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Mechanism of Action of Inhibition of Allergic Immune Responses by a Novel Antedrug TLR7 Agonist

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Triggering innate immune responses through TLRs is expected to be a novel therapeutic strategy for the treatment of allergic diseases. TLR agonists are able to modulate Th2 immune responses through undefined mechanisms. We investigated the mechanism of action of the suppression of Th2 immune responses with a novel antedrug TLR7 agonist. The antedrug is rapidly metabolized by plasma esterases to an acid with reduced activity to limit systemic responses. Topical administration of this compound inhibited features of the allergic airway inflammatory response in rat and murine allergic airways model. Type I IFN played a role in the suppression of Th2 cytokines produced from murine splenocytes. Inhibition of Th2 immune responses with the antedrug TLR7 agonist was shown to be via a type I IFN-dependent mechanism following short-term exposure to the compound, although there might be type I IFN-independent mechanisms following long-term exposure. We have demonstrated that local type I IFN signaling and plasmacytoid dendritic cells, but not Th1 immune responses, are required for *in vivo* efficacy against murine airway Th2-driven eosinophilia. Furthermore, migration of dendritic cell subsets into the lung was related to efficacy and is dependent on type I IFN signaling. Thus, the mechanism of action at the cytokine and cellular level involved in the suppression of Th2 allergic responses has been characterized, providing a potential new approach to the treatment of allergic disease. *The Journal of Immunology*, 2012, 189: 5194–5205.

The incidence of allergic diseases has increased in developed countries, and appears to correlate with the decrease in the incidence of infectious diseases as a result of antibiotics, vaccination, and improved sanitary conditions (1). The so-called hygiene hypothesis proposes that this decrease in the frequency of childhood infections that produce Th1 or regulatory T cell (Treg)-like responses results in the development of allergic (Th2-like) immune responses to common environmental Ags (1, 2). Asthma is a chronic disease characterized by eosinophilic airway inflammation due to a long-lasting Th2-like immune response to Ags (3), which could be prevented via induction of Treg phenotype cells (4). Studies of children growing up under different microbial exposures have shown that activation of innate immunity reduces Th2 immune responses such as IgE production (5).

TLRs are sensors of viral or microbial specific components and play a critical role in the innate immune response (6). Among these,

TLR7 is found on immune cells including B cells, monocytes/macrophages, and plasmacytoid dendritic cells (pDCs) (7). TLR7 recognizes synthetic ssRNA derived from RNA viruses such as HIV and influenza virus (8–10) and can be also triggered by low m.w. imidazoquinoline compounds (11). It has been shown that triggering TLR7 with a small m.w. compound can inhibit Th2-mediated immune responses in human PBMCs derived from allergic donors (12, 13). Many Th2 immune responses including IgE production, airway Th2 cytokine production, airway hyper-reaction, goblet cell metaplasia, and airway eosinophilia were suppressed by the systemic administration of a TLR7 agonist in a murine asthmatic model (14–18); this also involved induction of systemic proinflammatory cytokine production (17).

Recently, we reported the synthesis and biological evaluation of antedrug TLR7 agonists, which are designed for rapid metabolism to a product with substantially reduced activity (19, 20). Such compounds have the advantage over a metabolically stable TLR7 agonist by avoiding induction of potentially harmful systemic cytokine production. One novel antedrug TLR7 agonist suppressed airway eosinophilia in a rat allergic asthma model without causing systemic cytokine production.

In this article, we report our investigations into the mechanism of action of an antedrug TLR7 agonist (AZ12441970) in a murine airway eosinophilia model. We show that type I IFN has a role in the suppression of Th2 cytokine production *in vitro*. Inhibition of Th2 immune responses with a TLR7 agonist is via a type I IFN-dependent mechanism following short-term exposure to the compound. Subsequently, we provide evidence that type I IFN signaling is required for the efficacy observed with an antedrug TLR7 agonist *in vivo* (i.e., short-term exposure) using type I IFN receptor-deficient mice. Furthermore, inhibition of Th2 immune responses *in vivo* requires the presence of T cells. These findings help to characterize the mechanisms driving the efficacy of a potential new class of compounds for the treatment of allergic disease.

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Abbreviations used in this article: Alum, aluminum hydroxide; B6, C57BL/6; BAL, bronchoalveolar lavage; BchE, butyrylcholinesterase; BN, Brown-Norway; CpG, CpG-containing immunostimulatory oligodeoxynucleotide; DC, dendritic cell; FEV₁₀₀, forced expiratory volume in 100 ms; FVC, forced vital capacity; MLN, mediastinal lymph node; mPDCA-1, mouse plasmacytoid dendritic cell Ag-1; mTLR, mouse TLR; pDC, plasmacytoid dendritic cell; PEF, peak expiratory flow; Treg, regulatory T cell.

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Materials and Methods

Animals

All animal studies were performed according to the Animal Care and Use Committee of Dainippon Sumitomo Pharma Co., Ltd. or in accordance with UK Home Office legislation under license PPL40/2238, Protocol 5, and PPL40/2891, Protocol 2. Brown-Norway (BN) rats (200–250 g) were purchased from Harlan UK Ltd (Bicester, U.K.). C57BL/6 (B6) mice (6–8 wk old) were purchased from Charles River Japan Inc. (Hino, Japan). IFN- α/β receptor^{-/-} mice on the 129/Sv/Ev background were obtained from B & K Universal (Hull, U.K.) and backcrossed onto the B6 background for at least nine generations (IFN- α/β R^{-/-}). STAT1^{-/-} mice (21) on the 129S6 background and 129S6 mice, T-bet^{-/-} (22), Nude (*Homo*), Rag-2^{-/-} (23, 24), and OT-II $\times$ Rag-1^{-/-} (25, 26) mice on the B6 background were purchased from Taconic Farms (Germantown, NY). B cell-deficient mice on the B6 background (27) provided by MGC-Stiftung (Munich, Germany) were purchased from The Jackson Laboratory (Bar Harbor, ME). All of the animals were maintained under specific pathogen-free conditions.

Reagents

PE anti-mouse CCR3 (83103) was obtained from R&D Systems (Minneapolis, MN). Rat IgG2b isotype control Ab, anti-mouse CD16/CD32 (Fc γ III/IIIR) (2.4G2), FITC anti-mouse CD4 (H129.19), FITC anti-mouse CD8 α (53-6.7), FITC anti-mouse CD45R/B220 (RA3-6B2), Pacific Blue anti-mouse CD8 α (53-6.7), PerCP-Cy5.5 anti-mouse CD69 (H1.2F3), and PE-Cy7 anti-mouse CD11c (HL3), as well as allophycocyanin-Cy7 anti-mouse CD11b (M1/70), were purchased from BD Pharmingen (San Diego, CA). Allophycocyanin anti-mouse CD103 (2E7) and PE or nonconjugated anti-mouse pDC Ag 1 (mPDCA-1; JF05-1C2.4.1) were obtained from eBioscience (San Diego, CA) and Miltenyi Biotec (Bergisch Gladbach, Germany), respectively. The pDC-depleting Ab produced by the 120G8 hybridoma was purchased from AbCys S.A. (Paris, France). Anti-mouse IL-12 (C17.8) and anti-mouse IFN- γ (XMG1) were obtained from BD Pharmingen. Murine IL-4 and IL-2 were purchased from R&D Systems (Minneapolis, MN). OVA 323–339 peptide was obtained from Bio-Synthesis (Lewisville, TX). Aqua was obtained from Molecular Probes (Eugene, OR). Butyrylcholinesterase (BChE) was a product of Serotec (Kidlington, U.K.). AZ12441970 (Methyl 2-(3-(((3-(6-amino-2-butoxy-8-oxo-7H-purin-9(8H)-yl)propyl)(3-(dimethylamino)propyl)amino)phenyl)acetate), AZ12443988 (2-(3-(((3-(6-amino-2-butoxy-8-oxo-7H-purin-9(8H)-yl)propyl)(3-(dimethylamino)propyl)amino)phenyl)acetic acid), and R848 (1-[4-amino-2-(ethoxymethyl)imidazo[4,5-c]quinolin-1-yl]-2-methylpropan-2-ol) were synthesized in our laboratories (Medicinal Chemistry Facilities; AstraZeneca R&D Charnwood, Loughborough, U.K., and Dainippon Sumitomo Pharma, Osaka, Japan). Budesonide was obtained from Sigma-Aldrich (St. Louis, MO). OVA and aluminum hydroxide (Alum) were purchased from Sigma-Aldrich and Seikagaku Kogyo (Tokyo, Japan), respectively. Collagenase D was a product of Roche Diagnostics (Mannheim, Germany). Lympholyte M was purchased from Cedarlane Labs (Burlington, ON, Canada). PHA was obtained from Sigma (Dorset, U.K.). Human IFN- α Ab (MMHA2) and human IFN- α/β receptor Ab (MMHAR2) were purchased from R&D Systems.

TLR reporter assays

Human TLR7, rat TLR7, and human TLR8 reporter assay were performed as described previously (20). Mouse (m)TLR7, human TLR9, and mTLR9 reporter assays were carried out with HEK293-mTLR7 cells, HEK293-human TLR9 cells, and HEK293-mTLR9 cells, commercially available from InvivoGen. HEK293-TLR-Null cells, which were stably transfected with empty pUNO vector, were also purchased from InvivoGen as mock cells. These cells were transiently transfected with pNF- κ B-Luc reporter vectors (Stratagene, La Jolla, CA) using the transfection reagent FuGENE 6 (Roche Diagnostics) for 24 h. Then test compounds were incubated with these cells for 6 h at 37°C in 5% CO₂. The luciferase produced was quantified following addition of Bright-Glo Luciferase Assay System (E2610; Promega, Madison, WI).

Plasma stability determinations

The plasma stability of AZ12441970 was determined as described previously (20). Briefly, AZ12441970 (initial concentration of 1 μ M) was added to human, rat, or mouse plasma at 37°C. Supernatants were analyzed by liquid chromatography-tandem mass spectrometry for the remaining parent compound, and the half life was determined.

Ag-induced pulmonary inflammation in the OVA-sensitized BN rat

BN rats were sensitized by s.c. injection of 500 μ g OVA absorbed with 100 mg Alum in 0.4 ml at two sites (0.2 ml/site) on day 0. Animals

were challenged with aerosolized OVA solution for 15 min on day 14. AZ12441970 at 0.01, 0.1, and 1 mg/kg in a volume of 0.5 ml/kg was administered topically via the intratracheal route 2 h prior to and 24 h after OVA challenge. Animals were killed under anesthesia 48 h after the OVA challenge. The trachea was cannulated, and the airways were lavaged three times with 3 ml Isoton buffer (Coulter Electronics, Hialeah, FL). Cytospin slides were prepared in a Cytospin 3 centrifuge (Thermo Shandon, Runcorn, U.K.). Cellular differential was assessed on Wright-Giemsa-stained slides. Lung function was measured using a forced maneuvers system. Sensitized animals were challenged with aerosolized OVA solution on days 14 and 15. Budesonide at 1 mg/kg was administered 2 h prior to and 4 h after the OVA challenge. Animals were anesthetized, and the trachea was cannulated with a 5-cm yellow portex cannula securely tied in place with cotton. Animals were placed into a PLY 101 series plethysmograph (EMMS, Bordon, U.K.), which was connected to a CRFM 100 control panel (EMMS) and the tracheal cannula connected to the mouth port. The chamber was closed and the system allowed to equilibrate for 1 to 2 min. Between 48 and 96 h after the first OVA challenge, animals were anesthetized, and then the trachea was cannulated. Lung function parameters such as forced expiratory volume in 100 ms (FEV₁₀₀), peak expiratory flow (PEF), and forced vital capacity (FVC) were then assessed using the eDacq forced maneuvers system v1.6.0 (EMMS).

Th2 cytokine production from human PBMCs

Blood samples were obtained from volunteers in accordance with an ethically approved process, and all subjects gave written informed consent for their donations. PBMCs were isolated as described previously (20). The PBMCs were stimulated with PHA at a final concentration of 5 μ g/ml and then incubated for 2 d before the supernatants were removed for determination of the amount of IL-5 produced. BChE, the plasma esterase that cleaves AZ12441970, was added at indicated times after the PHA addition to give a final concentration of 1 U/ml.

Th1 and Th2 cytokine production from immunized murine splenocytes

Wild-type or IFN- α/β R^{-/-} mice were systemically sensitized by i.p. injection of 10 μ g OVA adsorbed in 4 mg Alum in 100 μ l on days 0 and 14. Spleens were removed from the immunized mice on day 21, and single-cell suspensions were obtained by homogenization with frosted glass slides; thereafter, they were passed through a 40- μ m nylon filter to remove larger aggregates of cells per tissue. RBCs were lysed by ammonium chloride (Invitrogen, Carlsbad, CA). The splenocytes (1 $\times$ 10⁶ cells/well in a 96-well plate) were stimulated with 0.5 mg/ml OVA for 3 d at 37°C in a 5% CO₂ incubator. The supernatant was removed for determination of the amount of IL-5, IL-13, and IFN- γ produced.

Allergic airway eosinophilia in mice model

Mice were sensitized by an injection (s.c.) of 10 μ g OVA adsorbed with 4 mg Alum in 100 μ l on days 0 and 14. The animals' lungs were challenged with an intranasal administration of OVA (0.25 mg/ml, 40 μ l) on day 22. AZ12441970 at 1 mg/kg in a volume of 40 μ l was administered to the lung via the intranasal route 24 h and 2 h prior to OVA challenge. Animals were killed under anesthesia 48 h after the OVA challenge. The trachea was exposed and cannulated with a 26-gauge needle, and the lungs were lavaged twice with 800 μ l PBS. Samples were centrifuged at 1,200 rpm for 5 min at 4°C, and supernatants were stored at -20°C for Th2 cytokine measurement. Cell pellets were resuspended in PBS containing 2% FCS, and the number of eosinophils in bronchoalveolar lavage (BAL) fluid was measured by flow cytometry, as described previously (28). BAL fluid cells were preincubated with anti-mouse CD16/CD32 Ab at 4°C for 15 min, and then incubated with FITC-CD4, FITC-CD8, FITC-CD45R/B220, and PE-CCR3 for 30 min at room temperature. Eosinophils were identified as CD4⁻CD8⁻CD45R/B220⁺CCR3⁺ cells within the small granulocytes gate (forward scatter^{low} versus side scatter^{high}) using a BD FACScan (BD Biosciences, San Jose, CA). The flow cytometry data obtained were analyzed using FlowJo software (Tree Star, San Carlos, CA). Mediastinal lymph nodes (MLN) were removed after BAL fluid collection. Single-cell suspensions of the MLN were obtained by homogenization with frosted glass slides; thereafter, they were passed through a 40- μ m nylon filter. The cells (1 $\times$ 10⁶ cells/well in a 96-well plate) were stimulated with 0.5 mg/ml OVA for 3 d at 37°C in an atmosphere of 5% CO₂. IL-5 or eotaxin in the BAL fluid and supernatant of the OVA-stimulated MLN cells was measured by ELISA kit, following the manufacturer's instructions.

Cytokines and chemokines measurement

Murine type I IFN concentration was measured using L929 cells transfected with luciferase reporter plasmid under the control of the promoter of the

type I IFN-inducible 2',5'-oligoadenylate synthetase gene (L929/2,5-OAS), as described previously (29). The luciferase reporter plasmids were constructed by inserting human 2',5'-oligoadenylate synthetase promoter fragments (−1262 to +108), which were isolated by PCR of human placenta genomic DNA (Oncor, Gaithersburg, MD), into the multicloning site of pGL3-basic plasmid (Promega). Samples were incubated with these cells for 20 h, and the luciferase produced was quantified following addition of Bright-Glo Luciferase Assay System, as described above, and the luminescence determined by comparison with a standard curve of mouse IFN- α (HC1040a; Hycult Biotech, Uden, The Netherlands). IFN- γ levels were determined using ELISA kit (OptiEIA Set; BD Biosciences). Murine IL-13 and IP-10 levels were assessed with ELISA Kit (R&D Systems). Mouse eotaxin-1 (CCL11) and eotaxin-2 (CCL24) levels in the BAL fluid were determined using ELISA kit (Ray Biotech, Norcross, GA). All other murine cytokines and rat IL-5, IL-13, and IFN- γ in the BAL fluid were determined using LINCOplex Cytokine Kit (Linco Research, St. Charles, MO) or BioSource Multiplex Kit (BioSource Division of Invitrogen, Camarillo, CA) on a Luminex 200 system (Luminex, Austin, TX) according to the manufacturer's instructions.

Murine DC subsets analysis in the lung tissue and MLN

Murine subsets of DCs were characterized as previously reported (30). The lung tissue and MLN were digested with 1 mg/ml collagenase D for 30 min at 37°C. Single-cell suspensions of the lung and MLN were obtained by homogenization with frosted-glass slides, following which they were passed through a 40- μ m nylon filter to remove larger aggregates of cells per tissue. RBCs were lysed by ammonium chloride. The lung lymphocytes were further recovered from RBCs, dead cells, or cell debris using Lympholyte M (Cedarlane Labs). DCs were enriched from the recovered lymphocytes with the Dynabeads Mouse DC Enrichment Kit (Invitrogen Dynal, Oslo, Norway), according to the manufacturer's protocol. The enriched DCs were preincubated with anti-mouse CD16/CD32 mAb at 4°C for 15 min, and then incubated with Pacific Blue-CD8a, PerCP-Cy5.5-CD69, PE-Cy7-CD11c, allophycocyanin-Cy7-CD11b, allophycocyanin-CD103, and PE-mPDCA-1 for 30 min at room temperature. Living cells were determined by staining with Aqua. The stained cells were quantified using flow cytometry on a BD FACSCanto II (BD Biosciences). Each DC subset was identified as mPDCA-1⁺CD11c⁺ (pDC), mPDCA-1[−]CD11c⁺CD8⁺ DC, mPDCA-1[−]CD11c⁺CD8[−]CD11b⁺CD103[−] (CD11b⁺ DC), and mPDCA-1[−]CD11c⁺CD8[−]CD11b[−]CD103⁺ cells (CD103⁺ DC). The data were analyzed using FlowJo software (Tree Star).

Depletion of murine pDC in vivo

Murine pDCs were depleted in vivo by i.v. injection of 300 μ g anti-mPDCA-1 and 40 μ g 120G8 Abs 1 d before AZ12441970 administration, as described previously (31, 32). Control mice were given an injection of 300 μ g isotype control Ab (rat IgG2b κ). pDC depletion was confirmed by flow cytometric analysis of mPDCA-1 and CD11c expression on splenocyte suspension and was consistent with previously reported data (33).

Statistical analysis

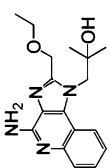
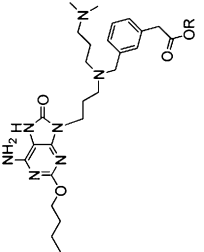
Results are presented as means $\pm$ SEM. Statistical analysis was performed using Dunnett's multiple comparison test or Student *t* test. The *p* values <0.05 were considered to be significant.

Results

Structure, TLR7 activation potency, and plasma stability of antedrug AZ12441970 and R848

The potency of AZ12441970 to activate TLRs was evaluated by measuring NF- κ B-dependent reporter gene expression in HEK293-expressing mouse, rat, or human TLR7 and compared with R848, a stable TLR7 agonist. The potency of the antedrug was comparable to that of R848 on human TLR7 activation and was slightly higher at mouse and rat TLR7 activation. There were no agonistic activities of AZ12441970 for TLR8 and TLR9 and no response in mock-transfected cells (Table I). These results demonstrate that the antedrug AZ12441970 is a specific agonist for TLR7. AZ12441970 was rapidly degraded in human, rat, and mouse plasma; the half life in human plasma was measured in minutes and seconds in rat and mouse plasma (Table I). AZ12443988, the acid metabolite converted from the methyl ester AZ12441970 in vivo, showed minimal activity in human, rat, or mouse TLR7. These data demonstrate

Table I. Structure, TLR activation potency, and plasma stability of R848, AZ12441970, and the metabolite AZ12443988

Compounds	Structure	TLR7 ^a pEC ₅₀		TLR8 ^a pEC ₅₀		TLR9 ^a pEC ₅₀		Mock ^a pEC ₅₀		Plasma t _{1/2} ^b (min)		
		Human	Mouse	Human	Rat	Human	Mouse	Human	Mouse	Human	Mouse	Rat
R848		6.8 $\pm$ 0.01	6.8 $\pm$ 0.04	6.4 $\pm$ 0.01	5.2 $\pm$ 0.03	<5.0	<5.0	<5.0	–	NT	NT	NT
AZ12441970 (R:Me)		6.8 $\pm$ 0.12	7.2 $\pm$ 0.10	6.6 $\pm$ 0.20	<5.0	<5.0	<5.0	<5.0	<5.0	1.2	0.03	0.07
AZ12443988 (R:H)		<5.0	5.4 $\pm$ 0.04**	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	NT	NT	NT

^aDose response curves of compounds (20 mM–10 μ M) were assayed to determine the potency of TLR activation, by measuring NF- κ B-dependent reporter gene expression in HEK293-expressing human, mouse, or rat TLRs. The activities were calculated from quantification of the activity of the reporter gene product secretory alkaline phosphatase from human TLR7, rat TLR7, and human TLR8-expressing cells or luciferase in mouse TLR7, human TLR9, and mouse TLR9-expressing cells, and mock-transfected cells. Data are mean $\pm$ SEM. ***p* < 0.01 compared with AZ12441970.

^bPlasma t_{1/2} was determined by incubating compound in human, rat, or mouse plasma and determining loss of parent compound by liquid chromatography-tandem mass spectrometry. NT, Not tested.

that AZ12441970 is a selective agonist for TLR7 and incorporates the antedrug concept, with the potential to have local activity via the active ester, which would be converted to a form with substantially reduced activity upon entry into the systemic circulation.

Suppression of various features of the asthmatic phenotype following AZ12441970 administration in a BN rat airway allergic model

AZ12441970 was evaluated for its ability to inhibit asthmatic end points in an in vivo model. Airway hyperresponsiveness was not induced in an allergic mice model with the described protocol. Therefore, various features of the asthmatic phenotype were assessed in a rat pulmonary inflammation model. Intratracheal administration of AZ12441970 showed a significant suppression of airway eosinophilia in a dose-dependent manner (Fig. 1A). Although Th2 cytokines such as IL-5 and IL-13 produced in BAL fluid were suppressed, IFN- γ was enhanced (Fig. 1B). In addition, AZ12441970 was compared with budesonide for its ability to reverse the lung function decline, as measured by FEV₁₀₀, PEF, and FVC. AZ12441970 significantly improved all of these lung function parameters; the effect was comparable to that of budesonide (Fig. 1C). These data confirm that the specific TLR7 agonist antedrug AZ12441970 suppresses various phenotypic features of asthma, such as airway eosinophilia, Th2 cytokine production in BAL fluid, and the declines in FEV₁₀₀, PEF, and FVC observed in this rat airway allergic model.

Type I IFN-independent mechanism of in vitro Th2 cytokine inhibition with long-term exposure of AZ12441970

In in vitro experiments, an antedrug compound is present in its active form throughout the incubation due to the absence of plasma esterases in the culture medium (long-term exposure). Therefore, AZ12441970 does not behave as an antedrug. AZ12441970 inhibited Th2 cytokine production in murine splenocytes, as determined by inhibition of IL-5 and IL-13 production, in a dose-dependent manner (Fig. 2A). In addition, AZ12441970 increased Th1 cytokine IFN- γ production. The potency was greater than that observed on NF- κ B-dependent reporter gene expression in TLR7-expressing HEK293 (Table I). To explore the factors playing a key role in the suppression, we determined whether type I IFN was involved in the inhibition of the Th2 cytokine production, as a TLR7 agonist is a powerful inducer of type I IFN (19). Results gained using splenocytes from IFN- α/β R^{-/-} mice showed that, although rIFN- α inhibited IL-5, IL-13, and IFN- γ production in murine splenocytes from wild-type mice in a dose-dependent manner, the suppression was completely lost in splenocytes from IFN- α/β R^{-/-} mice (Fig. 2B). These data show that IFN- α signaling was lost in cells from these animals. In contrast, inhibition of Th2 cytokine and increase in Th1 cytokine with AZ12441970 were not affected in splenocytes from IFN- α/β R^{-/-} mice (Fig. 2A). A similar phenomenon was observed in human PBMC, as IL-5 production was suppressed by AZ12441970, even in the presence of human type I IFN receptor-blocking Ab (Fig. 2C). Similar inhibition of IL-5 and IL-13 has been observed in an as-

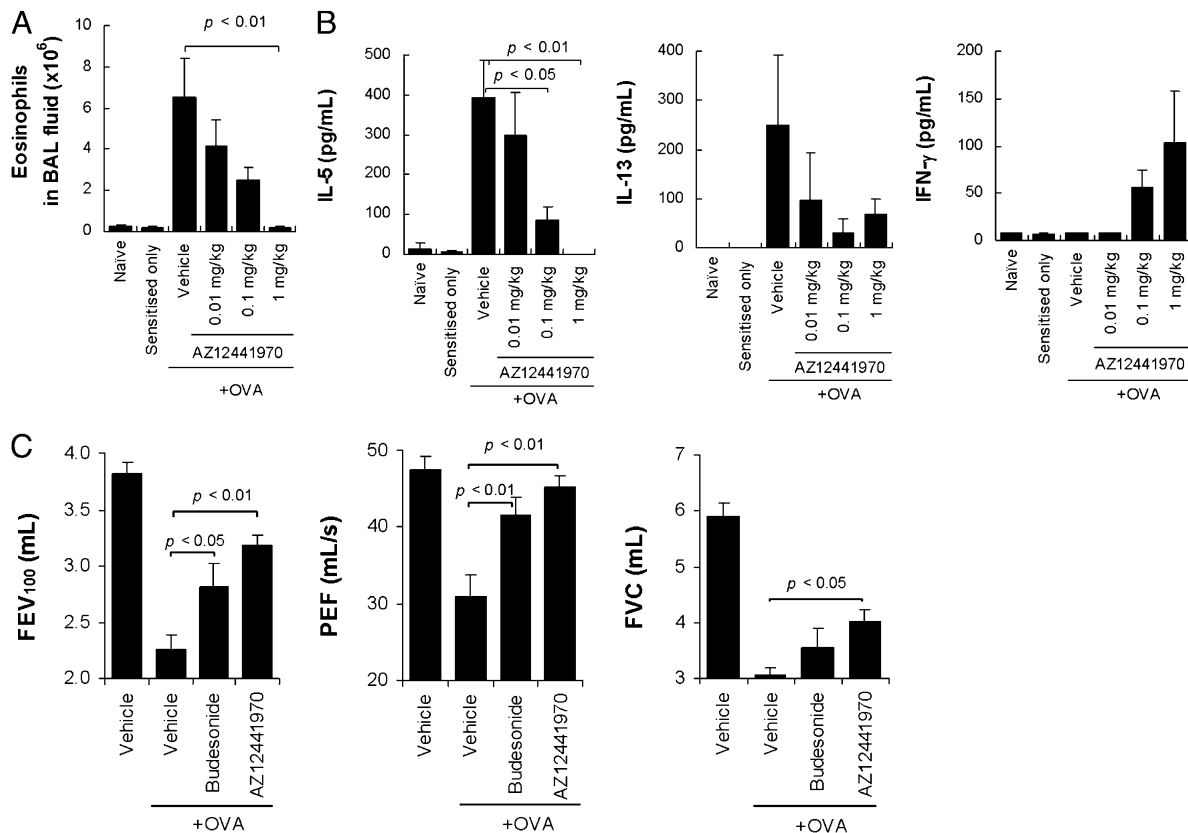


FIGURE 1. TLR7 agonist efficacy in the OVA-sensitized BN rat. OVA-sensitized BN rats were dosed intratracheally with AZ12441970 2 h prior to and 24 h after aerosolized OVA challenge. Two days after the challenge, the number of eosinophils (A) and concentration of IL-5, IL-13, and IFN- γ in BAL fluid (B) were determined. Lung function was measured using a forced maneuvers system after administration of 1 mg/kg of budesonide or 1 mg/kg of AZ12441970 to the OVA-challenged BN rat (C). Data are presented as mean $\pm$ SEM ($n = 6-8$ in each group). Similar results were obtained in two independent experiments.

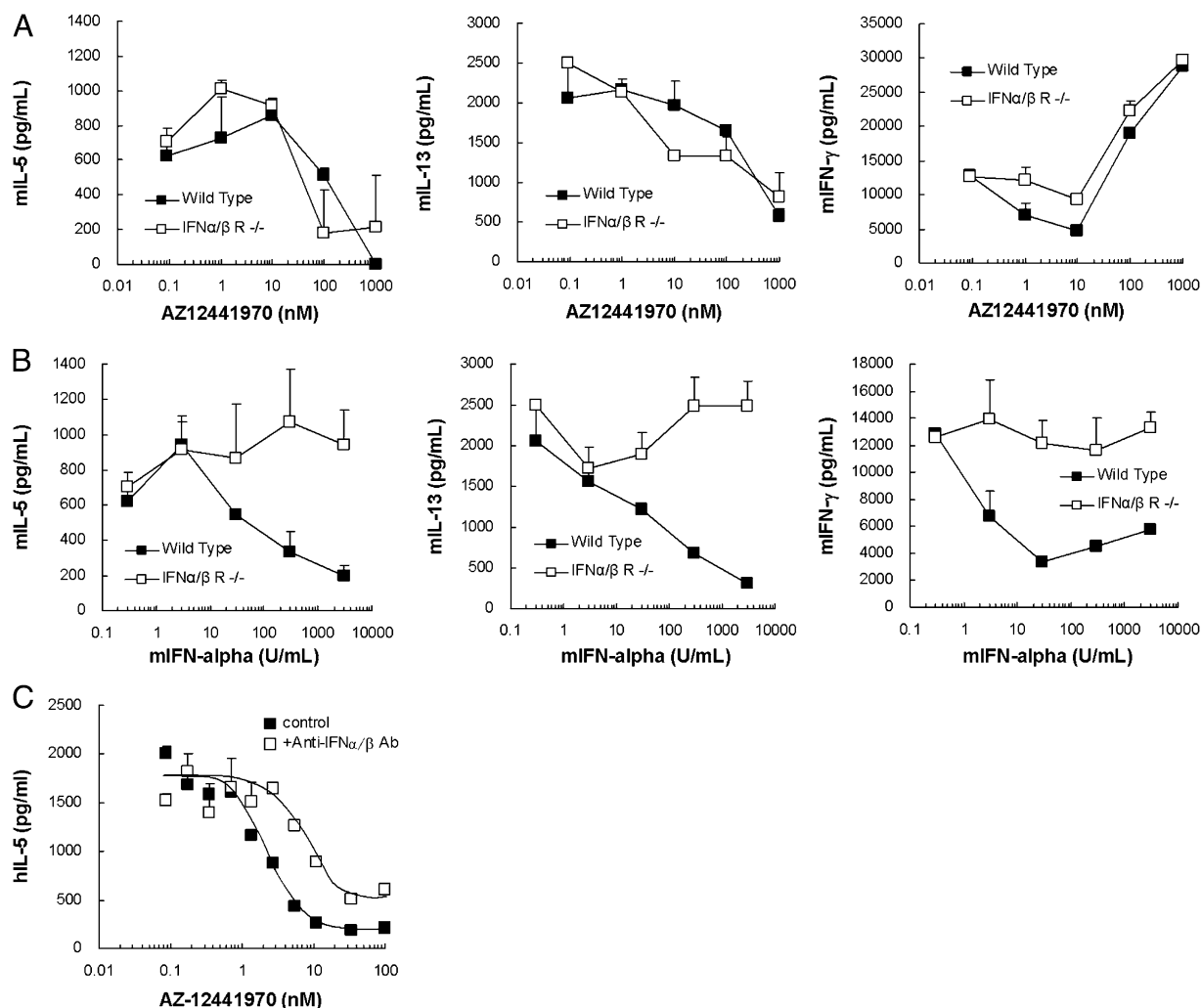


FIGURE 2. Type I IFN-independent mechanism of in vitro Th2 inhibition by long-term exposure of TLR7 agonist. Splenocytes from OVA-sensitized wild-type or IFN- α / β R $^{-/-}$ mice were incubated with AZ12441970 (A) or recombinant murine IFN- α (mIFN- α) (B) in the presence of OVA. Human PBMCs were incubated with AZ12441970 in the presence of 5 μ g/ml PHA $\pm$ anti-IFN- α Ab (C). Data are from a single experiment performed in triplicate. Similar results were obtained in two independent experiments.

assessment of 10 structurally related TLR7 agonists in human PBMCs (20). These observations revealed that although there is a clear IFN- α -mediated inhibition of Th2 cytokines, in vitro there is also a type I IFN-independent mechanism of both human and mouse Th2 cytokine inhibition with long-term exposure of AZ12441970.

Type I IFN-dependent mechanism of in vitro Th2 cytokine inhibition with short-term exposure of AZ12441970

The mechanism of Th2 cytokine inhibition by AZ12441970 in vitro might be different from the in vivo mechanism, in which esterases are present and exposure would be considerably shorter. To investigate this, Th2 cytokine inhibition by AZ12441970 was evaluated in vitro using human PBMCs in the presence of BChE to reduce the duration of exposure period of the active-form and mimic the short in vivo $t_{1/2}$ (short-term exposure). A BChE-specific inhibitor, ethephon, inhibited the hydrolysis of AZ12441970 by >90% in human plasma, and the level of BChE was sufficient to cause rapid hydrolysis of the antedrug to the inactive metabolite AZ12443988 (20). The potency of AZ12443988 to suppress Th2 cytokine production was evaluated, and the activity was far less than the parent compound, being inactive at 100 nM, a concentration at which AZ12441970 showed full inhibition (Fig. 3A). At an initial concentration of 100 nM, AZ12441970 still suppressed Th2 cytokine

in the presence of BChE, demonstrating that immune modulation was possible even with only short-term exposure to the active antedrug. When BChE was added to the incubation at the same time as AZ12441970, the addition of anti-IFN- α / β receptor Ab diminished the ability of AZ12441970 to suppress Th2 cytokine production, revealing a type I IFN component to the inhibition (Fig. 3B). As the time of addition of BChE was delayed, the influence of the anti-IFN- α Ab diminished and was largely lost at 60 min following the addition of AZ12441970. Taken together, these results suggest that Th2 cytokine inhibition by AZ12441970 under chemically unstable conditions, in which the exposure is very short, is predominantly dependent on the type I IFN signaling pathway.

Type I IFN dependence of airway eosinophilia suppression following intranasal AZ12441970 administration in a murine airway allergic model

Following the observation that short-term exposure of an antedrug TLR7 agonist could suppress Th2 cytokine production in vitro via type I IFN signaling, we examined whether AZ12441970-induced inhibition of Th2 immune responses in vivo was dependent on type I IFN signaling. The effect of type I IFN signaling on in vivo efficacy with AZ12441970 administered to the lung via the in-

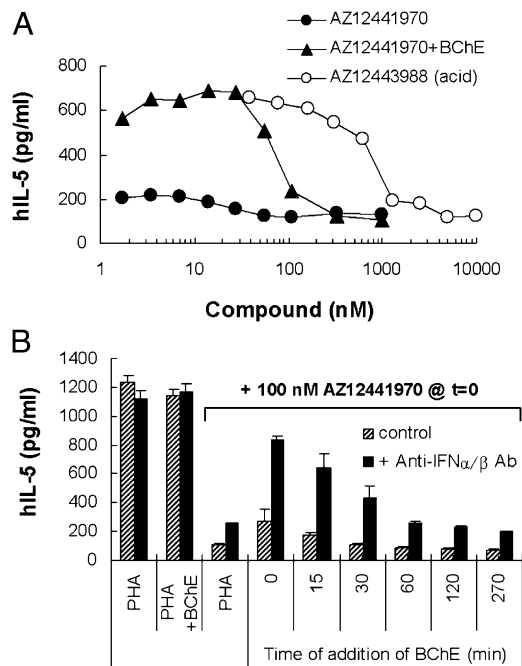


FIGURE 3. IFN-dependent mechanism of in vitro Th2 cytokine inhibition by short-term exposure to TLR7 agonist. Human PBMCs were incubated with antedrug TLR7 agonist AZ12441970 or the metabolite AZ12443988 in the presence or absence of BChE (**A**). In a further series of experiments, human PBMCs were incubated with AZ12441970 in the presence or absence of anti-IFN- α Ab, and BChE was added at the times indicated (**B**). IL-5 cytokine production from human PBMCs (hIL-5) was induced by stimulation with 5 μ g/ml PHA. The data are representative of two independent experiments.

transal route was assessed in an allergen-induced airway eosinophilia model using IFN- α/β R^{-/-} mice, in which type I IFN signaling is disrupted. As the eotaxin subfamily and its receptor play an important role in eosinophil recruitment into the pulmonary compartment (34, 35), CCL11 and CCL24 levels in the BAL fluid were measured. These eotaxin levels were not significantly increased after OVA challenge (Fig. 4A). Therefore, eotaxins are unlikely to be involved in the eosinophilic infiltration in this allergic mice model; a possible reason is that fewer OVA sensitizations and challenges were performed than in the other studies (34, 35). The efficacy of AZ12441970 against airway eosinophilia and IL-5 production was lost in IFN- α/β R^{-/-} mice compared with wild-type mice (Fig. 4B). Cellular signaling required for antiviral activity of the type I IFN is mediated through the IFN- α/β -STAT1 axis. As intranasal administration of AZ12441970 does not reduce airway eosinophilia and IL-5 in STAT1^{-/-} mice, the suppression of Th2 immune response in vivo by AZ12441970 is therefore expected to be mediated through the IFN- α/β -STAT1 axis (Fig. 4C). We next tested the effect of depletion of pDCs, the natural type I IFN-producing DCs, on efficacy following local treatment with AZ12441970 in an airway eosinophilia model. Efficacy with AZ12441970 was not observed in the pDC-depleting Ab treatment group, compared with the control Ab treatment group, again indicating a potential role for type I IFN derived from pDCs (Fig. 4D). Furthermore, we analyzed whether the suppression of Th2 immune responses with topical administration of AZ12441970 is dependent on the triggering of Th1 immune responses by using T-bet^{-/-} mice, in which the development of Th1 immune responses is defective. The efficacy of AZ12441970 against airway eosinophilia and IL-5 production in T-bet^{-/-} mice was comparable to that in wild-type animals (Fig. 4E). These findings indi-

cate that the efficacy against airway eosinophilia and production of Th2 cytokine by the local administration of antedrug TLR7 agonist is mediated by type I IFN released from pDCs acting through the IFN- α/β -STAT1 axis, but is independent of the modulation of Th1 immune responses. To identify what cells other than pDCs are required for efficacy against airway eosinophilia following local treatment with AZ12441970, the role of adaptive immune cells such as T cells or B cells was examined. Efficacy following local treatment of AZ12441970 against airway eosinophilia on the adoptive transfer model was largely lost in Rag-2^{-/-} mice, which lack both T cells and B cells (Supplemental Fig. 1A) as well as T cell-lacking Nude mice (Supplemental Fig. 1B), but was maintained in B cell-deficient mice (Supplemental Fig. 1C). These results imply that efficacy with an antedrug TLR7 agonist, when dosed locally, requires adaptive immune cells such as T cells, but not B cells.

Topical AZ12441970 triggers local cytokine production without systemic effects

Local versus systemic cytokine production was assessed following lung exposure of the antedrug TLR7 agonist AZ12441970 via the intranasal route and was compared with the stable TLR7 agonist R848. Although rapid systemic cytokine production of type I IFN, IP-10, and IL-12p40 occurred when R848 was administered to the lung by the intranasal route (Fig. 5B), these cytokines were not detected in the BAL fluid at the early time point of 90 min (Fig. 5A). In contrast, although a rapid systemic cytokine production following topical treatment of AZ12441970 did not occur (Fig. 5B), type I IFN, IP-10, IL-12p40, and IFN- γ were produced in BAL fluid at a later time point of 24 h (Fig. 5A). Topical administration of R848 did not induce local cytokine production even at this later time point (Fig. 5A). These observations indicate that local administration of a TLR7 agonist antedrug results in delayed local production of cytokines such as type I IFN, which is needed to suppress Th2 immune responses. This effect is achieved without causing systemic cytokine production and the subsequent induction of systemic events. In contrast, local administration of the stable TLR7 agonist R848 caused rapid systemic cytokine production with no delay in local cytokine production.

Type I IFN-dependent mechanism of delayed local cytokine production, CD69 expression on DC, and DC migration into the lung tissue

We next investigated whether a delayed local cytokine production was dependent on type I IFN signaling, using IFN- α/β R^{-/-} mice, and linked to the suppression of Th2 immune responses in vivo. Delayed local production of type I IFN, IP-10, and IFN- γ after local administration of AZ12441970 was suppressed in the absence of type I IFN signaling, but IL-12p40 production was not (Fig. 6A). IP-10 and IFN- γ production, which can be modulated by IL-12 production, were suppressed in IFN- α/β R^{-/-} mice following AZ12441970 treatment, even though IL-12 production was still observed. As type I IFN is required for expression of IL-12R β 2 (36), IL-12-mediated signal transduction would be defective, and subsequent events such as IFN- γ or IP-10 production would not be induced in IFN- α/β R^{-/-} mice. TNF- α was detected locally at 90 min after local administration of AZ12441970, and this rapid local production was not dependent on type I IFN signaling (Fig. 6B). These results suggest that the delayed local type I IFN production could be related to efficacy against airway eosinophilia, whereas rapid local TNF- α production or delayed local IL-12 production does not play a role in efficacy.

Subsequently, we evaluated the cellularity change in DC subsets in the lung or MLN following local treatment with AZ12441970.

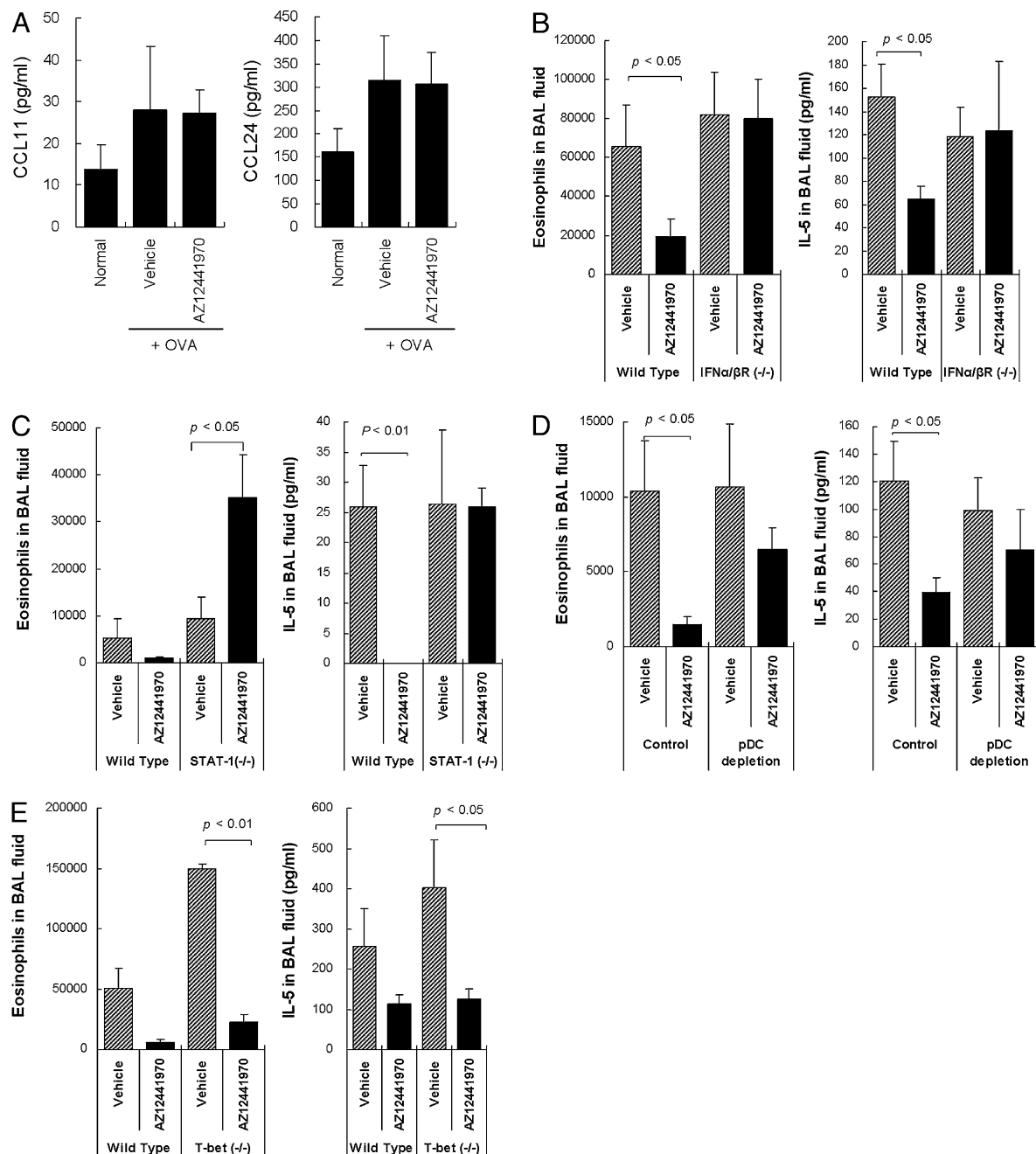


FIGURE 4. Type I IFN, STAT1, and pDC dependence of TLR7 agonist efficacy in vivo. CCL11 and CCL24 in BAL fluid were determined 24 h after the challenge (A). OVA-sensitized wild-type, IFN- α/β R $^{-/-}$ (B), STAT1 $^{-/-}$ (C), pDC-depleted (D), and T-bet $^{-/-}$ mice (E) were dosed intranasally with AZ12441970 (0.5 mg/ml, 40 μ l) 24 h and 2 h prior to intranasal challenge with OVA (0.25 mg/ml, 40 μ l). Two days after the challenge, the number of eosinophils and IL-5 concentration in BAL fluid were determined. Data are presented as mean $\pm$ SEM ($n = 4-6$ in each group). Similar results were obtained in two independent experiments.

To determine DC subsets in the lung or MLN, lymphocytes were prepared from the tissues, and DCs were negatively enriched using MACS technology and analyzed using flow cytometry. The number of DC subsets including CD103 $^{+}$ DC, CD11b $^{+}$ DC, CD8 $^{+}$ DC (only MLN), or pDC increased in the lung or MLN following the local treatment of AZ12441970 (Fig. 7A). Increases in CD103 $^{+}$ DC and pDC in the lung were largely lost in IFN- α/β R $^{-/-}$ mice. The number of CD11b $^{+}$ DC in the lung increased at 24 h but decreased at 48 h after administration of AZ12441970 in IFN- α/β R $^{-/-}$ mice (Fig. 7A). However, migration of DC subsets including CD103 $^{+}$ DC, CD11b $^{+}$ DC, CD8 $^{+}$ DC, or pDC into MLN following local administration of AZ12441970 were not affected

in IFN- α/β R $^{-/-}$ mice, compared with wild-type mice (Fig. 7A). Furthermore, we measured the upregulation of CD69, an activation marker mediated by type I IFN signaling, on DC subsets in the lung or MLN following local treatment with AZ12441970. CD69 upregulation on DC subsets in the lung and MLN by the local treatment of AZ12441970 was completely suppressed in IFN- α/β R $^{-/-}$ mice (Fig. 7A). These findings indicate that migration of DC subsets into the lung, along with CD69 upregulation, by local administration of AZ12441970 is dependent on type I IFN signaling and that this is not the case for the migration into MLN (Fig. 7A). To confirm whether these findings were dependent on type I IFN released from pDCs, the influence of pDC-

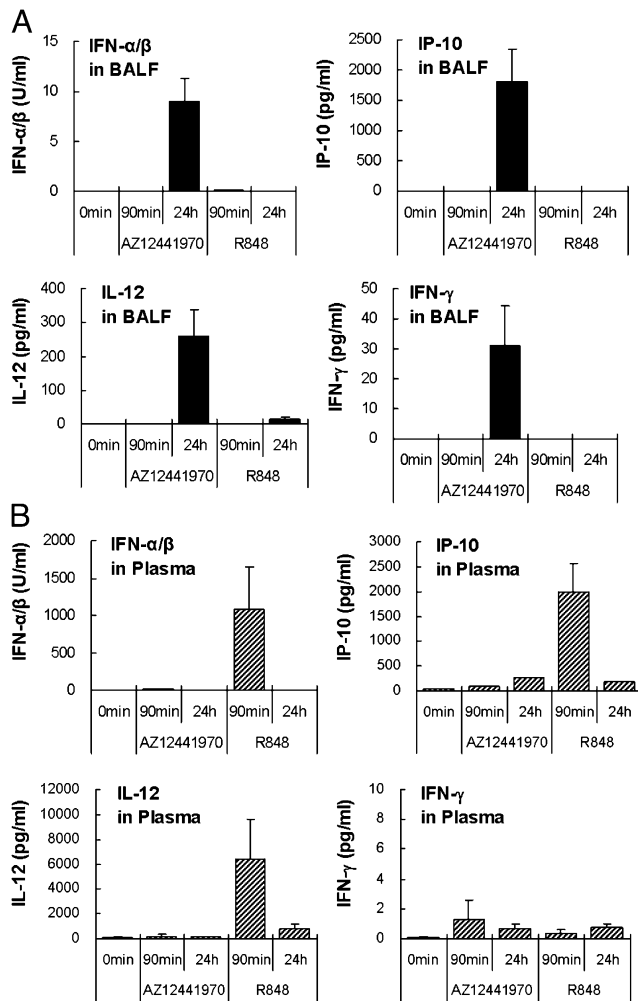


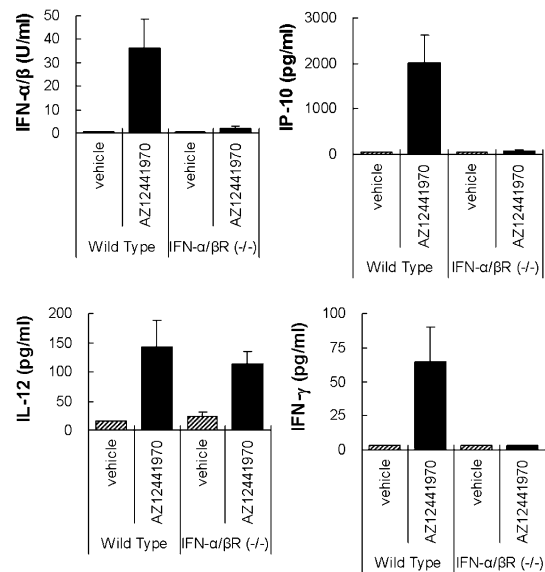
FIGURE 5. Delayed-type cytokine induction in BAL fluid 24 h after lung dosing of TLR7 agonist. Mice were administered AZ12441970 and R848 topically via the intranasal route (0.5 mg/ml, 40 μ l). After 90 min and 24 h, BAL fluid (BALF, **A**) and blood (**B**) were collected. Data are presented as mean $\pm$ SEM ($n = 3$ in each group). Similar results were obtained in two independent experiments.

depleting Abs on the lung migration of DC subsets was assessed. We confirmed that the pDC-depleting Ab effectively decreased the number of pDCs in the lung (Fig. 7B). Migration of DC subsets into the lung and CD69 upregulation on DC subsets in the lung following the local administration of AZ12441970 were completely lost in mice treated with pDC-depleting Abs compared with nondepleting Ab-treated mice (Fig. 7B). Taken together, these data demonstrate that the migration of DC subsets into the lung and the upregulation of CD69 on their cell surface are mediated by type I IFN released from pDCs, although there is a type I IFN-independent mechanism of migration into MLN that is not involved in CD69 upregulation. We also measured a Th2 response in the *in vitro* OVA-recall experiment using MLN cells from OVA-sensitized mice with or without AZ12441970 administration. No effect of AZ12441970 on IL-5 production was observed in MLN (Fig. 7C). This result suggested that the DCs that migrated into the MLN had no effect on the Th2 recall response in MLN.

Discussion

The incidence of allergic diseases has increased significantly in recent decades, particularly in western countries. Asthma, a chronic inflammatory disease of the airways, now affects 15–20% of the

A Delayed phase in BAL fluid (24h)



B Acute phase in BAL fluid (90 min)

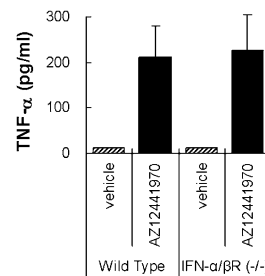
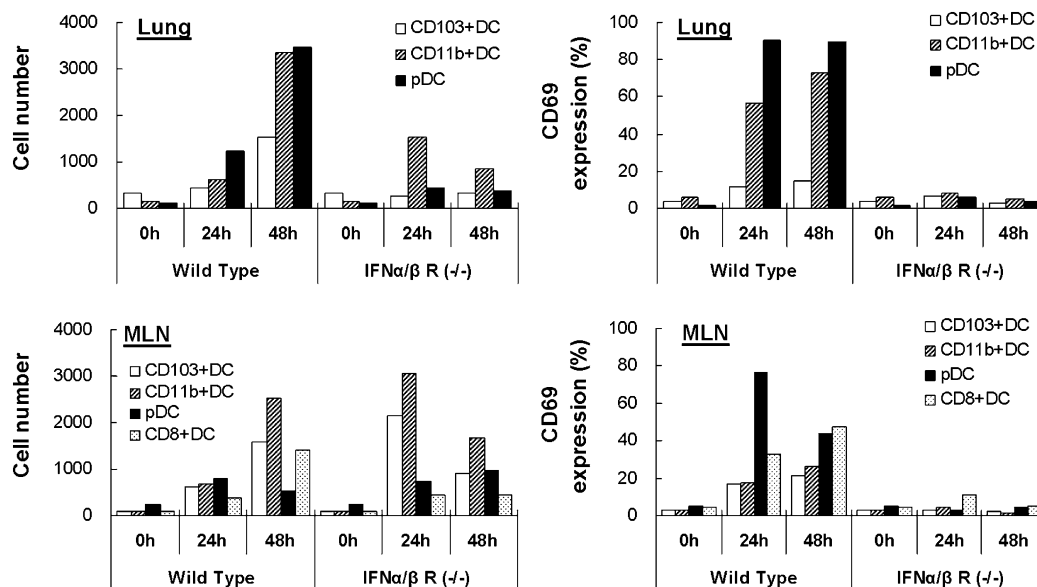


FIGURE 6. Delayed-type cytokine induction in BAL fluid 24 h after lung dosing of TLR7 agonist in IFN- α /BR $^{-/-}$ mice. Wild-type mice or IFN- α /BR $^{-/-}$ mice were administered AZ12441970 topically via the intranasal route (0.5 mg/ml, 40 μ l). BAL fluid was collected 24 h (**A**) or 90 min (**B**) after the administration. Data are presented as mean $\pm$ SEM ($n = 3$ in each group).

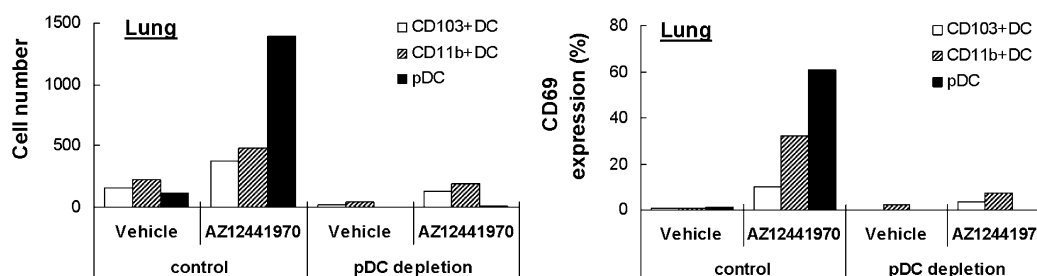
population in developed countries (37), whereas the prevalence of allergic rhinitis is estimated to range from 17 to 29% in Europe (38).

Triggering of TLRs (for example, TLR9) through the treatment of CpG-containing immunostimulatory oligodeoxynucleotides (CpG) (39), has shown promise for the treatment of allergic diseases. Results from the first clinical study of CpG therapy demonstrated that this treatment approach has limited efficacy when dosed to asthma patients prior to an Ag challenge (40). However, significant clinical benefits were observed in the subsequent allergy season when CpG was conjugated to an Ag and dosed to patients with allergic rhinitis (41, 42).

In animal models, triggering of TLR9 using CpGs has been shown to be effective against many features of allergic airway inflammatory responses (43–51). In recent mechanistic studies, this has been shown to be dependent on production of Th1 cytokines such as IFN- γ and IL-12 (43), induction of regulatory factors including upregulation of IDO (47, 48), and generation of Tregs (49), as well as functional impairment of APCs (50) and inhibition of DC migration (51). In terms of the dependency on Th1 cytokine generation, it is still unresolved as to whether IFN- γ or IL-12 is required for prevention of Th2 immune responses with CpG treatment (46). Many features of the mechanism of Th2 inhibition by CpG have been elucidated, but a unified theory has not been established.

A IFN- α/β R (-/-)

B pDC depletion



C IL-5 in MLN

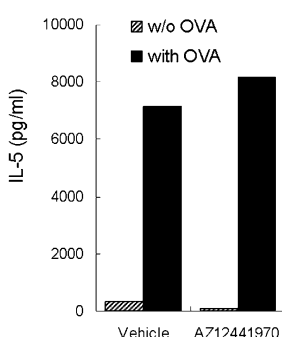


FIGURE 7. Changes in cellularity and CD69 expression on DC subsets in the lung and MLN following TLR7 agonist administration via intranasal route. Naive IFN- α/β R^{-/-} (A) and pDC-depleted mice (B) were administered AZ12441970 topically via the intranasal route (0.5 mg/ml, 40 μ l). Lung and MLN were removed at 24 and 48 h after the dosing of AZ12441970, and then DCs were enriched using Dynabeads Mouse DC Enrichment Kit (Invitrogen Dynal). CD69 expressions on DC subsets in the lung or MLN were characterized by FACS analysis. OVA recall responses were evaluated using MLN cells from OVA-sensitized mice following AZ12441970 administration. MLN cells were incubated with 0.5 mg/ml OVA for 3 d, and IL-5 in the supernatant was determined (C).

Both TLR7 and TLR9 are sensors for virus recognition following initiation of antiviral immune responses via type I IFN production from pDCs (7). Triggering TLR7 has been shown to inhibit Th2 immune responses (12–18), although this has also resulted in rapid systemic cytokine induction in a mouse model (17). In clinical studies, oral administration of R848 to hepatitis C virus-infected patients demonstrated efficacy with a reduction in viral titers, but caused severe adverse effects such as fever, headache, shivering, flulike symptoms, and lymphopenia (52). The TLR7 agonist 852A

caused a range of adverse effects including fever and fatigue when dosed i.v. to patients with cancer (53). Even though a lower frequency of adverse events was observed, i.v. injection of the TLR7 agonist isatoribine to patients chronically infected with hepatitis C virus resulted in fever, nausea, vomiting, and flulike symptoms as well (54).

To overcome these undesirable systemic events, Wu et al. (55) prepared a TLR7 agonist with low m.w. covalently coupled to a macro-molecule, mouse serum albumin. The molecule suc-

cessfully prolonged local cytokine production in the lung and antiviral efficacy in a pulmonary infectious disease model without causing systemic cytokine production when administered to the lung. An alternative approach may be to use an antedrug concept. An antedrug is defined as an active synthetic derivative that is designed to be readily metabolized to an inactive form upon entry into the systemic circulation.

We have previously reported the discovery of adenine derivatives as a novel class of IFN-inducing agents (56–59). Among these compounds, a representative compound has been identified as a novel class of TLR7 agonist (60). These compounds trigger undesirable systemic cytokine production. However, following an extensive research program, the introduction of a particular antedrug concept resulted in efficacy in the lung, in a rat allergic asthma model, without causing systemic cytokine production when dosed to the lung (19).

The present study demonstrated that type I IFN has a critical role in the suppression of Th2 cytokine production *in vitro* in both human and mouse. It has been reported that type I IFN inhibits IL-5 production from human PBMCs (61, 62). The IC_{50} values are ~ 100 IU/ml, which is ~ 1000 times higher than required for antiviral activity (~ 0.1 IU/ml) (63). The precise cellular events of IL-5 inhibition with type I IFN are unknown, but the mechanism does not seem to be the same as that of the antiviral activity, from the viewpoint of the extremely large difference in effective concentrations. Thus, the STAT1 requirement observed in the current study suggests that multiple cellular events under the control of type I IFN receptor/STAT1 signaling result in suppression of IL-5 production. In terms of the antedrug TLR7 agonist AZ12441970, type I IFN dependency of Th2 cytokine inhibition was most clearly observed *in vitro* following short-term drug exposure in the presence of BChE. There may be type I IFN-independent mechanisms of Th2 inhibition when the exposure of the TLR7 agonist is prolonged.

In this study, administration of AZ12441970 resulted in local cytokine production without inducing systemic cytokine production, confirming the utility of TLR7 antedrug development to reduce systemic effects. R848, a stable, nonantedrug TLR7 agonist, led to rapid systemic cytokine production at 90 min without local cytokine production at that time point. In contrast, local cytokine production after antedrug administration was observed at 24 h, but not at the earlier time point of 90 min. The delayed local cytokine production and migration of DC subsets into the lung were almost lost in IFN- $\alpha/\beta R^{-/-}$ mice compared with wild-type mice. These results suggest that interaction between infiltrating DC subsets could be required for the delayed local cytokine production. Cytokine production triggered by TLR9 agonist *in vivo* at later time points is dependent on the crosstalk between DC subsets (64). Thus, the delayed local cytokine production might be dependent on the crosstalk between DC subsets migrating from other compartments that are required for type I IFN signaling in the lung at an earlier time period. Delayed local IL-12 production was also observed following AZ12441970 treatment, but this production did not occur in a type I IFN-dependent manner despite *in vivo* conditions. TLR7 stimulation of pDC results in IFN- α production and subsequent type I IFN-dependent processes, whereas stimulation of TLR7 on other different cell types, such as cDCs, B cells, monocytes, and NK cells, can generate effects independent of type I IFN production, showing that not all of the *in vivo* TLR7 responses are type I IFN dependent.

The effect of AZ12441970 against airway eosinophilia and IL-5 required the type I IFN signaling and the natural type I IFN-producing cell, pDCs (65). Migration of DC subsets into the lung was lost in pDC-depleted mice compared with nondepleting Ab-

treated control animals. The lack of compound effect on eosinophil recruitment was consistent with that observed in IFN- $\alpha/\beta R^{-/-}$ mice. As delayed local cytokine production was lost in pDC-depleted mice, pDCs are required for crosstalk between DC subsets in the lung.

The Th2 response in MLN was not affected following AZ12441970 treatment, although pDCs were present in MLN. As the antedrug administered into the lung is rapidly metabolized in plasma and inactivated (20), it is unlikely the antedrug would act directly on the resident pDCs in the MLN. In addition, although migration of DCs was observed in both lung and MLN following TLR7 agonist, there could be some difference in characteristics of the DCs in the lung and MLN. Indeed, it has been reported that immature DCs, accumulated in the lung from the bloodstream under TLR7-stimulated inflammatory conditions, expressed various chemokine receptors such as CCR1, CCR2, CCR3, CCR5, CCR6, and CXCR4 (66). In contrast, the DCs that migrated into the MLN were matured in lung by TLR7 stimulation as shown by downregulation of CCR6 and upregulation of CCR7 (66). Therefore, it is likely that the DCs that migrated into the MLN could differ from the ones that accumulated in the lung. Our data suggest that the lung DCs mediate suppression of allergen-stimulated effects, whereas the MLN DCs do not. This offers the opportunity to further explore the mechanism of these two sets of DCs. Indeed, accumulation of the DCs into the lung was type I IFN dependent, whereas migration into the MLN was type I IFN independent. Regarding CD69 expression on DC subsets in MLN, there is a possibility that the DCs activated by TLR7 agonist in the lung could migrate into MLN, so type I IFN-dependent expression of CD69 on DCs would be also detected in MLN. Again, we consider that the delayed cytokine production and accumulation of the DCs in the lung, but not in MLN, following AZ12441970 treatment was obviously dependent on type I IFN signaling, and these local events in the lung are important for the efficacy by AZ12441970. The antitumor effect of TLR7 agonist 852A *in vitro* also been shown to require type I IFN and pDCs (67), and type I IFN and pDCs were required for *in vivo* adjuvant activities of TLR7 agonist ssRNA oligonucleotides (68). Taken together, these observations indicate that TLR7 agonists use pDC to release type I IFN, which elicits a variety of biological responses.

Although other TLR agonists have demonstrated Treg-generating ability (49, 69, 70), antedrug TLR7 agonists have not previously been reported to have this function *in vivo*. However, we have demonstrated that efficacy of AZ12441970 was lost in T cell-lacking mice. In addition, we also showed that type I IFN signaling is required for inhibition of airway eosinophilia. Correlation between the requirement of T cells and type I IFN signaling for efficacy of the compound was not still determined in the current studies. But lung pDC prevented disease in a mouse asthma model, possibly through Treg cell generation (71); therefore, further studies are needed to clarify this mechanism of action of an antedrug TLR7 agonist.

It has been reported that modulation of the Th1/Th2 balance can inhibit asthmatic features in allergen-induced murine asthma models (72, 73). T-bet, a member of the T-box family of transcription factors, is a master determinant of Th1 lineage (74). The current study examined whether Th1 immune responses are involved in the Th2 inhibition with the antedrug TLR7 agonist using T-bet $^{-/-}$ mice compared with wild-type mice on OVA/Alum-sensitized allergen-induced airway eosinophilia. Because efficacy with the antedrug TLR7 agonist against airway eosinophilia was maintained in T-bet $^{-/-}$ mice, we proved that the suppression of the Th2 immune response was not via the induction of Th1 immune responses.

It was reported previously that at least five different subsets of DCs can be found in the murine lungs (75). In steady-state conditions, resident DCs in the lung tissue are CD11b⁺ DC, CD103⁺ DC, and pDC, and alveolar DC exists in alveolar spaces in addition to alveolar macrophages. Under inflammatory conditions such as viral infection or LPS administration, additional subsets of CD11b⁺ monocyte-derived DCs are recruited from peripheral blood that rapidly express CD11c and are easily confused with resident CD11b⁺ conventional DCs (75). We showed that antedrug TLR7 agonist administration via the intranasal route resulted in the recruitment of DC subsets including CD11b⁺ DC, CD103⁺ DC and pDC that required type I IFN signaling, and pDC. The administration of the antedrug TLR7 agonist also induced an increase in the number of DC subsets in MLN. However, this increase was independent of type I IFN signaling. These observations suggested that there is a different mechanism for tissue infiltration and accumulation into draining LN of DC subsets in terms of the requirement for type I IFN signaling and pDC. CD69, the transmembrane C-type lectin, is essential for lymphocyte retention in lymphoid organs following viral infection or TLR3 agonist, dsRNA administration, and functions downstream of the type I IFN signaling (76). In contrast, we observed the recruitment of DC subsets into MLN following administration of the antedrug TLR7 agonist via the intranasal route without upregulation of CD69 on the DC subsets as well as type I IFN signaling.

In conclusion, the novel antedrug TLR7 agonist AZ12441970 has demonstrated efficacy against airway eosinophilia without causing undesirable systemic cytokine production in both OVA/Alum sensitization and challenge and Th2-polarized cell adoptive transfer models. Type I IFN signaling and pDC are required for efficacy following short-term exposure in vitro or in vivo. In longer exposure conditions, there might be a type I IFN-independent mechanism for the suppression of Th2 cytokines. Although Th1 immune responses or B cells are not required to achieve the suppression of eosinophil infiltration into the lung, at least T cells are required for the efficacy. We conclude that TLR7 antedrugs could have utility in the treatment of allergic airways disease.

Acknowledgments

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Disclosures

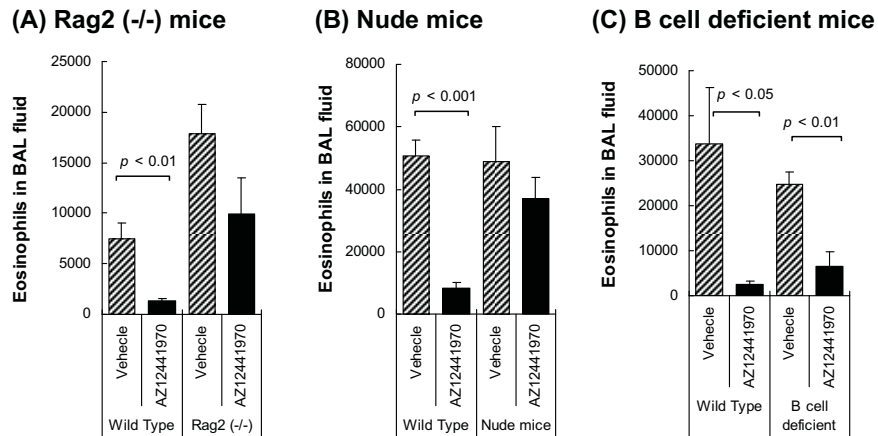
The authors have no financial conflicts of interest.

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Supplemental Fig. 1



Supplemental Fig. 1 Efficacy of antedrug TLR7 agonist, AZ12441970 against airway eosinophilia in Rag-2^{-/-} mice, Nude mice, or B cell-deficient mice.

In vitro Th2-polarized OVA-specific OT-II cells were adoptively transferred to wild type, Rag-2^{-/-} mice (A), nude mice (B), and B cell-deficient mice (C). Mice were dosed intranasally (0.5 mg/ml, 40 μ l) with AZ12441970 24 h and 2 h before intranasal challenge with OVA (0.25 mg/ml, 40 μ l). Two days after the challenge, the eosinophil number in BAL fluid was determined. Data are presented as mean $\pm$ SEM (n = 4–6 in each group). Similar results were obtained in two independent experiments.