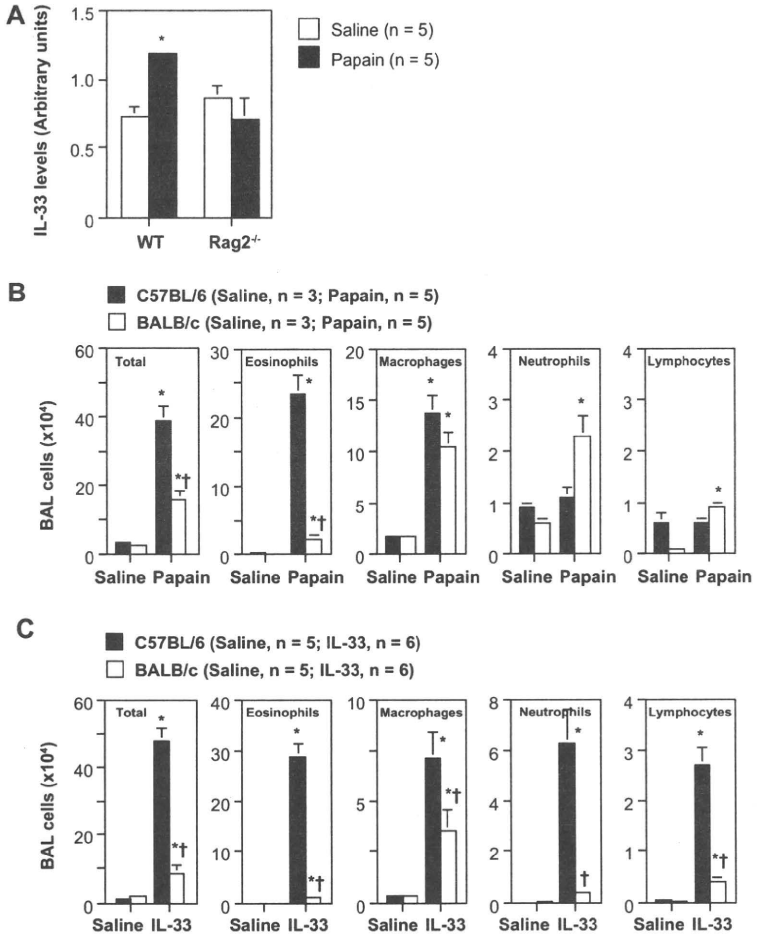

Fig. S4. The susceptibility to papain and IL-33 differs between BALB/c and C57BL/6 mice. (*A*) Expression of IL-33 mRNA in the lungs after papain inhalation. (*B*) The number of inflammatory cells in BAL fluids from C57BL/6- and BALB/c-wild-type mice at 24 h after the last papain or saline inhalation. (*C*) The number of inflammatory cells in BAL fluids from C57BL/6- and BALB/c-wild-type mice at 24 h after the last IL-33 or saline inhalation. Data show the mean ± SE. $^{*}P < 0.05$ vs. corresponding values for saline-treated mice, and $^{\dagger}P < 0.05$ vs. papain- or IL-33-treated C57BL/6-wild-type mice.

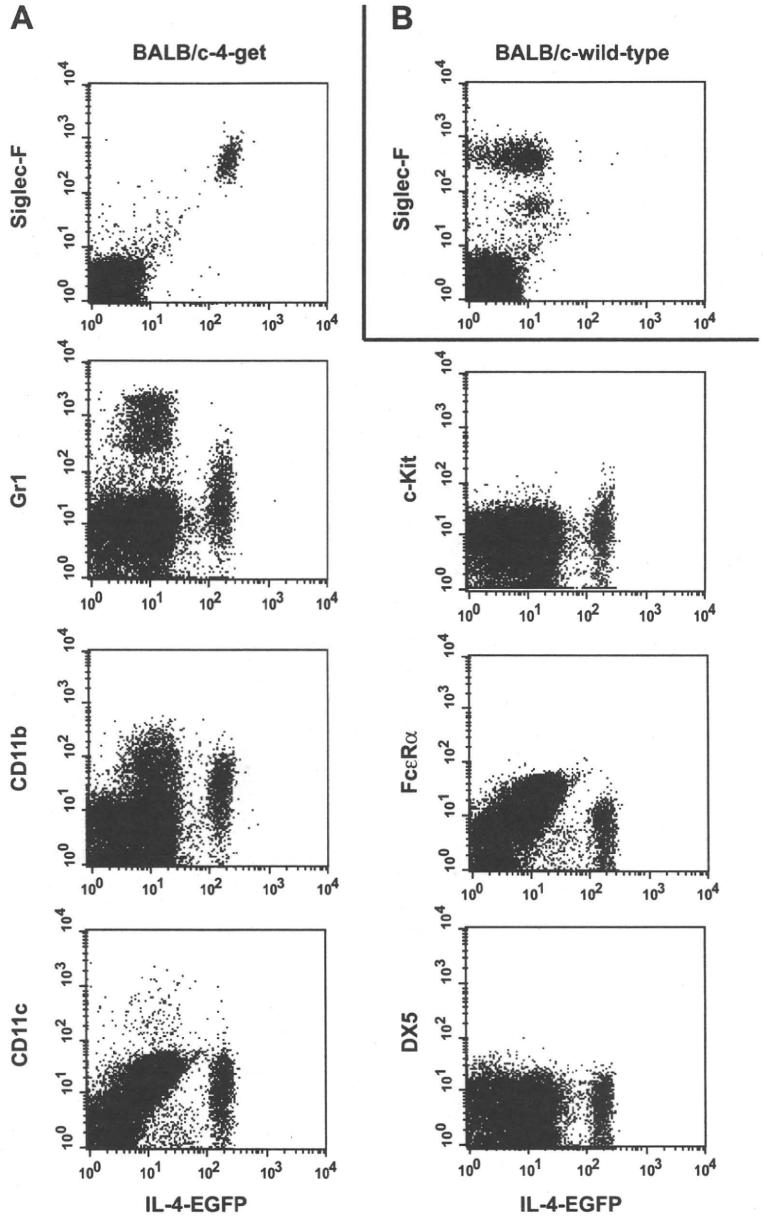

Fig. S5. Eosinophils are a producer of IL-4 in BAL cells after papain inhalation. BAL cells were harvested from BALB/c-4-get mice (*A*), which express EGFP with IL-4, and BALB/c-wild-type (*B*) at 24 h after the last papain inhalation. EGFP-IL-4-expressing cells in 7-aminoactinomycin D-, CD3- and B220-negative cells were detected by flow cytometry. Data show a representative result from two independent experiments.

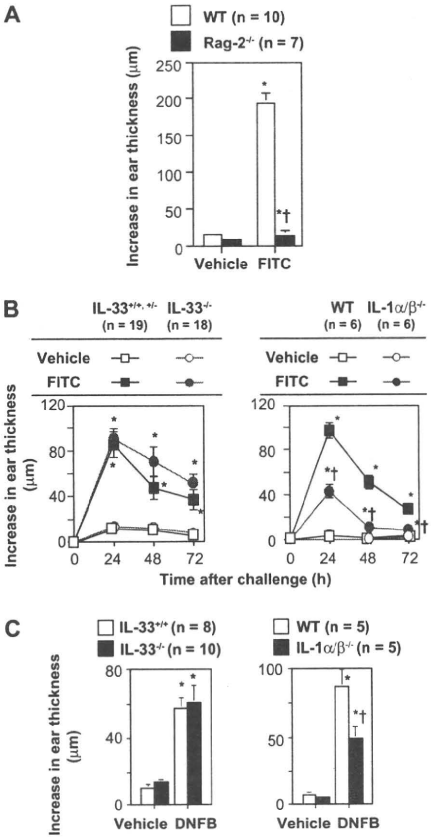

Fig. S6. The role of IL-33 in the development of CHS. Mice were sensitized with FITC or DNFB and then challenged with the same hapten. Ear thickness was measured before and after hapten challenge. (*A*) FITC-induced CHS in wild-type and Rag-$2^{-/-}$ mice on the C57BL/6 background. (*B*) FITC-induced CHS in IL-$33^{+/+}$ and IL-$33^{-/-}$ mice (*Left*) and in wild-type and IL-$1\alpha/\beta^{-/-}$ mice on the C57BL/6 background (*Right*). (*C*) DNFB-induced CHS in IL-$33^{+/+}$ and IL-$33^{-/-}$ mice (*Left*) and in wild-type and IL-$1\alpha/\beta^{-/-}$ mice on the C57BL/6 background (*Right*) at 24 h after the challenge. Data show the mean ± SE $^{*}P < 0.05$ vs. corresponding values for vehicle-treated mice, and $^{\dagger}P < 0.05$ vs. hapten-challenged wild-type mice.

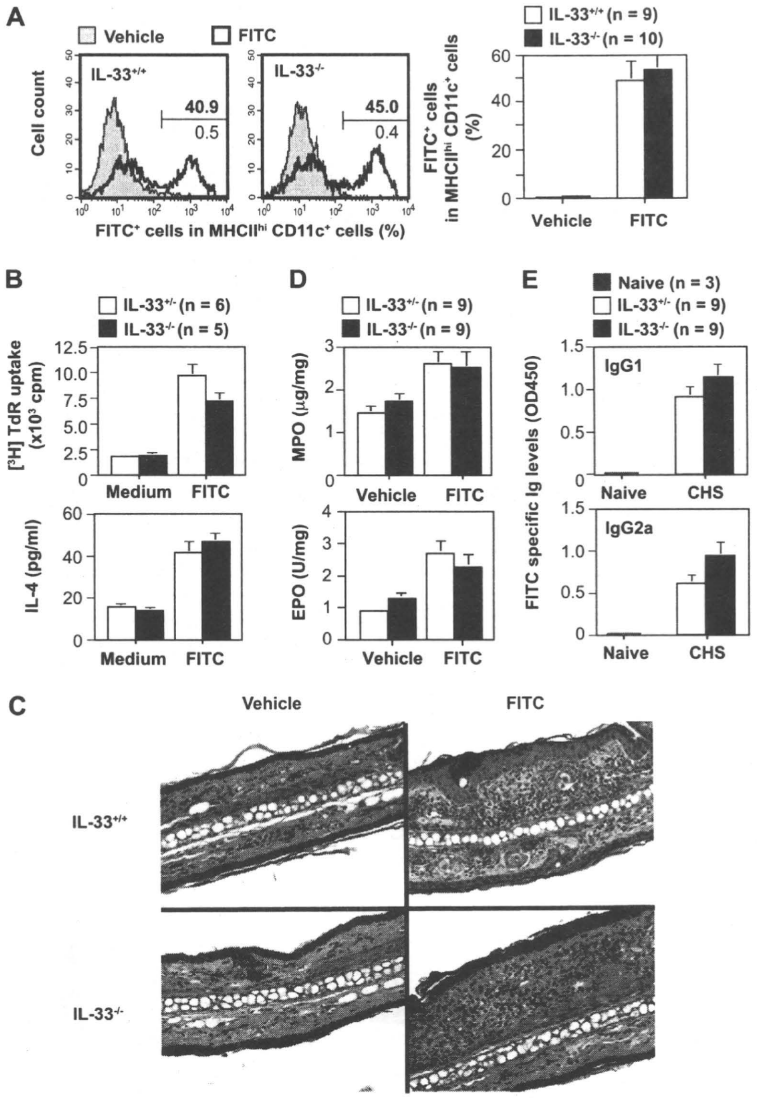

Fig. S7. IL-33 is not essential for hapten-specific T cell induction and activation. (*A*) Skin DC migration. The proportion of FITC$^+$ cells among 7-amino-actinomycin D-negative, MHC class II^{hi}, CD11c$^+$ cells was determined by flow cytometry. Shaded areas, LN cells from the vehicle-treated side; bold lines, LN cells from the FITC-treated side. Representative FACS results are shown. (*B*) Mice were treated epicutaneously with 2.0% FITC on both the left and right ears. Five days later, submaxillary LNs were collected, and LN cells were cultured in the presence and absence of 40 μg/mL FITC for 72 h. FITC-specific proliferative responses and IL-4 secretion of LN cells from mice sensitized with FITC are shown. (*C*) Histology (hematoxylin-eosin staining) of ear skin at 24 h after FITC challenge (200×; representative data from three to five mice are shown). (*D*) The levels of MPO and EPO activities in ear tissue homogenates at 24 h after FITC challenge. (*E*) Sera were collected 1 wk after FITC challenge. The levels of FITC-specific IgG1, IgG2a, and IgE in sera from naïve IL-33$^{+/+}$ mice and FITC-challenged IL-33$^{+/+}$ and IL-33$^{-/-}$ mice are shown. Data show the mean ± SE. No significant differences were found between the IL-33$^{+/+}$ and IL-33$^{-/-}$ groups.

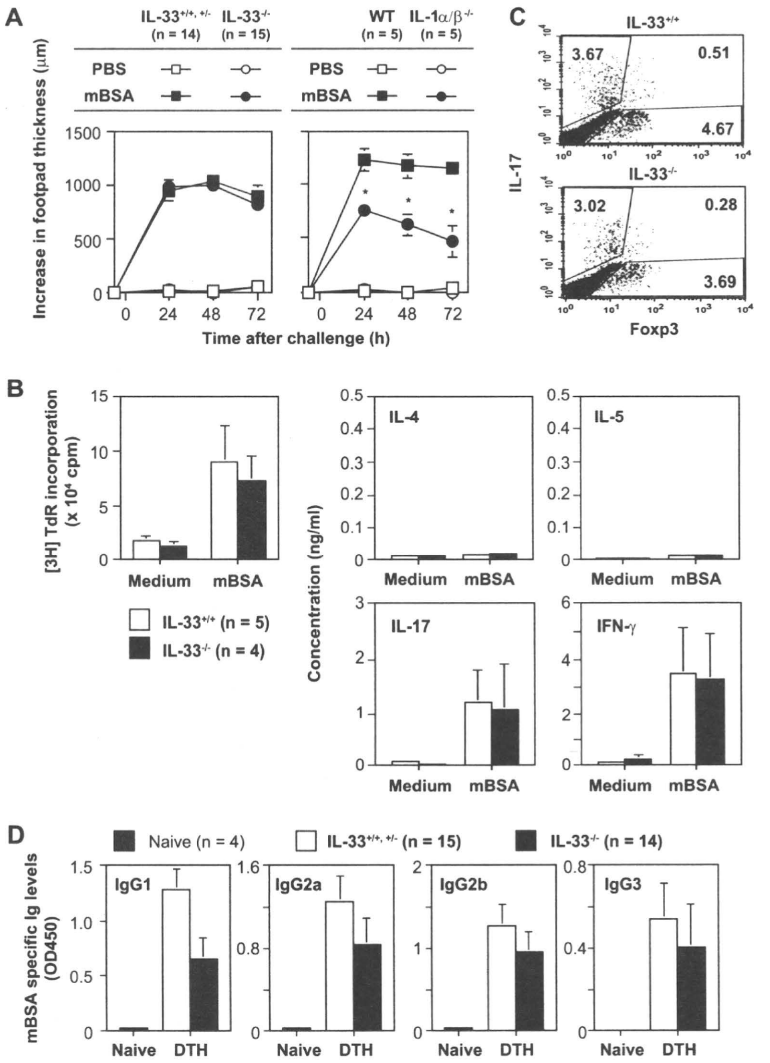

Fig. S8. The role of IL-33 in the pathogenesis of mBSA-induced DTH. (*A*) Footpad thickness was measured before and after mBSA or PBS challenge. (*B*) mBSA-specific proliferative responses and cytokine secretion of LN cells from mice sensitized with mBSA emulsified in CFA. (*C*) Profiles of IL-17- and Foxp3-expressing $CD4^+$ T cells in the culture with mBSA in *B*. Representative FACS results are shown. (*D*) mBSA-specific Ig levels in sera from mice 1 wk after mBSA challenge in *A* or naïve $IL\text{-}33^{+/+}$ mice. Data show the mean ± SE: $^*P < 0.05$ vs. corresponding values for WT mice.

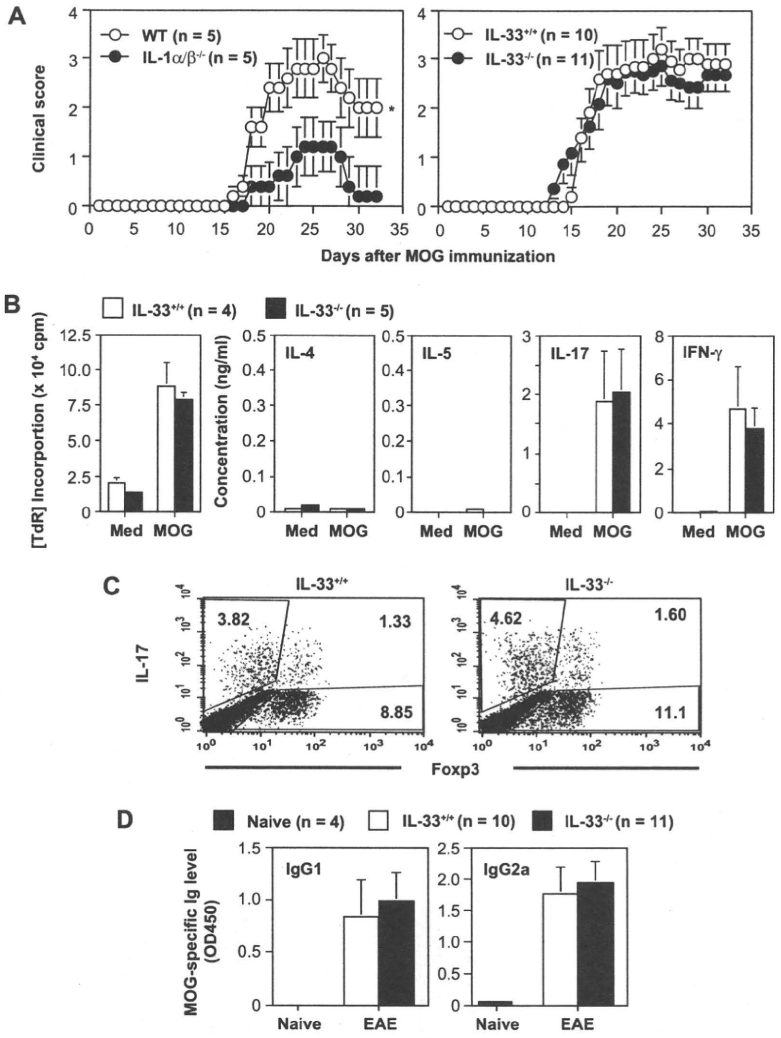

Fig. S9. The role of IL-33 in the development of EAE. (*A*) The severity of MOG-induced EAE. (*B*) MOG-specific proliferative responses and cytokine secretion of LN cells from mice sensitized with MOG emulsified in CFA. (*C*) Profiles of IL-17- and Foxp3-expressing $CD4^+$ T cells in the culture with MOG in *B*. Representative FACS results are shown. (*D*) MOG-specific Ig levels in sera from mice 33 d after the first MOG immunization in *A* or naïve $IL\text{-}33^{+/+}$ mice. The levels of MOG-specific IgG1 and IgG2a were measured by ELISA. Data show the mean ± SE: $^*P < 0.01$ (Mann–Whitney *u* test). No significant differences were found between the $IL\text{-}33^{+/+}$ and $IL\text{-}33^{-/-}$ groups.

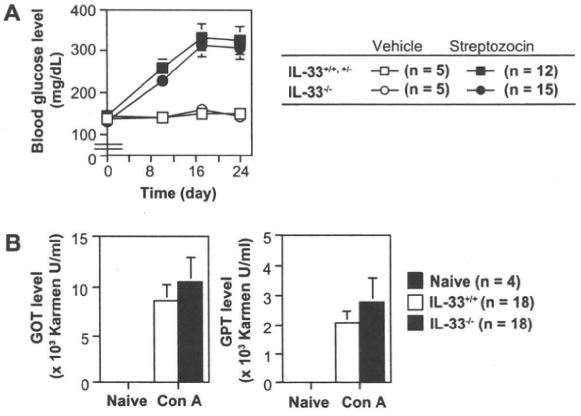

Fig. S10. The role of IL-33 in the development of T/NKT cell-dependent streptozocin-induced diabetes or Con A-induced hepatitis. (*A*) The levels of blood glucose in mice during streptozocin-induced diabetes. (*B*) GOT and GPT activity levels in sera from mice during Con A-induced hepatitis. Data show the mean ± SE. No significant differences were found between the $IL\text{-}33^{+/+}$ and $IL\text{-}33^{-/-}$ groups.

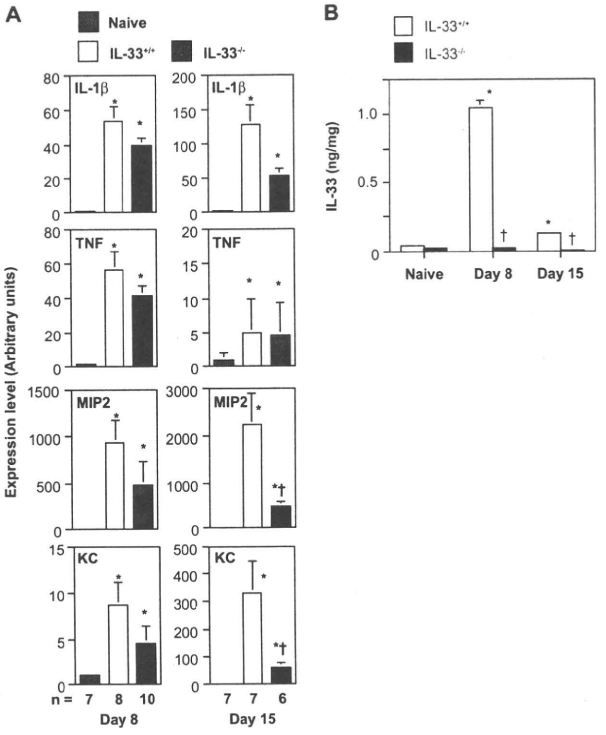

Fig. S11. IL-33 is involved in the expression of neutrophil chemoattractant factors during DSS-induced colitis. The colon was collected from mice on day 8 or 15 during 3.0% DSS-induced colitis or from naïve mice. (*A*) The levels of IL-1β, TNF, MIP2, and KC expression in the colon were determined by quantitative PCR. (*B*) The levels of IL-33 in the colon homogenates were determined by ELISA. Data show the mean ± SE: $*P < 0.05$ vs. corresponding values for naive mice, and $\dagger P < 0.05$ vs. DSS-treated IL-33$^{+/+}$ mice.

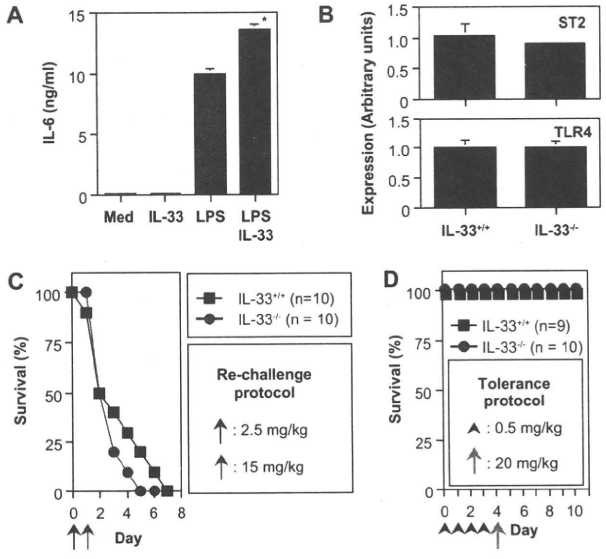

Fig. S12. IL-33 is not essential for secondary responses or tolerance to LPS. (*A*) TGC-induced peritoneal macrophages from C57BL/6 mice were cultured with plain medium or medium containing IL-33, LPS, or IL-33 + LPS for 48 h. IL-6 levels in the culture supernatants were determined by ELISA. Data show the mean + SE ($n = 3$). $*P < 0.05$ vs. medium alone, IL-33, and LPS stimulation. (*B*) The expressions of ST2 and TLR4 in peritoneal macrophages from IL-33$^{+/+}$ and IL-33$^{-/-}$ mice were determined by quantitative PCR. Data show the mean ± SE ($n = 3$). (*C*) The survival ratios in IL-33$^{+/+}$ and IL-33$^{-/-}$ mice, which were first sensitized with a low dose of LPS (2.5 mg/kg; black arrow), after treatment with a lethal dose of LPS (15 mg/kg; red arrow). (*D*) The survival ratios in IL-33$^{+/+}$ and IL-33$^{-/-}$ mice, which were first made tolerant to LPS by repeated injection of a low dose of LPS (0.5 mg/kg; black arrowhead), after treatment with a lethal dose of LPS (20 mg/kg; green arrow). No significant differences were found between the IL-33$^{+/+}$ and IL-33$^{-/-}$ groups (*B–D*).

Table S1. Primer design

Genes	Forward (5′ - 3′)	Reverse (5′ - 3′)
GAPDH	CCCACTCTTCCACCTTCGATG	AGGTCCACCACCCTGTTGCT
IL-1β	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
IL-33	CAGGCCTTCTTCGTCCTTCAC	TCTCCTCCACTAGAGCCAGCTG
TNF	GCCTCCCTCTCATCAGTTCT	CACTTGGTGGTTTGCTACGA
KC	CACGTGTTGACGCTTCCCTT	TGAACGTCTCTGTCCCGAGC
MIP-2	AACTGACCTGGAAAGGAGGAGC	ACTCTCAGACAGCGAGGCACAT
ST2	TCAACCGCCTAGTGAACACACC	CAAAGCCCAAAGTCCCATTCTC
TLR4	CGCTTTCACCTCTGCCTTCACTACAG	ACACTACCACAATAACCTTCCGGCTC

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ORIGINAL ARTICLE

Amphiregulin is Not Essential for Induction of Contact Hypersensitivity

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ABSTRACT

Background: Amphiregulin (AR) is expressed in Th2 cells, rather than Th1 cells, and plays an important role in Th2 cell/cytokine-mediated host defense against nematodes. We also found earlier that AR mRNA expression was strongly upregulated in inflamed tissue during Th2 cell/cytokine-mediated fluorescein isothiocyanate (FITC)-induced contact hypersensitivity (CHS), suggesting a contribution of AR to the induction of those responses.

Methods: To elucidate the role of AR in the induction of FITC- or dinitrofluorobenzene (DNFB)-induced CHS, AR-deficient mice were sensitized and/or challenged with FITC or DNFB epicutaneously. The levels of FITC-mediated skin dendritic cell (DC) migration and FITC-specific lymph node cell proliferation and cytokine production were assessed by flow cytometry, [3H]-thymidine incorporation and ELISA, respectively, after FITC sensitization. The degree of ear swelling, the activities of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) in inflammatory sites and the levels of FITC-specific immunoglobulin (Ig) in sera were determined by histological analysis, colorimetric assay and ELISA, respectively, after FITC challenge.

Results: DC migration and FITC-specific lymph node cell proliferation and cytokine production were normal in the AR-deficient mice. Ear swelling, tissue MPO and EPO activities and FITC-specific serum Ig levels were also similar in AR-deficient and -sufficient mice.

Conclusions: Amphiregulin is not essential for the induction of FITC- or DNFB-induced CHS responses in mice.

KEY WORDS

amphiregulin, contact dermatitis, EGF, mast cells, Th2 cell/cytokine

INTRODUCTION

Contact hypersensitivity (CHS) responses, which are induced by epicutaneous exposure to chemicals, are classically considered to be an IFN-γ-producing Th1 and Tc1 cell-mediated allergic disorder.[1] This is supported by the facts that increased levels of IFN-γ and accumulation of IFN-γ$^+$ Th1 cells were observed at local inflammatory sites in CHS.[2,3] However, results from studies using IFN-γ- and/or IFN-γR1-deficient mice did not always support the concept, since they showed normal development of CHS in response to oxazolone[4] and dinitrofluorobenzene (DNFB).[5,6] CHS induced by trinitrochlorobenzene (TNCB) in acetone and olive oil developed normally in IFN-γ- and IFN-γR 1-deficient mice,[4,7] while induction by TNCB in ethanol and acetone was attenuated in IFN-γ-deficient mice.[8] Although fluorescein isothiocyanate (FITC)-induced CHS was attenuated in IFN-γR2-deficient mice on a 129 × B6 mixed background,[9] a normal response was observed in IFN-γ-deficient mice on a C57 BL/6 background.[8,10] These observations suggest that IFN-γ may not to be crucial for induction of CHS. In contrast, it was shown that DNFB- and/or TNCB-

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induced CHS responses were significantly decreased in IL-4-deficient mice, while oxazolone-induced CHS developed normally in those mutant mice.[11-14] Although IL-13-deficient mice showed normal development of DNFB-induced CHS,[15] STAT-6-deficient mice, which lack both IL-4- and IL-13-mediated immune responses, exhibited decreased inflammation during DNFB-, TNCB-, oxazolone- and FITC-induced CHS responses.[16] Therefore, these observations indicate that Th2 cytokines/Th2 cells, rather than Th1 cytokines/Th1 and Tc1 cells, are important in the pathogenesis of CHS.

Recently, it was shown that amphiregulin (AR), which is a member of the EGF family of growth factors/cytokines and involved in mammary gland development and the progression of breast cancer,[17,18] is expressed in Th2 cells but not Th1 cells[19] and plays an important role in Th2 cell-mediated host defense against nematodes.[19] In addition, in our pilot study using DNA microarrays, we found that expression of AR mRNA was increased in local inflammatory sites during Th2 cell/cytokine-mediated FITC-induced CHS. These observations suggest that AR produced by Th2 cells and/or other immune cells may contribute to the induction of CHS responses. However, the precise role of AR in the pathogenesis of CHS is unknown. In the present study, we used AR-deficient mice and demonstrated that AR is not necessary for FITC- or DNFB-induced CHS.

METHODS

MICE

Amphiregulin-deficient ($AR^{-/-}$) mice on a 129 × B6 mixed background were originally generated as described elsewhere.[20] Littermates ($AR^{+/+}$, $AR^{+/-}$ and $AR^{-/-}$ mice) were used in all experiments. Mice were housed under specific-pathogen-free conditions in the 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.

SKIN DC MIGRATION

Skin DC migration was determined as described elsewhere.[21] In brief, mice were epicutaneously treated with 40 μl of 0.5% (w/v) FITC isomer I solution in a mixture of acetone and dibutylphthalate (1 : 1) (20 μl to the surface of each left ear) and the vehicle alone (20 μl to the surface of each right ear). Twenty-four hours later, submaxillary lymph nodes (LNs) were separately collected from both the FITC-treated left and vehicle-treated right ears. LN single-cell suspensions were prepared and incubated with anti-CD16/CD32 mAb (2.4 G2; BD Biosciences, San Jose, CA, USA) on ice for 15 minutes for FcR blocking. Then the cells were incubated with PE-anti-mouse CD11c mAb (N418; eBioscience) and APC-anti-mouse I-A/I-E mAb (M5/114.15.2; eBioscience, San Diego, CA, USA). The proportion of $FITC^+$ cells among 7-aminoactinomycin D-negative, MHC class II^{hi}, $CD11c^+$ cells was determined using a FACS Calibur (BD Biosciences).

FITC-SPECIFIC LN CELL RESPONSES

FITC-specific LN cell proliferative responses were measured as described elsewhere.[21] Briefly, mice were sensitized with 2.0% FITC on both the left and right ears (20 μl on one surface of each ear). Six days later, submaxillary LNs were collected, and single-cell suspensions were prepared. The LN cells were cultured in the presence or absence of 40 μg/ml FITC at 37℃ for 72 hours. FITC-specific LN cell proliferative responses were determined by pulsing with 0.25 μCi [^{3}H]-labeled thymidine for 6 hours.

MEASUREMENT OF CYTOKINES

Cytokine levels in the culture supernatants of FITC-specific LN cells were determined with mouse IFN-γ, IL-4, IL-10 and IL-17 ELISA kits obtained from BD Biosciences or eBioscience.

INDUCTION OF CHS

FITC- or DNFB-induced CHS was induced as described elsewhere.[21-23] Briefly, 2 days after shaving the back with clippers, mice were treated with 200 μl of a 0.5 or 2.0% (w/v) FITC isomer I (SIGMA) (0.5% FITC solution and 2.0% FITC suspension, respectively) in a mixture of acetone and dibutylphthalate (1 : 1) or 25 μl of a 0.5% (v/v) DNFB (Wako, Osaka, Japan) in a mixture of acetone and olive oil (4 : 1). Five days later, the animals were challenged with 40 μl of a 0.5% (w/v) FITC isomer I solution in a mixture of acetone and dibutylphthalate (1 : 1) (20 μl to the surface of each left ear) and 40 μl of the vehicle alone (20 μl to the surface of each right ear) or with 20 μl of a 0.2% (v/v) DNFB solution in a mixture of acetone and olive oil (4 : 1) (the left ear) and 20 μl of the vehicle alone (the right ear). Ear thickness was measured before and after FITC challenge using an engineer's caliper (Ozaki MFG. Co. Ltd., Tokyo, Japan).

HISTOLOGY

Twenty-four hours after FITC challenge, ear tissues were harvested, fixed in Carnoy's fluid and embedded in paraffin. Then sections were prepared and stained with hematoxylin-eosin.

MEASUREMENT OF MYELOPEROXIDASE (MPO) AND EOSINOPHIL PEROXIDASE (EPO) ACTIVITIES

Twenty-four hours after FITC challenge, ear tissues were harvested and homogenized in a 0.5% cetyltrimethylammonium chloride solution (Sigma-Aldrich, St Louis, MO, USA). After passing the homogenates through a nylon mesh and centrifuging at 3,000 rpm for 20 minutes at 4℃, the supernatants

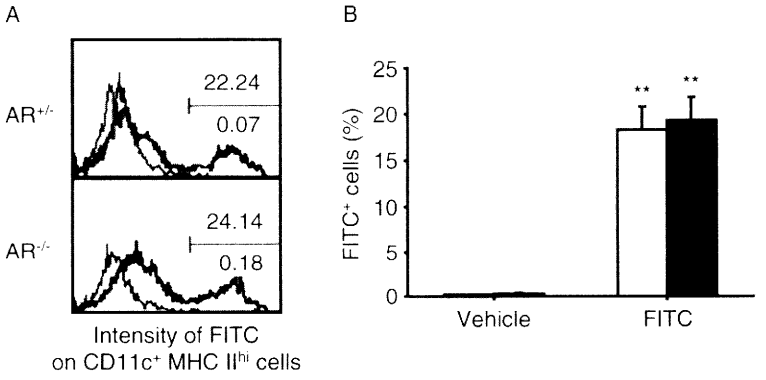

Fig. 1 AR is not required for migration of FITC-bearing LCs/DCs from skin to draining LNs. At 24 hours after epicutaneous treatment with FITC (left ear skin) and vehicle (right ear skin), the submaxillary LNs on the left and right sides were separately collected. The proportion of FITC-positive cells among 7-aminoactinomycin D$^-$ MHC class II^{hi} CD11c$^+$ cells was determined by flow cytometry. (**A**) Representative flow cytometry data. Shaded area = LNs draining vehicle-treated ears (lower number [%]); solid lines = LNs draining FITC-treated ears (upper number [%]). (**B**) Data show the mean + SEM of values for individual mice. Open columns = AR$^{+/-}$ mice (n = 7), and solid columns = AR$^{-/-}$ mice (n = 8). $^{**}p < 0.01$ *vs.* LNs draining vehicle-treated ears.

were collected. For measurement of MPO activity, the supernatants were diluted with 10 mM citrate buffer (pH 5.0), followed by addition of an equal volume of substrate solution for MPO assay (3 mM 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich), 120 mM resorcinol (Wako), 2.2 mM H_2O_2 (Wako) in distilled water). For measurement of EPO activity, the supernatants were diluted with 50 mM PIPES-NaOH (pH 6.5) containing 6 mM KBr, followed by addition of an equal volume of substrate solution for EPO assay (3 mM o-phenylenediamine (Sigma-Aldrich), 8.8 mM H_2O_2 (Wako) in 50 mM PIPES-NaOH (pH 6.5) containing 6 mM KBr). After 0.5 to 2 minutes, 2N H_2SO_4 was added to stop the reaction, and the absorbance was measured using a plate reader (MPO, 450 nm; EPO, 490 nm). The amount of total protein in ear tissue homogenate was measured using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). MPO and EPO activities were shown as the absorbance per gram of total protein in ear tissue homogenates.

MEASUREMENT OF FITC-SPECIFIC Ig LEVELS

Serum was collected from naïve mice and mice 8 days after FITC challenge during FITC-induced CHS responses. FITC-conjugated OVA was prepared as described elsewhere.[21,24] A 96-well ELISA plate (Nunc, 442404) was coated with 2 μg/ml FITC-OVA at 4℃ overnight. After the wells were blocked with PBS containing 10% FCS, optimally diluted serum samples (IgG1, 1/1000; IgG2a, 1/10; IgG2b, 1/10; IgG3, 1/10; and IgE, undiluted) were applied, and the plates were incubated at room temperature for 1 hour. After washing, biotinylated anti-mouse IgG1 (A 85-1; BD Biosciences), IgG2a (R19-15; BD Biosciences), IgG2b (R12-3; BD Biosciences), IgG3 (R 40-82; BD Biosciences) or IgE (R35-118; BD Biosciences) mAb was added, followed by incubation at room temperature for 1 hour. Then, after washing, HRP-conjugated streptavidin (BD Biosciences) was added, followed by incubation at room temperature for 1 hour. For enzymatic reaction, TMB substrate (KPL) was used as a substrate. The reaction was stopped by addition of 2N H_2SO_4, and then the absorbance was measured using a plate reader at 450 nm. Data show the absorbance value at 450 nm.

STATISTICAL ANALYSES

Data show the mean +/± SEM. Differences were evaluated by the two-tailed Student's t test and considered significant at a P value of less than 0.05.

RESULTS

AMPHIREGULIN IS NOT ESSENTIAL FOR MIGRATION OF SKIN DENDRITIC CELLS FROM SKIN TO DRAINING LNs AFTER EPICUTANEOUS FITC TREATMENT

Langerhans cells (LCs) and dermal dendritic cells (DCs) are important in the sensitization and elicitation phases of CHS. Activated keratinocyte-derived cytokines such as IL-1 and TNF are crucial for migration of LCs/dermal DCs from the skin to draining LNs.[2,3,7] AR can enhance activation of keratinocytes,[25] implying the ability of AR to promote LC/dermal DC

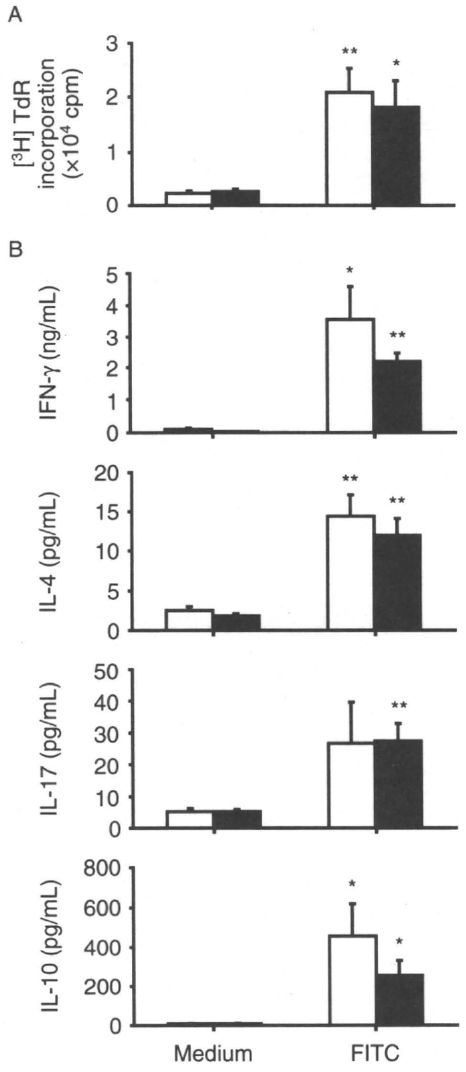

Fig. 2 AR deficiency did not influence FITC-specific LN cell responses. Mice were epicutaneously treated with 2.0% FITC on the ear skin. Six days later, submaxillary LN cells were collected and cultured in the presence and absence of 40 μg/ml FITC for 72 hours. (**A**) Incorporation of [3H]-thymidine and (**B**) the levels of IFN-γ, IL-4, IL-10 and IL-17 in the culture supernatants shown in (**A**) were determined. Data show the mean + SEM of values for individual mice. Open columns = $AR^{+/-}$ mice (n = 6), and solid columns = $AR^{-/-}$ mice (n = 6). *p < 0.05 and **p < 0.01 *vs.* LNs draining vehicle-treated ears.

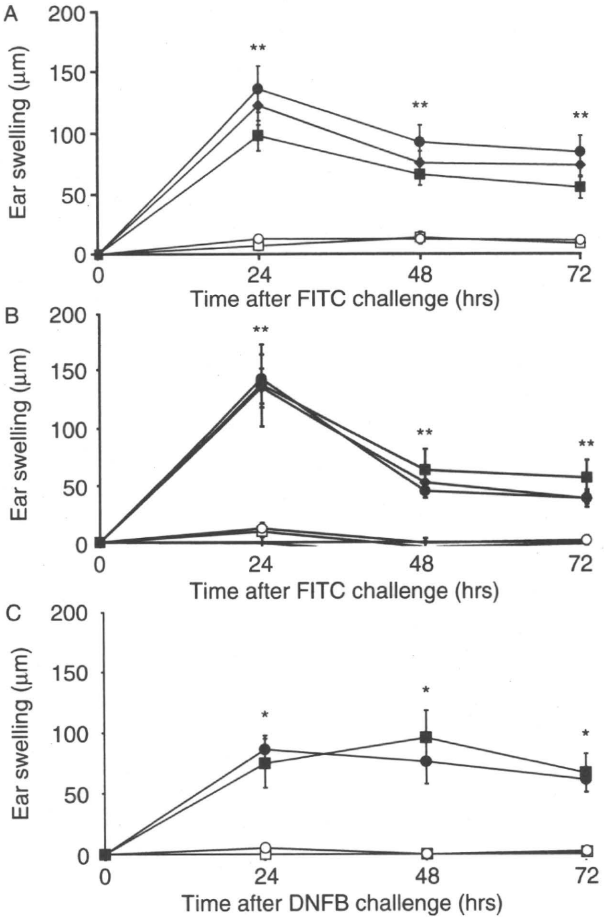

Fig. 3 AR is not required for development of FITC- and DNFB-induced CHS. Five days after epicutaneous sensitization with 0.5% (**A**), 2.0% FITC (**B**), and 0.5% DNFB (**C**), mice were challenged with 0.5% FITC (**A, B**) or 0.2% DNFB (**C**) (left ears) and vehicle alone (right ears). Before (0) and after challenge, ear thickness was measured using an engineer's caliper. Data show the mean ± SEM of values for individual mice. Open symbols = vehicle-treated ears, and closed symbols = FITC-treated ears. Diamonds = $AR^{+/+}$ mice (**A**; n = 7, **B**; n = 9), squares = $AR^{+/-}$ mice (**A**; n = 21, **B**; n = 8, and **C**; n = 6) and circles = $AR^{-/-}$ mice (**A**; n = 22, **B**; n = 7, and **C**; n = 4). *p < 0.05 and **p < 0.01 *vs.* vehicle-treated ears.

migration. To assess the role of AR in skin LC/DC migration, we treated AR-deficient and -sufficient mice with FITC epicutaneously. Twenty-four hours after FITC treatment, the proportion of FITC-positive cells among MHC class II^{hi} $CD11c^{+}$ cells in draining LNs was determined by flow cytometry. The proportion of FITC-positive cells in draining LNs from AR-deficient mice was equivalent to that from the littermate control mice (Fig. 1). These results indicate that AR is not essential for skin DC migration after epicutaneous FITC treatment.

AMPHIREGULIN IS NOT ESSENTIAL FOR DEVELOPMENT OF HAPTEN-SPECIFIC MEMORY T CELLS

After hapten-carrying skin DCs migrate from skin to draining LNs, these cells present the antigens to naïve T cells and induce allergen-specific effector and memory T cells in the sensitization phase of CHS.[2,3] To elucidate the role of AR in hapten-specific memory

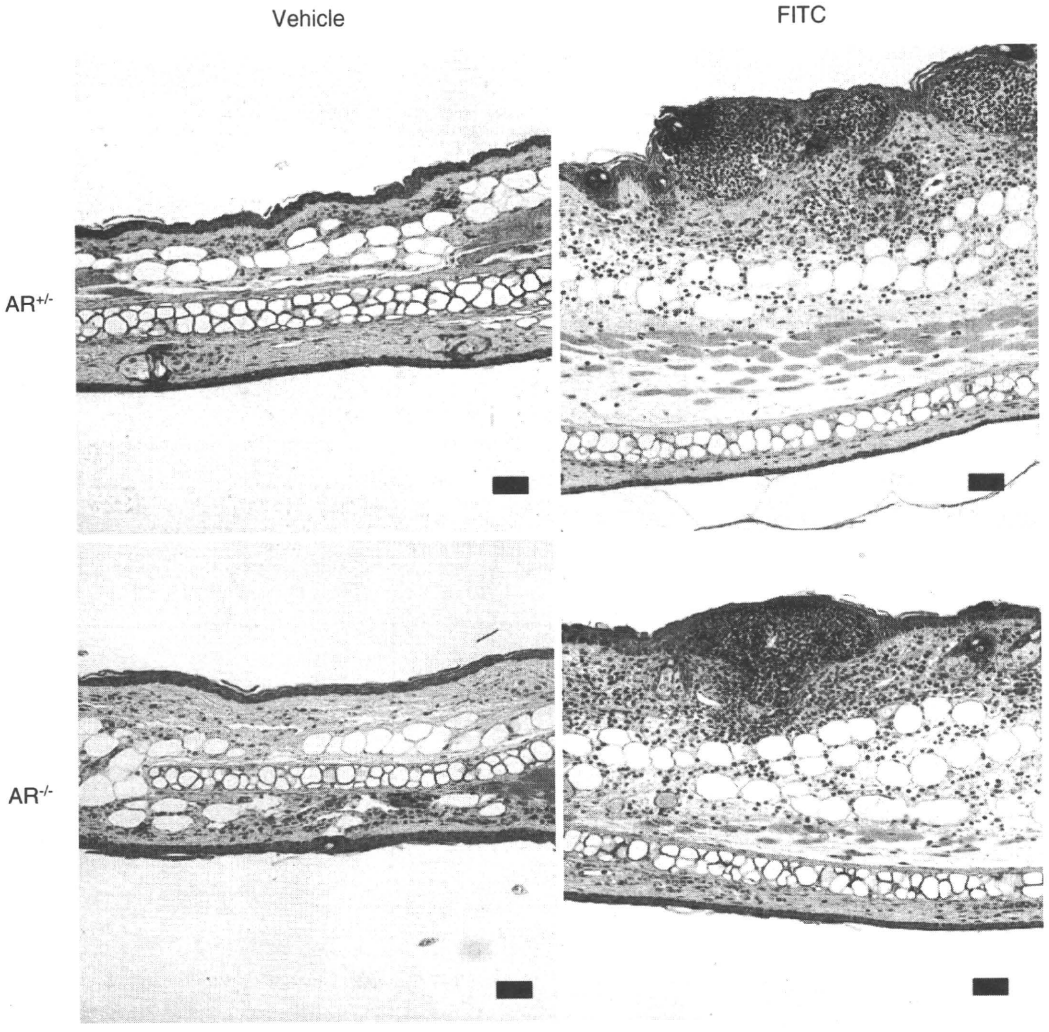

Fig. 4 Inflammation is normal in AR-deficient mice during FITC-induced CHS. At 24 hours after FITC and vehicle treatment, ear tissues were collected and embedded in paraffin. Sections of ear tissues were prepared and stained with hematoxylin and eosin. Representative data are shown. Scale bars = 100 μm.

T-cell induction, draining LN cells from FITC-treated mice were cultured in the presence of FITC *in vitro*. FITC-specific LN cell proliferative responses evaluated using [^{3}H]-labeled thymidine were comparable between AR-deficient and -sufficient mice (Fig. 2A). The AR-deficient and -sufficient mice also showed similar levels of IFN-γ, IL-4, IL-10 and IL-17 in their LN cell culture supernatants (Fig. 2B). These observations indicate that AR is not important for the generation of hapten-specific T cells in the sensitization phase of FITC-induced CHS.

AMPHIREGULIN IS NOT ESSENTIAL FOR DEVELOPMENT OF FITC-INDUCED CHS

It is known that Th2 cytokines such as IL-4 and IL-5 and mast cells are important for the development of FITC-induced CHS.[16,21] Moreover, in the microarray analysis, we found that mRNA expression of AR was increased 10.47-fold in FITC-challenged ears in comparison with vehicle-treated ears at 24 hours after the treatment (n = 3, data not shown). These observations suggest that AR may be involved in the development of FITC-induced CHS. However, AR-deficient mice showed similar levels of ear swelling as compared with AR-sufficient mice during both FITC- and DNFB-induced CHS (Fig. 3). Also, histological analysis found the same degree of inflammation in AR-deficient and -sufficient mice at 24 hours after FITC challenge (Fig. 4).

The inflammation in FITC-induced CHS was associated with infiltration of neutrophils and eosinophils.[16] We examined the activities of MPO and EPO in the ear tissue homogenates at 24 hours after FITC challenge. Consistently, MPO and EPO activities in the ear tissue homogenates did not differ significantly between the AR-deficient and -sufficient mice during FITC-induced CHS (Fig. 5).

We next used ELISA to examine the levels of FITC-

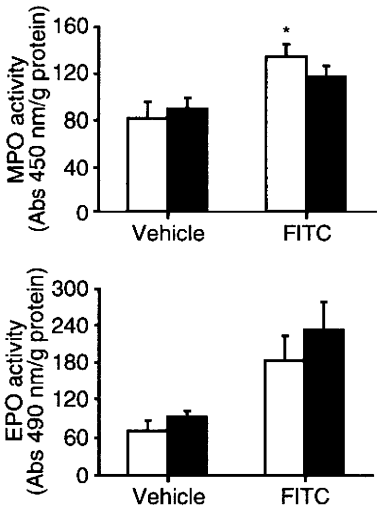

Fig. 5 MPO and EPO activities are normal in ear tissue homogenates from AR-deficient mice during FITC-induced CHS. At 24 hours after FITC and vehicle treatment, ear tissues were collected and homogenates were prepared. The activities of MPO and EPO in ear tissue homogenates were determined by colorimetric assay. Data show the mean + SEM of values for individual mice. Open columns = $AR^{+/-}$ mice (n = 3), and solid columns = $AR^{-/-}$ mice (n = 3). *p < 0.05 *vs.* vehicle-treated ears.

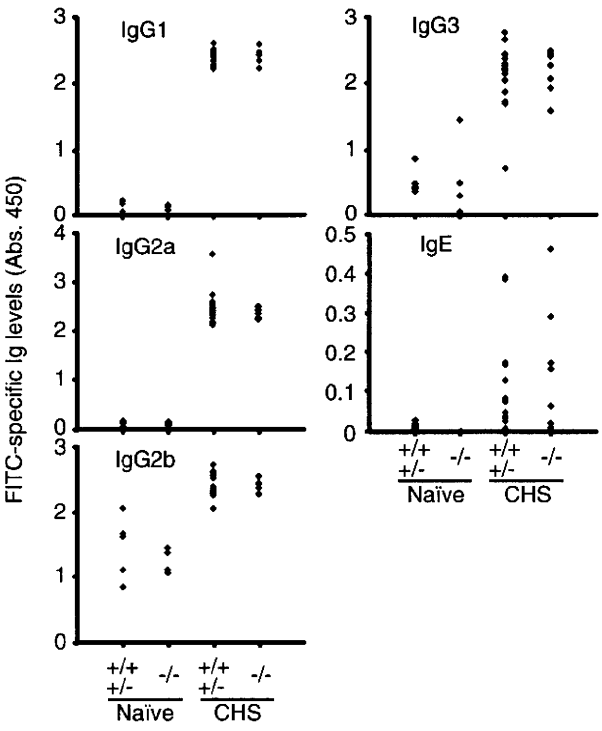

Fig. 6 Levels of FITC-specific Igs are normal in sera from AR-deficient mice during FITC-induced CHS. Blood was collected from naïve mice and mice 8 days after FITC challenge. The levels of FITC-specific IgG1, IgG2a, IgG2b, IgG3 and IgE in sera were determined by ELISA. Data show the mean + SEM of values for individual mice. Open columns = $AR^{+/-}$ mice (Naïve, n = 5; CHS, n = 8), and solid columns = $AR^{-/-}$ mice (Naïve, n = 4; CHS, n = 7). *p < 0.05 and **p < 0.01 *vs.* naïve mice.

specific Igs in sera from FITC-treated mice during FITC-induced CHS. The levels of FITC-specific IgG1, IgG2a, IgG2b, IgG3 and IgE in sera from AR-deficient mice were equal to those from AR-sufficient mice after FITC challenge (Fig. 6). Taken all together, these results indicate that AR is not essential for the development of FITC-induced CHS.

DISCUSSION

AR, a member of the EGF family of growth factors/cytokines, plays an important role in the enhancement and/or inhibition of cell growth and is involved in physiological responses such as mammary gland development, blastocyst implantation, bone formation and nerve regeneration as a growth factor.[17,18] As another functional aspect of AR, it was shown that Th2 cell-derived AR is involved in the pathogenesis of Th2 cell/cytokine-mediated host defense against nematodes.[19] In addition, we and others have demonstrated that mast cells can produce AR by IgE-FcεRI crosslinking, and AR enhanced not only mucin gene expression in human epithelial cells but also proliferation of human lung fibroblasts,[26,27] implying a contribution of AR to the pathogenesis of Th2 cell/cytokine- and mast cell-mediated immune responses, such as allergic diseases.

CHS is classically considered to be an IFN-γ-producing Th1- and Tc1-mediated allergic disease of the skin.[1] However, Th2 cells/cytokines rather than Th1/Tc1 cells/cytokines seemed to be potentially responsible for the development of CHS.[10,28] In particular, Th2 cytokines and mast cells are responsible for the development of CHS induced by FITC.[16,21] These observations suggest that AR may be involved in the development of Th2 cell/cytokine- and mast cell-associated CHS.

In the sensitization phase of CHS, activation of skin LCs/DCs is considered to be important for induction of hapten-specific effector/memory T cells.[2,3] Keratinocyte-derived proinflammatory cytokines such as TNF and IL-1 are crucial for LC/DC migration and maturation.[2,3,7] Since AR can enhance activation of keratinocytes,[25] it might be expected to play a role in that step during CHS. However, we found that the levels of skin DC/LC migration and the proliferation and cytokine production of hapten-stimulated LN cells were normal in AR-deficient mice. These observations indicate that AR is not crucial for migration of LCs/DCs from the skin into draining LNs or for induction of hapten-specific effector/memory T cells in the sensitization phase of CHS.

In the elicitation phase of CHS induced by FITC, we found (by DNA microarray analysis) that AR mRNA expression was approximately 10-fold increased in FITC-challenged ear skin at 24 hours after treatment in comparison with vehicle-treated ear skin. However, local inflammation, assessed by ear swelling, histological analysis and the levels of MPO and EPO activities in tissue homogenates at 24 hours after the FITC challenge, and the levels of hapten-specific serum Igs were also comparable between AR-deficient and -sufficient mice. Therefore, AR is not necessary for induction of inflammation in the elicitation phase of CHS.

Taken all together, we conclude that AR is not essential for the development of FITC-induced CHS in mice.

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Activation of human mast cells through the platelet-activating factor receptor

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Background: In human subjects platelet-activating factor (PAF) concentrations are markedly increased in the plasma after anaphylactic reactions, and these correlate strongly with the severity of the response. The mechanism for the systemic spread of mast cell (MC) activation in anaphylaxis is often assumed to relate to the hematogenous spread of allergen, but this is implausible, and amplification mechanisms need to be considered.

Objective: We have investigated the ability of PAF to induce human MC degranulation using skin, lung, and peripheral blood (PB)–derived cultured MCs and the signaling pathways activated in PB-derived MCs in response to PAF.

Methods: The expression of PAF receptor was investigated by means of RT-PCR and Western blot analysis. Cell-signaling pathways in PB-derived MCs in response to PAF were investigated by analyzing the effect of various inhibitors and the silencing of phospholipase C (PLC) mRNA on PAF-mediated histamine release.

Results: We show for the first time that PAF induces histamine release from human lung MCs and PB-derived MCs but not skin MCs. Activation of PAF receptor–coupled $G_{\alpha i}$ leads to degranulation through PLCγ1 and PLCβ2 activation in human MCs. PAF-induced degranulation was rapid, being maximal at 5 seconds, and was partially dependent on extracellular Ca^{2+}.

Conclusion: Our findings provide a mechanism whereby PAF mediates an amplification loop for MC activation in the generation of anaphylaxis. (J Allergy Clin Immunol 2010;125:1137-45.)

Key words: *Anaphylaxis, histamine, human lung mast cells, human peripheral blood–derived mast cells, human skin mast cells, platelet-activating factor receptor*

Mast cells (MCs) play a key role in IgE-dependent allergic diseases, such as asthma, rhinoconjunctivitis, and anaphylaxis, through the release of a variety of vasoactive and bronchospastic autacoid mediators and functionally diverse proteases, chemokines, and cytokines.[1,2] Anaphylaxis is a systemic life-threatening event characterized by local swelling, laryngeal edema, acute bronchospasm, urticaria, gut edema and dysmotility, and cardiovascular collapse. Respiratory embarrassment and cardiovascular collapse are the life-threatening events. Sometimes seemingly minute quantities of allergen can induce severe and fatal systemic reactions. MC activation is a central component of this anaphylactic response, a view supported by the finding of increased concentrations of MC-derived β-tryptase in the peripheral circulation.[3,4] The mechanism for the rapid systemic spread of MC activation in patients with anaphylaxis is often assumed to relate to the hematogenous spread of allergen, but this is perhaps implausible after oral exposure, and amplification mechanisms need to be considered (eg, through neurological reflexes).

Platelet-activating factor (PAF) has recently been implicated in the pathogenesis of anaphylaxis.[5-8] Not only does it make a major contribution to the anaphylactic response in murine models,[5-7] but also in human subjects PAF concentrations are markedly increased in the plasma after anaphylactic reactions, and these correlate strongly with the severity of the response.[8] Furthermore, the chief metabolizing enzyme for PAF, PAF acetylhydrolase, demonstrates reduced expression in patients with anaphylaxis.[8] There is evidence that PAF can activate human MCs through indirect mechanisms in both the skin and the lung. In the skin neural reflexes are required for the activation of MCs after administration of PAF,[9] and isolated human skin MCs do not degranulate in response to PAF directly.[10] In the lung one study reported that PAF induced the release of arachidonic acid metabolites from human lung fragments but not purified MCs, although cells from only 2 donors were studied.[11] Whether PAF induces human lung MC degranulation, arachidonic acid metabolism, or cytokine production is therefore not known.

In this study we have investigated the ability of PAF to activate MCs using primary cells isolated from the skin and lung and cells generated *in vitro* from adult peripheral blood (PB)–derived progenitors. We show for the first time that PAF induces histamine release from lung MCs and PB-derived MCs but not skin MCs and

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Abbreviations used
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
GPCR: G protein–coupled receptor
IMDM: Iscove modified Dulbecco medium
MC: Mast cell
MRGX2: Mas-related G protein–coupled receptor family member X2
PAF: Platelet-activating factor
PAF-R: Platelet-activating factor receptor
PAI-1: Plasminogen activator inhibitor 1
PB: Peripheral blood
PG: Prostaglandin
PKC: Protein kinase C
PLC: Phospholipase C
PTX: Pertussis toxin
shRNA: Short hairpin RNA

that this signals through PAF receptor (PAF-R)–coupled $G_{\alpha i}$ and activation of phospholipase C (PLC) γ1 and PLCβ2. Thus we provide a biologically plausible mechanism that links increased PAF concentrations in human anaphylaxis to an amplified airway MC response.

METHODS

Ethical considerations

The study was approved by the Ethics Committee of Nihon University, Dokkyo Medical University Koshigaya Hospital, and the Leicestershire Research Ethics Committee, and all subjects provided written informed consent in accordance with the Helsinki Declaration of the World Medical Association.

Isolation, purification, and culture of human lung and skin MCs

Macroscopically normal human lung or skin tissue was obtained during surgery. Lung and skin cells were dispersed from chopped lung specimens and foreskin specimens by means of an enzymatic procedure and purified by using magnetic bead affinity selection with the anti-CD117 mAb (clone AC126; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), respectively, as described previously.[12] The purity of human MCs, as assessed with metachromatic staining, was more than 97%. In some experiments the dispersed lung or skin cells were cultured in serum-free methylcellulose medium, as previously described.[13] After 8 weeks of culture, methylcellulose was dissolved in PBS. The purity of human MCs, as assessed with metachromatic staining, was greater than 98%.

Cell activation, mediator releases, and cytokine production

MCs were suspended in HBSS supplemented with 1% FCS and 1 mmol/L $CaCl_2$ for histamine and prostaglandin (PG) D_2 experiments. For cytokine experiments, MCs were cultured in Iscove modified Dulbecco medium (IMDM) containing stem cell factor (PeproTech EC Ltd, London, England) at 100 ng/mL and IL-6 (PeproTech EC Ltd) at 50 ng/mL with 2% FCS. MCs were challenged by means of incubation with PAF (Sigma-Aldrich, St Louis, Mo) or calcium ionophore A23187 (10^{-6} mol/L, Sigma-Aldrich) at 37°C for 30 minutes for measurement of histamine release and PGD_2 synthesis and for 16 hours for measurement of cytokine production. For aggregation of FcεRI, MCs were sensitized by means of incubation at 37°C for 16 hours with 1 μg/mL recombinant human myeloma IgE (Calbiochem, Gibbstown, NJ). After washing, the cells were challenged with rabbit anti-human IgE antibody (Dako, Glostrup, Denmark) at 37°C for the time indicated. After incubation, cell-free culture supernatants and cell pellets were harvested to measure mediator levels. To assess the effect of various inhibitors of signal molecules on PAF (10^{-7} mol/L)– or IgE/anti-IgE (30 μg/mL)–induced histamine release, MCs were preincubated with the PAF-R antagonists CV-6209 (BIOMOL International LP, Plymouth Meeting, Pa) and BN52021 (BIOMOL International LP), the PLC inhibitor U73122 (Sigma-Aldrich), the Ca^{2+}-dependent protein kinase C (PKC) inhibitor Go6976 (Calbiochem), or the intracellular Ca^{2+} chelating agent BAPTA/AM (10 μmol/L, Calbiochem) for 30 minutes or with pertussis toxin (PTX; Calbiochem) for 2 hours before addition of PAF or anti-IgE.

Statistical analysis

Data are expressed as means ± SEMs. Data were submitted to statistical analysis by means of 1-way ANOVA and the Tukey-Kramer test for multiple comparisons ($P < .05$).

See the Methods section of this article's Online Repository at www.jacionline.org for additional information about the experimental protocols.

RESULTS

Comparison of G protein–coupled receptor expression profile between lung MCs and skin MCs: *In silico* study

MCs express various G protein–coupled receptors (GPCRs), and because the function of human MCs is modulated by various GPCR ligands,[14,15] the GPCRs expressed on MCs might play an important role in allergic diseases and anaphylaxis. The GPCR ligands, such as anaphylatoxins C3a and/or C5a,[16] leukotrienes,[17] sphingosine-1-phosphate,[18] and PAF,[8] have been reported to be mediators of anaphylaxis, acting as signaling components within the MC and as circulating mediators. Our initial microarray analysis of GPCR expression in human lung and skin MCs revealed that 68 GPCR genes were expressed in lung MCs, skin MCs, or both. Both lung and skin MCs expressed 54 GPCRs, including *C3aR1*, *C5aR1*, *CYSLTR1*,[19,20] and *EDG1* (endothelial differentiation gene 1; sphingosine-1-phosphate receptor 1).[21] Six GPCRs were skin MC specific, including *GPRC5B*, *EBI2* (EBV-induced gene 2), and *MRGX2* (Mas-related GPCR family member X2). Eight GPCRs were lung MC specific, including *CRHR1* and *PAFR*. Table I shows lung and skin MC–specific GPCRs. Because PAF and MCs are implicated in the pathophysiology of anaphylaxis, we investigated further the expression and function of PAF-R on human MCs. The levels of PAF-R transcripts in human lung MCs, skin MCs, and PB-derived MCs were 12.7% ± 2.59% (n = 4), 2.83% (n = 2), and 15.6% ± 2.67% (n = 3) of levels of glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* mRNA, respectively (Table I).

Effect of PAF on histamine release from human MCs

In keeping with the expression of *PAFR* mRNA, lung MCs and PB-derived MCs released histamine in response to PAF in a concentration-dependent manner. However, skin MCs did not (Fig 1, *A-C*). The releasability of histamine from human lung MCs in response to PAF was variable and donor dependent (see Fig E1 in this article's Online Repository at www.jacionline.org). Therefore we used PB-derived MCs to examine further mediator production and the signaling pathway in response to PAF. The amount of PGD_2 released by PB-derived MCs in response to PAF was comparable with that released by IgE-sensitized MCs, which were stimulated with anti-IgE (Fig 1, *D*), whereas the amount of IL-8 and plasminogen activator inhibitor 1 (PAI-1) produced by PB-derived MCs in response to PAF was much lower than that produced by MCs after aggregation of FcεRI (Fig 1, *E* and *F*). To clarify the specific cytokine expression profile

TABLE I. Comparison of GPCR expression profiles between lung and skin MCs

Accession no.	GPCRs	Known/orphan	Lung MCs Mean	Lung MCs SEM		Skin MCs Mean		PB-MCs Mean	PB-MCs SEM		Lung/skin
Lung MC-specific GPCRs											
gb:AL031588	*CELSR1*	o	4.40	1.40	P	0.55	A	1.50	0.10	P	7.98
gb:AL567376	*GPR39*	o	6.00	4.02	P	1.03	A	0.20	0.10	A	5.85
gb:M80436.1	*PAFR*	k	12.7	2.59	P	2.83	A	15.6	2.67	P	4.46
gb:NM_017705.1	*PAQR5*	k	2.35	0.35	P	1.0	A	2.61	0.20	P	2.35
gb:NM_004382.1	*CRHR1*	k	0.51	0.05	P	0.46	M	0.76	0.18	A	1.13
gb:AI492234	*GPR92*	k	1.07	0.19	P	1.05	A	1.44	0.20	A	1.03
gb:NM_005288.1	*GPR12*	o	0.63	0.07	P	1.05	A	0.84	0.10	A	0.60
gb:AF348078.1	*GPR91*	k	0.39	0.07	P	0.76	A	6.50	4.12	P	0.52
Skin MC-specific GPCRs											
gb:M81778.1	*5-HT2C*	k	0.11	0.02	A	0.25	P	0.22	0.08	A	0.44
gb:AC002550	*GPRC5B*	o	1.04	0.11	A	2.33	P	0.43	0.19	A	0.44
gb:NM_000316.1	*PTHR1*	k	0.55	0.06	A	3.37	P	0.53	0.06	A	0.16
gb:NM_003991.1	*ETB*	k	1.24	0.37	A	12.0	P	2.60	0.13	P	0.10
gb:NM_004951.1	*EBI2*	o	0.43	0.10	A	4.50	P	0.58	0.13	A	0.09
gb:NM_054030.2	*MRGX2*	k	0.15	0.02	A	9.22	P	0.83	0.60	A	0.01

Data were expressed as means ± SEMs (lung MCs, n = 4; PB-derived MCs, n = 3) or means (skin MCs, n = 2) of mRNA expression (percentage GAPDH control). Lung/skin means a ratio of mRNA expression (percentage GAPDH control) of lung MCs to that of skin MCs.
A, Absent call; *M*, marginal call; *P*, present call; *k*, ligands are known; *o*, orphan receptor.

in MCs through PAF-R, we used a DNA array to compare cytokine expression levels between PAF-stimulated and anti-IgE–activated PB-derived MCs at 1 and 4 hours (see Fig E2 this article's Online Repository at www.jacionline.org). We found the cytokines induced in PAF-activated MCs were included in those induced by IgE/anti-IgE–activated MCs. Therefore there were no specific PAF-induced cytokines/chemokines. PAF upregulated mRNA for *CCL1*, *CCL3*, *CCL4*, *CSF1*, *CXCL3*, *EREG*, *IL8*, *INHBA*, and *SERPINE1* (PAI-1). Compared with FcεRI-mediated activation, PAF caused transient upregulation of mRNA for cytokines. The mRNAs for cytokines were upregulated in MCs by PAF at 1 hour but had waned at 4 hours, except for *CCL1* and *CCL3*, whereas they remained upregulated at 4 hours by IgE-mediated activation (see Fig E2). We next confirmed expression of mRNA for *PAFR* (Fig 2, *A*) and its protein in PB-derived MCs using RT-PCR and Western blotting (Fig 2, *B*), respectively. We confirmed that the sequence of *PAFR* expressed on human MCs was identical to that previously reported (data not shown).[22] We also confirmed the expression of *PAFR* in human bronchial MCs in bronchial biopsy specimens by using immunohistochemistry (Fig 2, *C*). *PAFR* was preferentially expressed in tryptase-positive chymase-negative MCs (Fig 2, *C* and *D*).

Ca^{2+} mobilization by PAF through PAF-R in PB-derived MCs

We then assessed the ability of PAF to signal through the PAF-R. Treatment of MCs loaded with the Ca^{2+}-sensitive dye Indo-1AM (Invitrogen, Carlsbad, Calif) with PAF resulted in a rapid increase in intracellular Ca^{2+} levels. This effect of PAF was concentration dependent (Fig 3, *A*). PAF also induced an intracellular Ca^{2+} flux in PB mononuclear cells (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). However, PAF did not induce a Ca^{2+} response in Jurkat cells (see Fig 3, *A*), which had no expression of PAF-R (Fig 2). As a positive control, treatment of MCs (Fig 3, *B*) and Jurkat cells (data not shown) with calcium ionophore A23187 also triggered an immediate increase in intracellular Ca^{2+} levels. Treatment of MCs with the PAF-R antagonists CV-6209 (Fig 3, *B*) and BN52021 (see Fig E3, *B*) inhibited PAF-induced intracellular Ca^{2+} flux in a concentration-dependent manner but not A23187-induced Ca^{2+} flux. These findings indicate that PAF can signal through the PAF-R on MCs.

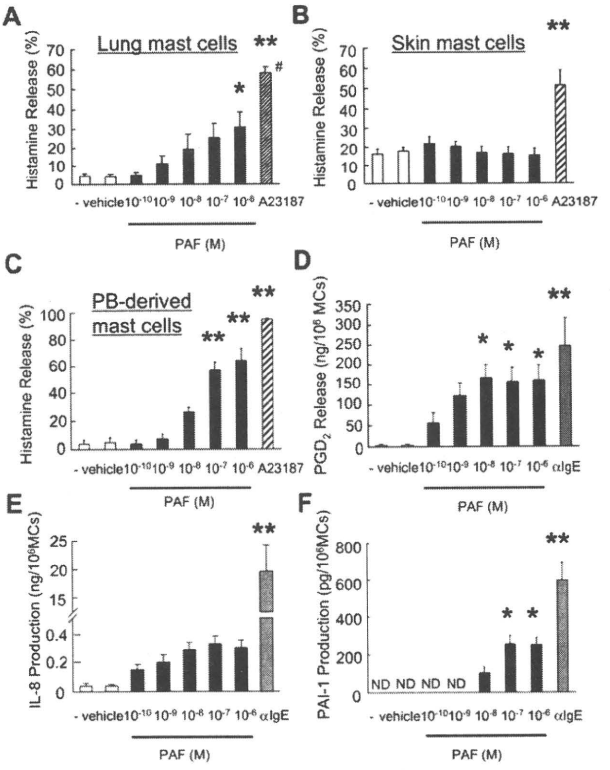

FIG 1. Mediators released by MCs in response to PAF. Histamine release by lung MCs (**A**, n = 7), skin MCs (**B**, n = 3), and PB-derived MCs (**C**, n = 3) in response to PAF and calcium ionophore A23187 (10^{-6} mol/L) are shown. PGD_2 (**D**, n = 5), IL-8 (**E**, n = 3), and PAI-1 (**F**, n = 3) production by PB-derived MCs in response to PAF and IgE/anti-IgE (α-IgE, 0.3 μg/mL) are also shown. Data shown are means ± SEMs. $*P < .05$ and $**P < .01$ compared with vehicle (0.1% chloroform). –, Buffer alone. #n = 3. *ND*, Not detected.

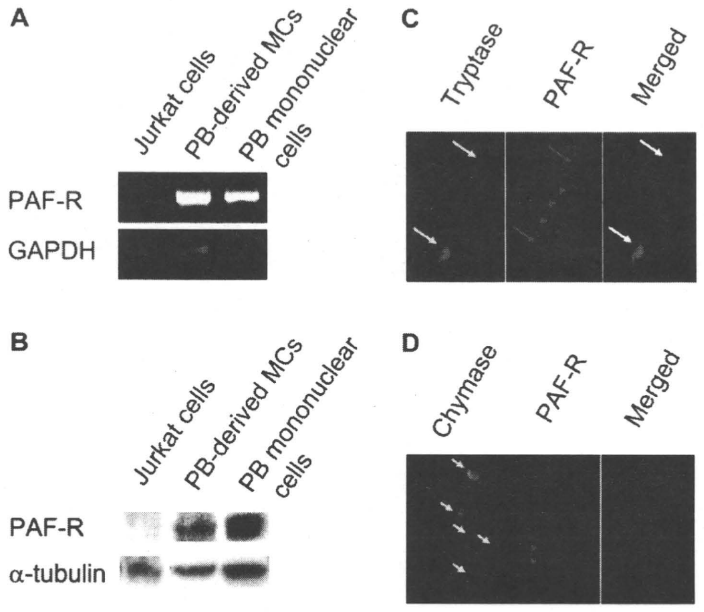

FIG 2. Expression of PAF-R in PB-derived MCs. **A**, *PAFR* mRNA expression was analyzed by means of RT-PCR. **B**, PAF-R protein expression was analyzed by means of Western blotting. Jurkat cells and PB mononuclear cells were used as negative and positive controls for PAF-R expression, respectively. The data are representative of similar results obtained from 3 independent experiments. **C** and **D**, Dual staining of bronchial MCs with anti–PAF-R antibodies (*red arrows* and *red arrowheads* indicate tryptase-positive PAF-R–positive cells and tryptase or chymase-negative PAF-R–positive cells, respectively; *middle*) and anti-tryptase mAb (**C**, *green arrows, left*) or anti-chymase mAb (**D**, *green arrows, left*). Yellow color indicates merged cells (Fig 2, *C, right*). The data are representative of similar results obtained from 6 independent experiments with MCs from different donors.

Effect of PAF on FcεRI-mediated histamine release and PGD_2 production by MCs

IgE-sensitized PB-derived MCs were simultaneously challenged with both PAF and anti-IgE to determine whether PAF synergizes with FcεRI-mediated histamine release and PGD_2 production. As shown in Fig E4, *A* (available in this article's Online Repository at www.jacionline.org), PAF at concentrations of 10^{-9} and 10^{-8} mol/L showed an additive effect on FcεRI-mediated histamine release, whereas PAF at higher concentrations than 10^{-8} mol/L showed a less than additive effect on FcεRI-mediated histamine release. PAF at concentrations of 10^{-10} and 10^{-6} mol/L showed a less than additive effect on FcεRI-mediated PGD_2 synthesis (see Fig E4, *B*), and even if suboptimal concentrations of PAF (10^{-9} mol/L) and the range of suboptimal concentrations of anti-IgE (0.03, 0.1, 0.3, and 1 μg/mL) were used in the experiment, simultaneous addition of PAF and anti-IgE to MCs showed a less than additive effect on PGD_2 synthesis (data not shown).

Time course of PAF-induced histamine release by MCs and effect of pretreatments of MCs with CV-6209 and PTX on PAF-induced histamine release

To clarify the PAF-R–mediated signaling pathways, we compared the kinetics of histamine release by MCs in response to PAF and IgE/anti-IgE and the effect of CV-6209 and PTX on PAF- or IgE/anti-IgE–induced histamine release. PAF induced rapid histamine release by MCs, reaching a maximum at 5 seconds (Fig 4, *A*). Compared with the kinetics of histamine release by PAF, FcεRI-mediated histamine release was relatively slow and reached a peak at 40 seconds. Treatment of MCs with CV-6209 and PTX significantly inhibited histamine release by PAF in a concentration-dependent manner, but they did not inhibit IgE/anti-IgE–dependent release (Fig 4, *B* and *C*). Therefore PAF induces degranulation through activation of a PTX-sensitive $G_{\alpha i}$ protein associated with the PAF-R.

Activation of PLC in PB-derived MCs by PAF

To investigate the involvement of activation of PLC, a downstream signaling molecule in PAF-R–mediated signaling,[23,24] we examined the effect of a PLC inhibitor, U73122, on PAF- and IgE/anti-IgE–induced histamine release by MCs. U73122 significantly inhibited PAF- and IgE/anti-IgE–induced histamine release by MCs in a concentration-dependent manner (Fig 5, *A*). Among the various isoforms of PLCβ and PLCγ, we observed that PLCβ2, PLCγ1, and PLCγ2 were dominantly expressed in PB-derived MCs, whereas the expression of PLCβ1 and PLCβ3 was not observed (Fig 5, *B*). PLCγ requires phosphorylation of tyrosine residues for activation, and both PLCβ and PLCγ isozymes require membrane localization for biological function.[25] Translocation of PLCβ2 to the membrane after activation was examined to investigate activation of PLCβ2 in MCs by IgE/anti-IgE and PAF. Both PAF and IgE/anti-IgE induced the translocation of PLCβ2 to the membrane (Fig 5, *C*). Densitometric analyses revealed that IgE/anti-IgE stimulation induced stronger phosphorylation of PLCγ1 than did PAF. PAF and IgE/anti-IgE stimulation did not have a significant effect on phosphorylation of PLCγ2 up to the 40-second time point (Fig 5, *D*). Therefore PAF and IgE/anti-IgE appeared to regulate activation of both PLCγ1 and PLCβ2, but the intensities of activation of PLC by PAF and IgE/anti-IgE were different.