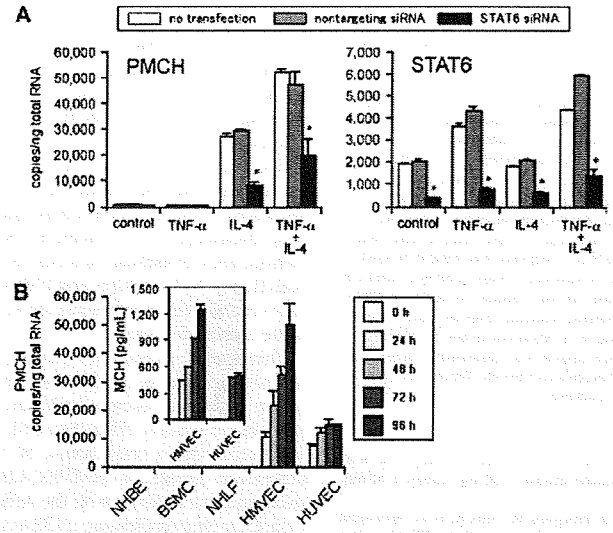

FIG 1. A, STAT6 is required for IL-4–induced expression of PMCH in HMVEC-LBl. siRNA for STAT6 or nontargeting control siRNA was transfected into HMVEC-LBl. The transfected cells were further grown for 48 hours and then stimulated with TNF-α, IL-4, or a combination of TNF-α and IL-4 for 24 hours. Data are shown as the means ± SDs of triplicate samples and are representative of 3 separate experiments. *$P < .05$ vs nontargeting siRNA, Mann-Whitney *U* test. B, MCH is characteristically expressed in vascular endothelial cells in response to IL-4 stimulation. Cells were treated with 10 ng/mL IL-4 for the indicated periods. The mRNA levels *(outer graph)* and the peptide levels *(inner graph)* are shown. Data are shown as the means ± SDs of triplicate samples and are representative of at least 3 separate experiments. *BSMC,* Bronchial smooth muscle cells; *HUVEC,* human umbilical vein endothelial cells; *NHBE,* normal human bronchial epithelial cells; *NHLF,* normal human lung fibroblasts.

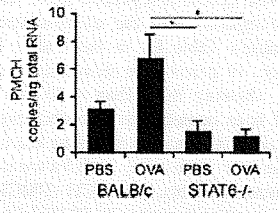

FIG 2. Mice were intraperitoneally injected with 10 μg ovalbumin *(OVA;* Sigma, St. Louis, Mo, grade V) in phosphate buffered saline (PBS) every other day for a total of 7 times. Two weeks after the last OVA sensitization, the mice were intranasally challenged with 200 μg OVA in PBS (BALB/c, n = 6; and STAT6$^{-/-}$, n = 6) or PBS alone (BALB/c, n = 3; STAT6$^{-/-}$, n = 4) every 3 days for a total of 3 times. The animals were sacrificed 24 hours after the last challenge. Total RNA in the lung was extracted with ISOGEN (Nippon Gene, Tokyo, Japan). Quantitative real-time PCR for mouse PMCH was performed as described in the text for human PMCH procedures except for replacing the antisense primer with the mouse PMCH sequence (5′-TTGCCAACATGGTCGGTAGA-3′). Data are shown as means ± SEMs. *P* values were obtained by the Mann-Whitney *U* test. *$P < .05$ ($P = .09$; BALB/c-PBS vs BALB/c-OVA).

control siRNA (QIAGEN) at 100 nmol/L was transfected into HMVEC-LBl by using HiPerFect transfection reagent (QIAGEN) following the manufacturer's instructions. The transfected cells were further grown for 48 hours and then stimulated with TNF-α, IL-4 or a combination of TNF-α and IL-4 for 24 hours. The efficiency of mRNA depletion was more than 70% compared with the level of STAT6 transcripts in nontargeting siRNA-transfected cells, which was confirmed by real-time PCR (Fig 1, *A, right graph*; sense, 5′-TCTGACCGGCTGATCATTGG-3′; antisense, 5′-CCAATCTCTGAGTCGCTGAAGC-3′). Transfection of STAT6 siRNA significantly reduced the IL-4–dependent upregulation of PMCH, suggesting that STAT6 is required for IL-4–induced expression of PMCH in HMVEC-LBl.

IL-13 is another T_H2 cytokine that plays a prominent role in the pathogenesis of allergic inflammation. IL-13 and IL-4 share many functional properties, stemming from the fact that they share a common receptor subunit, the α subunit of the IL-4 receptor. STAT6 is also activated by IL-13 via IL-4 receptor α, and we found that IL-13 also potently induced PMCH expression during 24 hours of culture to a similar degree with equivalent concentrations of IL-4 (see this article's Fig E1 in the Online Repository at www.jacionline.org). IFN-γ, a T_H1 cytokine, antagonizes many of the physiological responses mediated by IL-4/13, and this antagonism largely occurs at the level of transcription. As shown in Fig E1, IFN-γ significantly inhibited IL-4/13–induced expression of PMCH mRNA in HMVEC-LBl in a dose-dependent manner.

We next investigated whether IL-4–induced expression of PMCH mRNA is observed in other human airway structural cells, such as normal human bronchial epithelial cells, bronchial smooth muscle cells, and normal human lung fibroblasts. We found that upregulation of PMCH mRNA in response to IL-4 was specifically seen in HMVEC-LBl cells for as long as 4 days of culture (Fig 1, *B, outer graph*). These results suggest that PMCH expression is specific to vascular endothelial cells. IL-4–induced expression of PMCH was also seen in other

human endothelial cells, human umbilical vein endothelial cells (Fig 1, *B, outer graph*), and human coronary artery endothelial cells (data not shown). Next, using an MCH enzyme immunoassay kit (Phoenix, Burlingame, Calif), we further confirmed the accumulation of MCH peptide in the culture supernatants of vascular endothelial cells in response to IL-4 (Fig 1, *B, inner graph*), but never in the culture supernatants of IL-4–stimulated normal human bronchial epithelial cells, bronchial smooth muscle cells, or normal human lung fibroblasts (data not shown).

The current study reveals that human vascular endothelial cells are capable of producing an appetite-stimulating peptide, MCH, in response to T_H2 cytokines. The production of MCH is transcriptionally regulated via STAT6 (Fig 1, *A*). Recently, Sandig et al[4] demonstrated that human T_H2 cells, but not T_H1 cells, selectively produce MCH. Collectively, these findings support a novel hypothesis that MCH is a critical factor in the physiological interactions between allergic inflammation and obesity. The MCH system appears to be important for the regulation of stress and anxiety-related responses,[1] leading us to hypothesize that MCH may also be involved in the physiological interactions between allergic inflammation and depression.

Obesity and asthma may be dependent on shared genetic mechanisms. *PMCH* is located on chromosome 12q23-q24.[5] Chromosome 12q is one of the most frequently identified genomic regions linked to asthma and atopy.[6] Of note, this region also contains *IFN-γ* and *STAT6*, which are involved in the regulation of PMCH expression (Fig 1, *A and B*). In support of that concept of shared genetic mechanisms, interestingly, we also found that PMCH mRNA expression was increased in the lungs of BALB/cA–wild-type (Clea, Tokyo, Japan), but not STAT-6–deficient, mice after ovalbumin challenge during ovalbumin-induced airway inflammation[7] (Fig 2), which is considered to be an asthma model in rodents, suggesting that STAT-6–dependent PMCH expression may contribute to the pathogenesis of asthma in mice. These facts further support the physiological relevance of MCH to allergy and asthma.

Appetite is closely regulated by complex interactions between orexigenic and antiorexigenic factors originating in both the hypothalamus and the periphery. Unlike such orexigenic factors as neuropeptide Y, orexin, and agouti-related peptide, MCH and its receptor, MCH receptor 1, are the only molecules whose genetic ablation in mice results in obviously lean phenotypes, indicating that the MCH system is indispensable for energy balance and food intake in mammals. Numerous peripheral signals relating to energy metabolism are transmitted to the central nervous system via the circulation or afferent fiber sympathetic nerves. One important system is the vagus afferent neurons, in which both MCH and MCH receptor 1 were recently identified.[8] Both are negatively regulated by cholecystokinin, which suggests the peripheral MCH signal is involved in the appetite control system. Taken together with our findings, MCH produced in the vascular endothelium in response to T_H2 cytokines might be responsible for obesity in patients with asthma.

It would be of great interest to investigate the differences in the serum levels of MCH between patients with asthma and normal controls. Although it was reported that serum MCH levels were positively associated with the body mass index and fat mass, problems of specificity have also been reported in the measurement of serum MCH.[9] Therefore, it is necessary to develop a precise determination method for the serum MCH level. However, we anticipate that future studies will demonstrate MCH to be a key molecule in the molecular mechanisms that link allergic inflammation and obesity or depression, all of which are currently important public health issues.

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Caspase-1, Caspase-8, and Calpain Are Dispensable for IL-33 Release by Macrophages[1]

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In addition to IL-1 and IL-18, IL-33 was recently identified as a member of the IL-1 cytokine family. rIL-33 can promote production of Th2-type cytokines by Th2 cells and mast cells in vitro. Administration of rIL-33 to mice results in increases in IgE secretion and eosinophilic inflammation. However, the precise immune cell source of IL-33 remains unclear. Moreover, although recombinant pro-IL-33 is cleaved by recombinant caspase-1 in vitro, as are pro-IL-1β and pro-IL-18, the involvement of caspase-1 in pro-IL-33 cleavage remains controversial. In this study, we show that mouse peritoneal macrophages, but not splenic dendritic cells, produced IL-33 upon stimulation with LPS. Likewise, mouse bone marrow cell-derived cultured mast cells also produced a small, but significant amount of IL-33 via FcεRI cross-linking, but not in response to stimulation with LPS. To our surprise, IL-33 release was found even in caspase-1-deficient, caspase-8 inhibitor-treated, and calpain inhibitor-treated macrophages. These observations suggest that caspase-1-, caspase-8-, and calpain-independent IL-33 production by macrophages and/or mast cells may contribute to the pathogenesis of Th2-type allergic inflammation. *The Journal of Immunology*, **2009, 183: 7890–7897.**

Interleukin-33 (also called IL-1F11 (1), DVS27 (2), or nuclear factor from high endothelial venule (3)) is a member of the IL-1 family of cytokines that includes IL-1β and IL-18 (1). DVS27 and nuclear factor from high endothelial venule were, respectively, identified as an up-regulated gene in vasospastic cerebral arteries after subarachnoid hemorrhage (2) and as a NF expressed in endothelial cells (3). Then IL-33 was identified as a ligand for ST2 (also called T1, DER-4, Fit-1, or IL-1R4 (1, 4)), which is preferentially expressed in Th2 cells (5) and mast cells (6). High levels of constitutive IL-33 mRNA expression were found in various murine tissues, such as the stomach, lung, spinal cord, brain, and skin (1). In humans, smooth muscle cells and epithelial cells constitutively express IL-33 mRNA. Moreover, IL-33 mRNA expression was up-regulated in human fibroblasts and keratinocytes by stimulation with TNF and IL-1β (1).

IL-33 can promote Th2 cell and mast cell activation, leading to such functions as Th2-type cytokine secretion or chemotaxis (1, 7–11). IL-33 contributes to Th2-associated host defense against nematode infection (12). IL-33 administration results in IL-13-mediated eosinophilic inflammation in the gut and lung of mice (1). Moreover, inhibition of IL-33 by soluble IL-33R leads to attenuation of murine asthma models (13). In contrast, IL-33 ameliorates Th1-associated autoimmune-like atherosclerosis (14). Thus, IL-33 is considered to be involved in Th2-type immune responses and to suppress Th1-type responses. Based on these findings, it is clear that IL-33 contributes to the immune responses by acting as a proinflammatory cytokine. It is unclear what kinds of immune cell types can release IL-33 protein, although LPS-stimulated bone marrow cell-derived macrophages and resting bone marrow cell-derived dendritic cells (DCs)[3] were reported to express IL-33 mRNA (1). In a preliminary experiment, we examined the expression of IL-33 mRNA in various immune cells and detected it in activated mast cells as well as activated macrophages and DCs.

Similar to IL-1β and IL-18, IL-33 is considered to be produced intracellularly as pro-IL-33, which does not contain a signal peptide sequence for secretion, and then released extracellularly as mature IL-33 after cleavage (1). Caspase-1 and/or caspase-8 is required for the cleavage of pro-IL-1β and/or pro-IL-18 (15, 16), whereas calpain is important for the cleavage of pro-IL-1α (16–18). Although recombinant pro-IL-33 is cleaved by recombinant caspase-1 in vitro (1), the in vivo role of caspase-1 in the cleavage of pro-IL-33 remains controversial (19). In this study, we show that LPS-stimulated mouse peritoneal macrophages, but not splenic DCs, and IgE/Ag-stimulated bone marrow cell-derived cultured mast cells (BMCMCs) produced IL-33, and that caspase-1, caspase-8, and calpain were dispensable for IL-33 release.

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[3] Abbreviations used in this paper: DC, dendritic cell; BMCMC, bone marrow cell-derived cultured mast cell; Ct, threshold cycle; HSA, human serum albumin; LDH, lactate dehydrogenase; mIL, mouse IL; MUSTag, multiple simultaneous Tag; PEC, peritoneal exudate cell; PI, propidium iodide; qPCR, quantitative PCR; r.t., room temperature; TGC, thioglycolate.

Materials and Methods

Mice

BALB/cA mice (CLEA Japan) and BALB/c-caspase-1$^{-/-}$ mice (20) were housed under specific pathogen-free conditions in our institute (National Research Institute for Child Health and Development), and the animal protocols were approved by the Institutional Review Board of the National Research Institute for Child Health and Development.

Cells

For purification of CD4$^+$ T cells, mouse spleen and inguinal, axillary, brachial, and submaxillary lymph nodes were harvested and pooled. The pooled cells were incubated with biotinylated anti-mouse B220 (RA3-6B2), CD8 (53-6.7), CD11b (M1/70), CD11c (HL3), CD25 (PC61.5), CD49b (DX5), CD119 (c-*kit*, 2B8), FcεRIα (MAR-1), Gr-1 (RB6-8C5), Ter^{119}, and γδTCR (GL3) mAbs at 4°C for 20 min. These Abs were obtained from eBiosciences and BD Biosciences. After washing, the cells were incubated with Streptavidin Particles Plus-DM (BD Biosciences). Then CD4$^+$ T cells (>95%) were isolated by negative selection using a BD IMag system (BD Biosciences).

For purification of peritoneal macrophages and splenic DCs, mouse peritoneal exudate cells (PECs) and spleen cells were collected and incubated with anti-mouse CD16/CD32 mAb (93; eBiosciences) at 4°C for 20 min. The PECs were then incubated with biotinylated anti-mouse F4/80 mAb (BM8; eBiosciences) at 4°C for 20 min. After washing, the cells were incubated with streptavidin-Microbeads (Miltenyi Biotec) at 4°C for 20 min. Spleen cells were incubated with CD11c-Microbeads (Miltenyi Biotec) at 4°C for 20 min. F4/80$^+$ peritoneal macrophages (>92%) and CD11c$^+$ splenic DCs (>92%) were isolated by positive selection using a MACS system (Miltenyi Biotec).

For collection of thioglycolate (TGC)-induced mouse peritoneal macrophages (TGC-macrophages), mice were i.p. injected with 5 ml of 2% TGC (Nissui). Three days after injection, PECs were collected.

Mouse BMCMCs were generated, as described elsewhere (11). In brief, mouse femoral bone marrow cells were cultured in the presence of 10 ng/ml mouse rIL (rmIL)-3 (PeproTech) for 6–8 wk, at which time flow cytometry showed the cells to be a >98% c-*kit*$^+$ FcεRIα$^+$ population. Before using the cells, rmIL-3 was removed by washing.

Cell culture

CD4$^+$ T cells were cultured with plate-coated anti-CD3 mAb (1 μg/ml) (145-2C11; eBioscience). F4/80$^+$ macrophages, CD11c$^+$ DCs, or TGC-macrophages were stimulated with 100 ng/ml LPS (*Salmonella enterica* serotype typhimurium; Sigma-Aldrich), 100 ng/ml rmIL-33 (R&D Systems), or 0.1 μg/ml PMA (Sigma-Aldrich) plus 1 μg/ml ionomycin (Sigma-Aldrich). Naive BMCMCs were incubated with various concentrations of monomeric IgE (SPE-7; Sigma-Aldrich), LPS, and/or 0.1 μg/ml PMA plus 1 μg/ml ionomycin. For IgE/Ag-dependent mast cell activation, naive BMCMCs were sensitized overnight with 1 μg/ml anti-DNP IgE (SPE-7). After washing, IgE-sensitized BMCMCs were cultured with various concentrations of DNP-human serum albumin (DNP-HSA; Sigma-Aldrich). These cells were cultured for 1 h (RT-PCR), 6 h, or 24 h (ELISA, ELISPOT, and immunoprecipitation), respectively. For caspase-8 or calpain inhibition, F4/80$^+$ peritoneal macrophages were incubated with and without a caspase-8 inhibitor (30 μM caspase-8 inhibitor II (Calbiochem)) for 1 h, or calpain inhibitor (10, 30, or 100 μM MDL 28,170 (Calbiochem)) for 45 min, and then stimulated with and without LPS for 24 h. For induction of necrosis, F4/80$^+$ peritoneal macrophages and TGC macrophages were stimulated with 100 ng/ml LPS for 6 h, and then incubated with 1 M NaN_3 or treated by freeze thawing.

RT-PCR

IL-33 mRNA expression was analyzed by RT-PCR. The PCR primers were 5′-gaagatcccaacagaagacc-3′ and 5′-ttccggaggcgagacgtcac-3′ for mIL-33, and 5′-gccgcctggagaaacctgc-3′ and 5′-tgaggtccaccaccctgttg-3′ for murine GAPDH. The PCR conditions were (94°C for 30 s; 55°C for 30 s; 72°C for 60 s) × 35 cycles (IL-33) or 25 cycles (GAPDH).

ELISA/ELISPOT

For IL-33 detection, Nunc-Immuno plates (Nunc) for ELISA and MultiScreen-IP plates (MAIPS4510; Millipore) for ELISPOT were coated with anti-mIL-33 polyclonal Ab (R&D Systems; 2 μg/ml in PBS) as a capture Ab at 4°C overnight. After blocking with PBS containing 1% BSA (ELISA) or 10% FCS (ELISPOT), samples, and rmIL-33 as a standard cytokine, the cells were incubated at room temperature (r.t.) for 2 h for ELISA or cultured at 37°C for 24 h for ELISPOT. After washing the wells, biotinylated anti-mouse/human IL-33 mAb (Nessy-1; Alexis Biochemicals, 400 ng/ml in PBS containing 1% BSA) as a detection Ab was applied and incubated at r.t. for 1 h for ELISA and ELISPOT. Then, after washing the wells, HRP-conjugated streptavidin (BD Biosciences) was added to the wells at r.t. for 1 h. Tetramethylbenzidine (eBioscience) and 3-amino-9-ethylcarbazole (Sigma-Aldrich) were used as substrates in ELISA and ELISPOT, respectively. For ELISPOT, positive spots on Ab-coated MultiScreen-IP plates (MAIPS4510; Millipore) were analyzed with NIH Image software. For IL-1α, IL-1β, and IL-6 detection, mIL-1α DuoSet (R&D Systems) and mIL-1β and mIL-6 ELISA sets (eBiosciences) were used for ELISA and ELISPOT.

Immunocytochemistry

Immunocytochemistry for IL-33 was conducted by the polymer-immunocomplex method (DakoCytomation), according to the manufacturer's instructions. Briefly, cytospin samples were fixed with 4% performaldehyde at 4°C for 20 min and then blocked with peroxidase-blocking solution (DakoCytomation). The resultant complex was mixed with mouse anti-mouse/human IL-33 mAb (Nessy-2; Alexis Biochemicals) and EnVision detection reagent (DakoCytomation) at r.t. for 1 h, followed by addition of normal mouse serum and further incubation at r.t. for 1 h. Cytospin samples were incubated with the resultant complex at r.t. for 1 h, and then the IL-33/anti-IL-33/EnVision complex was detected with diaminobenzidine.

Immunoprecipitation and Western blot analysis

Cells that were stimulated with and without 100 ng/ml LPS or lungs that were harvested from mice at 6 h after inhalation of LPS (10 μg in 20 ml of sterile, pyrogen-free 0.9% NaCl (saline)) or saline were homogenized in cold lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS; 250 mg of lung tissue/ml) and held at 4°C for 30 min. Then, after centrifugation (16,000 × *g*) at 4°C for 30 min, the supernatants were collected. For immunoprecipitation, the lysates were incubated with anti-mIL-33 mAb (10 μg; Nessy-1; Alexis) or anti-mIL-1β mAb (2 μg; B122; BD Biosciences) at 4°C for 12 h. The immunoprecipitants, lung and cell lysates, rmIL-33, and rmIL-1β were analyzed by SDS-PAGE and blotted onto polyvinylidene difluoride membranes (Invitrogen). The membranes were blocked and then incubated consecutively with anti-mIL-33 mAb (4G4; MBL) and HRP-conjugated anti-mouse IgG (Amersham) for IL-33, and anti-mIL-1β (166926; R&D Systems) and anti-rat IgG (Amersham) for IL-1β. Then the immunoreactive proteins were visualized with ECL reagents (ECL Western Blotting Detection Reagents; Amersham).

Cell survival

Cell viability was assessed using the MEBCYTO-Apoptosis Kit (MBL) with flow cytometry or a colorimetric lactate dehydrogenase (LDH) assay kit (CytoTox 96; Promega).

Multiple simultaneous Tag (MUSTag) assay

For modified immune PCR, called MUSTag technology, anti-mIL-1β polyclonal Ab (R&D Systems) and anti-mIL-33 polyclonal Ab (R&D Systems), as detection Abs, were conjugated with DNA. Briefly, the detection Abs (15 μg) were incubated with 100 pmol biotinylated double-strand MUSTag DNA fragment (Synthera Technologies) and 100 pmol MUSTag adaptor protein in binding buffer (500 mM NaCl, 0.1 mM EDTA, and 10 mM Tris-HCl (pH 7.4)) at r.t. for 2 h. Then the mixture, in which the Abs are DNA conjugated via the MUSTag adaptor protein, was subjected to gel filtration chromatography on a Superdex 200 PC 3.2/30 column connected to a SMART System (GE Healthcare Bio-Science). The peak fractions were collected, and the yield of the DNA-conjugated Abs was determined by ELISA. The biotinylated double-strand MUSTag DNA fragment was designed as follows: 5′-[biotin]-CACTGCTTACTGGCTTATCGAAAT GGAATTCTGCATGCATCTAGAGGGCCCTATTCTATAGCATAGTG TCACCTAAATGCTAGGCACCTTCTAGTTGCCAGCCATCTGTTGC ACACCAAACGTGGCTTGCC-3′; the first box = *Eco*RI restriction site, the second box = MUSTag forward priming site, and the third box = MUSTag reverse priming site.

MUSTag assays were performed according to the manufacturer's instructions (Synthera Technologies). Briefly, Maxisorp Immunomodule 96-well plates (Nalge Nunc International) were coated with 50 μl/well anti-mIL-1β mAb (2 μg/ml; 30311; R&D Systems) or anti-human/mouse IL-33 mAb (3 μg/ml; Nessy-1; Alexis Biochemicals) in 50 mM sodium carbonate buffer (pH 9.6) at 4°C overnight. After blocking with a blocking buffer (Synthera Technologies) for 1 h at r.t., serially diluted standard rmIL-1β (R&D Systems) or rmIL-33 (PeproTech; 10 ng/ml to 0.64 pg/ml) in sample dilution buffer (Synthera Technologies) and samples (50 μl/well) were

incubated at r.t. for 1 h. After washing with wash buffer (Synthera Technologies), DNA-conjugated anti-mIL-1β or anti-mIL-33 polyclonal Ab in the dilution buffer (8 ng/ml, 30 μl/well) was added, followed by incubation at r.t. for 1 h. After washing, 7.5 U/ml *Eco*RI (30 μl/well; Nippon Gene) in digestion buffer was added, followed by incubation at r.t. for 15 min. A total of 3 μl of the solution in each well was subjected to real-time quantitative PCR (qPCR) analyses.

qPCR analyses were performed using the Mx3005P Real-Time PCR System (Agilent). The PCR mixtures consisted of 10 μl of 2× SYBR Premix EX *Taq* (Takara Bio), 100 nM MUSTag primers (Synthera Technologies), 0.4 μl of 50× 6-carboxy-X-rhodamine Reference Dye II (Takara Bio), 3 μl of each sample, and sterile distilled water added to a final volume of 20 μl. The temperature program for PCR was as follows: 1) 95°C for 10 s; 2) (95°C for 5 s) × 40 cycles; and 3) 60°C for 20 s. The fluorescence intensity was measured at the end of each cycle using excitation/emission filters of 492/516 nm for SYBR Green I and 585/610 nm for 6-carboxy-X-rhodamine (passive reference). The threshold cycle (Ct) values were calculated from amplification plots using MXPro qPCR software version 3.20 (Agilent) with adaptive baseline and amplification-based threshold algorithms, and further analyses were conducted with GraphPad Prism version 4.03 (GraphPad). Each standard was assayed in triplicate, and the mean Ct values with SD were plotted against the log of the concentration of each recombinant enzyme. The standard curves were fit to the following sigmoidal four-parameter logistic equation:

$$Ct = Ct_{min} + \frac{Ct_{max} - Ct_{min}}{1 + 10^{(LogEC_{50} - LogX) \times Hill\ Slope}} \quad (1)$$

where X is the enzyme concentration, Ct is the corresponding Ct value, Ct_{max} is the theoretical maximum Ct value (the minimum response), Ct_{min} is the theoretical minimum Ct value (the maximum response), EC_{50} is the concentration causing 50% of the maximum response, and *Hill Slope* is the slope of the linear portion of the sigmoidal curve. In all curve fittings, each Ct_{max} parameter was fixed to the mean Ct value of the blank concentration, and then Ct_{min}, EC_{50}, and *Hill Slope* were obtained by a nonlinear least-squares method. The concentrations of IL-33 and IL-1β in unknown samples were calculated from the respective standard curves.

Statistics

An unpaired Student's *t* test, two tailed, was used for statistical evaluation of the results.

Results

IL-33 mRNA is expressed in mouse macrophages, DCs, and BMCMCs

Macrophages, DCs, and mast cells express TLRs. These immune cells are considered to be important for host defense against various pathogens by activating innate immune responses via TLRs. TLRs are also important for the elicitation of acquired immune responses; for example, TLR signals contribute to, but are not essential for, the development of Th2-associated allergic responses (21). Thus, we used RT-PCR to examine IL-33 mRNA expression in those cells after LPS stimulation. Resting mouse F4/80$^+$ peritoneal macrophages, but not CD11c$^+$ splenic DCs or BMCMCs, constitutively expressed IL-33 mRNA (Fig. 1). After PMA plus ionomycin stimulation, IL-33 mRNA was increased in CD11c$^+$ splenic DCs and BMCMCs, but not in F4/80$^+$ peritoneal macrophages (Fig. 1). After LPS stimulation, IL-33 mRNA was up-regulated in F4/80$^+$ peritoneal macrophages and CD11c$^+$ splenic DCs, but not in BMCMCs (Fig. 1). However, BMCMCs expressed IL-33 mRNA after addition of monomeric IgE (22) and rmIL-33, and also after IgE/Ag stimulation (Fig. 2). IL-33 mRNA was not observed to be expressed by CD4$^+$ T cells under any conditions (Fig. 1).

IL-33 protein is produced by mouse macrophages, but not by DCs

In contrast to IL-33 mRNA expression, IL-33 protein levels were always below the limit of detection by ELISA in the culture supernatants of F4/80$^+$ peritoneal macrophages and CD11c$^+$ splenic DCs after stimulation with LPS or PMA plus ionomycin (Fig. 3),

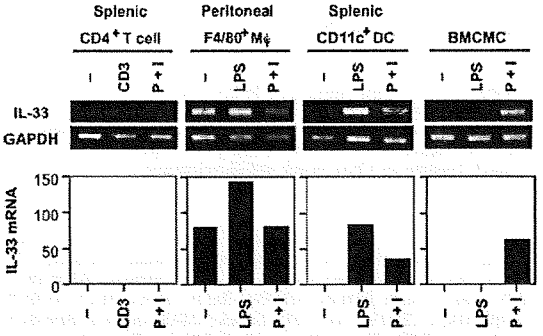

FIGURE 1. IL-33 mRNA expression in macrophages, DCs, and BMCMCs after LPS stimulation. Mouse splenic CD4$^+$ T cells, CD11c$^+$ DCs, peritoneal F4/80$^+$ macrophages (Mϕ), and BMCMCs were stimulated for 1 h with plate-coated anti-CD3 mAb (1 μg/ml), 100 ng/ml LPS, or 0.1 μg/ml PMA plus 1 μg/ml ionomycin. –, Indicates no stimulation. IL-33 mRNA expression was determined by RT-PCR. GAPDH mRNA expression was used as an internal control. Densitometric analysis was performed using NIH Image software, and IL-33 mRNA expression was normalized against GAPDH mRNA expression (the value of IL-33 mRNA expression/the value of GAPDH mRNA expression × 100). The data show representative results from at least two to three independent experiments. Semiquantitative PCR analysis was performed using the serially diluted cDNA templates during the exponential phase of PCR amplification.

even though IL-6 production was detected in the same supernatants (data not shown). However, IL-33 protein was detected in whole-cell lysates of F4/80$^+$ peritoneal macrophages, but not of CD11c$^+$ splenic DCs, after LPS and PMA plus ionomycin stimulation (Fig. 3). To detect released IL-33 protein, we next established an IL-33-specific MUSTag assay system (limit of detection:

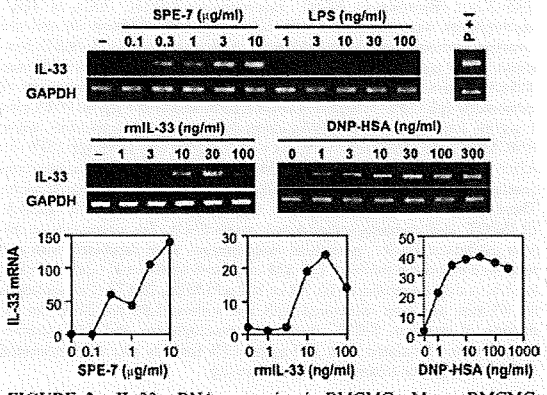

FIGURE 2. IL-33 mRNA expression in BMCMCs. Mouse BMCMCs were stimulated for 1 h with various concentrations of monomeric IgE (SPE-7), LPS, or rmIL-33 or 0.1 μg/ml PMA plus 1 μg/ml ionomycin. –, Indicates no stimulation. Mouse BMCMCs were sensitized overnight with 1 μg/ml anti-DNP IgE (SPE-7). After washing, IgE-sensitized BMCMCs were cultured for 1 h with various concentrations of DNP-HSA. IL-33 mRNA expression was determined by RT-PCR. GAPDH mRNA expression was used as an internal control. Densitometric analysis was performed using NIH Image software, and IL-33 mRNA expression was normalized against GAPDH mRNA expression (the value of IL-33 mRNA expression/the value of GAPDH mRNA expression × 100). The data show representative results from at least two to three independent experiments. Semiquantitative PCR analysis was performed using the serially diluted cDNA templates during the exponential phase of PCR amplification.

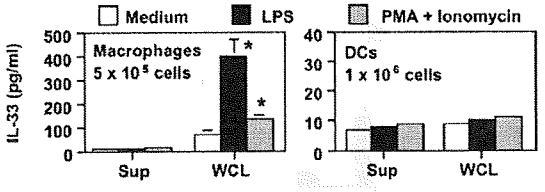

FIGURE 3. IL-33 production by LPS-stimulated macrophages. Mouse peritoneal F4/80$^+$ macrophages (5×10^5 cells/100 μl) and splenic CD11c$^+$ DCs (1×10^6 cells/100 μl) were stimulated for 6 h with 100 ng/ml LPS or 0.1 μg/ml PMA plus 1 μg/ml ionomycin. IL-33 levels in the culture supernatants (Sup) and whole-cell lysates (WCL; 5×10^5 macrophages or 1×10^6 DCs in 100 μl of lysis buffer) were determined by ELISA. Data show the mean + SEM ($n = 3$). *, $p < 0.05$ vs medium.

>0.64 pg/ml rmIL-33), which is much more sensitive than general ELISA (limit of detection: >30 pg/ml rmIL-33). Nevertheless, as shown in Fig. 3, any IL-33 that was present in the culture supernatants of LPS- or PMA plus ionomycin-stimulated F4/80$^+$ peritoneal macrophages was still below the limit of detection even with the MUSTag system, whereas IL-1β was able to be detected (data not shown).

Because IL-33 is localized in the nucleus as an intracellular NF (19), it has been unclear whether pro-IL-33 is cleaved and/or released. We next performed Western blot analysis for detection of processed-form IL-33. As shown in Fig. 4*A*, >1.2 ng of rmIL-33 was detectable with our Western blot system. However, both pro-IL-33 (32 kDa) and processed-form IL-33 (18 kDa) in whole-cell lysates or culture supernatants of LPS-stimulated F4/80$^+$ peritoneal macrophages (1×10^7 cells purified from pooled PECs from 20 mice) were below the limit of detection with Western blot analysis even after immunoprecipitation using anti-IL-33 Ab (data not shown). It has been shown that IL-33 mRNA is constitutively expressed in the lungs of mice (1). In support of this, pro-IL-33 (a 31-kDa protein), but not processed-form IL-33 (18 kDa), was detected in whole-lung homogenates from naive wild-type mice by the Western blot analysis (Fig. 4*B*). Pro-IL-33 expression was increased in whole-lung homogenates from LPS-inhaled wild-type mice in comparison with saline-inhaled wild-type mice by Western blot analysis, but processed-form IL-33 expression could not be detected in those homogenates by Western blot analysis irrespective of LPS treatment (Fig. 4*B*) or even by the Western blot analysis after immunoprecipitation using anti-IL-33 Ab (data not shown). Likewise, even though pro-IL-33 (32-kDa) expression was increased in whole-cell lysates, but not culture supernatants, of TGC macrophages after stimulation with LPS, processed-form IL-33 was below the limit of detection in both samples by Western blot analysis after immunoprecipitation using anti-IL-33 Ab (Fig. 4*C*). In contrast, released-form IL-1β could be detected under similar cell culture conditions by Western blot analysis after immunoprecipitation using anti-IL-1β Ab (Fig. 4*D*). However, in comparison with the amount of pro-IL-1β in whole-cell lysates of LPS-treated TGC-macrophages, the amount of released-form IL-1β in the culture supernatants was very small (Fig. 4*D*). These observations suggest that detection of low levels of processed-form IL-33, like released-form IL-1β, was difficult with our Western blot system.

Therefore, we established an IL-33-specific ELISPOT system to detect IL-33-releasing cells. Consistent with the IL-33 levels detected in whole-cell lysates by ELISA, ELISPOT analysis found

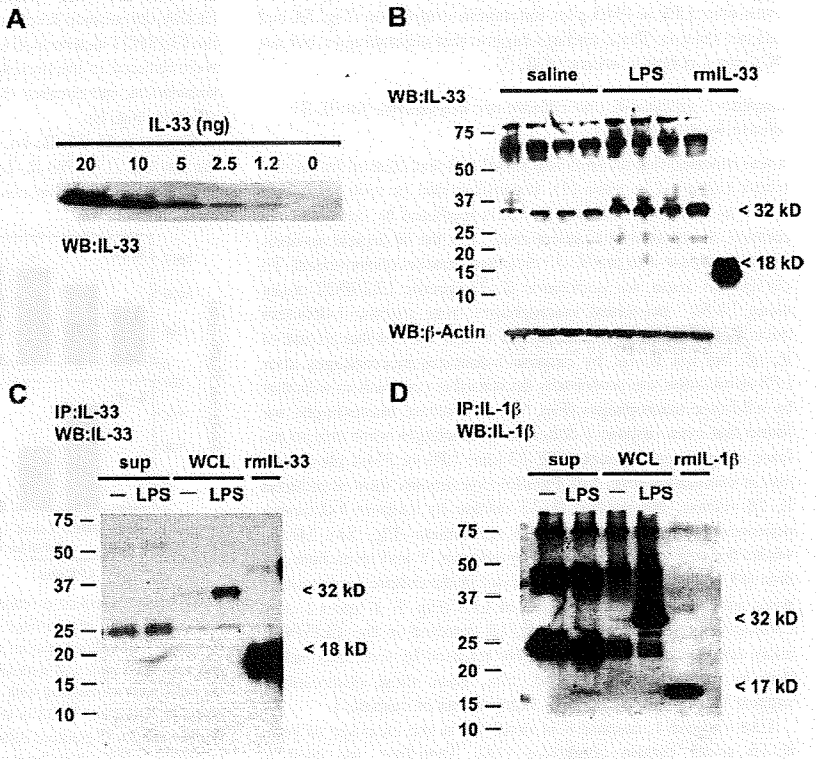

FIGURE 4. Detection of IL-33 by Western blot analysis. Western blot analysis for IL-33 detection was performed by loading various concentrations of rmIL-33 (*A*) and whole-lung lysates from LPS- or saline-inhaled wild-type mice (*B*) onto Western blot gels. A total of 20 ng of rmIL-33 was used as a control (*B*). Immunoprecipitants of culture supernatants and lysates from LPS-treated and untreated TGC-macrophages, 200 ng of rmIL-33, or 20 ng of rmIL-1β with anti-mIL-33 Ab or anti-mIL-1β Ab were analyzed by Western blotting for IL-33 or IL-1β detection (*C* and *D*). Pro-IL-33 = 32 kDa, and rIL-33 = 18 kDa. Pro-IL-1β = 31 kDa, and released-form IL-1β = 17 kDa.

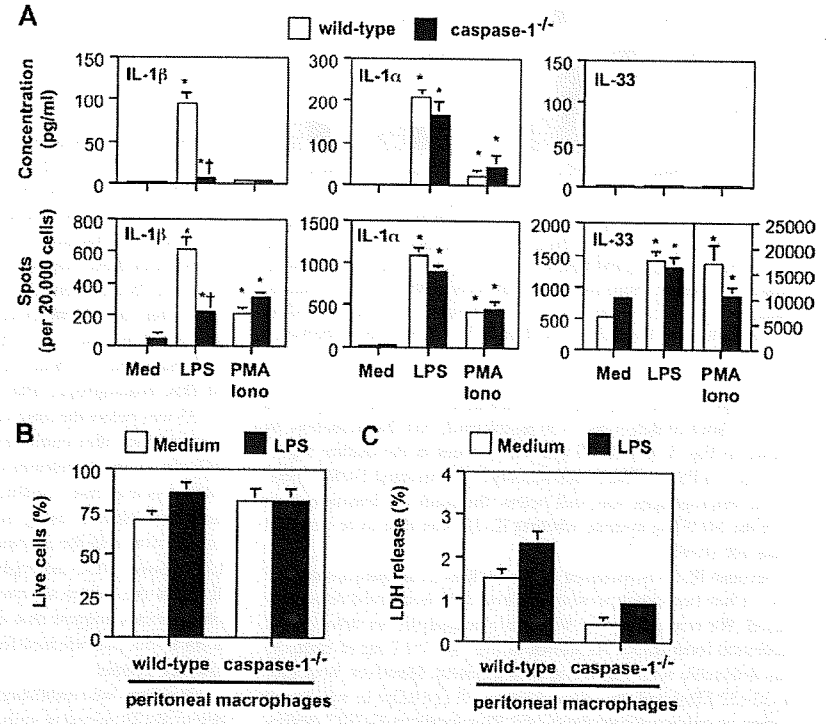

FIGURE 5. Caspase-1-independent IL-33 release by macrophages. Peritoneal F4/80$^+$ macrophages (2×10^4 cells/200 μl) from BALB/c wild-type mice or BALB/c-caspase-1-deficient mice were plated into an anti-mouse IL-1α, IL-1β, or IL-33 Ab-coated plate, and then stimulated with and without 100 ng/ml LPS or 0.1 μg/ml PMA plus 1 μg/ml ionomycin for 24 h. *A*, The levels of IL-1α, IL-1β, and IL-33 in the culture supernatants and the numbers of IL-1α-, IL-1β-, and IL-33-positive cells were determined by ELISA and ELISPOT, respectively. Data show the mean + SEM (ELISA, $n = 3$; ELISPOT, $n = 4$). *, $p < 0.05$ vs medium; †, $p < 0.05$ vs wild-type cells. *B*, The numbers of live cells in the cultures of *A* were determined by flow cytometry after staining with annexin V and PI. *C*, LDH levels in the culture supernatants shown in *A* were determined. Data show the mean + SEM ($n = 3$).

that F4/80$^+$ peritoneal macrophages and TGC-induced macrophages, but not CD11c$^+$ splenic DCs, released IL-33 at significant levels after LPS or PMA plus ionomycin stimulation (Fig. 5*A* and data not shown). These findings suggest that macrophages, but not DCs, are a potential source of IL-33.

Caspase-1, caspase-8, and calpain are dispensable for IL-33 release by macrophages

It was reported that recombinant pro-IL-33 was cleaved by recombinant caspase-1 in vitro (1), suggesting that in vivo release of IL-33, as well as IL-1β and IL-18, is mediated by caspase-1. In contrast, as reported by Carriere et al. (19), the biological involvement of caspase-1 in pro-IL-33 cleavage remains controversial. To clarify this issue, we performed IL-33-specific ELISPOT assay using F4/80$^+$ peritoneal macrophages from caspase-1-deficient mice. Both the ELISA and ELISPOT assays showed that release of IL-1β, but not IL-1α, by caspase-1-deficient macrophages after LPS stimulation was profoundly decreased in comparison with that by wild-type macrophages (Fig. 5*A*). In contrast, interestingly, we found that even caspase-1-deficient macrophages were able to release a significant amount of IL-33 after LPS or PMA plus ionomycin stimulation (Fig. 5*A*). Parallel experiments showed reduced IL-1β release, but normal IL-33 release by caspase-1-deficient peritoneal macrophages after LPS stimulation (Fig. 5*A*). Meanwhile, the cell viability assessed by annexin V and propidium iodide (PI) staining and the LDH activity in the culture supernatants were not affected by the addition of LPS (Fig. 5, *B* and *C*). Accordingly, both the IL-33 and IL-1β detected in the ELISPOT assay appeared to have been largely released by live cells rather than by apoptotic/necrotic/dead cells. Thus, these observations suggest that release of IL-33 is independent of caspase-1.

Like caspase-1, caspase-8 is involved in the cleavage of pro-IL-1β under certain conditions (18). Therefore, we used a caspase-8 inhibitor to examine whether caspase-8 is involved in IL-33 release by LPS-stimulated macrophages. Although both the ELISA-determined levels of IL-1β in the culture supernatants and the ELISPOT-determined number of IL-1β-releasing macrophages were reduced in the presence of the caspase-8 inhibitor after LPS stimulation, the number of IL-33-releasing macrophages was not affected (Fig. 6).

Similar to IL-33, IL-1α, but not IL-1β or IL-18, is localized in the nucleus (17). Pro-IL-1α is cleaved by calcium-dependent activation of a membrane-associated cysteine proteinase, calpain (16,

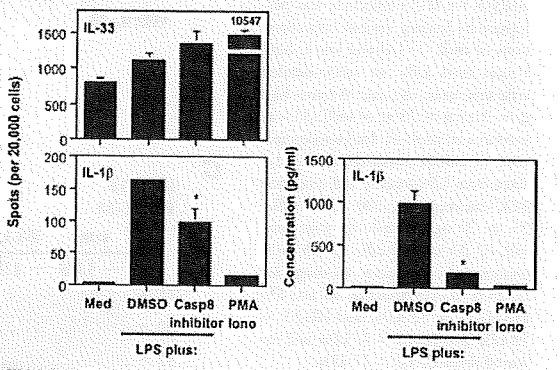

FIGURE 6. Caspase-8-independent IL-33 release by macrophages. Peritoneal F4/80$^+$ macrophages (2×10^4 cells/200 μl) from BALB/c wild-type mice were plated into an anti-mouse IL-1β or IL-33 Ab-coated plate and incubated with and without 30 μM caspase-8 inhibitor for 1 h. Then the cells were stimulated with and without 100 ng/ml LPS for 24 h. The levels of IL-1β and IL-33 in the culture supernatants and the numbers of IL-1β- and IL-33-positive cells were detected by ELISA and ELISPOT, respectively. Data show the mean + SEM ($n = 3$). *, $p < 0.05$ vs DMSO.

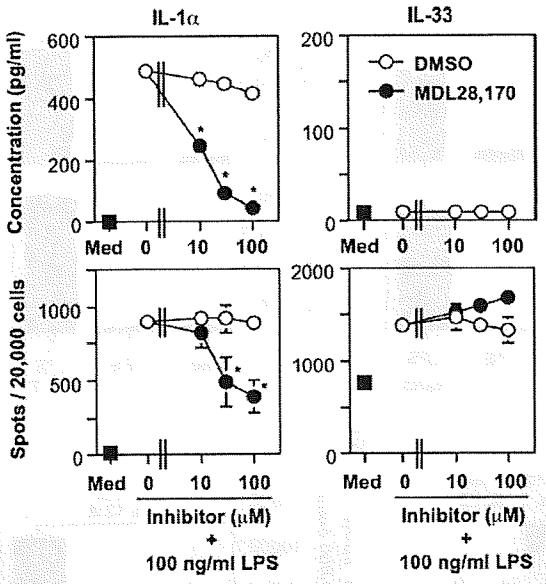

FIGURE 7. Calpain-independent IL-33 release by macrophages. Peritoneal F4/80$^+$ macrophages (2×10^4 cells/200 μl) from BALB/c wild-type mice were plated into an anti-mouse IL-1α or IL-33 Ab-coated plate and incubated with and without 10 μM calpain inhibitor, MDL 28,170. Then the cells were stimulated with and without 100 ng/ml LPS for 24 h. The levels of IL-1α and IL-33 in the culture supernatants and the numbers of IL-1α- or IL-33-positive cells were detected by ELISA and ELISPOT, respectively. Data show the mean + SEM (ELISA, $n = 3$; ELISPOT, $n = 4$). *, $p < 0.05$ vs DMSO.

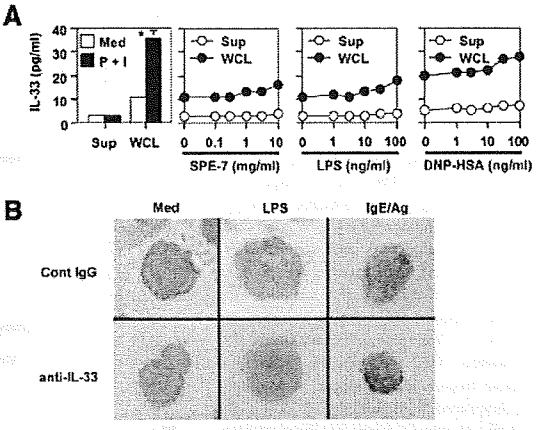

FIGURE 8. IL-33 protein production by mast cells. *A*, Naive BMCMCs (1×10^6 cells/100 μl) were stimulated for 6 h with various concentrations of monomeric IgE (SPE-7) and LPS or with 0.1 μg/ml PMA plus 1 μg/ml ionomycin, whereas IgE-sensitized BMCMCs (1×10^6 cells/100 μl) were stimulated with various concentrations of DNP-HSA. IL-33 levels in the culture supernatants (Sup) and whole-cell lysates (WCL; 1×10^5 BMCMCs in 100 μl of lysis buffer) were determined by ELISA. Data show the mean + SEM ($n = 3$ different batches of BMCMCs). *, $p < 0.05$ vs medium. *B*, BMCMCs were stimulated with and without LPS and IgE/Ag, as described above. IL-33-positive cells were detected by immunohistochemistry. Data show a representative result from three different batches of BMCMCs.

17). Indeed, in the presence of calpain inhibitors, the levels of IL-1α in the culture supernatants and the number of IL-1α-releasing macrophages were reduced in ELISA and ELISPOT, respectively, after LPS stimulation (Fig. 7). In contrast, the number of IL-33-releasing macrophages detected by ELISPOT was not influenced by the addition of calpain inhibitors to the culture (Fig. 7). Taken together, these observations indicate that neither caspase-1, caspase-8, nor calpain is essential for IL-33 release by macrophages and suggest that the mechanism of cleavage of pro-IL-33 is different from the cleavage mechanisms for pro-IL-1α, pro-IL-1β, and pro-IL-18.

IL-33 production is induced in BMCMCs after IgE/Ag stimulation

As shown in Figs. 1 and 2, the expression of IL-33 mRNA was up-regulated in BMCMCs upon stimulation with monomeric IgE, rmIL-33, or PMA plus ionomycin, but not LPS, and after IgE/Ag-FcεR cross-linking. However, IL-33 protein in the culture supernatants was below the limit of detection by ELISA under all conditions, whereas IL-6 in the supernatants was detectable in the same settings (data not shown). In contrast, with ELISA, IL-33 protein was detectable and increased in the whole-cell lysates of BMCMCs after PMA plus ionomycin stimulation (Fig. 8*A*). Therefore, we surmised that mast cells were producing IL-33. However, IL-33 was not dramatically increased even in the whole-cell lysates of BMCMCs after stimulation with monomeric IgE, LPS, or IgE/Ag (Fig. 8*B*). IL-33 release could not be detected by ELISPOT even after PMA plus ionomycin stimulation (data not shown). However, IL-33 protein was detected around the nuclear membrane of BMCMCs by immunohistochemistry after stimulation with IgE/Ag, but not LPS (Fig. 8*B*).

Discussion

IL-33 can promote Th2 cell and mast cell activation, suggesting that it contributes to the development of such Th2 cytokine- and mast cell-associated disorders as allergic diseases (1, 7–11). Indeed, administration of rIL-33 results in dramatic pathological changes in mice, as follows: splenomegaly, elevated numbers of blood eosinophils, increased serum IgE and IgA levels, and IL-5 and IL-13 levels, and eosinophil-dominant inflammation in the gut and lung (1). Thus, it is clear that IL-33, like IL-1β and IL-18, acts as a proinflammatory cytokine in immune responses. In contrast, the precise immune cell source of IL-33 remains poorly understood, although IL-33 mRNA was increased in the lungs of mice in an OVA-induced asthma model (13).

Schmitz et al. (1) demonstrated that LPS-stimulated bone marrow-derived macrophages and resting bone marrow-derived DCs expressed IL-33 mRNA in mice. We have now demonstrated that IL-33 mRNA was constitutively expressed in resting mouse F4/80$^+$ peritoneal macrophages and up-regulated in those cells after LPS or PMA plus ionomycin stimulation. In contrast to the resting bone marrow-derived DCs reported by Schmitz et al. (1), CD11c$^+$ DCs freshly isolated from mouse spleen did not express IL-33 mRNA. However, as in the case of F4/80$^+$ peritoneal macrophages, IL-33 mRNA expression was strongly induced in CD11c$^+$ splenic DCs by LPS or PMA plus ionomycin stimulation. We also found that mouse BMCMCs expressed IL-33 mRNA upon stimulation with monomeric IgE and rmIL-33, but not LPS, and after IgE/Ag cross-linking. Thus, these observations suggest that macrophages, rather than DCs or mast cells, may be a producer of IL-33 during bacterial infections.

Schmitz et al. (1) reported that recombinant pro-IL-33 was cleaved by recombinant caspase-1 in vitro. In contrast, Carriere et al.

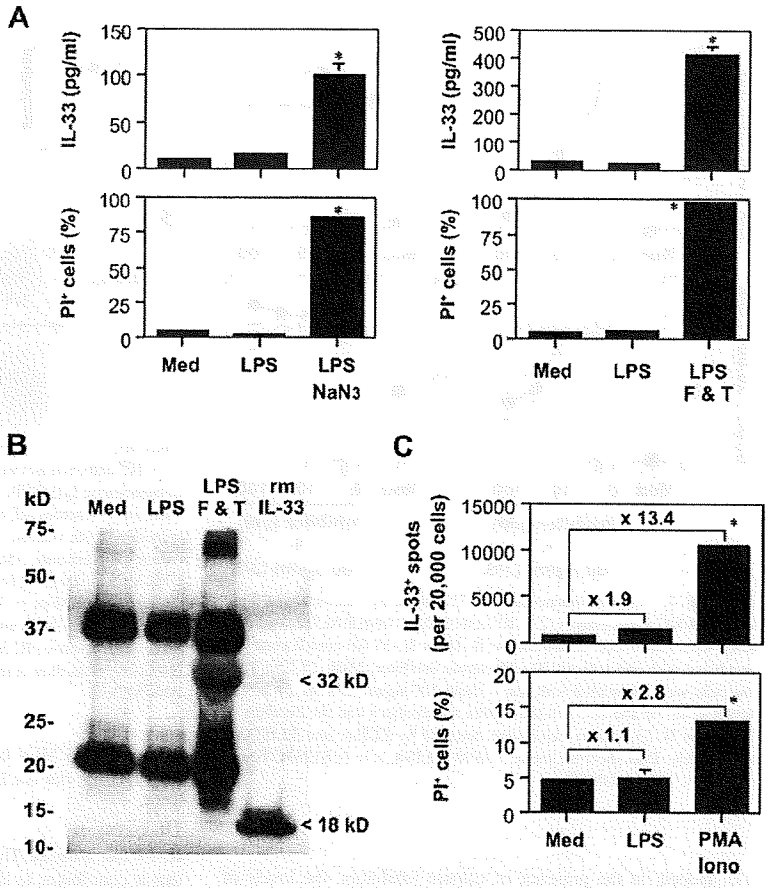

FIGURE 9. Necrotic cells release pro-IL-33. *A*, Peritoneal F4/80$^+$ macrophages (5×10^5 cells/200 μl) from BALB/c wild-type mice were stimulated for 6 h with LPS. Cells were then treated with and without NaN_3 (*left panel*) for 1 h or treated by freeze thawing (F & T; *right panel*). IL-33 levels in the culture supernatants were determined by ELISA. *B*, Culture supernatants from LPS-stimulated TGC-macrophages, with and without freeze-thaw treatment, were immunoprecipitated with anti-mouse IL-33 Ab, and rIL-33 was analyzed by Western blotting for IL-33 detection. Pro-IL-33 = 32 kDa, and rIL-33 = 18 kDa. *C*, Peritoneal F4/80$^+$ macrophages (2×10^4 cells/200 μl) from BALB/c wild-type mice were plated into an anti-mouse IL-33 Ab-coated plate and then stimulated with and without 100 ng/ml LPS or 0.1 μg/ml PMA plus 1 μg/ml ionomycin (PMA + Iono) for 6 h. *A* and *C*, The proportion of necrotic cells was determined by flow cytometry after staining with annexin V and PI. Data show the mean + SEM ($n = 3$). *, $p < 0.05$ vs medium.

(19) noted that biological involvement of caspase-1 in pro-IL-33 cleavage remains controversial. Pro-IL-33 was recently demonstrated to show biological activity in inducing mast cell activation (23), but cleavage of pro-IL-33 by caspase-1 in vitro resulted in loss of that biological activity (24, 25). In addition, it was very recently reported that pro-IL-33 was released by necrotic cells independent of caspase-1 (25). We also confirmed that IL-33 was detected in the culture supernatants of LPS-treated F4/80$^+$ peritoneal macrophages in the presence, but not absence, of NaN_3, and also in the supernatants of LPS-treated F4/80$^+$ peritoneal macrophages after freeze thawing (Fig. 9*A*). Such treatments induced profound PI-positive necrosis (Fig. 9*A*). In addition, pro-IL-33, but not a protease-cleaved form of IL-33, was detected in the culture supernatants from that setting (Fig. 9*B*). The number of IL-33-positive F4/80$^+$ peritoneal macrophages was dramatically increased after PMA plus ionomycin stimulation (13.4-fold increase vs the control condition, medium alone) in comparison with LPS stimulation (1.9-fold increase vs the control condition, medium alone) by ELISPOT (Fig. 9*C*). Compared with this, the proportion of PI-positive necrotic macrophages was slightly increased after PMA plus ionomycin stimulation (2.8-fold increase vs the control condition, medium alone; Fig. 9*C*). Although we cannot rule out the possibility that pro-IL-33 is released only by necrotic cells, our observations suggest that it is released by live cells in addition to necrotic cells.

In our present study, we also clearly demonstrated for the first time that neither caspase-1, caspase-8, nor calpain is essential for IL-33 release by F4/80$^+$ peritoneal macrophages after LPS stimulation. That is, whereas IL-1β release or IL-1α release was significantly impaired in caspase-1-deficient, caspase-8 inhibitor-treated, or calpain inhibitor-treated macrophages after stimulation, respectively, IL-33 release was unaffected by these treatments. It is also known that caspase-1-deficient mice show significantly reduced IL-1β and IL-18 release, but they still release significant amounts of IL-1β and IL-18 (18, 26), suggesting a caspase-1-independent alternative pathway(s) for IL-1β and IL-18 release. In support of this notion, pro-IL-18 is known to be cleaved predominantly by chymase, which is a proteinase expressed in mast cells, but not macrophages (27). These observations also suggest the existence of an alternative mechanism(s), independent of caspase-1, caspase-8, and calpain, for IL-33 release.

We also demonstrated that BMCMCs are a potential producer of IL-33 protein. In contrast to IL-33 release by F4/80$^+$ peritoneal macrophages, IL-33 release by BMCMCs was hardly detected after stimulation with monomeric IgE, LPS, and IgE/Ag. However, the IL-33 protein was detected in whole-cell lysates of BMCMCs by ELISA after PMA plus ionomycin stimulation and around the nuclear membrane of BMCMCs by immunohistochemistry after IgE/Ag cross-linking. Therefore, we cannot rule out the possibility that mast cells release a very small amount of IL-33 protein, which might be below the limits of detection with ELISPOT and ELISA.

In the present study, we demonstrated that macrophages and mast cells are potential producers of IL-33, and that neither caspase-1, caspase-8, nor calpain is essential for the release of

IL-33. These observations imply that caspase-1- and calpain-independent IL-33 production by macrophages and/or mast cells may contribute to the pathogenesis of certain infections and/or Th2-type allergic inflammation.

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Disclosures

The authors have no financial conflict of interest.

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Cupressaceae Pollen Grains Modulate Dendritic Cell Response and Exhibit IgE-Inducing Adjuvant Activity In Vivo[1]

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Pollen is considered a source of not only allergens but also immunomodulatory substances, which could play crucial roles in sensitization and/or the exacerbation of allergies. We investigated how allergenic pollens from different plant species (Japanese cedar and Japanese cypress, which belong to the Cupressaceae family, and birch, ragweed, and grass) modulate murine bone marrow-derived dendritic cell (DC) responses and examined the effect of Cupressaceae pollen in vivo using mice. DCs were stimulated with pollen extracts or grains in the presence or absence of LPS. Cell maturation and cytokine production in DCs were analyzed by flow cytometry, ELISA, and/or quantitative PCR. Pollen extracts suppressed LPS-induced IL-12 production and the effect was greatest for birch and grass. Without LPS, pollen grains induced DC maturation and cytokine production without IL-12 secretion and the response, for which TLR 4 was dispensable, was greatest for the Cupressaceae family. Intranasal administration of Cupressaceae pollen in mice induced an elevation of serum IgE levels and airway eosinophil infiltration. Co-administration of ovalbumin with Cupressaceae pollen grains induced ovalbumin-specific IgE responses associated with eosinophil infiltration. The results suggest that modulation of DC responses by pollen differs among the plant families via (1) the promotion of DC maturation and cytokine production by direct contact and/or (2) the inhibition of IL-12 production by soluble factors. The strong DC stimulatory activity in vitro and IgE-inducing activity in mice support the clinical relevance of Cupressaceae pollen to allergies in humans. *The Journal of Immunology*, **2009, 183: 6087–6094.**

Pollen is an important trigger of seasonal rhinitis, conjunctivitis, and/or asthma and an exacerbating factor in atopic dermatitis (1–4). Pollen grains of trees of the Cupressaceae family including the Taxodiaceae, such as Japanese cedar (*Cryptomeria japonica*), Japanese cypress (*Chamaecyparis obtuse*), *Cupressus* species, and *Juniperus* species, are relevant sources of allergens (2, 3, 5). In Europe and North America, birch of *Betula* species is the most important allergenic tree (5). Pollen grains of ragweed of *Ambrosia* species (6) and grasses of the Poaceae family, such as timothy (*Phleum pratense*), rye (*Lolium* spp.), Kentucky blue grass (*Poa pratensis*), orchard grass (*Dactylis glomerata*), Bermuda grass (*Cynodon dactylon*), and others (7), are also among the most clinically relevant sources of allergens.

In addition to the function of pollen grains as carriers of allergen proteins, pollen-derived substances could exhibit immunomodulatory effects. Ragweed pollen-derived NADPH oxidase (8–10) increases levels of reactive oxygen species in the epithelium and has critical roles in both sensitization to and the development of allergies in mouse models (8, 9). Very recently, we have demonstrated that allergenic pollen grains showed NADPH oxidase activity that differed in intensity and localization according to the plant families and that the activity was mostly concentrated within insoluble fractions (11). Pollen grains release substances that have structural similarity with the inflammatory lipid mediators (12, 13). Lipid fractions from birch and grass pollen extracts induce chemotaxis and the activation of neutrophils (14) and eosinophils (15). Pollen extracts of birch, hazel, lilac, maple, and mugwort and birch pollen phytoprostanes inhibit LPS-induced IL-12 production (16) and birch pollen extract, and phytoprostanes enhance migratory and Th2-attracting capacities (17) in dendritic cells (DCs).[3] CD1-restricted T cells and IgE in blood samples obtained from allergic subjects during the pollinating season have been reported to recognize cypress pollen-derived phospholipids (18). We have demonstrated that pollen grains of members of the Cupressaceae family, and birch, ragweed, and grass, release proteases (13, 19) that might be involved in the pathogenesis of allergic diseases, similar to house dust mite-derived and other protease allergens (20–29). Pollen extracts of grass, birch, giant ragweed, and Easter lily degrade tight junctions, and grass pollen extract does so in a protease-dependent manner (30). However, how these innate responses to allergenic pollen differ among plant species is unclear.

DCs are crucial for the initiation and maintenance of T cell-mediated adaptive immune responses (31, 32). Immature DCs, which reside in peripheral tissues, take up pathogens or allergens or are exposed to the milieu of proinflammatory cytokines provided by accessory cells, leading to the induction of DC maturation, which is characterized by the cell-surface expression of co-stimulatory and MHC class II molecules. Having matured, DCs migrate to lymph nodes, where they activate T cells. The type of

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[3] Abbreviations used in this paper: DC, dendritic cell; BAL, bronchoalveolar lavage; MFI, mean fluorescence intensity; OVA, ovalbumin; rmGM-CSF, recombinant mouse GM-CSF.

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pathogen or proinflammatory cytokine milieu determines the phenotype of the mature DCs, which in turn determine the Th phenotype that naive Th cells adopt. DC-derived IL-12 is a crucial Th1-polarizing cytokine.

Inhalation of house dust mite extract (33) or fungal culture extract (34) with ovalbumin (OVA) induces production of IgE against OVA in mice. In mice sensitized i.p. with ragweed pollen extract plus alum, intranasal challenge with extract that includes ragweed pollen NADPH oxidase induces more specific IgE than does challenge with that without this enzyme (8). However, as far as we know, in vivo IgE-inducing adjuvant activity of inhaled pollen-derived substances without the use of alum has not been reported. Although one paper (35) described that the intranasal coadministration of birch pollen extract with OVA in OVA-specific TCR-transgenic mice resulted in Th2-skewed cytokine production in restimulated OVA-specific T cells in vitro, IgE production in vivo was not described.

In the present study, we compared how pollen grains and extracts of different plant species (Japanese cedar and Japanese cypress, which belong to the Cupressaceae family, and birch, ragweed, and grass) affect cell maturation and cytokine production in murine bone marrow-derived DCs in vitro. Additionally, we examined the activity of Cupressaceae pollen grains administered intranasally to induce elevated serum IgE levels and airway eosinophil infiltration and also its adjuvant activity to induce an IgE response specific to a coadministered protein in vivo using mice.

Materials and Methods

Mice

Seven- to 10-wk-old female C57BL/6 and BALB/c mice purchased from Charles River Japan and TLR4-deficient mice (C57BL/6 background), which were a gift from S. Akira (Osaka University, Osaka, Japan), (36) were maintained in a specific pathogen-free animal facility at Juntendo University and used in accordance with the guidelines of the institutional committee on animal experiments.

Pollens

Pollen grains of Japanese cedar (*Cryptomeria japonica*) were purchased from Wako Pure Chemical or generously provided by Torii Pharmaceutical. Pollen grains of Japanese cypress (*Chamaecyparis obtusa*) were purchased from Wako Pure Chemical. Pollen grains of white birch (*Betula alba*), water birch (*Betula fontinalis occidentalis*), and Kentucky bluegrass (*Poa pratensis*) were purchased from Sigma-Aldrich. Pollen grains of short ragweed (*Ambrosia artemisiifolia*) were purchased from Polysciences.

Preparation of pollen extracts

Pollen grains were suspended in Dulbecco's PBS without calcium and magnesium (pH 7.4) (100-mg grains/10 ml in 15-ml tubes). The suspension was rotated gently at 37°C for 1 h and centrifuged for 5 min at 490 × *g*. The supernatant was collected and filtered (0.22 μm). The filtered supernatants were stored at −80°C until used.

Measurement of endotoxin

Endotoxin contained in the pollen extracts and pollen grain suspensions was measured by using Endospecy (Seikagaku).

Generation and stimulation of DCs

C57BL/6 mouse bone marrow-derived DCs were generated as described (37). In brief, 2×10^6 bone marrow cells prepared from tibia and femur of mice were cultured in 10 ml of RPMI 1640 medium (Sigma-Aldrich) supplemented with 200 U/ml recombinant mouse GM-CSF (rmGM-CSF) (Wako), 2 mM L-glutamine, 10% (v/v) heat-inactivated FCS, 0.05 mM 2-ME, and antibiotics (day 0). At day 3, another 10 ml of medium containing rmGM-CSF was added. At days 6 and 8, half the medium was exchanged for fresh medium containing rmGM-CSF. At day 9, DCs suspended in fresh medium containing rmGM-CSF were plated onto 24-well culture plates (5×10^5 cells/400 μl/well). Finally, 100 μl of the suspension of LPS (Sigma-Aldrich or List Biological Lab), pollen extracts, or pollen grains was added to each of the wells.

Cell viability after the culture for 24 h with LPS, pollen extracts, or pollen grains, or without them was determined by the trypan blue exclusion test.

Flow cytometry

DCs stimulated for 24 h were collected and washed three times with PBS, then incubated with anti-mouse Fcγ receptor (CD16/CD32) mAb (2.4G2) (BD Biosciences) for 30 min at 4°C to avoid nonspecific binding of labeled mAbs. Cell-surface molecules were then stained by incubation of DCs with PE-conjugated anti-mouse CD11c (HL3) and FITC-conjugated anti-mouse I-A^b (AF6-120.1), CD80 (16-10A1) (BD Biosciences), or CD86 (GL1) (eBioscience) mAb for 20 min at 4°C. After being washed with PBS, the stained cells (live-gated on the basis of forward and side scatter profiles) were analyzed on a FACSCalibur (BD Biosciences), and data were processed using the CellQuest program (BD Biosciences).

Cytokine ELISA

After stimulation for 24 h, culture supernatants were recovered by centrifugation at 490 × *g* for 5 min. Cytokine concentrations were measured with ELISA kits (Ready-SET-Go (eBioscience) for IL-23 and Quantikine or DuoSet (R&D Systems) for other cytokines).

Quantitative PCR

After stimulation for 3 h, total RNA was extracted from DCs using an RNeasy Plus Micro Kit (Qiagen). First-strand cDNA was synthesized from total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time quantitative PCR was performed with the TaqMan method using an ABI 7500 (Applied Biosystems). The mRNA levels of the target gene were normalized to the gene expression of GAPDH and are shown as relative expression levels to the control group.

Intranasal administration of Cupressaceae family pollen grains to mice

C57BL/6 or BALB/c mice were lightly anesthetized with an i.p. injection of pentobarbital (Nembutal; Abbott Laboratories) and allowed to inhale 20 μl of the Japanese cedar or Japanese cypress pollen grain suspension with or without OVA (grade V; Sigma-Aldrich) following application to the nares with a pipette twice per week for 6 wk for a total of 13 administrations. The day after the last intranasal administration, sera and bronchoalveolar lavage (BAL) cells were collected.

Bronchoalveolar lavage

At 24 h after the last intranasal administration, mice were terminally anesthetized, the tracheas were cannulated, and internal airspaces were lavaged with 500 μl of PBS with 10% FCS, followed by another 500-μl wash. Fluids were centrifuged at 1200 × *g* and the pellets were recovered for cellular analysis. Specimens were prepared on glass slides by Cytospin 4 (Thermo Shandon) followed by Diff-Quick (Sysmex) staining. Differential cell counts were performed with a minimum of 200 cells.

ELISA for serum total IgE and OVA-specific Abs

Serum total IgE was measured by a sandwich ELISA as described previously (38). OVA-specific Abs were detected on plates coated with 1 mg/ml OVA and blocked with BlockAce (Snow Bland) and developed with HRP-conjugated Abs specific to the murine IgE and IgG subclasses as described previously (39, 40) with some modifications as follows. Sera and detection Abs were diluted with solutions 1 and 2 of CanGetSignal (Toyobo), respectively. Serum dilutions were 1/200, 1/40,000, 1/200, and 1/4,000 for detecting OVA-specific IgE, IgG1, IgG2a, and IgG2b, respectively. For detecting OVA-specific IgE, incubation with serum or a detection Ab was for 15 h at 4°C or for 5 h at room temperature, respectively. For detecting OVA-specific IgGs, incubation with serum or a detection Ab was for 1.5 h at 37°C.

Statistical analysis

A one-way ANOVA with the Tukey post hoc test and the Mann-Whitney *U* test (two-tailed) were used to analyze the data obtained in the in vitro and in vivo experiments, respectively. A value of $p < 0.05$ was regarded as statistically significant.

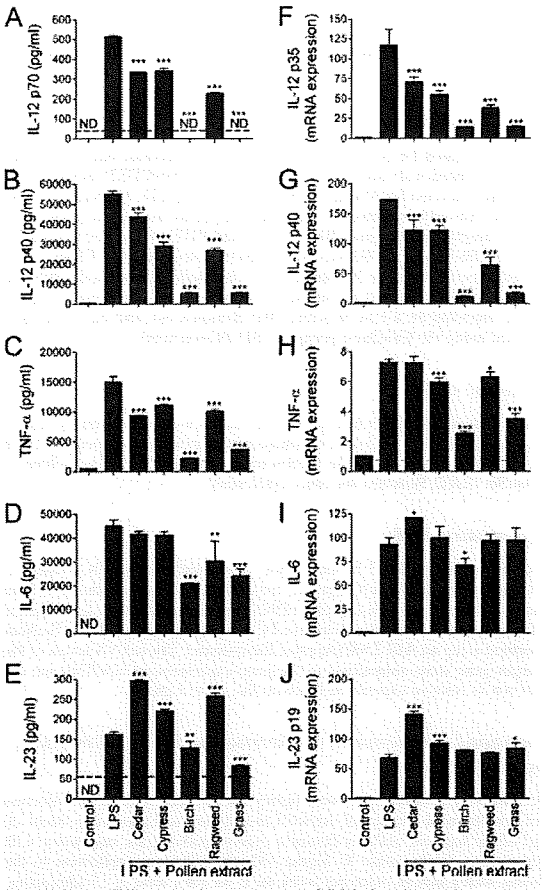

FIGURE 1. Effect of pollen extracts on LPS-induced cytokine expression in DCs. C57BL/6 mouse bone marrow-derived immature DCs were stimulated with LPS (Sigma-Aldrich) (100 ng/ml) in the absence or presence of pollen extracts. *A–E*, At 24 h, culture supernatants were collected and cytokine concentrations were measured by ELISA. *F–J*, At 3 h, DCs were collected and mRNA expression was examined by quantitative real-time PCR. ND, Not detected. Broken line inicates minimum detection limit. Data are indicated as the means ± SD for three wells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs cells stimulated with 100 ng/ml LPS alone by one-way ANOVA with the Tukey post hoc test. Data shown are representatives of three independent experiments with similar results.

Results

Pollen extracts modulated LPS-induced cytokine production by DCs

We examined the effect of extracts containing substances released from pollen on the response of DCs to LPS. LPS-induced cytokine production in DCs was analyzed (Fig. 1). The extracts of birch and grass pollen diminished the LPS-induced production of IL-12 (IL-12 p70), which is a heterodimer composed of the IL-12 p40 and IL-12 p35 subunits (Fig. 1*A*), and inhibited IL-12 p40 and TNF-α remarkably (Fig. 1, *B* and *C*) and IL-6 moderately (Fig. 1*D*). The pollen extracts other than those of birch and grass moderately inhibited the LPS-induced production of IL-12 p70, IL-12 p40, and TNF-α (Fig. 1, *A–C*). Pollen extracts exhibited little or no inhibition of the LPS-induced production of IL-23, a heterodimer composed of the IL-12 p40 and IL-23 p19 subunits (Fig. 1*E*). The results for protein levels (Fig. 1, *A–E*) were supported by the analyses at the mRNA level (Fig. 1, *F–J*). LPS-induced DC maturation in terms of the expression of CD80, CD86, and a MHC class II molecule ($I\text{-}A^b$) was not affected by the addition of pollen extracts (data not shown).

In the absence of LPS, the pollen extracts had no effect on the production of the cytokines examined, although they slightly enhanced the cell surface expression of a MHC class II molecule, and Japanese cedar or Japanese cypress pollen extracts marginally enhanced CD80 expression (data not shown). Endotoxin amounts released into the pollen extract samples were 26, 32, 2.3, 440, and 510 pg of endotoxin from 1 mg of pollen grains of Japanese cedar, Japanese cypress, birch, ragweed, and grass, respectively. Therefore, endotoxin concentrations in the DC stimulation, where the pollen extracts originally prepared at 10 mg of grains/ml were used at the dilution factor of 5 (2 mg of grains/ml), were 52, 64, 4.6, 870, and 1000 pg of endotoxin/ml, respectively.

The viabilities of DCs after the 24-h culture with LPS, pollen extracts, or both, or without them, were similarly ~95% (data not shown), indicating that the pollen extracts showed no toxicity at the concentrations tested and suggesting that the inhibitory effect of birch and grass pollen extracts (Fig. 1), particularly on the IL-12 p70 production, is not due to their cytotoxicity.

Thus, soluble substances released from pollen grains inhibit the LPS-induced production of IL-12 p70 in DCs with an efficiency that differs among plant species, and the effect was greatest for birch and grass pollen extracts. These substances themselves, at least at the concentration tested, have little or no activity to induce DC maturation or cytokine production.

Pollen grains promoted DC maturation in the absence of LPS

To mimic the contact of DCs with pollen grains in peripheral tissues during the initial sensitization process, we tested the effect of pollen on immature DCs in the absence of LPS (Figs. 2 and 3 and supplemental Fig. S1).[4] Pollen grains induced the cell-surface expression of CD80, CD86, and a MHC class II molecule (Fig. 2*A*). Pollen of Japanese cedar and Japanese cypress showed maturation-inducing activity at much lower doses than pollen of birch, ragweed, and grass (Fig. 2*B*).

The viabilities of DCs after the 24-h culture with or without the pollen grains were similarly ~95% with an exception of 2.5 mg of birch pollen grains/ml that showed a low viability (~25%) (data not shown), indicating that the pollen grains other than birch at the highest density and birch at lower densities (0.5 mg of grains/ml and 0.1 mg of grains/ml) showed no toxicity.

Pollen grains induced cytokine production in DCs in the absence of LPS

Next, we analyzed the production of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) and cytokines related to the differentiation of Th cells (IL-12 and IL-23) (Fig. 3 and supplemental Fig. S1). Pollen grains induced production of TNF-α, IL-6, and IL-1β (Fig. 3, *A–C*). Similar to the results of the analysis of DC maturation (Fig. 2), Cupressaceae family (Japanese cedar and Japanese cypress) pollen had a strong stimulatory effect at much lower doses than did the other pollen species tested (Fig. 3, *A–C*). Birch pollen grains induced a lesser response (Fig. 3 and supplemental Fig. S1). None of the pollen grains induced IL-12 p70 production (Fig. 3*D*), although all of the species induced production of IL-12 p40 (Fig. 3*E* and supplemental Fig. S1). Pollen grains induced production of IL-23 (Fig. 3*F*) in accordance with expression of the IL-12 p40 subunit (Fig. 3*E*).

[4] The online version of this article contains supplemental material.

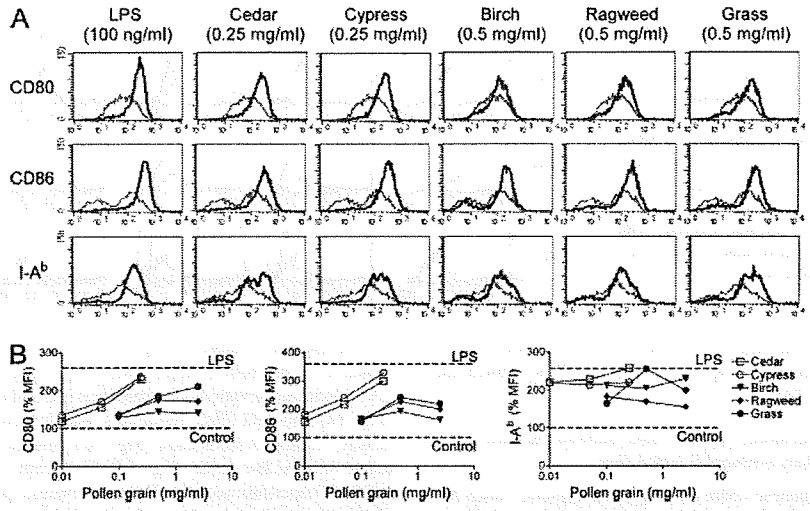

FIGURE 2. Effect of pollen grains on maturation of DCs. C57BL/6 mouse bone marrow-derived immature DCs were stimulated for 24 h in the presence of pollen grains. Cell-surface expression of costimulatory molecules (CD80 and CD86) and a MHC class II molecule ($I\text{-}A^b$) were analyzed by flow cytometry. *A*, Histograms of pollen grain- or LPS-stimulated cells (bold lines) are overlaid on histograms of control cells (fine lines). The histograms indicated are gated on the $CD11c^+$ population. *B*, Relative cell-surface expression indicated as a percentage of mean fluorescence intensity (MFI). Values were calculated as follows: (% MFI) = 100 × (MFI of stimulated cells/MFI of control cells without stimulation). LPS indicates cells stimulated with LPS (Sigma-Aldrich) (100 ng/ml); broken lines, values of negative (Control) and positive (LPS) controls. Data shown are representatives of three independent experiments with similar results.

Endotoxin contents in the pollen grains were 100, 260, 23, 1500, and 1100 pg of endotoxin/mg of pollen grains of Japanese cedar, Japanese cypress, birch, ragweed, and grass, respectively.

Pollen grains induced maturation and cytokine production in TLR4-deficient DCs

In TLR4-deficient DCs, pollen grains other than birch induced maturation (Fig. 4) and cytokine production but not IL-12 p70 production (Fig. 5), and LPS induced no response (Figs. 4 and 5, LPS). Pollen of Japanese cedar and Japanese cypress induced the similar levels of DC maturation (Fig. 4) and ~50–100% production of TNF-α, IL-12 p40, and IL-23 (Fig. 5, *A*, *E*, and *F*), and <50% but significant production of IL-6 and IL-1β (Fig. 5, *B* and *C*) compared with those in wild-type DCs (Figs. 2 and 3 and supplemental Fig. S1), indicating that most of the DC-maturating activity and the activity to induce TNF-α, IL-12 p40, and IL-23 and a part of the activity to induce IL-6 and IL-1β are not via the TLR4 pathway in Japanese cedar and Japanese cypress.

Pollen of ragweed and grass induced the similar levels of DC maturation (Fig. 4) and lower cytokine production (Fig. 5) compared with those in wild-type DCs (Figs. 2 and 3 and supplemental Fig. S1), indicating that the TLR4 pathway is dispensable for the DC maturation but partially contributes to the cytokine response in ragweed and grass. Endotoxin concentrations in the DC stimulation were 25 and 65 pg of endotoxin/ml for 0.25 mg of pollen grains/ml in Japanese cedar and Japanese cypress and 3800 and 2800 pg of endotoxin/ml for 2.5 mg of pollen grains/ml in ragweed and grass. The higher endotoxin concentrations in ragweed and grass at the tested pollen grain

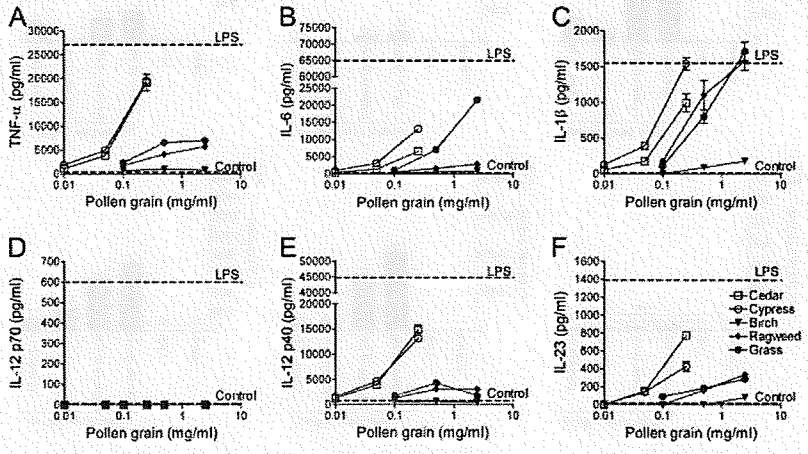

FIGURE 3. Effect of pollen grains on cytokine production in DCs. C57BL/6 mouse bone marrow-derived immature DCs were stimulated for 24 h in the presence of pollen grains. Cytokine concentrations in the culture supernatant were measured. LPS indicates cells stimulated with LPS (Sigma-Aldrich) (100 ng/ml); broken lines, values of positive (LPS) and negative (Control) controls. Data are indicated as the means ± SD for three wells. Data shown are representatives of three independent experiments with similar results. Results of statistical analyses are shown in supplemental Fig. S1.

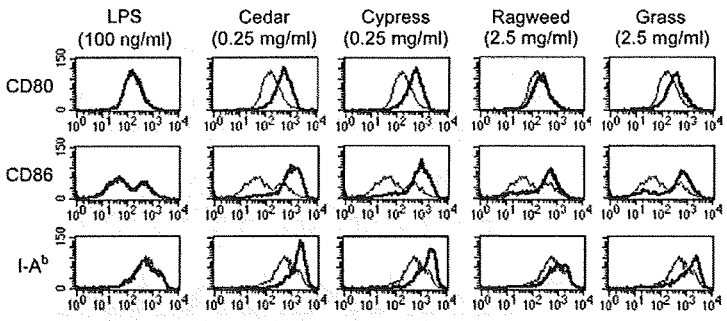

FIGURE 4. Effect of pollen grains on maturation of TLR4-deficient DCs. TLR4-deficient mouse bone marrow-derived immature DCs were stimulated for 24 h in the presence of pollen grains. Cell-surface expression of costimulatory molecules (CD80 and CD86) and a MHC class II molecule (I-A^b) were analyzed by flow cytometry. Histograms of pollen grain- or LPS -(List Biological Lab) -stimulated cells (bold lines) are overlaid on histograms of control cells (fine lines). The histograms indicated are gated on the CD11c$^+$ population.

dose could explain the partial contribution of the TLR4 pathway to the cytokine response in ragweed and grass (Figs. 3 and 5 and supplemental Fig. S1).

Inhalation of Cupressaceae pollen grains induced increases in serum IgE and airway eosinophil infiltration

Pollen grains of Japanese cedar and Japanese cypress, which belong to the Cupressaceae family, induced DC maturation (Fig. 2) and cytokine production (Fig. 3 and supplemental Fig. S1) more efficiently than did pollen of the other plant species tested. Next, we examined the effect of Cupressaceae pollen on IgE induction and airway eosinophil infiltration in vivo (Figs. 6 and 7).

In C57BL/6 mice, inhalation of Japanese cedar pollen grains resulted in an elevation of serum total IgE levels (Fig. 6*A*) and the infiltration of inflammatory cells, mainly eosinophils, into the airway (Fig. 6*B*). Similar results were obtained in BALB/c mice (Fig. 6, *C* and *D*). Infiltration of macrophages/monocytes was also observed in BALB/c mice exposed to Japanese cedar pollen (data not shown). Inhalation of Japanese cypress pollen grains induced similar effects (data not shown).

Coadministration of Cupressaceae pollen grains with OVA induced an OVA-specific IgE response in vivo

In C57BL/6 mice, while the administration of OVA alone induced little or no elevations of total IgE and OVA-specific Abs and airway eosinophil infiltration (Fig. 7*A–C*, OVA), coadministration of Japanese cedar pollen grains with OVA (Fig. 7*A–C*, Cedar + OVA) induced elevations of serum total IgE and OVA-specific IgE (Fig. 7*A*) and IgG (Fig. 7*B*) levels, which were associated with airway eosinophil infiltration (Fig. 7*C*). Similar results were obtained in BALB/c mice (Fig. 7*D–F*) except for OVA-specific IgG2a. Coadministration of Japanese cypress pollen grains with OVA induced similar effects (data not shown).

Discussion

Exposure to pollen or house dust mite triggers a Th2-skewed immune response toward allergic diseases, which are associated with IgE production and eosinophilic inflammation. Although little is known about the initial sensitization process after first contact with innocuous environmental allergens, recent studies have suggested that molecules produced by allergen-producing organisms are involved in the pathogenesis through sensitization and/or exacerbation via IgE-independent mechanisms and the modification of IgE-dependent responses (8–30, 41–43). In the present study, we compared the response of DCs to pollen of different plant species. We found that pollen grains of members of the Cupressaceae family, Japanese cedar and Japanese cypress, exhibited the greatest stimulatory effect (Figs. 2 and 3 and supplemental Fig. S1), for which the TLR4 is dispensable (Figs. 4 and 5), while birch and grass pollen extracts exhibited the most prominent inhibition of

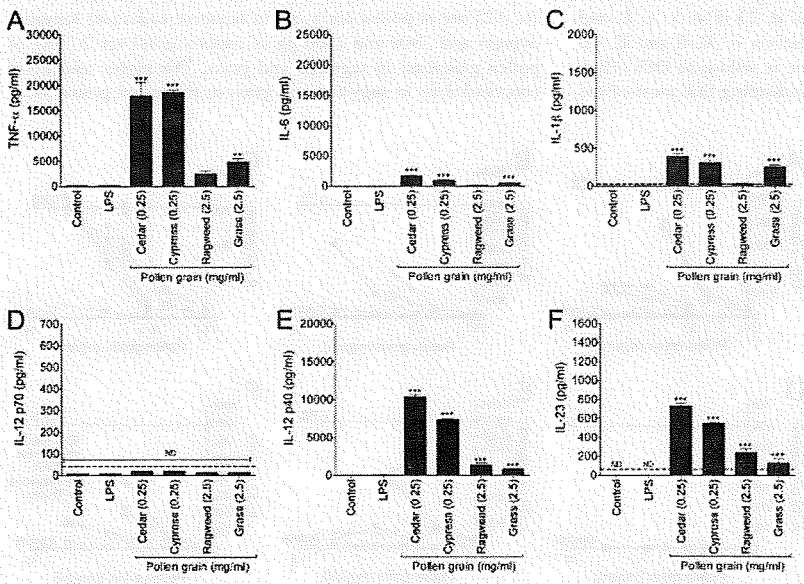

FIGURE 5. Effect of pollen grains on cytokine production in TLR4-deficient DCs. TLR4-deficient mouse bone marrow-derived immature DCs were stimulated for 24 h in the presence of pollen grains. Cytokine concentrations in the culture supernatant were measured. LPS indicates cells stimulated with LPS (List Biological Lab) (100 ng/ml); ND, not detected; broken line, minimum detection limit. Data are indicated as the means ± SD for three wells. **, $p < 0.01$ and *** $p < 0.001$ vs control by one-way ANOVA with the Tukey post hoc test.

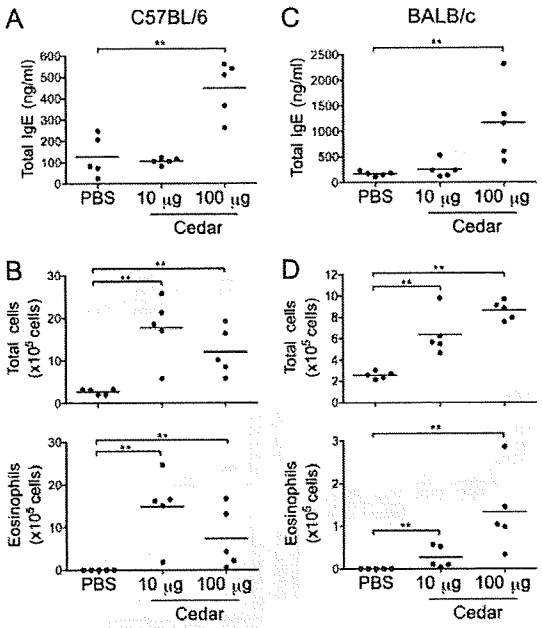

FIGURE 6. Induction of IgE production and airway eosinophil infiltration by inhalation of Japanese cedar pollen grains. C57BL/6 (*A* and *B*) or BALB/c (*C* and *D*) mice were intranasally administered with Japanese cedar pollen grains (10 or 100 μg/head) twice a week for 6 wk. Serum and BAL fluid were collected at 1 day after the last intranasal administration. *A* and *C*, Serum total IgE. *B* and *D*, Total number of cells and number of eosinophils in BAL fluid. Data are values for five mice per group. Bars indicate means. **, $p < 0.01$ vs PBS by the Mann-Whitney *U* test. Data shown are representatives of three independent experiments with similar results.

LPS-induced IL-12 (IL-12 p70) production (Fig. 1). Furthermore, we demonstrated that inhaled Cupressaceae pollen grains increased levels of serum IgE and airway eosinophilic infiltration (Fig. 6) and had adjuvant activity to induce production of IgE specific to coadministered OVA in mice (Fig. 7).

A research group reported that birch pollen extract reduced LPS-induced IL-12 p70 production in DCs, leading to Th2-biased priming of naive $CD4^+$ T cells in both human (16) and mouse (35) in vitro systems. Consistent with their results, birch pollen extract strongly suppressed the LPS-induced production of IL-12 p70 and p40 at the protein level (Fig. 1*A*), although a difference was observed between the murine bone marrow-derived DCs used in the present study and human monocyte-derived DCs (16), namely, the birch pollen extract inhibited both IL-12 p40 and p35 mRNA expression in the present study (Fig. 1) while it inhibited p40 but not p35 mRNA expression in the human monocyte-derived DCs (16). We demonstrated that grass pollen also inhibited LPS-induced IL-12 production. Birch and grass pollen extracts inhibited LPS-induced production of TNF-α significantly and IL-6 moderately. Pollen extracts of Japanese cedar, Japanese cypress, and ragweed showed moderate suppression of LPS-induced IL-12 p70 and TNF-α production, although the possibility that they could show more effective suppression if concentrated cannot be excluded. These results suggest that pollen-derived substances released into pollen extracts act as suppressors of immune responses by inhibiting production of IL-12 p70, TNF-α, and so on, although the extent of the effect differs among the plant families.

Stimulation of DCs with pollen grains in the absence of LPS promoted the maturation of DCs (Fig. 2) and induced the production of cytokines (Fig. 3*A–C*), but not the secretion of IL-12 p70 (Fig. 3*D*). Pollen grains of Japanese cedar and Japanese cypress, part of the Cupressaceae family, exhibited the greatest capacity to induce DC responses even at lower doses compared with the other pollen species tested (Figs. 2 and 3 and supplemental Fig. S1). As stimulation with pollen extracts alone had little effect on the response (data not shown), contact with the surface of pollen grains seemed crucial in the induction of maturation and cytokine production. Grass and ragweed pollen-induced maturation of human monocyte-derived DCs has been reported to be contact-dependent (44). APCs, including DCs, express a diversity of surface receptors, such as scavenger receptors, mannose receptors, and C-type lectins, for binding to exogenous ligands (45, 46). In addition to these receptors, the interaction of CD1 molecules on human monocyte-derived DCs and phospholipids on the surface of pollen has been reported (18). The mechanisms behind the induction of the DC responses by pollen grains (Figs. 2 and 3) should be addressed in future studies.

The exposure of DCs to pollen grains induced no detectable production of IL-12 p70 (Fig. 3*D*), but it significantly induced the production of IL-12 p40 (Fig. 3*E*). The p40 subunit seemed to contribute to the formation of IL-23, a heterodimer of the IL-12 p40 and IL-23 p19 subunits (Fig. 3*F*). IL-23 and IL-6 are cytokines involved in the differentiation of Th17 cells (47). Although it was reported that serum IgE levels and airway eosinophil infiltration were reduced in IL-17 receptor-deficient mice (48), several studies indicated that IL-17 is related to neutrophil-dominant, rather than eosinophil-dominant, inflammation (47, 49). DCs stimulated with pollen grains produced both IL-23 and IL-6 (Fig. 3, *B* and *F*). Whether the inhalation of pollen grains leads to Th17 cell-mediated inflammation via the stimulation of DCs is yet to be investigated.

In Japan, a common seasonal allergic disease posing a major public health problem is caused by inhalation of Cupressaceae pollen of Japanese cedar and Japanese cypress, affecting ~20% of the total population (2, 3). Cupressaceae pollen has also been identified as a source of pollinosis in Mediterranean countries (2, 50) and the United States (51, 52). Large studies on unselected young adults in France and Italy estimated the prevalence of allergies to cypress pollen to be ~2.4–8% of the general population (1, 2, 50). As Cupressaceae pollen exhibited the greatest stimulatory effect on DCs, even at lower doses (Figs. 2 and 3), we examined their effect in vivo using mice (Figs. 6 and 7). Inhalation of Cupressaceae pollen grains increased serum IgE levels and airway eosinophil infiltration (Fig. 6). The coadministration of OVA with Cupressaceae pollen grains induced increases in OVA-specific IgE and IgGs associated with airway eosinophil infiltration (Fig. 7). The lack of IL-12 p70 production by DCs in vitro (Fig. 3) suggests a contribution to the elevation in levels of IgE (Fig. 7, *A* and *D*), IgG1, and IgG2b rather than IgG2a (Fig. 7, *B* and *E*) in mice exposed to pollen of the Cupressaceae family. The results indicate that pollen grains of the Cupressaceae family have adjuvant activity in vivo that promotes Ag-specific IgE production associated with airway eosinophil infiltration. As far as we know, this is the first demonstration of in vivo IgE-inducing adjuvant activity of inhaled pollen-derived substances without using alum.

Hashiguchi et al. (53) reported that artificial exposure to Japanese cedar pollen at 2500 pollen grains/m^3, which is equivalent to the airborne pollen amount in the early pollinating season, caused penetration of 250 and 14 grains/h to the nose and eyes in humans. Airborne pollen amount during the middle and late pollinating season is ~5000 pollen grains/m^3 (53) and it could be estimated to

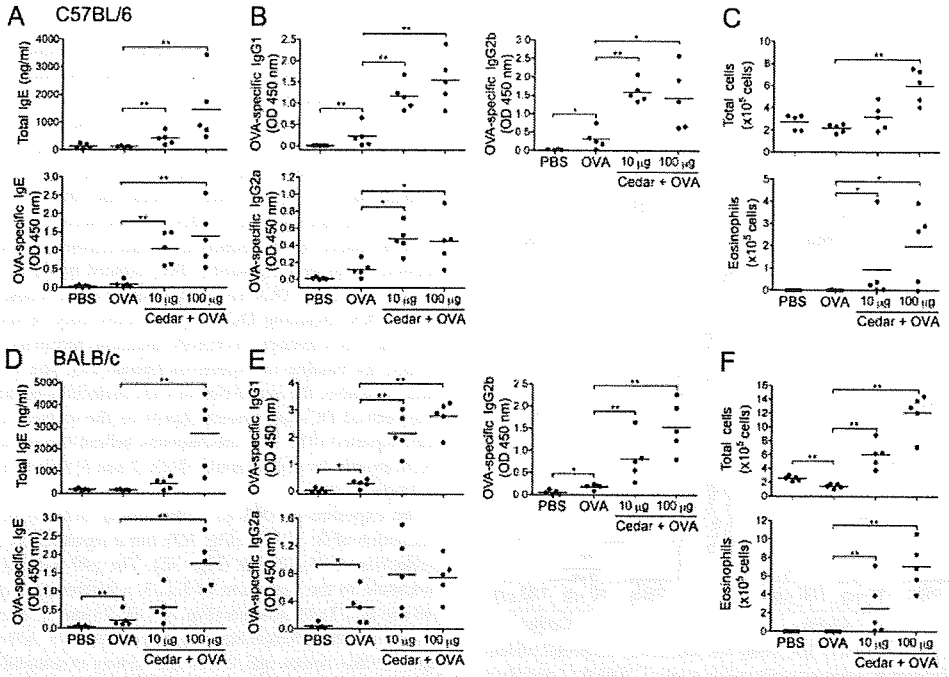

FIGURE 7. In vivo IgE-inducing adjuvant effect of Japanese cedar pollen grains. C57BL/6 (*A–C*) or BALB/c (*D–F*) mice were intranasally administered with OVA (25 μg/head) and Japanese cedar pollen grains (10 or 100 μg/head) twice a week for 6 wk. Serum and BAL fluid were collected at 1 day after the last intranasal administration. *A* and *D*, Serum total IgE and OVA-specific IgE. *B* and *E*, OVA-specific IgGs. *C* and *F*, Total number of cells and number of eosinophils in BAL fluid. Data are values for five mice per group. Bars indicate means. *, $p < 0.05$ and **, $p < 0.01$ vs OVA by the Mann-Whitney *U* test. Data shown are representatives of three independent experiments with similar results.

cause penetration of 500 grains/h to the nose, which is equivalent to 5 μg of grains/h (13, 54). Therefore, everyday exposure for 3 h/day could achieve inhalation of 100 μg of grains/wk in the middle and late pollinating season. In mice, administration of 20 μg of grains/wk with OVA exhibited adjuvant activity to induce OVA-specific IgE (Fig. 7, *A* and *D*) and eosinophil infiltration (Fig. 6, *B* and *D*, and Fig. 7, *C* and *F*). In our preliminary experiments, lower dose administration of 2 or 0.2 μg of grains/wk without OVA-induced eosinophil infiltration in mice (data not shown), suggesting the relevance of the natural exposure during the pollinating season to the sensitization toward Cupressaceae pollen allergy in humans. Although administration of 20 μg (or less) of grains/wk without OVA did not increase serum total IgE levels (Fig. 6, *A* and C, 10 μg; and data not shown), it does not exclude the possibility that Ag-specific IgE was increased (39). An assay system for IgE specific to pollen-derived Ags with high sensitivity should be established in a future study.

In summary, the results of the present study suggest that the modulation of DC responses to pollen differs among plant families in two ways: (1) the promotion of maturation and cytokine production through direct contact, which is greatest for Cupressaceae pollen, and (2) the inhibition of IL-12 production by soluble factors, which is greatest for birch and grass pollen. The strong stimulatory effect on DCs in vitro and IgE-inducing adjuvant activity in mice supports the clinical relevance of Cupressaceae pollen to allergies in humans, which are prominent in diverse geographic areas (2, 3, 50–52). Pollen grains contain various releasable or insoluble substances including lipids (12–18), proteases (13, 19, 30), NADPH oxidase (8–11), subpollen particles (10), and starch granules (55), which could be involved in the pathogenesis of allergic diseases. Mechanisms of the induction of DC responses by pollen and induction of Th2-inducing cytokines in other types of cells (22, 56, 57) by pollen-derived substances should be addressed in future studies.

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Disclosures

The authors have no financial conflicts of interest.

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Cytokine milieu modulates release of thymic stromal lymphopoietin from human keratinocytes stimulated with double-stranded RNA

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Background: Thymic stromal lymphopoietin (TSLP) plays a key role in allergic diseases, such as atopic dermatitis (AD) and asthma. TSLP is highly expressed by keratinocytes in skin lesions of patients with AD, but environmental triggers for its release from keratinocytes with endogenous factors are not well understood. Patients with AD, in whom allergic sensitization is already established, are susceptible to viral dissemination.
Objectives: We investigated TSLP's release from primary human keratinocytes stimulated with a Toll-like receptor (TLR) 3 ligand, polyinosinic-polycytidylic acid, which mimics viral double-stranded RNA (dsRNA), and its modulation by cytokines.
Methods: Primary human keratinocytes were stimulated with TLR ligands, cytokines, or both. TSLP released into culture supernatants was measured by means of ELISA.
Results: Stimulation of keratinocytes with dsRNA induced release of TSLP and upregulated gene expression of TSLP and other cytokines and chemokines. The release of TSLP was enhanced by the addition of IL-4, IL-13, and/or TNF-α. With or without the T_H2/TNF cytokines, the dsRNA-induced release of TSLP was upregulated by IFN-α and IFN-β and suppressed by IFN-γ, TGF-β, or IL-17.
Conclusions: The effect of the TLR3 ligand on keratinocytes suggests contribution of viral dsRNA to skin inflammations under the influence of a cytokine milieu. The results imply that viral dsRNA and a T_H2 cytokine milieu might promote T_H2-type inflammation through an induction of TSLP expression, suggesting that a vicious cycle exists between AD with T_H2-type inflammation and viral infections and a possible blockade of this cycle by other cytokine milieus provided by cells, such as T_H1, regulatory T, and T_H17 cells. (J Allergy Clin Immunol 2009;123:179-86.)

Key words: *Thymic stromal lymphopoietin, keratinocyte, double-stranded RNA, cytokine milieu, atopic dermatitis, viral exposure, viral infection, vicious cycle, promotion and blockade*

Abbreviations used
AD: Atopic dermatitis
DC: Dendritic cell
dsRNA: Double-stranded RNA
Pam_3CSK_4: (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH, 3HCl
poly I:C: Polyinosinic-polycytidylic acid
TLR: Toll-like receptor
Treg: Regulatory T
TSLP: Thymic stromal lymphopoietin

Recent findings indicate that thymic stromal lymphopoietin (TSLP), an IL-7–like cytokine, plays a key role in allergic inflammation.[1,2] TSLP is highly expressed by keratinocytes in skin lesions of patients with atopic dermatitis (AD)[3] and airway epithelial cells of patients with asthma.[4] TSLP was found in keratinocytes of skin lesions of patients with acute and chronic AD but not in their nonlesional skin and lesions of patients with nickel-induced allergy contact dermatitis or cutaneous lupus erythematosus.[3] TSLP-activated dendritic cells (DCs) secrete T_H2-recruiting chemokines but not IL-12 and induce naive T cells to differentiate into inflammatory T_H2 cells, producing IL-4, IL-5, IL-13, and TNF-α through OX40 ligand.[1,2,5] TSLP similarly activates epidermal Langerhans cells, a subset of DCs.[6] The TSLP DCs can induce allergen-specific T_H2 memory cells to undergo homeostatic expansion and further T_H2 polarization and to mediate recall responses.[7] In support of the findings in human subjects, mice with keratinocyte- or lung-specific overexpression of TSLP have an AD- or asthma-like phenotype,[8,9] and mice lacking the TSLP receptor have considerably attenuated disease in asthma models.[9,10] TSLP can also directly act on human mast cells synergistically with IL-1 and TNF-α to produce IL-5 and IL-13[11] and on human $CD4^+$ T cells activated with T-cell receptor stimulation to markedly proliferate.[12] Thus TSLP represents a critical factor linking responses at interfaces between the body and environment to allergic type 2 immune responses.

In human primary airway epithelial cells, the release of TSLP *in vitro* is induced on stimulation with polyinosinic-polycytidylic acid (poly I:C), mimicking viral double-stranded RNA (dsRNA), and the proinflammatory cytokines TNF-α and IL-1, mimicking an inflammatory microenvironment.[11] The T_H2 cytokines IL-4 or IL-13, mimicking an allergic microenvironment, upregulate the poly I:C–induced release of TSLP and the level of *TSLP* mRNA synergistically with rhinovirus infection,[13] suggesting that viral infections in a T_H2 cytokine milieu amplify airway

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allergic reactions. Poly I:C also induces TSLP's release from human tonsillar epithelial cells.[14]

In human keratinocytes the environmental factors triggering TSLP's release and its modulation with endogenous factors are not well investigated, although Bogiatzi et al[15] have recently reported that proinflammatory and T_H2 cytokines act synergistically to induce the release of TSLP from human skin explants obtained from healthy donors. Patients with AD tend to have widespread disseminated viral infections.[16] Disturbed skin barrier[17-19] and decreased expression of LL-37,[20] which has innate immune function to control viral replication,[21] in AD skin are considered to provide easier access for the virus and permit its replication, respectively. In the present study we analyzed TSLP's release from primary human keratinocytes stimulated with dsRNA in the absence or presence of cytokines.

METHODS

Reagents

The following recombinant human cytokines and concentrations were used to stimulate keratinocytes: 20 ng/mL TNF-α, 100 ng/mL IL-4, 100 ng/mL IL-13, 100 ng/mL IFN-γ, 100 ng/mL IL-10, 10 ng/mL TGF-β, 100 ng/mL IL-17, 100 ng/mL IL-22 (R&D Systems, Minneapolis, Minn), 10 ng/mL (2000 U/mL) IFN-α, and 100 ng/mL (2000 U/mL) IFN-β (PeproTech, London, United Kingdom). The following Toll-like receptor (TLR) ligands and concentrations were used to stimulate keratinocytes: 0.1 to 100 μg/mL poly I:C; 5 μg/mL (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys_4-OH, 3HCl (Pam_3CSK_4; Calbiochem, San Diego, Calif); 20 μg/mL peptidoglycan from *Staphylococcus aureus*; 20 μg/mL LPS from *Escherichia coli* serotype 0111:B4 (Sigma, St Louis, Mo); 20 ng/mL flagellin purified from *Salmonella typhimurium* strain 14028 (Alexis Biochemicals, San Diego, Calif); 20 μg/mL loxoribine; 10 μg/mL CL097 (InvivoGen, San Diego, Calif); and 5 μmol/L human CpG (Hycult biotechnology, Uden, The Netherlands).

Cell culture and stimulation of keratinocytes

Primary human keratinocytes (Cascade Biologics, Portland, Ore) were cultured in EpiLife KG2 (Kurabo, Osaka, Japan) supplemented with 0.1 ng/mL epidermal growth factor, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, 50 μg/mL gentamycin, 50 ng/mL amphotericin B, and 0.4% vol/vol bovine brain pituitary extract. Cells were seeded at 1×10^5 cells/well in flat-bottomed, 96-well microculture plates and cultured until they reached 80% confluence, and then the medium was renewed. After further cultivation for 24 hours, cells were stimulated with TLR ligands, cytokines, or both in experiments, except for quantitative PCR for gene expression at 4 and 8 hours, in which the medium used for the stimulation and 24-hour cultivation before the stimulation did not contain hydrocortisone.

ELISA

Concentrations of TSLP and IL-8 protein were measured by using diluted (1:2 for TSLP and less than 1:20 for IL-8) culture supernatants collected at 48 hours after the stimulation with ELISA kits (DuoSet, R&D Systems). In this study the minimum detection limits for TSLP and IL-8 in the supernatants were 7.8 and 156 pg/mL, respectively.

Real-time quantitative PCR

Total RNA was extracted from cells with RNAspin Mini (GE Healthcare, Buckinghamshire, United Kingdom) and treated with DNase I (GE Healthcare) in Fig 1, *C*, and RNeasy Plus Micro Kit (QIAGEN, Hilden, Germany) in Fig 2. cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, Calif) and random primers. Real-time quantitative PCR was performed with a TaqMan method with an ABI 7500 (Applied Biosystems, Piscataway, NJ). The mRNA level was normalized to the gene expression of glyceraldehyde-3-phosphate dehydrogenase in Fig 1, *C*, and β-actin in Fig 2.

Statistical analysis

An unpaired Student *t* test (2-tailed) or 1-way ANOVA with the Tukey multiple comparison test was used. *P* values of less than .05 were regarded as statistically significant. Data shown are the means ± SDs for 3 wells, except for Fig 2, and are representative of 3 independent experiments.

RESULTS

dsRNA-induced TSLP release from keratinocytes

Primary human keratinocytes were treated with TLR ligands, including synthetic bacterial lipoprotein Pam_3CSK_4 (TLR2 ligand), peptidoglycan (TLR2 ligand), poly I:C (TLR3 ligand), LPS (TLR4 ligand), flagellin (TLR5 ligand), loxoribin (TLR7 ligand), CL-097 (TLR7/8 ligand), and CpG (TLR9 ligand), to test for a possible role of the regulation of TSLP's production by TLR ligands in keratinocytes. ELISA of the culture supernatants showed that keratinocytes treated with poly I:C, mimicking viral dsRNA, released detectable amounts of TSLP (Fig 1, *A*). The other ligands tested did not induce TSLP's release, whereas peptidoglycan and flagellin induced IL-8's release, although less effectively than poly I:C (Fig 1, *B*), which is consistent with other reports.[22,23] The gene expression for *TSLP* mRNA was significantly upregulated by stimulation with poly I:C (Fig 1, *C*).

Stimulation of keratinocytes with poly I:C induced a more than 10-fold increase of gene expression of TSLP and other cytokines (TSLP, IFN-β, TNF-α, IL-6, and GM-CSF) and chemokines (CXCL8/IL-8, CXCL9/monokine induced by IFN-γ, CXCL10/IFN-inducible protein 10, CXCL11/IFN-inducible T-cell α chemoattractant, CCL2/monocyte chemoattractant protein 1, CCL5/RANTES, CCL20/macrophage inflammatory protein 3α, and CCL22/macrophage-derived chemokine; Fig 2).

T_H2 cytokines and TNF-α upregulated dsRNA-induced release of TSLP from keratinocytes

TSLP is highly expressed by keratinocytes in skin lesions of patients with AD.[3] AD skin is characterized by the overexpression of the T_H2 cytokines IL-4 and IL-13.[24,25] To test whether a cytokine milieu that mimics the atopic environment modulates poly I:C–induced release of TSLP, we examined whether a combination of TNF-α together with the T_H2 cytokines IL-4 and IL-13 (T_H2/TNF cytokines) would enhance TSLP's release from keratinocytes (Fig 3, *A-C*, and Fig 4, *A-C*). The dose- and time-dependent poly I:C–induced release was markedly upregulated by costimulation with the T_H2/TNF cytokines (Fig 3, *A-C*). A low level of TSLP was detectable on stimulation with the T_H2/TNF cytokines without poly I:C (Fig 4, *A*), supporting a recent study with skin explants obtained from healthy donors.[15] Poly I:C significantly induced TSLP's release and was most effective in the presence of both TNF-α and one of the 2 T_H2 cytokines IL-4 and IL-13 (Fig 4, *B* and *C*). Removing TNF-α or both T_H2 cytokines decreased the amount of TSLP released, suggesting that signaling pathways through receptors for TNF-α and the T_H2 cytokines synergistically upregulate the poly I:C–induced release. Removing one of the T_H2 cytokines had no effect (Fig 4, *B* and *C*), as supported by the finding that the IL-4 receptor α-chain is shared by receptors for IL-4 and IL-13 and is essential for activation of the central transcription factor signal transducer and activator of transcription 6.[26] TNF-α or poly I:C upregulated the release of IL-8, but IL-4 and IL-13 made little or no contribution to IL-8 levels (Fig 4, *D-F*).