cells of different subsets and from different organs.

Lung ILC2s enhance B cell Ab production

To investigate the effects of ILC2s on B cell Ig production, we cultured splenic B cells with or without ILC2s and quantitated Ig levels in the culture supernatants. B cells were stimulated with anti-CD40 or LPS in the presence or absence of IL-4. At 1.5 $\mu\text{g}/\text{ml}$, anti-CD40 did not induce detectable amounts of IgM or IgG1 in the absence of IL-4, and the addition of ILC2s did not affect Ig production (Fig. 2A). When IL-4 was added to the culture, anti-CD40 induced low levels of IgM and IgG1 production; the addition of ILC2s dramatically enhanced production of these Ig subtypes

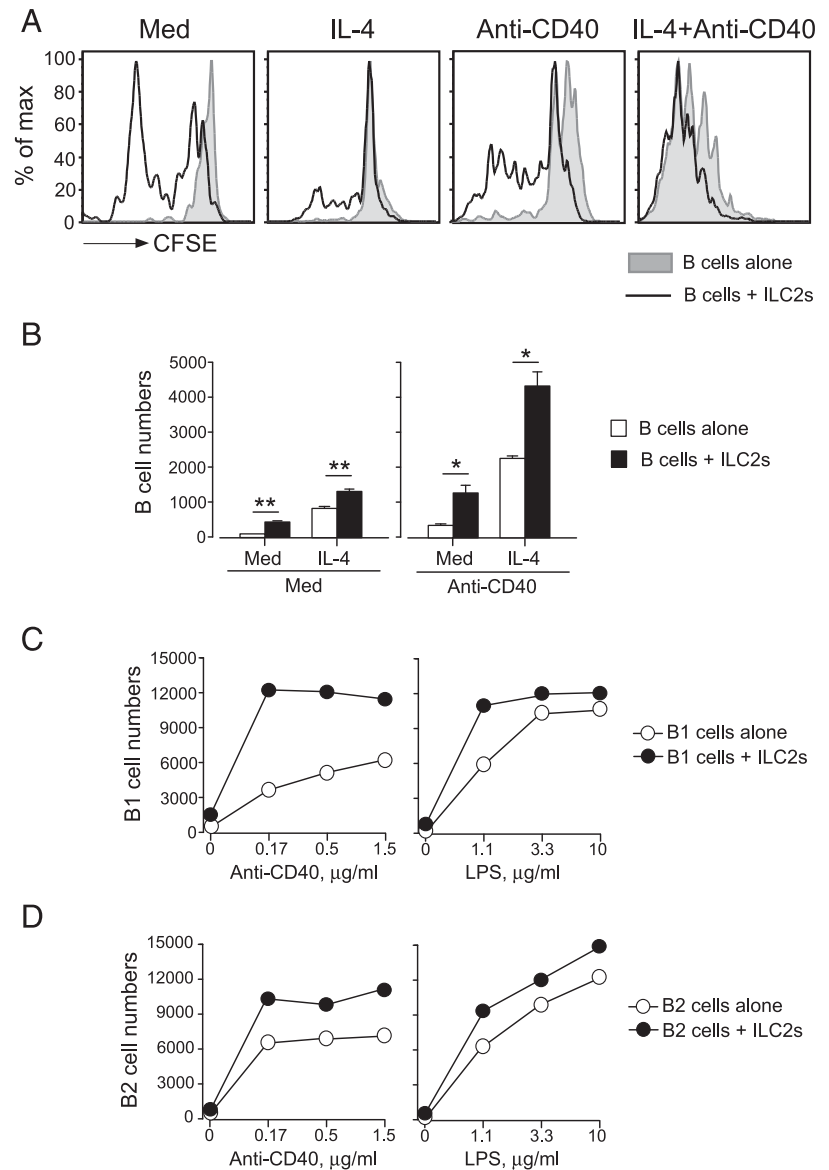


FIGURE 1. Lung ILC2s enhance the proliferation of B lymphocytes. Lung ILC2s and splenic or peritoneal B cells were isolated from naive BALB/c mice. **(A)** CFSE-labeled splenic B cells were cultured at 2×10^4 cells/well with or without ILC2s (10^4 cells/well) for 4 d. IL-4 (10 ng/ml) and/or anti-CD40 (1.5 µg/ml) were added to some cultures, as indicated. The proliferation profiles of B cells were analyzed by FACS. **(B)** Splenic B cells were cultured with or without ILC2s for 4 d with Med alone or in the presence of IL-4 and/or anti-CD40. The numbers of CD19⁺ B cells were determined by FACS. Data (mean $\pm$ SEM, $n = 3$) are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$ between the groups indicated by horizontal lines. **(C)** and **(D)** Peritoneal B1 cells and B2 cells were cultured at 2×10^4 cells/well with or without ILC2s (10^4 cells/well) in the presence of serial dilutions of anti-CD40 Ab or LPS plus IL-4 (10 ng/ml) for 4 d. The number of B220⁺ cells was determined by FACS. Each data point represents the cell numbers from one well. Data are representative of two independent experiments. Med, medium.

(Fig. 2A, $p < 0.01$ and $p < 0.05$). When B cells were stimulated with LPS at 5 µg/ml in the absence of IL-4 (Fig. 2B), IgM, but not IgG1, was produced, and ILC2s significantly increased IgM levels. The addition of IL-4 showed no apparent effect on IgM production, but enhanced IgG1 production. ILC2s robustly increased both IgM and IgG1 levels in the presence of IL-4. Collectively, these findings suggest that exogenous IL-4 is most likely required to reveal the full spectrum of ILC2's effects on B cell Ig production. Thus, we added IL-4 to all cultures in subsequent experiments.

To examine whether ILC2s affect production of other Ig subtypes by B cells, we analyzed the levels of IgE and IgA in the supernatants of B cells that were cultured with or without ILC2s in the presence of IL-4 (Fig. 2C). Similarly to IgM and IgG1, IgE and IgA production by B cells stimulated with LPS or anti-CD40 was significantly enhanced in the presence of ILC2s.

Because B1 and B2 cells are most likely involved in distinct immune responses (9), we compared the ILC2's effects on Ig production by B1 and B2 cells. When stimulated with anti-CD40 plus IL-4 in the absence of ILC2s, B1 cells produced IgM, but little IgG1, IgA, or IgE (Fig. 3A). ILC2s dramatically enhanced the production of all Ig classes, including IgM, IgG1, IgA, and IgE. Indeed, although IgG1, IgA, and IgE were nearly undetect-

able in the supernatants without ILC2s, the concentration-dependent response to anti-CD40 was apparent in the presence of ILC2s. Similarly, ILC2s enhanced the production of IgM, IgG1, IgA, and IgE by B2 cells stimulated with anti-CD40 (Fig. 3B); the concentration of Ig produced by B2 cells was approximately two to five times higher with ILC2s as compared with no ILC2s. In addition, both B1 and B2 cells stimulated with LPS plus IL-4 without ILC2s produced modest amounts of IgM, IgG1, and IgA (Fig. 3C, 3D); a >2 -fold increase in the levels of these Igs was observed in the presence of ILC2s. Additional replicate data are provided in Supplemental Fig. 4. Altogether, these findings suggest that ILC2s robustly promote production of various Ig subtypes by both B1 and B2 cells that are activated by immunological stimuli, such as LPS and anti-CD40, whereas ILC2s by themselves do not appear to induce Ig production.

Secretory product of ILC2s promotes B cell Ig production

We next investigated the mechanisms involved in the stimulatory effects of ILC2s on B cells. Previously, we found that direct cellular contact mediated by the OX40/OX40L interaction plays a critical role in the ILC2 promotion of CD4⁺ T cell functions (5). However, the addition of blocking anti-OX40L Ab to ILC2–B cell culture

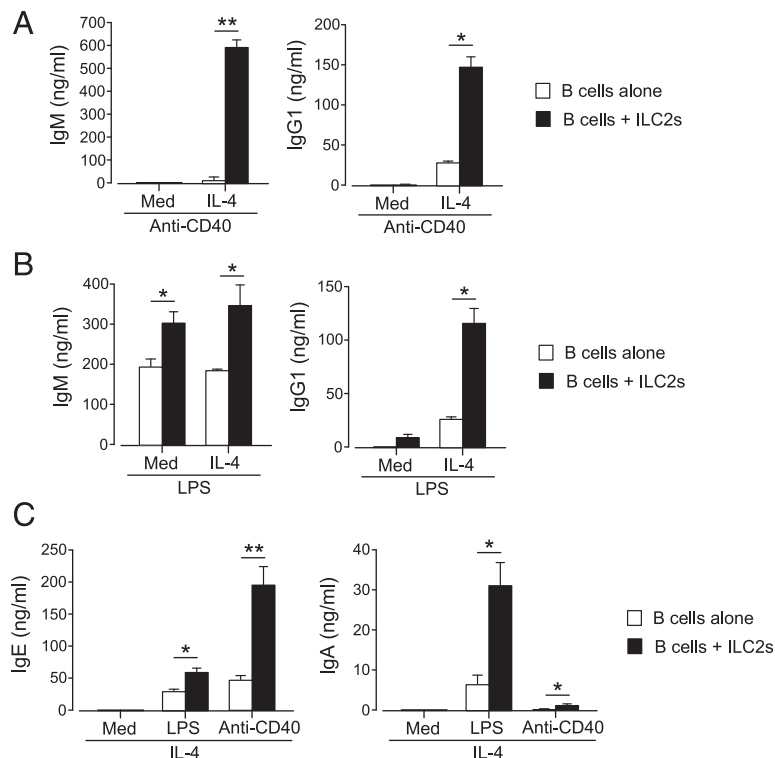


FIGURE 2. Lung ILC2s enhance Ig production by B lymphocytes. Lung ILC2s and splenic B cells were isolated from BALB/c mice. B cells were cultured at 2×10^4 cells/well with or without ILC2s (10^4 cells/well) for 4 d. IL-4 (10 ng/ml) (A–C), anti-CD40 (1.5 μ g/ml) (A and C), or LPS (5 μ g/ml) (B) was added to some cultures, as indicated. Levels of IgM and IgG1 (A and B) and IgE and IgA (C) in the supernatants were analyzed by ELISA. Data (mean $\pm$ SEM, $n = 3$) are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$ between the groups indicated by horizontal lines.

did not affect IgM and IgG1 production (Supplemental Fig. 5). Lung ILC2s also express high levels of ICOS (12), and B cells express the binding partner, ICOS ligand (15, 16). Furthermore, the ICOS:ICOS ligand interaction in ILC2s promotes ILC2 survival and cytokine production (17). However, the anti-ICOS Ab showed no effect on the ability of ILC2s to enhance B cell IgM and IgG1 production (Supplemental Fig. 5).

These findings led us to hypothesize that cytokines and other soluble factors that are secreted by ILC2s may play a key role. To test this hypothesis, we collected cell-free supernatants of ILC2s that had been cultured with IL-33 plus IL-7 for 3 or 4 d and added them to the culture of splenic B cells. No or minimal IgM, IgG1, or IgA was produced by splenic B cells in the absence of anti-CD40 or ILC2 supernatants (Fig. 4). ILC2 supernatant alone without anti-CD40 significantly induced the production of IgA and IgE ($p < 0.05$), but not IgM or IgG1. In the presence of anti-CD40 plus IL-4, ILC2 supernatants potently promoted the production of IgM and IgG1 ($p < 0.05$ and $p < 0.01$, respectively), suggesting a synergistic effect of anti-CD40 and ILC2 supernatants.

ILC2-derived IL-5 is involved in promoting IgM production

We performed subsequent mechanistic studies by focusing on the effects of ILC2s on anti-CD40-mediated IgM and IgG1 production, because these classes of Abs responded most robustly to the addition of ILC2s. When stimulated with IL-33, lung ILC2s produce IL-5, IL-6, IL-9, IL-13, and GM-CSF (12). To identify which cytokines are involved, we added neutralizing Abs to the B cell–ILC2 culture. Anti-IL-5 Ab significantly inhibited the ILC2-mediated upregulation of IgM production, but had no effect on IgG1 levels (Fig. 5A, $p < 0.05$). Anti-IL-6, anti-IL-9, anti-IL-13, and anti-GM-CSF Abs did not have any effects on either IgM or IgG1 production (Fig. 5A–C).

To verify the role of IL-5, we took a genetic approach. We isolated lung ILC2s from wild-type (WT) mice or *Il5*^{−/−} mice and cultured them with splenic B cells from WT mice. WT ILC2s significantly enhanced IgM production by B cells stimulated with either anti-CD40 or LPS (Fig. 6, $p < 0.05$). ILC2s from *Il5*^{−/−}

mice exhibited a significantly decreased ability to promote IgM production as compared with WT ILC2 ($p < 0.05$). In contrast, *Il5* deficiency did not affect IgG1 production. These findings suggest that ILC2-derived IL-5 plays a key role in promoting IgM production by B cells, whereas other molecules are most likely involved in promoting IgG1 production.

ILC2s enhance Ag-specific IgM responses in vivo

We took an adoptive transfer approach to examine whether lung ILC2s promote the production of specific Abs to respiratory Ags in vivo. IL-7R-deficient mice are defective in both B and T lymphopoiesis and lack ILC2s (12, 18); however, they have sufficient B1 cells to generate IgM Ab responses (18). Therefore, we sought to investigate the IgM responses against a polysaccharide model Ag NP-Ficoll in IL-7R-deficient mice. Administration of innocuous protein via the airway is generally tolerogenic, and coadministration of adjuvants, such as proteases, is necessary to sensitize naive animals (19, 20). Indeed, TNP-Ficoll, an Ag similar to NP-Ficoll, previously induced Ag-specific Ab responses only when it was administered to the airway together with the bacteria *Brucella abortus*, but not by itself (21). Thus, we took a similar approach to induce NP-Ficoll-specific Ig responses by exposing mice i.n. to NP-Ficoll together with a cysteine protease, bromelain, as an adjuvant (22). We isolated lung ILC2s from nonsensitized WT C57BL/6 mice that had been injected i.p. with IL-33 and IL-25 and immediately transferred them i.v. to naive *Il7r*^{−/−} mice. These mice were then exposed i.n. to NP-Ficoll and bromelain. To examine early Ab responses, serum Ab concentrations were analyzed 6 d after initial Ag exposure (Fig. 7A).

Sera of *Il7r*^{−/−} mice that were exposed to PBS contained ~400 and 80 μ g/ml total IgM or IgG1, respectively, without ILC2 transfer (Fig. 7B). Intranasal exposure to NP-Ficoll or adoptive transfer of ILC2s showed no significant effects on the levels of total IgM and IgG1, suggesting that ILC2s are unlikely to be involved in steady state production of these Igs. *Il7r*^{−/−} mice displayed low levels of NP-Ficoll-specific IgM Ab responses when exposed to NP-Ficoll without ILC2 transfer (Fig. 7C). The

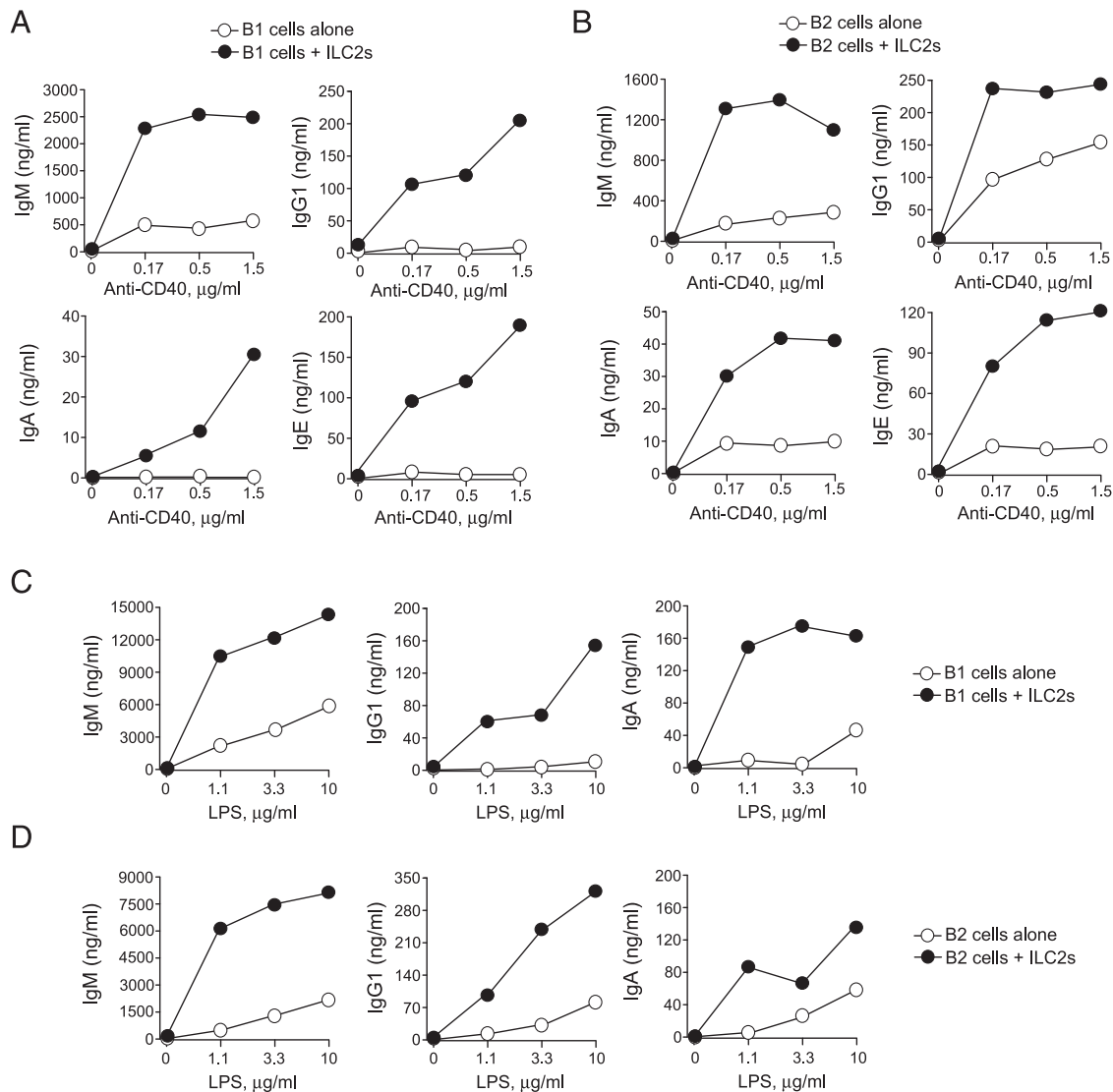


FIGURE 3. Lung ILC2s enhance Ig production by B1 and B2 cells. Lung ILC2s and peritoneal B1 and B2 cells were isolated from BALB/c mice. B1 cells (**A** and **C**) and B2 cells (**B** and **D**) were cultured at 2×10^4 cells/well with or without ILC2s (10^4 cells/well) in the presence of serial dilutions of anti-CD40 or LPS plus IL-4 for 4 d. Ig levels in the supernatants were analyzed by ELISA. Each data point represents the Ig concentration from one well. Data are representative of three independent experiments.

adoptive transfer of ILC2s significantly increased the serum levels of NP-Ficoll-specific IgM Ab ($p < 0.05$). In contrast, no significant effects of exposure to NP-Ficoll or ILC2 transfer were observed in the levels of NP-Ficoll-specific IgG1 Ab. Collectively, these findings suggest that ILC2s promote the generation of IgM Abs to a respiratory Ag even in the absence of T cells in vivo.

Discussion

Innate immunity and adaptive immunity are intimate partners. Besides performing their specialized functions, innate immune cells and adaptive immune cells also interact with each other during the immune response. Previously, we and others reported that the new innate immune cell ILC2s interact with CD4⁺ T cells and subsequently enhance Th2-type immune responses (4–6). ILC2s also act on other innate immune cells, such as DCs, and indirectly promote Th2-type immune responses (3, 7). This manuscript adds to this knowledge of the effects of ILC2s on adaptive immunity by demonstrating that ILC2s activate B cells and enhance the early Ab response to a respiratory Ag in vivo. Thus, the regulatory functions of ILC2s are not limited to the

cellular arm of adaptive immunity, but most likely extend to humoral immunity as well.

Mature peripheral B cells consist of three different subsets, including innate-like B1 cells, follicular B2 cells, and marginal zone B cells (23). A previous study showed that adipose tissue-derived ILC2s support the self-renewal of B1 cells and enhance IgA production (11). The observations in this manuscript show that lung ILC2s enhance both B1 and B2 cell proliferation and promote the production of IgM, IgG1, IgA, and IgE. Indeed, by using a model polysaccharide Ag NP-Ficoll, ILC2s adoptively transferred to *Il7r*^{−/−} mice promoted Ag-specific IgM Ab responses to a respiratory Ag. Because *Il7r*^{−/−} mice do not have ILC2s, mature T cells, or B2 cells (12, 18), the stimulating effects of ILC2s on IgM Ab production (Fig. 7) are most likely independent of T cells and are mediated by the interaction between ILC2s (or their products) and B1 cells. This speculation is consistent with the in vitro observation that ILC2s potentially enhance B1 cell proliferation and promote IgM production (Figs. 1, 3).

Because B1 and B2 cells are considered to mediate T cell-independent and T cell-dependent Ab responses, respectively, our

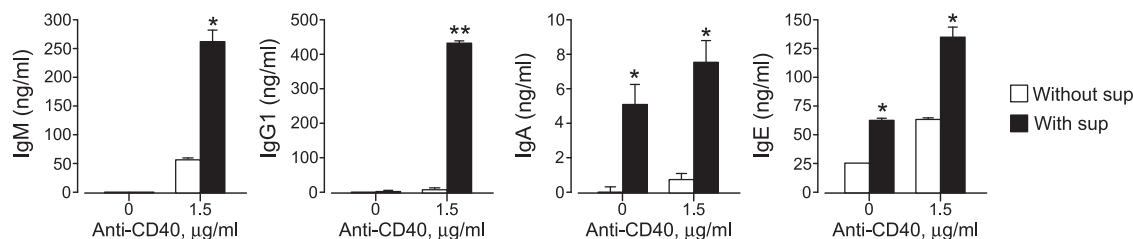


FIGURE 4. Supernatants of ILC2s promote B cell Ig production. Lung ILC2s and splenic B cells were isolated from BALB/c mice. B cells were cultured with or without pooled supernatants (sup) of ILC2s in the presence or absence of anti-CD40 Ab (1.5 µg/ml) plus IL-4 (10 ng/ml) for 4 d. Ig concentrations in the culture supernatants were analyzed by ELISA. Data (mean ± SEM, $n = 3$) are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$ compared with cells cultured in the absence of ILC2 supernatants.

in vitro results would suggest that ILC2s may regulate both T cell-dependent and T cell-independent Ab responses. We have not examined specifically whether ILC2s promote Ab responses to T cell-dependent Ags in vivo; however, this scenario can be reasonably expected, as judged by the fact that ILC2s enhance Th2-type CD4⁺ T cell functions (3–7). Indeed, in a papain-induced airway inflammation model, ILC2-deficient mice showed reduced levels of total serum IgE (3), suggesting the ability of ILC2s to promote T cell-dependent Ab responses.

ILC2s facilitate adaptive immune cell functions directly through several mechanisms, including cellular interactions through TCR/MHC class II and OX40/OX40L, and the paracrine secretion of soluble factors, such as IL-4 (1, 5, 8). The potent B cell stimulatory effects of ILC2 culture supernatant suggest that soluble factors

secreted by ILC2s are sufficient to mediate the ILC2 effects on B cells. Activated lung ILC2s produce a variety of cytokines, including IL-4, IL-5, IL-6, IL-9, IL-13, and GM-CSF (1, 12). All of these cytokines have been shown to be involved in regulating B cell functions (14, 24–28). In particular, IL-4 plays an important role in regulation of B cell growth and Ig class switch and production (14). Our finding that ILC2s promote anti-CD40-induced IgM or IgG1 production only in the presence of exogenous IL-4 (Fig. 2A) suggests that ILC2-derived IL-4 is unlikely to be sufficient to upregulate Ig production in this experimental setting. IL-5 is another important cytokine that regulates B cell growth and Ab production (29). Indeed, mice deficient in IL-5R α showed decreased numbers of B1 cells in the peritoneal cavity and decreased serum levels of IgM, but not IgG1 (29, 30). IL-5 transgenic mice

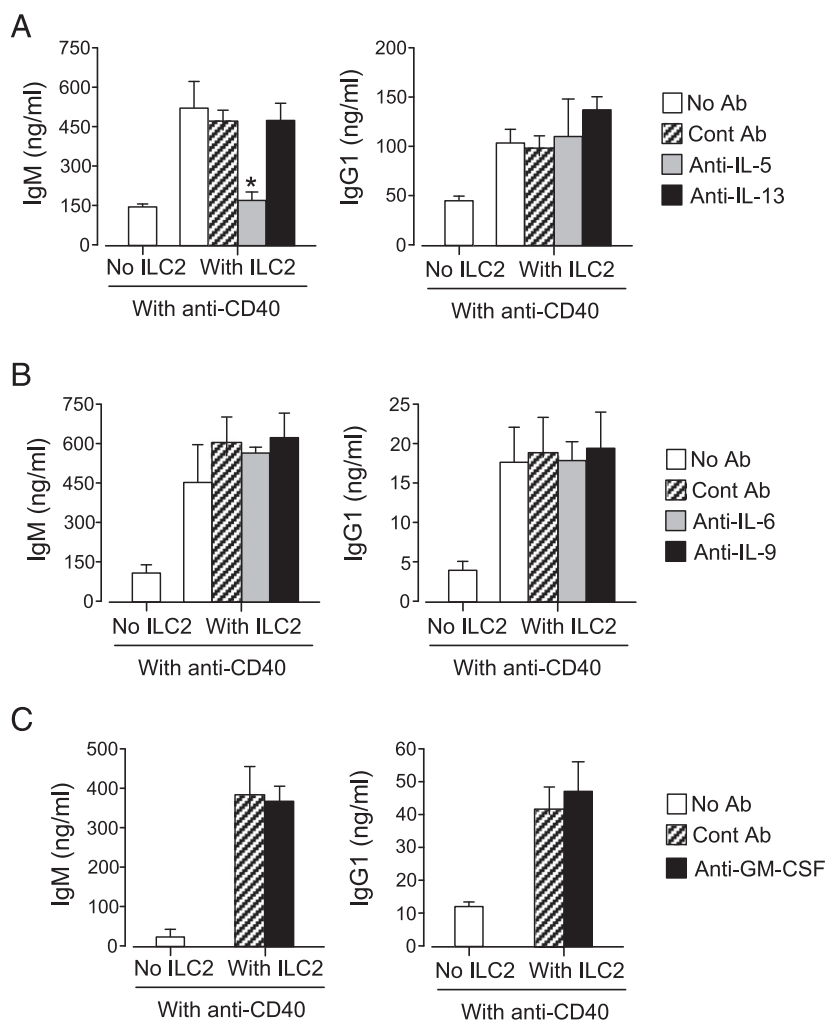


FIGURE 5. IL-5 is involved in enhanced Ig production by B cells cultured with ILC2s. Lung ILC2s and splenic B cells were isolated from BALB/c mice. B cells (2×10^4 cells/well) were cultured with or without ILC2s (10^4 cells/well) in the presence of anti-CD40 Ab (1.5 µg/ml) plus IL-4 for 4 d. Neutralizing Abs to IL-5 or IL-13 (A), to IL-6 or IL-9 (B), or to GM-CSF (C), or control Ab (Cont Ab, 2 µg/ml) were added to the culture. In (A), data (mean ± SEM, $n = 3$) are a pool of three independent experiments. In (B) and (C), data (mean ± SEM, $n = 3$) are representative of two to three independent experiments. * $p < 0.05$ compared with no Ab.

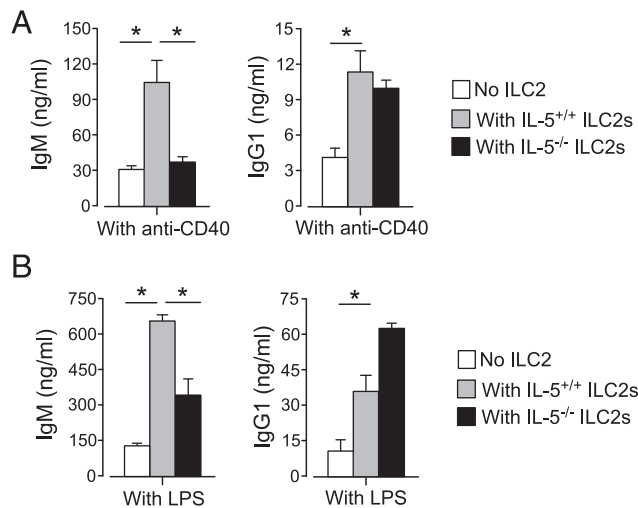


FIGURE 6. Lung ILC2s from IL-5-deficient mice fail to promote IgM production by B cells. Splenic B cells were isolated from C57BL/6 mice. ILC2s were isolated from C57BL/6 or C57BL/6 *IL5*^{-/-} mice. B cells (2×10^4 /well) were cultured with or without ILC2s (10^4 /well) in the presence of anti-CD40 Ab [$1.5 \mu\text{g/ml}$ (**A**) or LPS ($5 \mu\text{g/ml}$ (**B**)] plus IL-4 for 4 d. Ig concentrations in the culture supernatants were analyzed by ELISA. Data (mean $\pm$ SEM, $n = 3$) are representative of two independent experiments. * $p < 0.05$ between the groups indicated by horizontal lines.

showed increased serum levels of polyreactive autoantibodies of IgM class (31). Consistent with these previous observations, the results of our Ab-blocking experiments suggest that IL-5 produced by lung ILC2s promotes B cell IgM production (Fig. 5). This conclusion was further supported by experiments involving the culture of B cells with IL-5-deficient ILC2s (Fig. 6).

Currently, we do not know what soluble factors are involved in the enhanced production of IgG1 and other Ab classes. Our Ab-blocking experiments (Fig. 5) suggest that ILC2-derived IL-5, IL-13, IL-6, IL-9, and GM-CSF are unlikely to be responsible for this, although we cannot rule out the possibility that these cytokines may work additively or redundantly to promote IgG1 production. It has been shown that cytokine B cell-activation factor (BAFF), a proliferation-inducing ligand (APRIL), and IL-21 are involved in neutrophil-induced Ig production (32). However, IL-33-activated lung ILC2s are an unlikely source for these cytokines because microarray gene analysis revealed no or extremely low level expression of BAFF, APRIL, and IL-21 in these cells (K. Bartemes, unpublished observations). Thus, further studies are necessary to identify the molecule(s) that is produced by ILC2s and responsible for enhanced production of IgG1 and other Ig classes.

T cell-independent Ab responses play a crucial role in the rapid clearance of pathogens because these responses can develop as early as 1–3 d after exposure to blood-borne microorganisms (33). In the case of lung infection, a subset of B1 cells in the pleural space migrates to the lung, where they produce protective IgM against pneumonia in the early phase of infection (28). Furthermore, IL-33, a key cytokine involved in the activation of ILC2s, promoted the production of IgM Abs to oxidized low-density lipoprotein in an IL-5-dependent manner and reduced the development of atherosclerosis in mice (34). As innate immune cells, ILC2s react promptly to infection, injury, stress, and metabolic changes (1, 2, 35). Therefore, the findings of our study suggest a model in which activated lung ILC2s interact with B1 cells, possibly those in the pleural cavity or lung parenchyma (36), to promote rapid Ag-specific IgM Ab production. Accordingly, the interaction between ILC2s and B1 cells may strengthen the first

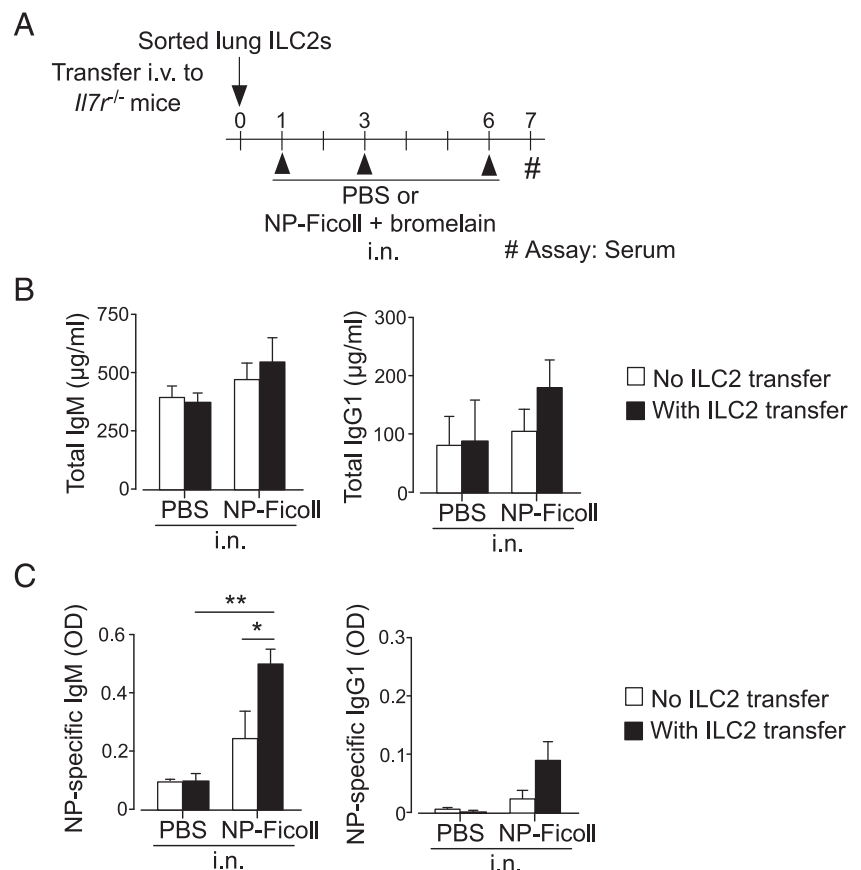


FIGURE 7. ILC2s promote Ag-specific IgM Ab production in IL-7R-deficient mice. (**A**) Experimental protocol: Lung ILC2s were isolated from C57BL/6 mice and transferred into C57BL/6 *IL7R*^{-/-} mice. Mice were then exposed i.n. to NP-Ficoll plus bromelain on days 1, 3, and 6, and sera were analyzed on day 7. The levels of total IgM or IgG1 (**B**) or NP-Ficoll-specific IgM or IgG1 (**C**) in sera were analyzed by ELISA. Data (mean $\pm$ SEM, $n = 2$ in PBS and $n = 6$ in NP-Ficoll) are representative of two independent experiments. * $p < 0.05$, ** $p < 0.01$ between the groups indicated by horizontal lines.

line of defense against certain pathogens. Achieving a better understanding of the molecular and cellular mechanisms involved in the interaction between ILC2s and B cells will be of central importance in furthering our knowledge of innate and adaptive immunity to pathogens and tissue homeostasis against insults.

Disclosures

The authors have no financial conflicts of interest.

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