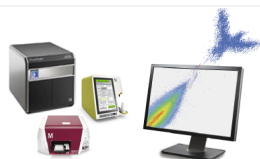




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B Cell–Intrinsic MyD88 Signaling Is Essential for IgE Responses in Lungs Exposed to Pollen Allergens

Kazufumi Matsushita* and Tomohiro Yoshimoto*[†]

Allergen-specific IgE is linked to asthma pathogenesis, but the underlying mechanisms of IgE production in response to allergen exposure are poorly understood. In this article, we show that B cell–intrinsic MyD88 is essential for IgE/IgG1 production evoked by ragweed pollen instilled into lungs. MyD88-deficient mice showed defective IgE/IgG1 production and germinal center responses to lung instillation of ragweed pollen. However, MyD88 was dispensable for dendritic cell activation and Th2 cell development. B cell–specific deletion of MyD88 replicated the defective Ab production observed in MyD88-deficient mice. Although ragweed pollen contains TLR ligands, TLR2/4/9-deficient mice developed normal allergic responses to ragweed pollen. However, anti-IL-1R1 Ab-treated mice and IL-18-deficient mice showed decreased IgE/IgG1 production with normal Th2 development. Furthermore, B cell–specific MyD88-deficient mice showed reduced IgE/IgG1 production in response to lung instillation of OVA together with IL-1 α , IL-1 β , or IL-18. Thus, pollen instillation into lungs induces IL-1 α/β and IL-18 production, which activates B cell–intrinsic MyD88 signaling to promote germinal center responses and IgE/IgG1 production. *The Journal of Immunology*, 2014, 193: 000–000.

Antigen-specific IgE mediates an inflammatory pathway in the pathogenesis of allergic diseases, including asthma (1, 2). The cross-linking of IgE bound to mast cells by specific Ags induces mast cell degranulation and rapid release of histamine, leukotrienes, and PGs (2). These mast cell–derived molecules contribute to the development of early- and late-phase responses and chronic inflammation in asthma (2, 3). Although several immunological pathways other than IgE also participate in allergic inflammation (4, 5), a deeper understanding of the generation of Ag-specific IgE might help to develop new therapeutic options for the treatment of allergic diseases.

Recent studies revealed that MyD88-mediated signaling promoted Ab production to T-dependent (TD) Ags (6, 7). MyD88-mediated signaling in TD Ab responses has two pathways. In myeloid APCs, such as dendritic cells (DCs) and macrophages, MyD88 upregulates costimulatory molecules and cytokine production that are essential for CD4⁺ T cell priming (8). The second role for MyD88 in TD Ab responses is the direct ligation of TLRs on B cells, allowing their interaction with cognate Th cells (9).

Furthermore, combined signaling of BCR, CD40, and TLR induces maximal activation of human naive B cells (10). MyD88 and TIR domain-containing adapter-inducing IFN- β (TRIF)-mediated signals are dispensable for Ab production to hapten Ags immunized with major adjuvants, including alum, indicating that MyD88/TRIF-independent innate signals can evoke TD Ab responses (11, 12). However, the addition of TLR ligands and activation of MyD88 signaling can improve adjuvanticity of vaccines and enhance Ab production to TD Ags (6, 7).

The exact role of B cell–intrinsic MyD88 signaling in TD Ab responses is not completely understood. Specific deletion of MyD88 in B cells results in defective germinal center (GC) responses and IgG2b and IgG2c production in virus infection or immunization of virus-like particles containing CpG oligodeoxynucleotides, a TLR9 ligand, or ssRNA, a TLR7 ligand (13–16). Specific involvement of B cell–intrinsic TLR7/9 signaling in IgG2b and IgG2c production is explained by direct upregulation of T-bet in B cells (17, 18) and B cell–mediated stimulation of CD4⁺ T cells to drive IFN- γ secretion (16). GC B cells express high levels of MyD88 and are highly responsive to TLR ligands (12). MyD88 signaling in GC B cells enhances proliferation, class-switch recombination, the differentiation of B cells into Ag-secreting plasma cells, and the maintenance of long-term humoral immunity (12). In addition, B cell–intrinsic MyD88 signaling has an essential role in autoantibody production. MRL-*lpr* mice produce high levels of autoantibodies (19), and B cell–specific deletion of MyD88 in MRL-*lpr* mice ameliorated autoantibody production (20).

Early studies reported that MyD88 signaling was specifically involved in Th1-type immune responses but not Th2-type responses (21). However, it is now recognized that MyD88 signaling has an important role in allergic disease development (22, 23), and several allergens were reported to activate MyD88 signaling. Lung sensitization of Ags with low-dose LPS is a well-characterized model for inducing airway allergic symptoms (23). Natural airborne allergens, such as house dust mite (24) or the fungus *Alternaria alternata* (25), contain TLR ligands that mediate their allergenic activity. Thus, *Myd88*^{−/−} mice show ameliorated allergic responses to these allergens when exposed to lungs and develop

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Abbreviations used in this article: BM, bone marrow; DC, dendritic cell; GC, germinal center; LN, lymph node; PNA, peanut agglutinin; TD, T dependent; Tfh, follicular helper T; TRIF, TIR domain-containing adapter inducing IFN- β ; WT, wild-type.

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decreased Ag-specific IgE production together with defective Th2 cytokine production and inflammatory cell lung infiltration (24, 25). Therefore, MyD88 signaling, which initiates or exacerbates allergic asthma, might be mediated by TLRs on DCs or other innate cell populations (23). However, the role of B cell-intrinsic MyD88 signaling in the production of allergen-specific IgE in lung allergic responses has not been determined. Recently, many studies pointed out the ability of particulate or crystalline matter to induce type-2 immune responses (26). However, mechanisms underlying IgE production induced by particulate matter are poorly understood.

In this study, we show that airway instillation of ragweed pollen, a major airborne particulate allergen in Central Europe and North America, elicits MyD88-dependent total and ragweed-specific IgE and IgG1 production in mice. In contrast to other soluble allergens that activate MyD88 signaling, *Myd88*^{-/-} mice developed normal Th2 responses to ragweed pollen. B cell-intrinsic MyD88 signaling has an essential role in Ab production in lungs exposed to ragweed pollen. Furthermore, IL-1R- and IL-18R-mediated, rather than TLR-mediated, signaling was essential for ragweed-induced Ab production. This study provides new insight into the mechanisms of particulate allergen-induced IgE production in lungs.

Materials and Methods

Mice

Wild-type (WT) BALB/c and C57BL/6 mice were purchased from Charles River Laboratories Japan (Yokohama, Japan). *Il18*^{-/-} and *Myd88*^{-/-} mice (27) were backcrossed onto C57BL/6 and BALB/c mice over eight generations. BALB/c-background *Il33*^{-/-} mice were generated as described (28). C57BL/6-background *Tlr2/4/9*^{-/-} mice were provided by Dr. Shizuo Akira (Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan). BALB/c-background *Ighm*^{-/-} and *Rag2*^{-/-} mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were maintained under specific pathogen-free conditions, where they received sterilized food and water ad libitum. All animal experiments were performed with the approval of, and in accordance with the guidelines of, the Institutional Animal Care Committee of Hyogo College of Medicine (No. A11-235 and No. 28028).

Reagents

Ragweed pollen was purchased from PolyScience (Niles, IL). Ragweed extract was purchased from LSL (Tokyo, Japan). CL075 was purchased from Invitrogen (San Diego, CA). Aluminum hydroxide hydrate, LPS, and OVA were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Recombinant human IL-2 and mouse GM-CSF were purchased from PeproTech (Hamburg, Germany). Recombinant mouse IL-1 α and IL-1 β were purchased from eBioscience (San Diego, CA). Recombinant mouse IL-18 was purchased from MBL (Nagoya, Japan). Recombinant mouse IL-33 was prepared in our laboratory (28). mAbs used in this study were specific for mouse B220 (RA3-6B2), CD3 ϵ (145-2C11), CD4 (RM4-5), CD11c (N418), CD28 (37.51), CD95 (Jo2), CD121a (35F5), CD278 (RMP1-30), CXCR5 (2G8), GL-7 (GL-7), IgD (11-26c), and MHC class II (M5/114.15.2). These Abs were purchased from eBioscience, BioLegend (San Diego, CA), or BD Biosciences (San Jose, CA). DuoSet ELISA for mouse IL-1 β , IL-4, IL-5, IL-6, IL-13, IL-33, and IFN- γ were purchased from R&D Systems (Minneapolis, MN). The IL-1 α ELISA kit was purchased from Biovendar (Brno, Czech Republic), and the IL-18 ELISA kit was purchased from MBL.

Mouse models

Mice were anesthetized and received 500 μ g/50 μ l ragweed pollen suspended in PBS, 20 μ g/50 μ l alum mixed with 10 μ g/50 μ l OVA suspended in PBS, or cytokines (IL-1 α , IL-1 β , IL-18, or IL-33; 50 ng/50 μ l) mixed with 10 μ g/50 μ l OVA suspended in PBS intranasally at days 0, 4, 8, and 12. Mice were sacrificed at day 13 and analyzed. For anti-IL-1R1 Ab treatment, mice were injected i.v. with 250 μ g anti-mouse IL-1R1 Ab (35F5) at day -1, followed by 250 μ g i.p. injection at days 3, 7, and 11. In some experiments, mice received heat-treated ragweed pollen (500 μ g/50 μ l) with OVA (10 μ g/50 μ l). For this, ragweed pollen was suspended in PBS and heated at 100°C for 10 min; after cooling, the ragweed pollen was

mixed with OVA. For i.p. immunization, mice were injected i.p. with ragweed pollen (1 mg/200 μ l) or alum (1 mg/200 μ l) mixed with OVA (100 μ g/200 μ l) at days 0 and 7. The sera were obtained at day 14. To examine early responses to lung ragweed pollen exposure, mice were given a single intratracheal administration of 500 μ g ragweed pollen. Mice were sacrificed at 1, 2, 3, or 7 d after ragweed pollen administration and analyzed. Mediastinal lymph nodes (LNs) were dissected from mice, and single-cell suspensions were prepared by sieving and gentle pipetting. Lungs were obtained and frozen at -80°C for preparation of total RNAs and lung homogenates. Lungs were homogenized in 500 μ l cold lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40). Then, lysates were centrifuged to remove debris, and clear lysates were quantified for protein concentration with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL).

B cell transfer to μ MT mice

Spleens were dissected from WT or *Myd88*^{-/-} mice, and single-cell suspensions were prepared by sieving and gentle pipetting. Cells were washed in ice-cold staining buffer (1% BSA in PBS), incubated with MicroBeads conjugated with an anti-B220 Ab (Miltenyi Biotec, Gladbach, Germany) for 15 min, and washed twice with staining buffer. B220⁺ cells were sorted using an AutoMACS Separator (Miltenyi Biotec). A total of 1×10^7 B220⁺ cells was injected i.v. into each *Ighm*^{-/-} mouse 24 h before the first ragweed pollen administration.

Mixed bone marrow transfer

To generate B cell-specific *Myd88*^{-/-} (B-*Myd88*^{-/-}) mice, bone marrow (BM) cells were prepared from WT, *Myd88*^{-/-}, and *Ighm*^{-/-} mice. BM cells from *Ighm*^{-/-} mice and WT or *Myd88*^{-/-} mice were mixed at a 4:1 ratio. The cells were injected i.v. into lethally irradiated (9 Gy) *Rag2*^{-/-} (BALB/c) mice. To generate CD45.1/CD45.2 mixed BM chimeric mice, BM cells were prepared from WT, *Myd88*^{-/-}, and CD45.1⁺ mice. BM cells from CD45.1⁺ mice and WT or *Myd88*^{-/-} mice were mixed at a 1:1 ratio. The cells were injected i.v. into lethally irradiated (10 Gy) WT (C57BL/6) mice. The chimeric mice were given neomycin (1 mg/ml) and Polymyxin B (1000 U/ml) in their drinking water for 4 wk. The mice were used from 8 wk after reconstitution.

In vitro cell culture

Mediastinal LN cells were isolated from mice and cultured for 5 d in 96-well plates at 2×10^5 cells/200 μ l/well with IL-2 (100 pM) and ragweed extract (5 μ g/ml) in the presence of 1×10^5 irradiated conventional APCs (T cell-depleted BALB/c splenic cells) in complete medium (RPMI 1640 supplemented with 10% FBS, 2-ME [50 μ M], L-glutamine [2 mM], penicillin [100 U/ml], and streptomycin [100 μ g/ml]). In some experiments, mediastinal LN cells were cultured with plate-bound anti-CD3 mAb (1 μ g/ml for coating) in the presence of soluble anti-CD28 mAb (1 μ g/ml) for 2 d. To prepare BM-derived DCs, BM cells were isolated from the femora and tibia. Then, cells were cultured in complete medium together with murine GM-CSF (10 ng/ml). Medium was changed every 2 d, and cells were collected at day 6. BM-derived DCs were washed twice with RPMI 1640 medium and cultured for 24 h in 96-well plates at 1×10^5 cells/200 μ l/well with ragweed pollen (100 or 500 μ g/well), ragweed extract (100 or 500 μ g/ml), LPS (1 μ g/ml), or CL075 (1 μ g/ml) in complete medium.

Flow cytometry

Cell suspensions of mediastinal LNs were maintained in the dark at 4°C. Cells were washed in ice-cold staining buffer (1% BSA in PBS), incubated with each Ab for 15 min, and washed twice with staining buffer. Data were acquired using a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software (version 7.6.1; TreeStar, Ashland, OR).

Immunohistochemical examination

Frozen sections of freshly isolated mediastinal LNs were cold-fixed in 4% paraformaldehyde for 5 min at 4°C and blocked with 1% BSA-PBS. Then, the samples were incubated with rhodamine-labeled peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA) and allophycocyanin-conjugated anti-IgD mAb and mounted with Prolong Antifade Gold with DAPI (Invitrogen). The immunohistochemical samples were evaluated under a microscope (Zeiss LSM 510; Carl Zeiss, Oberkochen, Germany).

Determination of serum Ig levels

Mouse serum IgM, IgG1, IgG2a, and IgE levels were measured by ELISA, as previously described (29). Polyvalent goat anti-mouse IgM, IgG1, or IgG2a Abs or rat anti-mouse IgE mAb (23G3) (SouthernBiotech,

Birmingham, AL) were used as capture Abs, and polyclonal goat anti-mouse IgG1 Abs biotin-conjugated (SouthernBiotech) or rat anti-mouse IgM (R6-60.2), IgG2a (R1G-15), or IgE (R35-118) mAbs biotin-conjugated (BD Biosciences) were used as secondary Abs. For ragweed- or OVA-specific Ab levels, ELISA plates were coated with polyclonal goat anti-mouse

IgG1 or rat anti-mouse IgE mAb (23G3) (SouthernBiotech). Then, homemade biotinylated ragweed extract (28) or biotinylated OVA (DS Pharma Biomedical, Osaka, Japan) was used as secondary reagents. Biotinylated ragweed extract was generated by incubating the ragweed extract with (+)-Biotin *N*-hydroxysuccinimide ester (Sigma-Aldrich). We con-

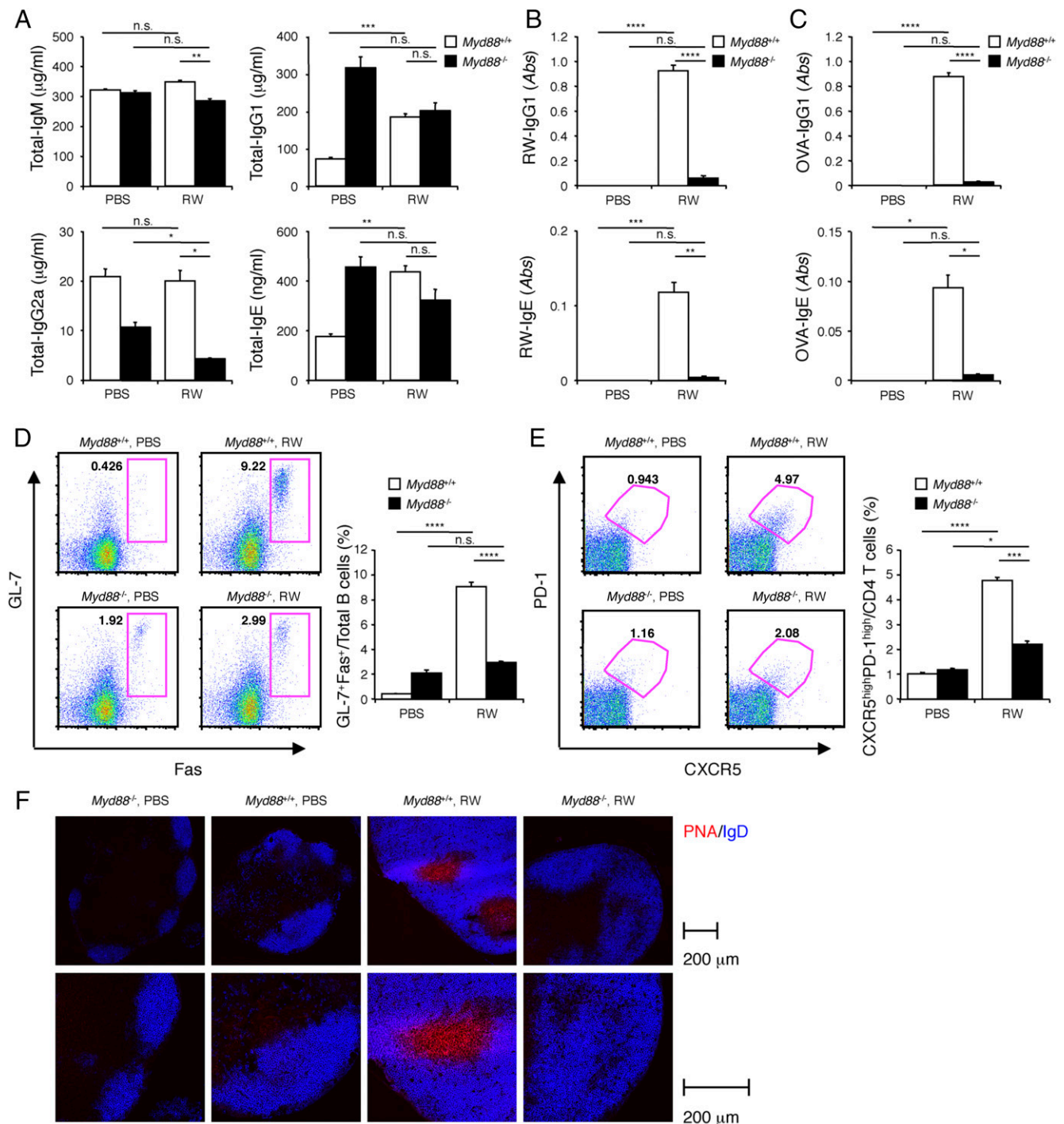


FIGURE 1. MyD88 is indispensable for IgE and IgG1 production in response to particle allergen exposure of lungs. (A and B) *Myd88*^{+/+} or *Myd88*^{-/-} mice were administered ragweed (RW) pollen or PBS intratracheally four times at 3-d intervals. Mouse sera were collected 1 d after the final RW administration. Total IgM, IgG1, IgG2a, and IgE (A) and RW-specific IgG1 and IgE (B) levels in the sera were determined by ELISA. (C) *Myd88*^{+/+} or *Myd88*^{-/-} mice were administered alum plus OVA intratracheally as in (A). OVA-specific IgG1 and IgE levels in the sera were determined by ELISA. Data (mean + SEM) are representative of three independent experiments [*n* = 5 (A) and *n* = 6 (B and C)]. Results represent three (A) or two (B and C) independent experiments. (D and E) Mediastinal LNs were obtained from mice 7 d after a single intratracheal administration of RW pollen. Expression of GL-7 and Fas in B220⁺ B cells (D) and PD-1 and CXCR5 in CD4⁺ T cells (E) was examined. Representative flow cytometry plots and quantified graphs for three independent experiments are shown (*n* = 4 [PBS], *n* = 5 [RW]). (F) Immunohistochemistry of mediastinal LNs obtained from mice 7 d after a single intratracheal administration of RW pollen. Representative images from three mice are shown. Red, peanut agglutinin (PNA); Blue, IgD. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001. n.s., not significant.

firmed that the ragweed-specific ELISA system did not react with OVA-immunized sera or mouse DNP-IgE standard.

Quantitative PCR

Total RNAs from CD4⁺ T cells in mediastinal LNs were prepared using Sepasol (Nakarai, Kyoto, Japan), and cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan). The expression of genes was quantified with TaqMan Gene Expression Assays (Applied Biosystems) and the Thermal Cycler Dice RT-PCR system (Takara Bio, Otsu, Japan), according to the manufacturer's instructions. The results are shown as the relative expression standardized to the expression of a gene encoding eukaryotic 18S rRNA. The specific primers and probes used for quantitative RT-PCR were TaqMan probes for *Il4*, *Il5*, *Il13*, *Il21*, and 18S rRNA (Applied Biosystems).

Statistical analysis

Statistical significance was determined by the two-tailed Student *t* test. The *p* values < 0.05 were considered statistically significant.

Results

MyD88 signaling is essential for IgE/IgG1 production in lungs instilled with ragweed pollen

To investigate the role of MyD88-mediated signaling in airway allergic responses elicited by lung exposure to ragweed pollen, we anesthetized mice and intratracheally administered ragweed pollen suspended in PBS. Mice received ragweed pollen every 4 d, for a total of four times, and were sacrificed 24 h after the final ragweed administration. As reported previously (11), *Myd88*^{-/-} mice showed spontaneously high serum IgE and IgG1 and low IgG2a levels compared with WT mice (Fig. 1A, see PBS group). In the ragweed pollen-instilled group, WT, but not *Myd88*^{-/-}, mice showed increased serum IgE and IgG1 levels (Fig. 1A). IgM and IgG2a levels were not elevated by lung exposure to ragweed pollen in WT or *Myd88*^{-/-} mice (Fig. 1A). Ragweed-specific IgE and IgG1 production was induced in WT mice (Fig. 1B). In contrast, the production of ragweed-specific IgE and IgG1 was completely abrogated in *Myd88*^{-/-} mice (Fig. 1B). To investigate whether defective Ab production in *Myd88*^{-/-} mice was ragweed pollen specific, we intratracheally administered WT and *Myd88*^{-/-} mice with OVA plus alum. Alum evokes MyD88-independent IgE and IgG1 production when immunized in footpads (21) or i.p. (11). OVA-specific IgE and IgG1 production was completely MyD88 dependent in the intratracheal administration model (Fig. 1C). Thus, the requirement of MyD88-mediated signaling in Ab production might be common for a set of particulate allergens exposed to lungs.

Because GC responses are essential and characteristic for Ab production to TD Ags (6, 7), we examined GL-7⁺Fas⁺ GC B cell and CXCR5⁺PD-1⁺ follicular helper T (Tfh) cell expansion in draining LNs. Seven days after a single ragweed exposure, GC B cells increased ~18-fold and Tfh cells expanded ~5-fold in WT LNs (Fig. 1D, 1E). However, the expansion of those cell populations was severely abrogated in *Myd88*^{-/-} mice, indicating defective GC responses (Fig. 1D, 1E). In addition, GC formation was evaluated by immunofluorescent staining of draining LNs with PNA (red, GC B cells) and anti-IgD Ab (blue, follicular mantle B cells). Although ragweed pollen-treated *Myd88*^{+/+} mice clearly showed the development of PNA⁺ GCs, *Myd88*^{-/-} mice showed low PNA staining and defective GC formation (Fig. 1F).

MyD88 signaling is essential in evoking allergic responses to several allergens. Ragweed pollen contains TLR4 ligands and triggers Th2 inflammation in a MyD88-dependent manner in an allergic conjunctivitis model (30). However, in *Myd88*^{-/-} mice, DCs migrated normally into the draining LNs (Fig. 2A), and Th2 cytokine production, including IL-4, IL-5, and IL-13, was normal or rather higher than in WT mice (Fig. 2B, Supplemental Fig. 1A). Furthermore, in CD4⁺ T cells from *Myd88*^{-/-} mouse draining

LNs 7 d after ragweed pollen exposure, mRNA levels for Th2 cytokines, *Il4*, *Il5*, and *Il13*, were comparable or higher, whereas the level of a Tfh cytokine *Il21* was reduced compared with WT mice (Supplemental Fig. 1B). Thus, although MyD88 is required for optimal Th2 responses in conjunctival application of ragweed pollen into ragweed-sensitized mice, MyD88 is dispensable for Th2 development induced by ragweed pollen introduced directly to lungs. The initial activation of myeloid APCs and T cell priming is not abrogated in *Myd88*^{-/-} mice in the lung ragweed pollen exposure model. Furthermore, mechanisms for Ab production evoked in lungs might be unique and different from that in other tissues.

B cell-intrinsic MyD88 signaling is essential in ragweed-induced Ab production in lungs

To examine the role of B cell-intrinsic MyD88 signaling, we reconstituted mature B cell-deficient *Ighm*^{-/-} (μMT) mice with WT or *Myd88*^{-/-} B cells, followed by intratracheal administration of ragweed pollen. μMT mice reconstituted with *Myd88*^{-/-} B cells produced severely reduced amounts of total and ragweed-specific IgE and IgG1 compared with mice reconstituted with WT B cells (Fig. 3A). We also used a mixed BM transfer system, in which lethally irradiated *Rag2*^{-/-} mice were administered mixed

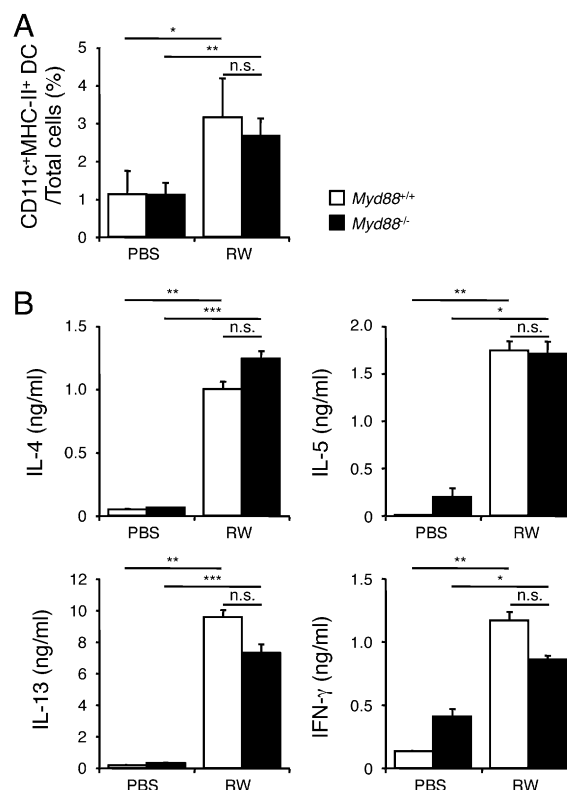


FIGURE 2. MyD88 is dispensable for T cell activation induced by lung exposure to ragweed (RW) pollen. **(A)** Mediastinal LNs were obtained from *Myd88*^{+/+} or *Myd88*^{-/-} mice 2 d after a single intratracheal administration of RW pollen or PBS. Expression of CD11c and MHC class II in total LN cells was determined by flow cytometry. **(B)** Mediastinal LNs were obtained from *Myd88*^{+/+} or *Myd88*^{-/-} mice 3 d after a single intratracheal administration of RW pollen or PBS. LN cells were incubated with T cell-depleted and irradiated splenocytes in the presence of IL-2 and RW extract for 5 d. Culture supernatants were harvested, and the levels of IL-4, IL-5, IL-13, and IFN-γ were determined by ELISA. Data (mean + SEM) are representative of two (A) or three (B) independent experiments [*n* = 3 (A), *n* = 5 (B)]. **p* < 0.05, ***p* < 0.01, ****p* < 0.001. n.s., not significant.

BM cells comprising 80% μ MT BM and 20% WT or *Myd88*^{-/-} BM. In these chimeric mice, normal hematopoietic cells develop mainly from μ MT BM, but B cells that mediate TD Ab responses originate only from mixed WT or *Myd88*^{-/-} BM. In this article, the chimeric mice are referred to as B-*Myd88*^{+/+} and B-*Myd88*^{-/-} mice, respectively. B-*Myd88*^{-/-} mice showed defective total and ragweed-specific IgE and IgG1 compared with B-*Myd88*^{+/+} mice (Fig. 3B). The Ab responses in B-*Myd88*^{-/-} mice immunized i.p. with ragweed pollen or OVA plus alum were comparable to those in B-*Myd88*^{+/+} mice (Fig. 3C), again showing the unique immune system that lungs harbor. Although μ MT mice possess B-1 B cells and can produce IgE in certain conditions, the Abs have no Ag affinity (31). Thus, the production of ragweed-specific IgE and IgG1 in chimeric mice should be by B-2 cells originating from WT or *Myd88*^{-/-} BM. We generated another BM chimeric mouse strain in which *Myd88*^{+/+} or *Myd88*^{-/-} CD45.2⁺ BM cells were transferred into lethally irradiated WT mice with WT CD45.1⁺ BM cells. Although CD45.1⁺ and CD45.2⁺ B cells were differentiated equally into GC B cells in response to lung ragweed exposure in the *Myd88*^{+/+}CD45.1⁺/*Myd88*^{+/+}CD45.2⁺ chimeras,

WT CD45.1⁺ cells were the dominant GC B cells in the *Myd88*^{+/+}CD45.1⁺/*Myd88*^{-/-}CD45.2⁺ chimeras (Fig. 3C, left panels). The CD45.1⁺/CD45.2⁺ ratio of Tfh cells was comparable between *Myd88*^{+/+}CD45.1⁺/*Myd88*^{+/+}CD45.2⁺ and *Myd88*^{+/+}CD45.1⁺/*Myd88*^{-/-}CD45.2⁺ chimeric mice, although the ratio was consistently high in CD45.2⁺ cells for an unknown reason (Fig. 3C, right panels). Thus, B cell-intrinsic MyD88 signaling is indispensable for Ab production against pollen allergen introduced to lungs.

TLR2/4/9 signaling is dispensable for Ab production to ragweed pollen introduced to lungs

It was reported that ragweed pollen contains TLR4 ligand(s) (30, 32). Thus, we next examined whether MyD88 signaling required for Ab production is evoked by ligation of TLRs. Indeed, the ragweed pollen that we used in this study activated GM-CSF-induced BM-derived DCs in vitro in a TLR-MyD88-dependent manner (Supplemental Fig. 2A). However, when ragweed pollen was administered intratracheally, *Tlr2/4/9*^{-/-} mice developed normal IgE and IgG1 production (Fig. 4). Thus, although we

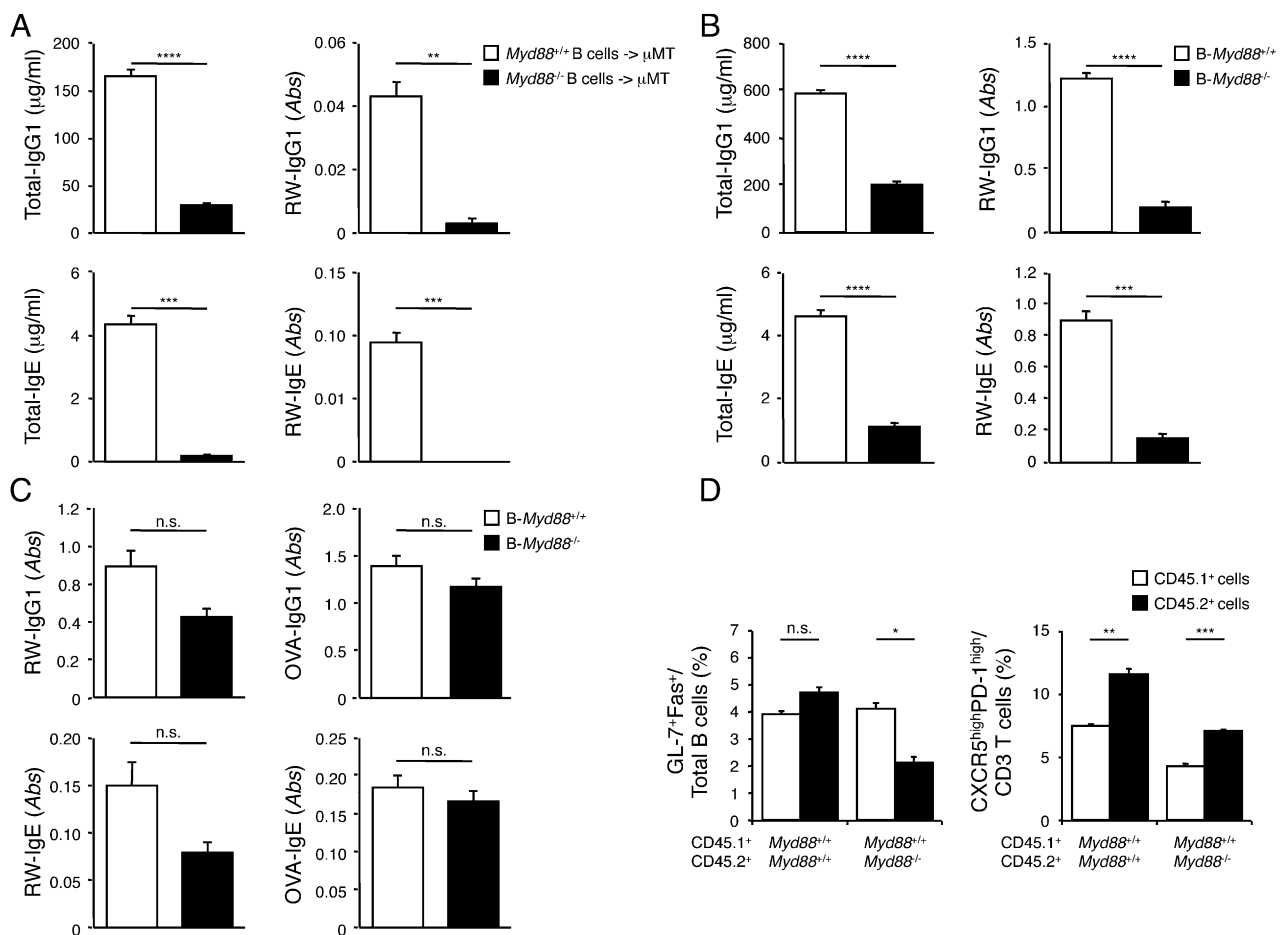


FIGURE 3. B cell-intrinsic MyD88 has an essential role in IgE and IgG1 production in response to ragweed (RW) pollen exposure of lungs. (A) *Ighm*^{-/-} (μ MT) mice were reconstituted with splenic B cells from *Myd88*^{+/+} or *Myd88*^{-/-} mice 24 h before intratracheal administration. Mice were administered RW pollen as in Fig. 1A. Total and RW-specific IgG1 and IgE levels in the sera were determined by ELISA. (B) Lethally irradiated *Rag2*^{-/-} mice were reconstituted with BM cells from μ MT mice together with BM cells from *Myd88*^{+/+} (B-*Myd88*^{+/+}) or *Myd88*^{-/-} (B-*Myd88*^{-/-}) mice. The chimeric mice were administered RW pollen intratracheally, as in Fig. 1A. Total and RW-specific IgG1 and IgE levels in the sera were determined by ELISA. (C) B-*Myd88*^{+/+} or B-*Myd88*^{-/-} mice were immunized i.p. with RW pollen or alum plus OVA. RW-specific or OVA-specific IgG1 and IgE levels in the sera were determined by ELISA. (D) Lethally irradiated WT mice were reconstituted with BM cells from CD45.1⁺*Myd88*^{+/+} mice together with BM cells from CD45.2⁺*Myd88*^{+/+} or CD45.2⁺*Myd88*^{-/-} mice. Mediastinal LNs were obtained from mice 7 d after a single intratracheal administration of RW pollen. Expression of GL-7 and Fas in CD45.1⁺B220⁺ and CD45.2⁺B220⁺ B cells (left panel) and PD-1 and CXCR5 in CD45.1⁺CD3⁺ and CD45.2⁺CD3⁺ T cells (right panel) was examined. Data (mean + SEM) are representative of two (A and B) or three (D) independent experiments [$n = 7$ (A), $n = 6$ (B), $n = 5$, RW group in (C) and (D), $n = 4$, OVA group in (C)]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. n.s., not significant.

cannot rule out the involvement of MyD88-dependent TLRs that were not investigated in this study, ragweed pollen activates TLR2, TLR4, or TLR9, but they are dispensable for inducing allergic responses elicited by direct exposure of ragweed pollen to lungs. Importantly, B cell-intrinsic MyD88 signaling that is essential for IgE and IgG1 production may not be evoked by the TLRs expressed on B cells. Although ragweed extract reportedly has enzymatic activities (33–35), heat-treated ragweed pollen induced Th2 and Ab responses to bystander Ag OVA, thus excluding the involvement of enzymatic activity in the induction of Th2 and Ab production by ragweed (Supplemental Fig. 2B, 2C).

Endogenous IL-1 α / β and IL-18 production is essential for Ab production to ragweed pollen introduced to lungs

Because *Tlr2/4/9*^{-/-} mice showed normal IgE/IgG1 production, we next examined endogenous factors that activate MyD88 signaling. We measured protein levels of IL-1 family cytokines in the lungs after ragweed pollen exposure. All IL-1 family cytokines examined in ragweed pollen-exposed lungs—IL-1 α , IL-1 β , IL-18, and IL-33—were upregulated (Fig. 5). Therefore, IL-1 family cytokines are likely candidates for endogenous ligands that evoke B cell-intrinsic MyD88 signaling.

To clarify the involvement of IL-1 family cytokines in the production of IgE/IgG1 elicited by ragweed pollen exposure to lungs, we used anti-IL-1R1 Ab treatment, which dampens IL-1 α and IL-1 β signaling (36), and *Il18*^{-/-} and *Il33*^{-/-} mice. For neutralizing Ab treatment, mice were administered anti-IL-1R1 Ab i.v. 1 d before the first ragweed administration. Subsequently, mice were administered the Ab i.p. 1 d before each ragweed dose. We found a significant decrease in total and ragweed-specific IgG1 production from mice treated with anti-IL-1R1 Ab (Fig. 6A). Total and ragweed-specific IgE showed decreased trends, but they were not statistically significant (Fig. 6A). *Il18*^{-/-} mice showed more severe defects in total and ragweed-specific IgE production (Fig. 6B). Ragweed-specific IgG1 production also was significantly decreased in *Il18*^{-/-} mice, but total IgG1 levels were comparable to those in WT mice (Fig. 6B). Total and ragweed-specific IgE and IgG1 production was not abrogated in *Il33*^{-/-} mice (Fig. 6C). Of

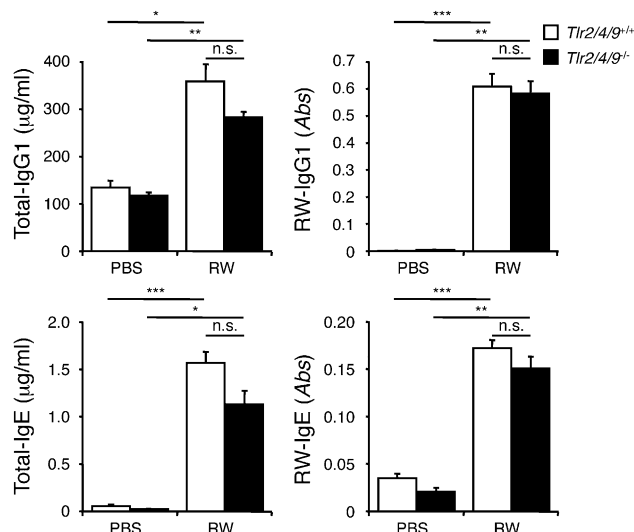


FIGURE 4. TLRs are dispensable for IgE and IgG1 production induced by lung exposure to ragweed (RW) pollen. *Tlr2/4/9*^{+/+} or *Tlr2/4/9*^{-/-} mice were administered RW pollen or PBS intratracheally, as in Fig. 1A. Total and RW-specific IgG1 and IgE levels in the sera were determined by ELISA. Data (mean + SEM) are representative of two independent experiments ($n = 5$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s., not significant.

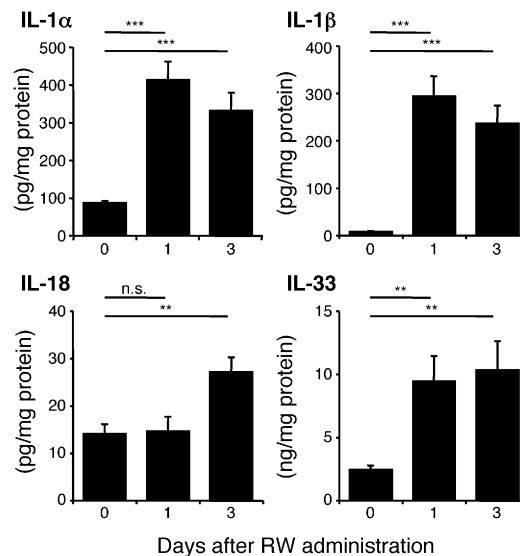


FIGURE 5. IL-1 family cytokines are induced in lungs by ragweed (RW) pollen exposure. Lungs were obtained from BALB/c mice 1 or 3 d after a single intratracheal administration of RW pollen. Lungs were homogenized, and the IL-1 α , IL-1 β , IL-18, and IL-33 contents in the lysates were determined by ELISA. Data (mean + SEM) are representative of two independent experiments ($n = 3$). ** $p < 0.01$, *** $p < 0.001$. n.s., not significant.

note, Th2 cell development was not abrogated by anti-IL-1R1 Ab treatment or in *Il18*^{-/-} or *Il33*^{-/-} mice (Supplemental Fig. 3).

B cell-intrinsic MyD88 signaling is required for optimal IgE/IgG1 production elicited by IL-1 family cytokines in lungs

Finally, we sought to investigate whether the IL-1 family cytokines are directly linked to the B cell-intrinsic MyD88 signaling that is essential for Ab responses in lungs. To this end, we immunized B-Myd88^{+/+} and B-Myd88^{-/-} BM chimeric mice intratracheally with OVA together with IL-1 α , IL-1 β , or IL-18. Of note, intratracheal administration of IL-1 α , IL-1 β , or IL-18, but not IL-33, with OVA elicited OVA-specific IgE and IgG1 production in WT mice (Supplemental Fig. 4). In B-Myd88^{-/-} mice, the Ab responses elicited by lung instillation of OVA plus IL-1 α , IL-1 β , or IL-18 were significantly reduced compared with those observed in control B-Myd88^{+/+} mice (Fig. 7). Thus, in addition to the effect of IL-1 family cytokines on epithelial cells, APCs, or T cells (37–40), they stimulate B cells to fully activate the Ab responses in the lung Ag instillation model. Taken together, lung exposure of pollen allergens induces the production of IL-1 α / β and IL-18, which, in turn, activate B cell-intrinsic MyD88 signaling to facilitate Ab production.

Discussion

This study shows that B cell-intrinsic MyD88 signaling has an essential role in Ab production induced by lung instillation of a particulate allergen. The role of B cell-intrinsic MyD88 signaling has been well studied in virus infection (13–16) and autoimmunity (20). The stimulation of TLR7 or TLR9 on B cells promotes B cells to produce IgG2 class Abs with decreasing amounts of IgG1 Ab (13–16). However, B cell-intrinsic MyD88 signaling also contributes to GC responses, and plasma cell differentiation, and thus can globally augment humoral immunity (12). Indeed, studies showed that B cell-specific deletion of MyD88 results in a comprehensive loss of Ab production, including IgG1, in some conditions (9, 20). Our results also dem-

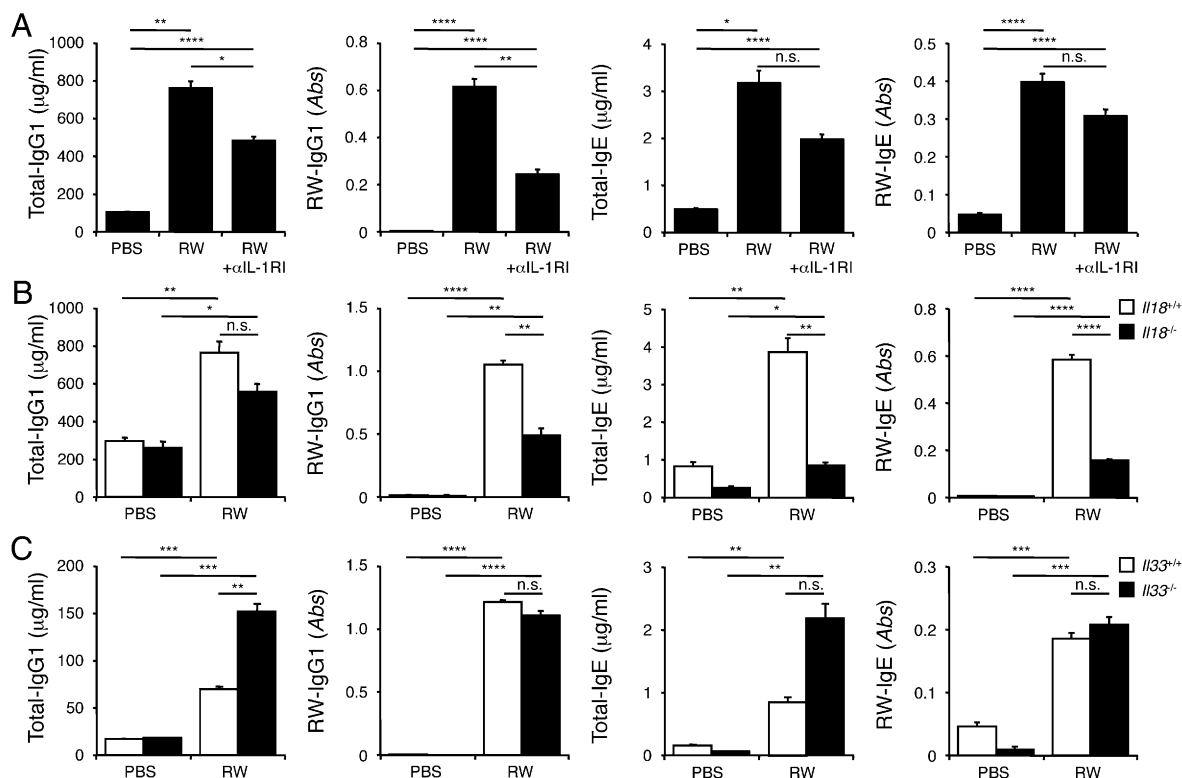


FIGURE 6. IL-1 α/β and IL-18, but not IL-33, facilitate IgE and IgG1 production in response to ragweed (RW) pollen exposure of lungs. Anti-IL-1R1 Ab-treated mice (A), *Il18*^{+/+} or *Il18*^{-/-} mice (B), or *Il33*^{+/+} or *Il33*^{-/-} mice (C) were administered RW pollen or PBS intratracheally, as in Fig. 1A. Total and RW-specific IgG1 and IgE levels in the sera were determined by ELISA. Data (mean + SEM) are representative of two independent experiments [$n = 6$ (A), $n = 4$ (B), $n = 5$ (C)]. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. n.s., not significant.

onstrate that *Myd88*^{-/-} mice do not develop GC B cells and Tfh cells upon ragweed pollen instillation. Because ragweed pollen instillation in the lung strongly induced IgE and IgG1, but not IgG2, production in WT mice, defective IgE and IgG1 production may reflect defective Ab production rather than the specific loss of Th2-type Ab production. B cell–intrinsic MyD88 signaling may be required for GC-mediated TD Ab responses induced by pollen Ags introduced into lungs. The defective IgE/IgG1 production in *Myd88*^{-/-} mice is not attributable to a defective response to IL-4 in MyD88-deficient B cells, because purified MyD88-deficient B cells can respond normally to anti-CD40 Ab plus IL-4 stimulation in vitro (41, 42).

Our data demonstrate that MyD88 signaling evoked by ragweed pollen instilled into lungs participates only in Ab production and not in the activation of Th2 cells. In classical immunology, it was thought that Th2 cells and IgE production should occur concurrently. However, recent advances revealed that Tfh cells, rather than Th2 cells or other Th subsets, are important for controlling humoral immunity (43). In addition, previous studies reported the differential regulation of Th2 cell development and Tfh cell–controlled IgE production (43, 44). In the current study, Tfh cell development was abrogated in *Myd88*^{-/-} mice with ragweed pollen exposure in the lung. It was reported that both priming by DCs and sustained interactions with GC B cells were essential for the full development of Tfh cells (45). Because B cell–specific deletion of MyD88 reproduced the defective Ab production to ragweed pollen, and *Myd88*^{-/-} T cells differentiated normally into Tfh cells in the presence of *Myd88*^{+/+} B cells, the defective Tfh cell development observed in *Myd88*^{-/-} mice could be the result of the lack of GC B cells rather than priming by DCs.

Although the exact significance of GCs in IgE production is still under debate, GC responses may be an important contribution to

IgE production (46, 47). However, IgE also can be produced from GC-independent sources, because B cell lymphoma 6–deficient or lymphotoxin- α –deficient mice produce IgE, despite having defective formation of GCs or lymph nodes, respectively (48, 49). Furthermore, although local production of IgE in mucosal tissues is important in allergic disease, and some studies suggested the local formation of GC-like structures (50, 51), it is not clear how GC responses are important for local IgE production (46). Our results demonstrate that MyD88 also may mediate GC-independent IgE production, if any, in lungs exposed to ragweed pollen, because IgE responses were totally abrogated in *Myd88*^{-/-} mice.

Several allergens, especially airborne allergens, were reported to contain TLR ligands that activate host MyD88 signaling (24, 25, 30, 32). Lung epithelia or DCs activated by allergen-associated TLR ligands initiate type-2 immune responses (24, 25). Ragweed pollen also strongly activated Th2 responses when instilled into lungs. Although ragweed pollen contains TLR ligands (30, 32), our data also demonstrates that, these TLR ligands were not essential for evoking IgE and IgG1 production. We showed that IL-1R and IL-18R, but not TLRs, were essential for IgE and IgG1 production. Because inhibition of IL-1R ligation or IL-18 production resulted in a decrease in IgE and IgG1 production with a milder phenotype than in *Myd88*^{-/-} mice, these cytokines seem to stimulate B cells cooperatively. Furthermore, we cannot rule out the possibility of the involvement of other signals not investigated in this study. Pollens are particulate matter; recently, studies on the immune-stimulatory properties of particulate matter 2.5—diesel exhaust particles and sand dust—demonstrated their involvement in pulmonary inflammation and asthma as environmental pollutants (26). These particles and micrometer-sized crystals, such as alum and silica, which are considered Th2-inducing adjuvants,

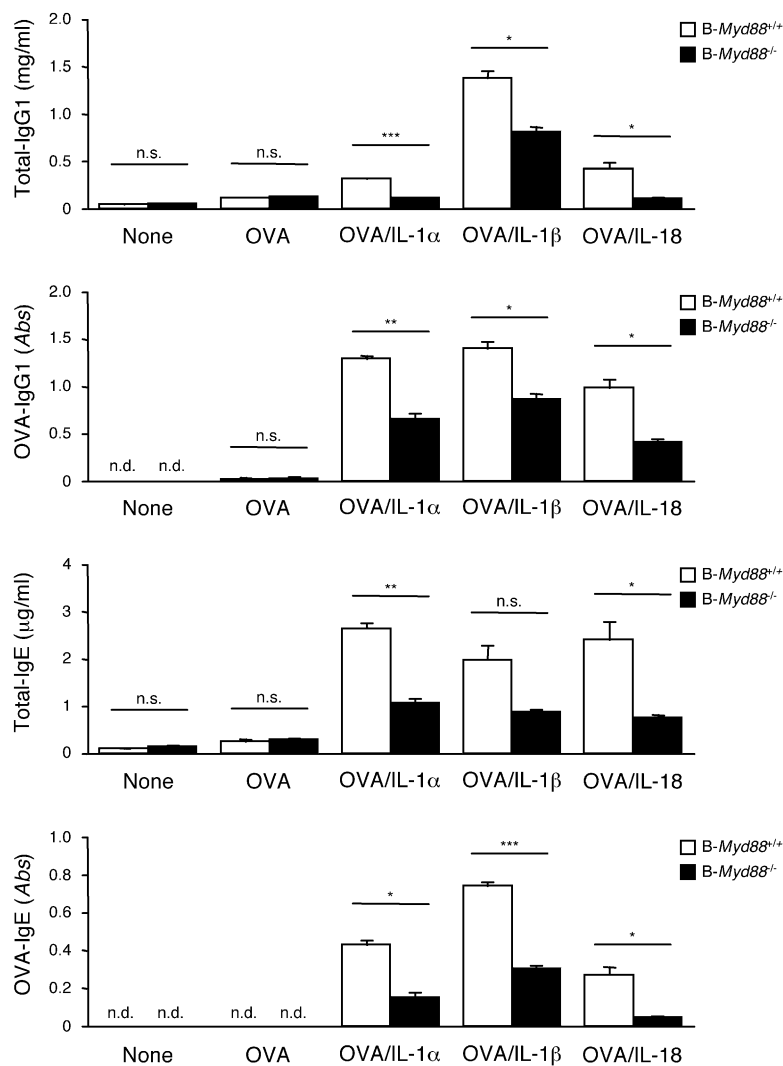


FIGURE 7. B cell-intrinsic MyD88 signaling participates in lung IL-1α-, IL-1β-, or IL-18-elicited induction of IgE and IgG1. Lethally irradiated *Rag2*^{-/-} mice were reconstituted with BM cells from μMT mice together with BM cells from *Myd88*^{+/+} (B-*Myd88*^{+/+}) or *Myd88*^{-/-} (B-*Myd88*^{-/-}) mice. The chimeric mice were not treated or were administered OVA alone, IL-1α plus OVA, IL-1β plus OVA, or IL-18 plus OVA (all intratracheally), as in Fig. 1A. Total and OVA-specific IgG1 and IgE levels in the sera were determined by ELISA. Data (mean + SEM) are representative of two independent experiments ($n = 4$, OVA/IL-1α and OVA/IL-1β groups, B-*Myd88*^{+/+}; $n = 5$, OVA/IL-1α and OVA/IL-1β groups, B-*Myd88*^{-/-}; $n = 5$, None, OVA, and OVA/IL-18 groups). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.d., not detected; n.s., not significant.

induce inflammasome-dependent IL-1β and IL-18 production (26). Furthermore, particles can induce cellular damage, resulting in the release of alarmins, including IL-1α (52). Although the involvement of IL-1 family cytokines in particulate matter-induced Th2 responses is controversial (26), IL-1 family cytokines might participate in IgE and IgG1 production in the lungs (37–40, 53).

Exogenous administration of IL-18 into mice stimulates innate type B cells, such as B-1 B cells and marginal zone B cells, in vivo (54). Furthermore, the administration of IL-18 induced strong GC responses and the production of IgE (54). In humans, IL-18Rα is expressed on GC and memory B cells at higher levels than on naive B cells (55), suggesting involvement of IL-18 in GC Ab production. These studies demonstrate that direct stimulation of B cells by IL-18 can enhance the GC response and IgE production. The intratracheal administration of IL-18, together with ragweed pollen, augmented Th2 development and serum IgE levels (37). IL-1α and IL-1β might also participate in the pathogenesis of lung allergic diseases. IL-1R1- or IL-1α/β-deficient mice develop significantly reduced Th2 cell responses and IgE/IgG1 production in an OVA-induced asthma model (38, 39). IL-1α is a critical mediator of house dust mite-induced pulmonary inflammation (40). Although the contribution of IL-1α/β and IL-18 in lung Th2 responses has been interpreted as an adjuvant effect through epithelial cells and APCs or a direct effect on CD4⁺ T cells, the role of these cytokines in B cells was previously unknown. However,

recapitulating our results from this study and the fact that functional IL-1R and IL-18R are expressed on B cells (54–56), IL-1α/β and IL-18 also stimulate B cells to promote GC responses and IgE/IgG1 production during the airway instillation of pollen allergens. The results demonstrated in this study are not contradictory to the roles for IL-1 family cytokines on epithelia, myeloid APCs, or T cells that were described previously (37–40). B cell-intrinsic MyD88 signaling may cooperatively activate Ab responses with MyD88 signaling in other cells, because the defective Ab responses were greater in systemic *Myd88*^{-/-} mice than in B-*Myd88*^{-/-} mice.

In this study, we did not determine how Th2 cell priming is induced following ragweed pollen instillation, because Th2 cell development was intact in *Myd88*^{-/-} mice. Pollens possess enzymatic activity, and ragweed contains NADPH oxidase (33, 34) and protease (35) activities. Although these enzymatic activities have important roles in Th2 development and lung pathology in ragweed extract instillation models (33, 34), they were not important for the induction of Th2 responses in our pollen instillation model, because heat-treated ragweed pollen induced Th2 activity. Thus, the particulate property of ragweed pollen may bypass the role of enzymatic activities, which mediate the allergenicity of some soluble allergens. How particles induce Th2 responses is a complex subject for immunologists (26). The lipid mediator PGE₂ (57), self nucleic acids (58), and uric acid (59) are considered to be involved in alum-induced Th2 responses. It would be

intriguing to investigate these pathways in pollen-induced lung allergic diseases. Furthermore, the lung-specific environment might affect the induction of Th2 cells and IgE production. Indeed, our results observed in lungs are different from those observed in other tissues. We showed that TLR ligands are not involved in ragweed pollen-induced allergic responses, despite having a role in conjunctival tissue responses (30). Furthermore, although MyD88 is dispensable for IgE and IgG1 production induced by alum immunized in the footpad (21) or i.p (11), we showed that MyD88 was essential when alum is applied into lungs. These differences could be explained by the unique immune system present in the lungs. Although healthy lungs were previously regarded as a sterile environment, accumulating evidence demonstrated the presence of a microbiome in lungs and its association with lung diseases (60, 61). Thus, lungs may have a distinct immune system reminiscent of that in the intestine or skin. An understanding of lung-specific immune responses will aid in the development of new therapies for airway diseases, including allergic asthma.

In conclusion, we demonstrated that B cell-intrinsic MyD88 signaling is essential for IgE and IgG1 production in lung-mediated pollen allergen immunization. Host production of IL-1 α / β and IL-18, rather than allergen-associated TLR ligands, induces IgE and IgG1 production to ragweed pollen. Our data also suggest that lung-specific mechanisms promote IgE and IgG1 production. Further study of the lung immune system and mechanisms involved in Th2 development and IgE production will help to develop new therapies for allergic asthma with low systemic side effects.

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Disclosures

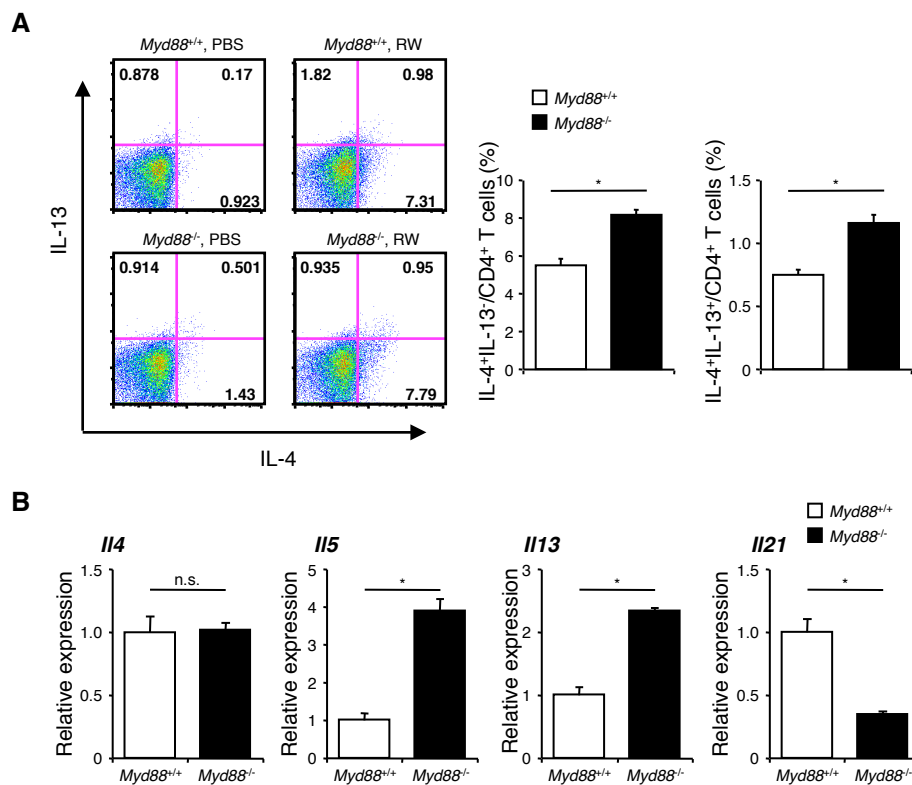
The authors have no financial conflicts of interest.

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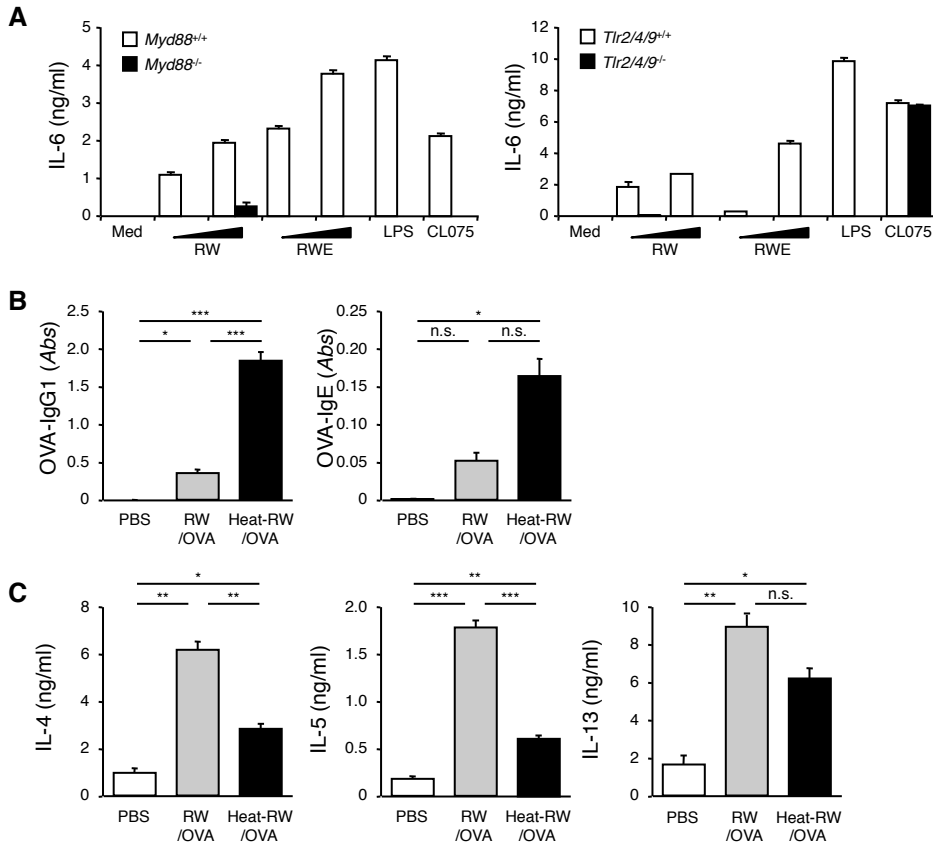
Supplementary Figure 1



Supplementary Figure 1. Cytokine production by CD4⁺ T cells in mediastinal lymph nodes.

(A) Presence of IL-4 and IL-13 producing CD4⁺ T cells in mediastinal LNs 7 days after ragweed exposure. (B) Cytokine mRNA levels in mediastinal LNs 7 days after ragweed pollen exposure. Data representative of two independent experiments. (mean, SEM, n=5 (A) n=3 (B)). *P<0.05. n.s. not significant.

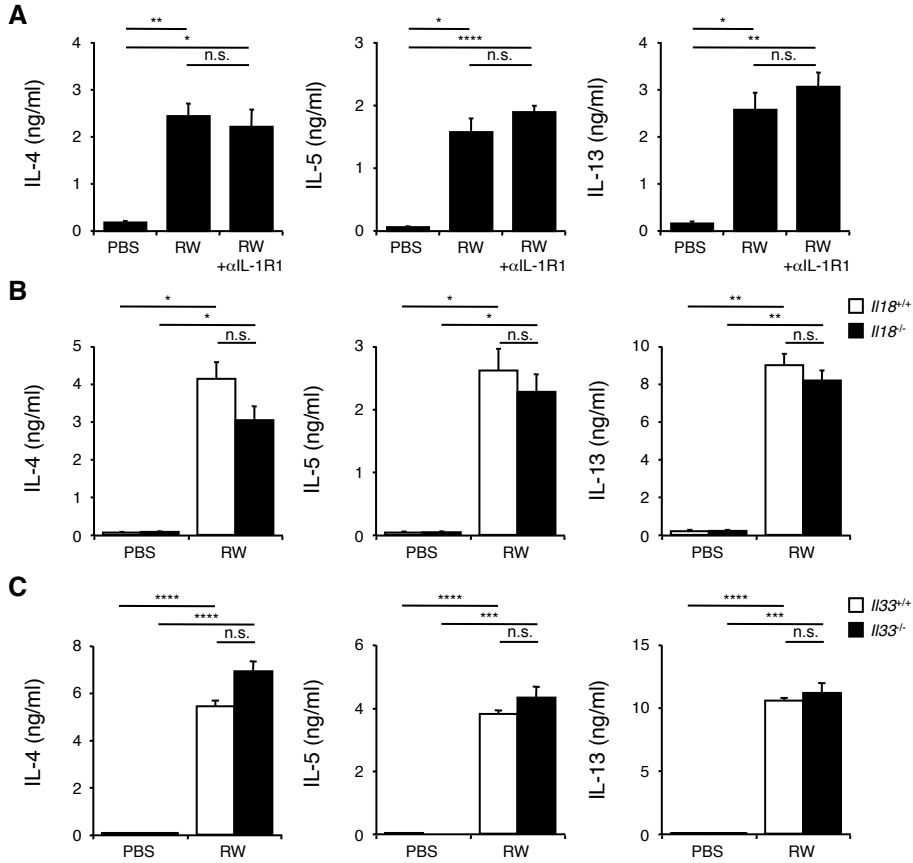
Supplementary Figure 2



Supplementary Figure 2. TLR-ligand(s) and enzymatic activity in ragweed pollen.

(A) GM-CSF-induced bone-marrow-derived dendritic cells (BM-DCs) were prepared from *Myd88*^{+/+} or *Myd88*^{-/-} mice or *Tlr2/4/9*^{+/+} or *Tlr2/4/9*^{-/-} mice. BM-DCs were incubated with ragweed pollen (RW), ragweed extract (RWE), LPS (a TLR4 ligand), or CL075 (a TLR7 ligand) for 24 h. Culture supernatants were harvested and the levels of IL-6 were determined by ELISA. Data representative of two independent experiments. (mean, SD, triplicate culture). (B and C) Mice were intratracheally administered native or heat-treated ragweed pollen together with OVA as in Fig. 1A. (B) OVA-specific IgG1 and IgE levels in the sera were determined by ELISA. (C) Mediastinal LNs were obtained from mice 3 days after antigen exposure. LN cells were incubated with plate-bound anti-CD3 Ab and soluble anti-CD28 Ab for 2 days. Culture supernatants were harvested and the levels of IL-4, IL-5, and IL-13 were determined by ELISA. Data representative of two independent experiments. (mean, SEM, n=6). *P<0.05, **P<0.01, ***P<0.001.

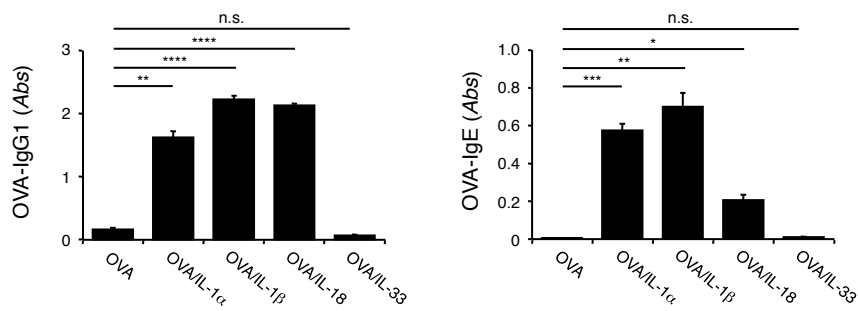
Supplementary Figure 3



Supplementary Figure 3. IL-1 α/β , IL-18, and IL-33 are dispensable for Th2-cell development in response to ragweed pollen exposure of lungs.

Anti-IL-1RI antibody-treated mice (A), *Il18*^{+/+} or *Il18*^{-/-} mice (B), or *Il33*^{+/+} or *Il33*^{-/-} mice (C) were intratracheally administrated with ragweed (RW) pollen or PBS. Mediastinal LNs were obtained from mice 3 days after ragweed exposure. LN cells were incubated with T-cell-depleted and irradiated splenocytes in the presence of IL-2 and RW extract for 5 days. Culture supernatants were harvested and the levels of IL-4, IL-5, and IL-13 were determined by ELISA. Data representative of two independent experiments. (mean, SEM, n=3 (A), n=4 (B), n=5(C)). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. n.s. not significant.

Supplementary Figure 4



Supplementary Figure 4. Intratracheal administration of OVA together with IL-1 α , IL-1 β , or IL-18 induce OVA-specific IgE/IgG1 responses.

Mice were intratracheally administered OVA alone or OVA together with IL-1 α , IL-1 β , IL-18, or IL-33 as in Fig. 1A. OVA-specific IgG1 and IgE levels in the sera were determined by ELISA. (mean, SEM, n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. n.s. not significant.