

# Effects of Aryl Hydrocarbon Receptor Signaling on the Modulation of Th1/Th2 Balance<sup>1</sup>

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An orally active antiallergic agent, M50367, skews the Th1/Th2 balance toward Th1 dominance by suppressing naive Th cell differentiation into Th2 cells in vitro. Administration results in the suppression of IgE synthesis and peritoneal eosinophilia in vivo. In this report, we determined that M50354 (an active metabolite of M50367) was a ligand for the aryl hydrocarbon receptor (AhR); the immunological effects of this compound on in vitro Th1/Th2 differentiation from naive Th cells and Th1/Th2 balance in vivo were manifested through binding to AhR. These effects were completely abolished in AhR-deficient mice. AhR expression in the naive Th cell was significantly up-regulated by costimulation of TCR and CD28. Suppression of naive Th cell differentiation into Th2 cells via binding of M50354 to AhR was associated with inhibition of GATA-3 expression in Th cells. In addition, forced expression of a constitutively active form of AhR or activation of AhR by the addition of representative ligands suppressed naive Th cell differentiation into Th2 cells. Based on these results, we conclude that AhR functions as a modulator of the in vivo Th1/Th2 balance through activation in naive Th cells. *The Journal of Immunology*, 2005, 175: 7348–7356.

The aryl hydrocarbon receptor (AhR)<sup>3</sup> is a ligand-activated transcription factor belonging to the basic-helix-loop-helix (bHLH)/period-AhR nuclear translocator-single-minded (PAS) transcription factor superfamily (1). AhR normally exists within the cytoplasm as part of a complex with multiple proteins, including heat shock protein (HSP) 90 (2), AhR-activated 9 (3), and p23 (4). Once a ligand enters the cell and binds AhR, the ligand-bound AhR complex translocates to the nucleus. AhR then forms a heterodimer with another bHLH-PAS protein, AhR nuclear translocator (Arnt), after dissociating from the HSP90-containing complex. Within the nucleus, the AhR/Arnt heterodimer binds to a specific sequence within the promoter of various target genes, designated a xenobiotic responsive element (XRE), to activate their expression (5).

Although the natural ligand of AhR is not known, polycyclic aromatic hydrocarbons, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds, can bind and activate the AhR. The interaction of these chemicals with AhR mediates a variety of toxic effects, including immune suppression, thymic atrophy, endocrine disruption, tumor promotion, and cell differentiation (6–10). The T cell-mediated immunological response is one of the most sensitive targets of TCDD toxicity (11).

We previously reported that oral administration of the synthetic compound M50367 (Fig. 1) inhibited the production of plasma IgE and pulmonary eosinophilia, resulting in the suppression of airway hyperresponsiveness in murine models of atopic asthma. In ex vivo experiments, while oral administration of M50367 reduced the production of IL-4 and IL-5 by Ag-restimulated splenocytes, it enhanced the production of IFN- $\gamma$  (12). Treatment of naive Th cells with M50354, a hydrolyzed form of M50367 (Fig. 1), suppressed their in vitro differentiation into Th2 cells with a concomitant increase in the Th1 cell population (13). Thus, the antiallergic effects of this compound may be explained by a direct influence on the Th1/Th2 differentiation of naive Th cells. Although we have found M50367 induces expression of the cytochrome P450 1A1 enzyme (CYP1A1) in mouse liver (our unpublished data), the molecular mechanism underlying this immunomodulatory effect remains unclear.

In this study, we demonstrated that M50354 is an AhR agonist; the AhR signaling pathways activated by M50354 binding skews the Th1/Th2 balance toward Th1 dominance, resulting in immunological responses with antiallergic effects. Furthermore, we determined that the modulatory effects of M50354 on the Th1/Th2 balance were associated with reduced expression of GATA-3, a key factor for Th2 differentiation.

Materials and Methods

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### Chemicals

M50367 and M50354 (Fig. 1) were synthesized in our laboratory (12). M50354 was used for in vitro studies, and M50367 was used for in vivo studies because M50354 is an active metabolite of M50367 and has low bioavailability (12, 13). [<sup>3</sup>H]M50354 was synthesized by Amersham Biosciences. 3-Methylcholanthrene (3MC),  $\alpha$ -naphthoflavone ( $\alpha$ NF), and  $\beta$ -naphthoflavone ( $\beta$ NF) were purchased from Wako Pure Chemical. Resveratrol was obtained from Calbiochem.

### Antibodies

Anti-mouse-CD3 (145-2C11), FITC-conjugated anti-CD4 (GK1.5), PE-conjugated anti-IL-4 (11B11), and allophycocyanin-conjugated anti-IFN- $\gamma$  (XMGI.2) Abs were obtained from BD Pharmingen. An anti-CD28 mAb

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<sup>3</sup> Abbreviations used in this paper: AhR, Aryl-hydrocarbon receptor; bHLH, basic-helix-loop-helix; PAS, period-AhR nuclear translocator-single-minded; XRE, xenobiotic responsive element; Arnt, AhR nuclear translocator; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 3MC, 3-methylcholanthrene;  $\alpha$ NF,  $\alpha$ -naphthoflavone;  $\beta$ NF,  $\beta$ -naphthoflavone; CA, constitutively active; wt, wild type; m, murine; PSL, photo-stimulated luminescence; HPMC, hydroxyl-propyl-methylcellulose; PCB, polychlorinated biphenyl; CYP, cytochrome P-450.

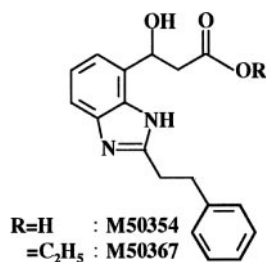


**FIGURE 1.** Chemical structures of M50354 and M50367.

(PV-1) was purchased from Southern Biotechnology Associates. Microbead-conjugated anti-FITC and anti-CD62L Abs were purchased from Miltenyi Biotec.

### Construction of plasmids

A CYP1A1 luciferase reporter plasmid was constructed as follows. A full-length rat *CYP1A1* transcriptional regulatory region (−6300 to +2566) was prepared from pMC6.3k (14) by digestion with *Bgl*II and *Nor*I and, after blunting, was end-cloned into the *Sma*I site of the pGL3-Basic vector (Promega) to give pMC6.3k-luc (see Fig. 2A).

A recombinant mouse AhR expression vector, wild-type (wt)-murine (m) AhR/pcDNA was constructed as follows. Mouse AhR cDNA fragment was amplified from C57BL/6 mouse liver cDNA by using the primers 5′-GGGTCTAGA<sup>−15</sup>AGGGCGCGGGGACCATTGAG<sup>+5</sup>-3 and 5′-GGGCTCGAG<sup>+2463</sup>GAAACACCTCAACTCTGCACCTTG<sup>+2427</sup>-3′. The resultant PCR fragment was digested with *Xba*I and *Xho*I, and ligated to the *Xba*I and *Xho*I site of pcDNA3.1<sup>+</sup> (Invitrogen Life Technologies).

A constitutively active AhR (CA-AhR) was constructed as described by McGuire et al. (Ref. 15; see Fig. 6A). Two cDNA fragments of AhR, corresponding to aa 1–276 and 419–805, were amplified by PCR. Primers were designed to carry restriction sites for *Xba*I and *Bam*HI (1–276), and *Bam*HI and *Xho*I (419–805). The resulting fragments were digested with *Xba*I and *Bam*HI (1–276) or *Bam*HI and *Xho*I (419–805), and subcloned into *Xba*I-*Xho*I sites of pBluescript II KS<sup>+</sup> to give CA-mAhR/pBS.

pMSCV retroviral vector was a kind gift of Dr. A. Kume (Jichi Medical School, Tochigi, Japan). CA-mAhR/pBS or wt-mAhR/pcDNA was digested with *Xba*I and *Xho*I, and then blunt-ended with T4 DNA polymerase. The resulting fragments were ligated to the blunt *Xba*I site of pMSCV to generate wt-mAhR-RV or CA-mAhR-RV.

pBSK-mAhR was constructed by inserting the 2.5-kb *Xho*I-*Xba*I fragment of mAhR cDNA described in Ema et al. (16) into the *Xho*I/*Xba*I sites of pBluescript II SK<sup>+</sup>.

### Luciferase reporter assay

Before transfection, Hepa-1c1c7 cells were seeded in 96-well plates at  $0.6 \times 10^4$  cells/well, then incubated at 37°C for 24 h. The cells were then transfected with the pMC6.3k-luc plasmid (30 ng/well) using FuGENE 6 transfection reagent (Roche Diagnostics). After 24 h of transfection, cells were treated for 24 h with a variety of compounds at indicated concentrations. Luciferase activity was then measured by using a PicaGene assay system (Wako Pure Chemical Industries).

### EMSA

A double-stranded oligonucleotide XRE probe (17) was end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP (Amersham Bioscience) by T4 polynucleotide kinase (NEB) and purified by using ProbeQuant G-50 microcolumns (Amersham Biosciences). In vitro-translated mouse AhR and Arnt were prepared by the TnT T7 quick-coupled transcription/translation system (Promega), according to the manufacturer's instruction, using pBSK-mAhR and pBSK-mArnt (18), respectively.

An unprogrammed transcription/translation system (9  $\mu$ l) or in vitro-translated mouse AhR and Arnt (4.5  $\mu$ l) were mixed with vehicle (1  $\mu$ l of DMSO) or chemicals and incubated for 2 h at 30°C. The reaction mixtures were combined with 10  $\mu$ l of 2 $\times$  binding buffer (200 mM HEPES-KOH (pH 7.9), 1 M KCl, 2 mM EDTA, 60 mM MgCl<sub>2</sub>, 6% glycerol, 20 mM DTT, 0.2 mg/ml sonicated salmon sperm DNA, and one of the chemicals). After incubation for 20 min at 25°C, the radiolabeled XRE probe ( $2 \times 10^4$  cpm) was added and incubated for 20 min at 25°C. The reaction mixtures were applied onto 4.8% acrylamide gel in 0.5 $\times$  Tris-borate-EDTA buffer. After the electrophoresis, the gel was processed on a gel dryer and then protein-DNA interaction was visualized and quantified with the BAS-1500

bio-image analyzing system (Fujifilm). The intensities of the bands were expressed as units of photostimulated luminescence (PSL).

### Binding assay

Hepatic cytosols were prepared essentially as described previously (19). Mouse hepatoma cells, Hepa 1c1c7, were grown in modified  $\alpha$ MEM supplemented with 10% FBS, sodium bicarbonate, and penicillin/streptomycin. The cells were harvested homogenized in HEDG buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM DTT, 10% glycerol, pH 7.6 adjusted). For preparation of cytosols, membranes were removed by a 45 min centrifugation at  $100,000 \times g$ , and then the supernatant was stored at  $-70^\circ\text{C}$  until use.

Specific binding of M50354 to AhR was assessed by trapping of the AhR complex using anti-AhR Ab. Briefly, 1  $\mu$ l of <sup>3</sup>H-labeled M50354 (60  $\mu$ M in ethanol) and a competitor (60 mM in DMSO) were added to 100- $\mu$ l aliquots of cytosols (2.0 mg/ml), and then incubated for overnight at 4°C. These mixtures were added to 96-well microplates coated with the anti-AhR Ab (10  $\mu$ g/ml), and incubated for 2 h at 4°C. These microplates were washed three times with HEDG buffer, and the retained radioactivities were measured using a scintillation counter.

### Mice

C57BL/6 mice were obtained from Japan SLC. AhR-deficient (*AhR*<sup>−/−</sup>) mice (20) were backcrossed with the C57BL/6 strain by 10 generations. The deficient mice were maintained as heterozygous mice in our laboratory. Mating *AhR*<sup>+/−</sup> males with *AhR*<sup>+/−</sup> females generated *AhR*<sup>+/+</sup>, *AhR*<sup>+/−</sup> and *AhR*<sup>−/−</sup> mice. In the following experiments, we used the wt (*AhR*<sup>+/+</sup>) and homozygous mutant mice (*AhR*<sup>−/−</sup>) of the littermates. The neonates were genotyped by PCR of DNA from the tail. The sense primer for the wt at the 5′ region of the *AhR* gene was 5′-CGCGGGCACCATTGAGCAG-3′. The antisense primer for intron 1 was 5′-GAGACTCAGCTCCTGGATGG-3′. The same 5′ primer was used as for the mutant type, while the antisense primer for the *LacZ* gene was 5′-CGCCGAGTTAACGCCATCAA-3′. All in vivo animal experiments were approved by the Institutional Animal Use Committee of our institute.

### Evaluation of Th1/Th2 balance in vivo

Mice were given 10  $\mu$ g of DNP-*Ascaris* adsorbed with 4 mg of alum i.p. on day 0. The sensitized mice were orally treated from days 0 to 9 with 100 mg/kg M50367 or vehicle alone (0.5% hydroxyl-propyl-methylcellulose (HPMC): 10 ml/kg). On day 10, plasma samples were obtained to measure total plasma IgE levels by the sandwich ELISA method described by Hirano et al. (21). Subsequently, spleens were collected, and homogenized using a glass homogenizer under sterile conditions. Homogenates were centrifuged at  $300 \times g$  for 7 min at 4°C and cell pellets were resuspended in RPMI 1640 medium. The cell suspensions were filtered through 40  $\mu$ m pore size nylon sieves to remove large cell aggregates. The isolated splenocytes were washed twice in the modified RPMI 1640 medium (S-Clone SF-B; Sanko Junyaku) and resuspended in S-Clone SF-B. One milliliter containing  $5 \times 10^6$  cells was seeded onto 48-well plates and cultured in the presence of 10  $\mu$ g/ml DNP-*Ascaris* for 18 h at 37°C. The culture supernatants were harvested and stored at  $-80^\circ\text{C}$  until cytokine determination. IL-4, IL-5, and IFN- $\gamma$  concentrations in the supernatants were measured using a commercial ELISA kit.

### Ag-induced cell infiltration to the peritoneal cavity

Mice were given 10  $\mu$ g of DNP-*Ascaris* i.p. on day 0. This injection was repeated on day 7, and the sensitized mice were orally given 100 mg/kg/day M50367 or vehicle alone (0.5% HPMC, 10 ml/kg/day) from days 0 to 9. On day 10, the peritoneal cells were harvested by lavage (3 ml of saline containing 1% EDTA), and the total cell number was counted with a hemocytometer (Sysmex).

### Naive Th cell preparation and evaluation of Th1/Th2 balance in vitro

Naive CD4<sup>+</sup>CD62L<sup>+</sup> Th cells were prepared from murine spleens as described (22, 23). Briefly, splenocytes were treated with FITC-conjugated anti-CD4 mAb (GK1.5) and microbead-conjugated anti-FITC Ab, and then the microbead-labeled CD4<sup>+</sup> Th cells were separated by MACS. After washing, the microbeads were cleaved enzymatically. CD4<sup>+</sup>CD62L<sup>+</sup> Th cells were then isolated using microbead-conjugated anti-CD62L mAb (MEL-14) using the MACS system. The obtained cells were confirmed to be >95% pure CD4<sup>+</sup>CD62L<sup>+</sup> Th cells by flow cytometry.

The naive Th cells were seeded at  $0.25 \times 10^6$  cells/ml in the plates containing immobilized anti-CD3 mAb (5  $\mu$ g/ml). These cells were cultured in RPMI 1640 medium containing 10 mM HEPES (pH 7.4), 10%

FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 50  $\mu$ M 2-ME, and 1  $\mu$ g/ml anti-CD28 mAb. Three days after the stimulation, a portion of the culture supernatant was sampled for ELISA, and then the cells were expanded 3-fold in a fresh medium. On day 6, Th cells were stimulated again with immobilized anti-CD3 mAb for 6 h in the presence of 4  $\mu$ M monensin to prevent the release of cytokines. After being stained with FITC-conjugated anti-CD4 mAb (GK1.5/4), the restimulated Th cells were sequentially permeabilized and fixed with Cytofix/CytoPerm (BD Pharmingen). Intracellular cytokines were detected with allophycocyanin-conjugated anti-IFN- $\gamma$  (XMG1.2) and PE-conjugated anti-IL-4 (11B11) Abs, as described by the manufacturer's protocol (BD Pharmingen). Flow cytometric analysis was performed using FACSCalibur and CellQuest software (BD Biosciences). The axes were set such that the total positive population of isotype control may be below 1%.

### Retroviral transduction

A retroviral packaging cell line, phenix (eco) cell, was transfected with CA-mAhR-RV or wt-mAhR-RV using FuGENE6. After 4 or 5 days of culture, PT67 cells (24) were infected with the supernatants from transfected phenix cells. Bright GFP-positive PT67 cells were selected for the preparation of high titer retroviral production cells.

Naive CD4<sup>+</sup>CD62L<sup>+</sup> Th cells were activated as described above and infected on days 2 and 4 with solutions containing viral supernatant and 6  $\mu$ g/ml polybrene (Sigma-Aldrich) at a 1:1 volume ratio. The cells were grown for 5 days after the primary activation, and intracellular cytokine staining was performed as described above.

### Western blot analysis of AhR

Naive CD4<sup>+</sup> Th cells were activated as described above. At 24, 48, and 72 h after activation, the cells were harvested, washed with PBS<sup>-</sup>, and lysed in SDS sample buffer (Daiichi Pure Chemicals) for 10 min at 99°C. The protein concentrations were measured by DC Protein Assay (Bio-Rad). Total proteins (10  $\mu$ g) were separated by 7.5% SDS-PAGE, and then transferred to Fluorotrans W membrane filters (Pall Gelman Laboratory). The membrane filters were incubated with a rabbit polyclonal anti-AhR Ab (BIOMOL) or a rabbit polyclonal anti-Zap70 Ab (Santa Cruz Biotechnology), followed by detection with secondary peroxidase-conjugated anti-rabbit immunoglobulins (DakoCytomation). Signals on the filters were visualized with ECL plus Western blot detection reagent (Amersham Bioscience).

### Quantitative transcript analysis

Total RNA was prepared from cultured cells using TRIzol reagent. Quantitative real-time PCR assay was performed using gene-specific primers and SYBR Green RT-PCR Reagent or TaqMan One-step RT-PCR Master Mix Reagent (Applied Biosystems) on a 7000 Sequence Detector (Applied Biosystems). The following pairs of primers and TaqMan probe were used for the RT-PCR assay: *GATA-3* in TaqMan RT-PCR, forward 5'-CA GAACCGGCCCTTATCA-3', reverse 5'-CAGGATGTCCCTGCTCTC CTT-3', and TaqMan probe, 5'-6FAM-TGGCCCCAGCATGCGACCTC-TAMRA-3'; *T-bet*, in SYBR Green RT-PCR, forward 5'-TGCCAGG GAACCGCTTATAT-3' and reverse 5'-GTTGGAAGCCCCCTTGTGT-3'. *c-maf*, in SYBR Green RT-PCR, forward 5'-AAGGAGGAG GTGATCCGACT-3' and reverse 5'-TCTCCTGCTTGAGGTGGTCT-3'. Results were normalized to expression of  $\beta$ -actin: forward 5'-GCTCTG GCTCCTAGCACCAT-3', reverse 5'-GTGGACAGTGAGGCCAGGAT-3', and TaqMan Probe, 5'-6FAM-TCAAGATCATGCTCCTCCT GAGCGC-TAMRA-3'. The means  $\pm$  SD of triplicate determinations are shown.

### Statistical analysis

Results were evaluated by Dunnett's procedure for multiple comparisons or Student's *t* test using STAT LIGHT 1997 (Yukms). A difference among groups was considered to be significant when *p* < 0.05.

## Results

### Binding of M50354 to AhR and enhancement of CYP1A1 promoter activity

As oral administration of M50354 or M50367 induced CYP1A1 expression in mouse liver, we hypothesized that M50354 was an AhR ligand. To confirm this assumption, we examined the in vitro effect of M50354 on CYP1A1 induction, a common property of AhR agonists, in mouse hepatocytes. Hepa1c1c7 cells transiently transfected with pMC6.3k-luc were treated with a variety of con-

centrations of either M50354 or control compounds for 24 h. We then determined the expression levels of the inducible reporter gene. We observed that M50354 was a relatively weak inducer of CYP1A1, with an EC<sub>50</sub> greater than  $3 \times 10^{-4}$  M (Fig. 2A). Due to a low solubility, we could not test the induction activities of M50354 at concentrations >300  $\mu$ M. The EC<sub>50</sub> values for the reference compounds  $\beta$ NF and  $\alpha$ NF were estimated to be  $\sim 3 \times 10^{-7}$  M and  $\sim 1 \times 10^{-5}$  M, which were 3 and 2 orders of magnitude less than that of M50354, respectively. In contrast, resveratrol (25), a control AhR antagonist, had no effect on CYP1A1 promoter activity at any of the concentrations tested.

To examine whether the addition of M50354 transformed AhR into an active form allowing the interaction with the XRE enhancer sequence, we performed EMSA. In vitro-translated mouse AhR and Arnt were mixed with the radiolabeled XRE probe and several concentrations of M50354. Addition of M50354 induced the formation of an AhR/Arnt/XRE complex in a dose-dependent manner at concentrations exceeding 10  $\mu$ M (359 PSL at 10  $\mu$ M, 1204 PSL at 30  $\mu$ M, and 3601 PSL at 100  $\mu$ M) (Fig. 2B). This result correlates with the induction activity of M50354 in luciferase assays. The reference compounds, 3MC and  $\beta$ NF, also promoted the interaction of AhR/Arnt complex with the XRE at 1  $\mu$ M (3005 PSL at 1  $\mu$ M 3MC, 1985 PSL at 0.3  $\mu$ M  $\beta$ NF, and 2570 PSL at 1  $\mu$ M  $\beta$ NF); these shifted bands disappeared with competition from excess unlabeled XRE probe. The ability of M50354 to form an AhR/Arnt/XRE complex was relatively weak, which is consistent with its ability to induce CYP1A1 expression.

To investigate whether M50354 binds AhR, Hepa-1c1c7 cytosol was mixed with 0.6  $\mu$ M [<sup>3</sup>H]M50354, then incubated in 96-well plates with an immobilized anti-AhR Ab. After washing, the retained radioactivity was measured. Higher levels of radioactivity were detected in plates with immobilized anti-AhR Ab; this radioactivity could be abolished by the addition of excess competitor, such as unlabeled M50354,  $\alpha$ NF,  $\beta$ NF, or 3MC (Fig. 2C). This result clearly demonstrated that M50354 specifically binds the AhR protein in a manner similar to other representative AhR ligands.

These results indicate that AhR was activated by M50354 binding. An enhancement of CYP1A1 promoter activity followed this activation, although this enhancement was not as potent as that induced by  $\alpha$ NF, a known partial agonist.

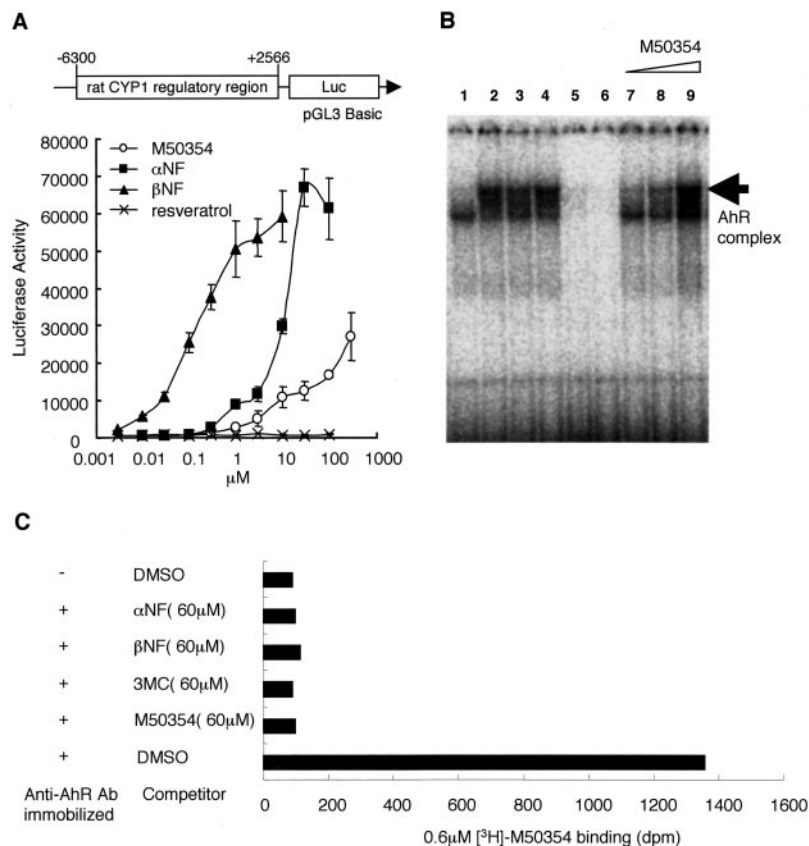
### Requirement of AhR for the antiallergic effects of M50367 in vivo

To examine the requirement of AhR in the antiallergic effects mediated by M50367, we administered M50367 to *AhR*<sup>-/-</sup> mice.

We first investigated the effects of M50367 on plasma IgE levels in Ag-sensitized mice. *AhR*<sup>-/-</sup> and wt mice were sensitized by an i.p. injection of 10  $\mu$ g of DNP-*Ascaris*/Almu on day 0. Sensitized mice were then treated orally with M50367 (100 mg/kg, daily) for 10 days, beginning on day 0. Orally administered M50367 inhibited IgE production with an EC<sub>50</sub> value of 1–3 mg/kg (12). Despite the efficacy of low concentrations in this model, however, we used a dosage of 100 mg/kg/day to make the effects of M50367 in *AhR*<sup>-/-</sup> mice clear.

Irrespective of AhR genotype, plasma IgE levels in sensitized mice were elevated to levels  $\sim$ 2-fold higher than those observed in nonsensitized mice (Fig. 3A). Although the administration of M50367 to wt mice lowered the plasma IgE levels significantly (from 423 ng/ml to 67 ng/ml, *p* < 0.01), this suppression was not observed in *AhR*<sup>-/-</sup> mice. The Ag-sensitized *AhR*<sup>-/-</sup> mice exhibited 2.5-fold higher serum IgE levels than the wt animals (Fig. 3A), although the underlying mechanism remains unclear.

**FIGURE 2.** M50354 binds to AhR to enhance CYP1A1 promoter activity. **A**, Before transfection, Hepa-1c1c7 cells were seeded in 96-well plates at  $0.6 \times 10^4$  cells/well, then incubated at  $37^\circ\text{C}$  for 24 h. The cells were then transfected with the pMC6.3k-luc plasmid (30 ng/well), the rat CYP1A1 promoter region ( $-6300$  to  $+2566$ ) ligated with the pGL3 Basic vector, using FuGENE 6 transfection reagent. Twenty-four hours after transfection, cells were treated for 24 h with a variety of compounds at the indicated concentrations. Luciferase activity was then measured using a PicaGene assay system. The means  $\pm$  SD of quadruplicate determinations are shown. M50354 ( $\circ$ ),  $\beta$ NF ( $\blacktriangle$ ),  $\alpha$ NF ( $\blacksquare$ ), resveratrol ( $\times$ ). Data are representative of four independent experiments. **B**, In vitro-translated mouse AhR and Arnt were mixed with either vehicle ( $1 \mu\text{l}$  of DMSO) or the indicated chemicals, then incubated for 2 h at  $30^\circ\text{C}$ . We then performed EMSA with radiolabeled XRE probe ( $2 \times 10^4$  cpm). **Lanes:** 1, DMSO control; 2,  $1 \mu\text{M}$  3MC treatment; 3,  $0.3 \mu\text{M}$   $\beta$ NF treatment; 4,  $1 \mu\text{M}$   $\beta$ NF treatment; 5–6,  $1 \mu\text{M}$   $\beta$ NF in the presence of a 30- or 100-fold excess of unlabeled XRE probe, respectively; 7–9, 10, 30, and  $100 \mu\text{M}$  M50354 treatment, respectively. **C**, Cytosols prepared from Hepa-1c1c7 cells were incubated with  $0.6 \text{ mM}$  [ $^3\text{H}$ ]M50354 ( $37\text{kBq}$ ) and competitor ( $100$ -fold excess) overnight at  $4^\circ\text{C}$ . Samples were added to anti-AhR Ab-coated 96-well plates and incubated for 2 h at  $4^\circ\text{C}$ . After washing with PBS $^-$ , the retained radioactivity was measured by scintillation.



We then investigated the effects of M50367 on the increases in peritoneal cells induced by repeated Ag sensitization.  $\text{AhR}^{-/-}$  and wt mice were sensitized twice by i.p. injection of  $10 \mu\text{g}$  of *Ascaris* extract on days 0 and 7. Mice were given M50367 orally ( $100 \text{ mg/kg}$ , daily) for 10 days beginning on day 0, after which the number of peritoneal exudate cells was counted. Although administration of M50367 significantly suppressed the total number of peritoneal exudate cells in the wt mice (from  $13.8 \times 10^6$  cells to  $7.9 \times 10^6$  cells,  $p < 0.05$ ), no suppression was observed in  $\text{AhR}^{-/-}$  mice. Ag-sensitized  $\text{AhR}^{-/-}$  mice exhibited a small, but significant, increase in the number of peritoneal exudate cells from the levels observed in their wt counterparts (Fig. 3B). This increase in cell number may be related to the elevated serum IgE levels in  $\text{AhR}^{-/-}$  mice.

To evaluate the effects of M50367 on Th1/Th2 balance, we measured cytokine production by ex vivo restimulated splenocytes. In wt splenocytes, the enhanced levels of IL-5 induced by DNP-*Ascaris* sensitization were reduced by treatment with M50367 (from  $332 \text{ pg/ml}$  to  $107 \text{ pg/ml}$ ,  $p < 0.01$ ), while IFN- $\gamma$  production was enhanced by M50367 treatment (from  $334 \text{ pg/ml}$  to  $3310 \text{ pg/ml}$ ,  $p < 0.01$ ) (Fig. 3, C and D). This result indicated that M50367 administration skewed the Th1/Th2 balance toward Th1 dominance in wt mice. In contrast, M50367 had no effect on either IL-5 or IFN- $\gamma$  production in  $\text{AhR}^{-/-}$  mice. Interestingly, sensitized  $\text{AhR}^{-/-}$  mice exhibited 3-fold higher IL-5 levels than wt animals treated in a similar manner (Fig. 3, C and D). Even higher increases in IFN- $\gamma$  production were observed in sensitized  $\text{AhR}^{-/-}$  mice than those seen in wt animals.

The results of these in vivo experiments indicate that AhR is responsible for the antiallergic and modulatory effects of M50367 on the Th1/Th2 balance.

#### Modulation effects of M50354 on in vitro Th1/Th2 differentiation from naive Th cells

The addition of M50354 suppressed IL-4 production by naive Th cells and modulated the naive Th cell differentiation into Th1/Th2 cells toward Th1 dominance (13).

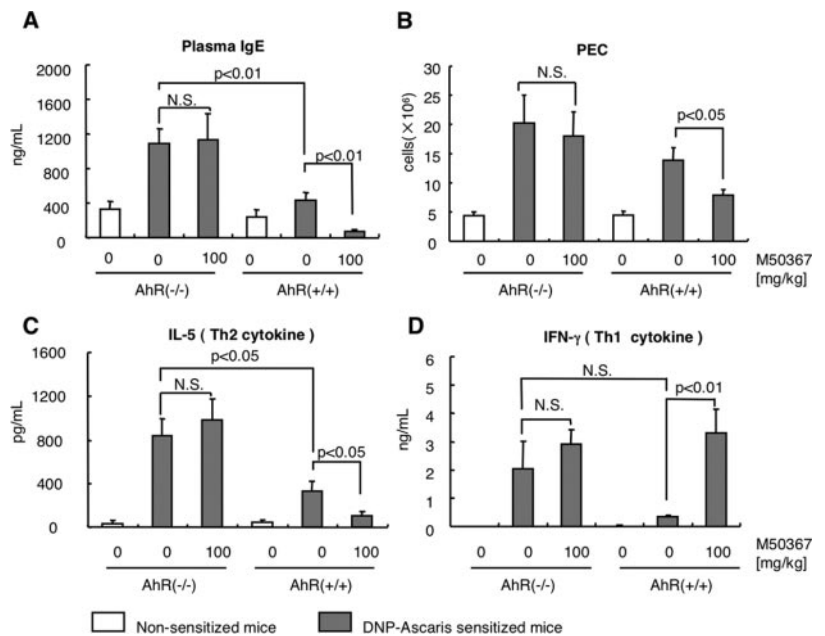
Naive Th cells isolated from  $\text{AhR}^{-/-}$  or wt mice were stimulated with anti-CD3 and anti-CD28 mAbs, and then incubated for 6 days in the presence or absence of M50354. We measured the levels of IL-4 and IFN- $\gamma$  in culture medium by ELISA on day 3 to evaluate the effects of M50354 on early cytokine production, and conducted intracellular cytokine staining on 6 days after treatment to determine the proportions of differentiated Th1/Th2 cells. Addition of M50354 significantly suppressed IL-4 production ( $1.1$ – $0.4 \text{ ng/ml}$ ) in wt mice (Fig. 4A). M50354 also significantly reduced the population of Th2 cells from  $16.4$  to  $4.5\%$  in these animals (Fig. 4B). In  $\text{AhR}^{-/-}$  mice, however, neither IL-4 production nor the proportion of Th2 cells were altered by the addition of M50354 (Fig. 4).  $\text{AhR}$ -deficient mice exhibited 2- to 3-fold higher levels of both IL-4 and IFN- $\gamma$  than those seen in wt mice (Fig. 4A), a phenotype that is likely associated with enhanced IgE production.

The results of these in vitro experiments indicated that the modulatory effects of M50354 on Th1/Th2 differentiation were mediated by AhR signaling and AhR is likely involved in regulation of the differentiation of naive Th cells into Th1/Th2 cells.

#### Involvement of AhR signaling in the modulation of Th1/Th2 differentiation

To investigate the role of AhR in the differentiation of naive Th cells, we investigated the expression of AhR throughout the course of Th cell differentiation. Naive Th cells prepared from C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 mAbs. After

**FIGURE 3.** Effects of M50367 in in vivo allergic models using *AhR*<sup>-/-</sup> and wt mice sensitized with DNP-*Ascaris*. **A**, *AhR*<sup>-/-</sup> and wt mice sensitized with 10  $\mu$ g of DNP-*Ascaris* were given 100 mg/kg M50367 orally. Ten days later, we assessed the IgE concentrations present in plasma samples collected on day 10 by mouse IgE ELISA. **B**, Mice were given 10  $\mu$ g of DNP-*Ascaris* on day 0. This injection was repeated on day 7; the sensitized mice were treated orally from days 0 to 9 with 100 mg/kg M50367. On day 10, we measured the total number of peritoneal cells collected. **C** and **D**, *AhR*<sup>-/-</sup> and wt mice sensitized with 10  $\mu$ g of DNP-*Ascaris* were given 100 mg/kg M50367 orally for 10 days. On day 10, isolated splenocytes were cultured at  $5 \times 10^6$  cells/ml in the presence of 10  $\mu$ g/ml DNP-*Ascaris* in the absence of M50367 for 18 h. We then estimated the concentrations of cytokines present in the supernatants by ELISA. Values are expressed as the means  $\pm$  SEM of eight animals.  $p < 0.05$ ;  $p < 0.01$ , calculated by the Student *t* comparison test.



harvesting the cells at various time points after stimulation, cell lysates were prepared for immunoblot analysis. AhR expression was strongly induced at the earliest time point, and then gradually decreased over the next 72 h (Fig. 5).

To confirm the modulation of Th1/Th2 differentiation by activated AhR, we investigated the effects of a CA-AhR on naive Th cells. Deletion of the ligand-binding domain (PAS B domain) converts AhR into a constitutively active molecule, even in the absence of ligand (15). The CA-AhR and wt-AhR expression vectors were constructed as described in *Materials and Methods* (Fig. 6A). After stimulation with anti-CD3 and anti-CD28 mAbs, naive Th cells from *AhR*<sup>-/-</sup> mice were infected with a retrovirus encoding wt-AhR or CA-AhR. Cells were cultured in the presence of exogenous IL-4 (10 ng/ml), and then the populations of Th1 and Th2 cells were assessed on day 6 (Fig. 6B). Despite the presence of exogenous IL-4, CA-AhR expression induced significant Th1 polarization (3.6–8.8%) with a concomitant decrease in Th2 differentiation (38.5–16.7%). In contrast, wt-AhR transduction did not significantly affect Th1/Th2 differentiation, indicating that activation of AhR modulates Th1/Th2 differentiation toward Th1 dominance.

We examined the modulatory effects of representative AhR ligands on Th cell differentiation. Naive Th cells isolated from wt C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of one of the following compounds: 3MC and  $\beta$ NF as AhR agonists,  $\alpha$ NF as a partial antagonist, and resveratrol as a complete antagonist. We assessed the Th1 and Th2 cell populations by intracellular cytokine staining with flow cytometry on day 6 (Fig. 6C). M50354, 3MC, and  $\beta$ NF suppressed Th2 differentiation (from 16.2 to 8.0, 9.5, and 7.6%, respectively) with concomitant increases in Th1 differentiation (from 21.3 to 25.7, 23.6, and 25.1%, respectively), whereas  $\alpha$ NF did not affect Th1/Th2 differentiation. Resveratrol treatment increased both the Th1 and Th2 populations, similar to the result observed in the Ag-sensitized *AhR*<sup>-/-</sup> mice. This result indicates that immunological responses following AhR antagonist treatment mimic the AhR deficiency status of mice. The estimated IC<sub>50</sub> value of M50354 ( $\sim 3$   $\mu$ M),  $\beta$ NF ( $\sim 3$   $\mu$ M), and  $\alpha$ NF ( $> 10$   $\mu$ M) for the inhibition of IL-4 production (Fig. 6D) did not agree with the observed EC<sub>50</sub> for the induction of CYP1A1 expression (Fig. 2A), suggesting that AhR uses different molecular mechanisms in these processes.

#### Effects of M50354 on expression of genes in Th cell differentiation

Taken together with the result that M50354 treatment and CA-AhR transduction of naive Th cells suppressed Th2 differentiation, even in the presence of excess exogenous IL-4 (Ref. 13 and Fig. 6B), we presumed that the effect of AhR on Th1/Th2 differentiation likely occurs through modulation of a signaling component upstream of IL-4 action, such as GATA-3 or c-maf. GATA-3 is a master transcriptional factor controlling the differentiation of Th2 cells; impairment of GATA-3 activity by either antisense RNA or a dominant-negative form of the protein potentially suppressed Th2 cytokine production and Th2 cell differentiation (26).

To test our hypothesis, we compared the expression of *GATA-3* in *AhR*<sup>-/-</sup> mice with that observed in the wt animals. Naive Th cells from *AhR*<sup>-/-</sup> and wt littermate mice were stimulated with anti-CD3 and anti-CD28 mAbs. Using RNAs prepared from these cells 24 h after stimulation, we measured the mRNA levels of *GATA-3* and other transcription factors by quantitative RT-PCR (Fig. 7A). *GATA-3* expression in naive Th cells isolated from *AhR*<sup>-/-</sup> mice exhibited  $\sim 3$ -fold higher *GATA-3* mRNA levels than cells derived from wt mice, while the levels of *IL-4* $\alpha$ -chain and *STAT6* mRNA were not affected.

From these observations, we hypothesized that the suppression of *GATA-3* expression by activated AhR modulated Th1/Th2 differentiation. Thus, we examined the effect of M50354 on the time course of *GATA-3* mRNA expression. Naive Th cells isolated from C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 mAbs in the presence or absence of M50354. At various time points after treatment, we used quantitative RT-PCR to measure the mRNA levels of *GATA-3* and other transcription factors within RNA samples prepared from these Th cells (Fig. 7B). In the absence of M50354, expression of *GATA-3* mRNA began to increase at 48 h, continuing to increase throughout the course of the experiment until 96 h. In the presence of M50354, however, *GATA-3* mRNA expression was already suppressed at 48 h, remaining low at  $\sim 10\%$  of control levels at 96 h. mRNA expression levels of *c-maf* and *T-bet* were similarly increased in either the presence or absence of M50354. IL-4 protein production, quantitated by ELISA, began to increase at 72 h in untreated controls; M50354

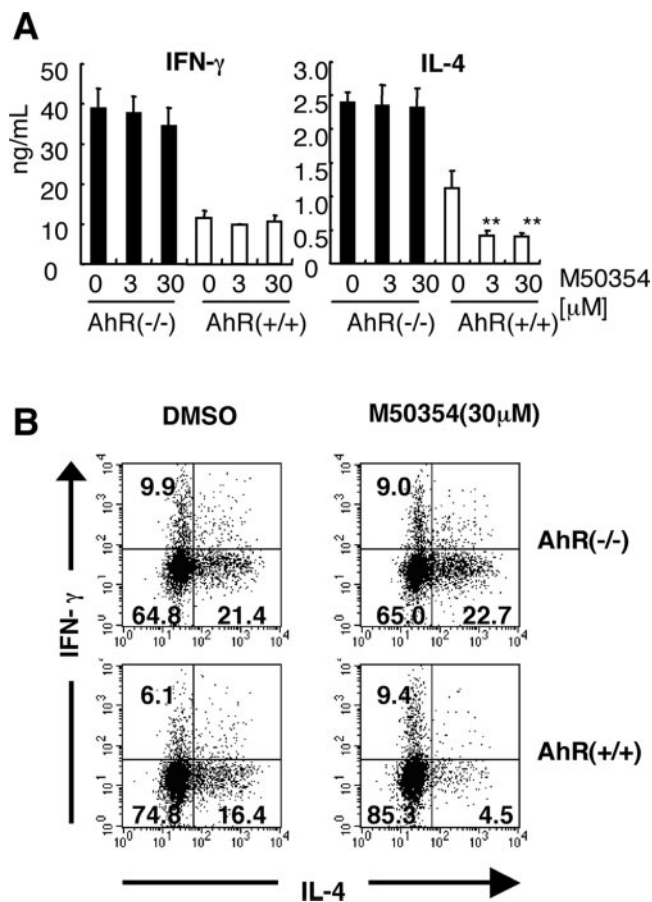


**FIGURE 4.** Effect of M50354 on the in vitro differentiation of naive Th cells of *AhR*<sup>-/-</sup> and wt mice into Th1 and Th2 cells. Naive Th cells prepared from *AhR*<sup>-/-</sup> or wt mice were stimulated with immobilized anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) mAbs, then cultured with 3 μM or 30 μM M50354. **A**, On day 3, culture supernatants were harvested; the levels of IL-4 and IFN-γ in these samples were determined by ELISA. Values are expressed as the means ± SD of four independent experiments. \*\*, *p* < 0.01, calculated by Dunnett's procedure for multiple comparisons. **B**, On day 6, cells were restimulated with anti-CD3 mAb in the presence of monensin for 6 h. The proportions of IL-4- and IFN-γ-producing cells were then determined by intracellular cytokine staining, as described in *Materials and Methods*. The percentages of cells present in each quadrant are indicated.

treatment reduced these levels to 30% of the observed control levels. These results suggest that M50354 skewed Th1/Th2 differentiation toward Th1 dominance by inhibiting the expression of *GATA-3* in naive Th cells.

## Discussion

Recently, there have been an increasing number of reports detailing the various effects of AhR signaling on the immune system, including thymic atrophy and suppression of Ab production, by an AhR ligand, TCDD. Thymic atrophy has been observed in several experimental animal models following exposure to TCDD, reportedly due to the direct effect of TCDD on thymocytes and lymphocyte precursors (27). Furthermore, TCDD exposure suppresses Ag-specific Ab production in mouse models (28, 29). Kerkvliet et al. (30) have also described the marked suppression of CTL responses following TCDD exposure. All of these effects of TCDD have been confirmed to be mediated by AhR signaling by experiments using AhR-deficient mice. These results strongly suggest

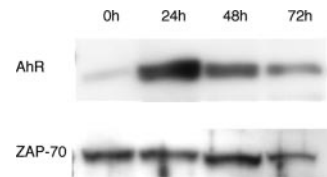
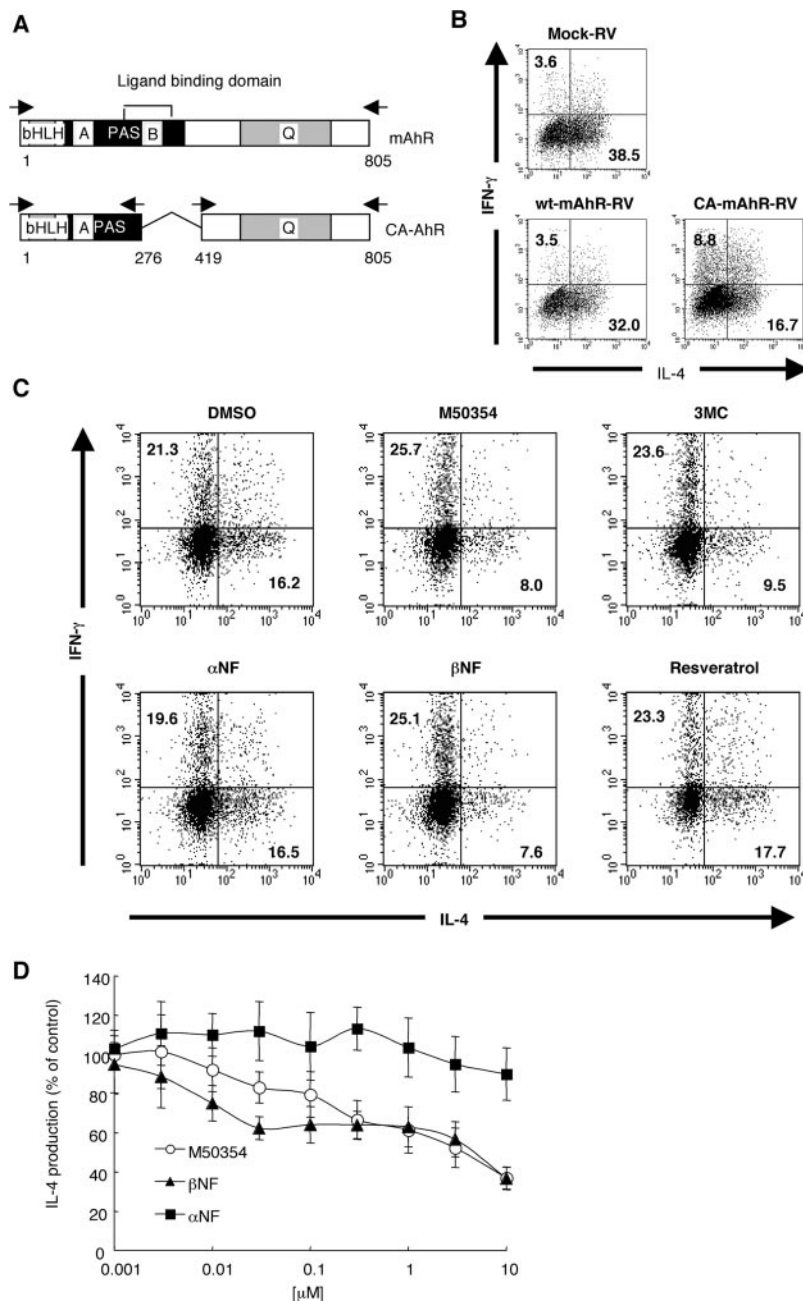


**FIGURE 5.** Time course of AhR expression during the in vitro differentiation of naive T cells. Naive Th cells prepared from C57BL/6 mice were costimulated with immobilized anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) mAbs. After 24, 48, and 72 h, cells were harvested and analyzed for the expression of AhR proteins by Western blotting using a specific anti-AhR Ab. The image shown is representative of three independent experiments.

that AhR is expressed in the T lineage cells, likely functioning in their differentiation and effector function.

The synthetic compound M50367 modulates the Th1/Th2 balance by influencing Th cell differentiation from naive T cells, resulting in the suppression of IgE production and eosinophilic infiltration into sites of inflammation in an in vivo allergy model (12, 13). The modulatory effect of M50354 on Th1/Th2 differentiation from naive Th cells is unique; to our knowledge, there have not been any previous reports detailing the effect of a small molecule, such as this chemical, on the modulation of the Th1/Th2 balance. Therefore, elucidating the mechanism of M50354 action would greatly contribute to our understanding of the mechanisms underlying Th1/Th2 differentiation, potentially facilitating the development of novel antiallergic agents. In this study, we demonstrate that the antiallergic compound M50354 bound and activated AhR, enhancing the expression of a reporter gene driven by the *CYP1A1* promoter in transient DNA-transfection assays, gel mobility shift assays, and ligand-binding experiments. We also revealed that M50354 exerted inhibitory effects on Th2 cell differentiation and IgE production in both in vitro and in vivo experiments. As M50354 also suppressed the production of Th2 cytokines, including IL-4 and IL-5, the inhibition of IgE production by M50354 was likely caused by suppression of the differentiation of naive Th cells into Th2 cells, leading to a reduced production of Th2 cytokines. Although the inhibitory effects of this chemical were completely lost in *AhR*<sup>-/-</sup> mice, retroviral CA-AhR expression potently inhibited naive Th cell differentiation into Th2 cells in both *AhR*<sup>-/-</sup> and wt mice (data not shown), even in the presence of exogenous IL-4. These results indicate that M50354 suppressed Th2 cell differentiation from naive Th cells via activation of AhR signaling, exerting antiallergic effects. 3MC and βNF, representative AhR ligands, also inhibited naive T cell differentiation into Th2 cells and the production of the Th2 cytokines. These findings clearly indicate that AhR signaling regulates the Th1/Th2 balance by influencing Th cell differentiation. The potency of these chemicals to induce *CYP1A1* expression, however, differs from their immunomodulatory activities. M50354 exerted a greater immunomodulatory activity than the examined representative AhR ligands, βNF and 3MC, while the opposite was true for *CYP1A1* induction. Judging from the *IC*<sub>50</sub> (~3 μM) for Th2 differentiation and the *IC*<sub>50</sub> (> 300 μM) for *CYP1A1* induction, M50354 is specifically more effective in comparison to other AhR ligands at modulating Th cell differentiation than inducing *CYP1A1* expression. The conformation of the AhR complex when bound to M50354 may differ from that bound to other AhR ligands. The molecular basis for the differential activities of these AhR ligands will be intriguing to investigate. TCDD, a high affinity AhR ligand, exerts pleiotropic immunosuppressive effects on the production of various Ab isotypes (IgE, IgG1, IgG2, and IgM) (31) and cytokines (IL-2,

**FIGURE 6.** Effects of AhR signaling on in vitro Th1/Th2 differentiation. **A**, Schematic representation of the wt AhR and CA-AhR constructs. Q, glutamine rich domain. The positions of the primers used in the plasmid construction are indicated by arrows. **B**, The effects of CA-AhR transduction on Th1/Th2 differentiation. Naive Th cells prepared from *Ahr*<sup>-/-</sup> mice were stimulated with immobilized anti-CD3 and anti-CD28 mAbs in the presence of IL-4. Cells were then infected with retroviruses encoding the wt AhR, CA-AhR, or control sequences at 24 and 72 h after stimulation. On day 6, cells were harvested and restimulated with anti-CD3 and anti-CD28 mAbs in the presence of monensin. Six hours later, cells were harvested to determine the proportions of IL-4- and IFN- $\gamma$ -producing cells by intracellular cytokine staining, as described in the *Materials and Methods*. The percentages of cells present in each quadrant are indicated. **C**, Effects of AhR agonists and antagonists on Th1/Th2 differentiation. Naive Th cells prepared from C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of each AhR ligand (3  $\mu$ M). Six days after stimulation, the cells were collected and restimulated with anti-CD3 and anti-CD28 mAbs in the presence of monensin. Six hours later, cells were harvested to determine the proportions of IL-4- and IFN- $\gamma$ -producing cells by intracellular cytokine staining. The percentages of the cells present in each quadrant are indicated. **D**, Dose-dependent effect of each chemical on IL-4 production. Naive Th cells prepared from C57BL/6 mice were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of varying concentrations of the indicated chemicals. On day 3, culture supernatants were harvested. The levels of IL-4 were then determined by ELISA. Values are expressed as the means  $\pm$  SD of four experiments.



IL-4, IL-5, and IL-6). The majority of the effects of TCDD effects, if not all, are thought to be mediated by AhR. In contrast, M50367 did not affect the production of IgG2a, IgM, IL-2, or IL-6; its effects appear to be limited to Th2-mediated immune responses (12). Although M50364 treatment suppressed the expression of *GATA-3*, a key transcription factor for Th2 cell differentiation, it is not known whether activated AhR is directly involved in *GATA-3* expression. As the molecular mechanisms underlying T cell-specific *GATA-3* expression are complex and difficult to investigate (32), we are now investigating the mechanism of *GATA-3* expression inhibition by activated AhR. In the AhR-deficient mice, plasma IgE levels were significantly increased in comparison to those observed in wt mice; the production of both IL-5 and IFN- $\gamma$  was 2- to 3-fold higher in the primary cultures of *Ahr*<sup>-/-</sup> Th cells than that seen in wt animals. In AhR-deficient mice, the populations of both Th1 and Th2 cells were increased. Moreover, treatment with resveratrol,

an AhR antagonist, enhanced cytokine production in a manner similar that seen in *Ahr*<sup>-/-</sup> mice. These results indicate that AhR suppresses immune responses under normal conditions; ablation of AhR activity by either gene disruption or antagonist treatment also enhances immune responses. In the first description of AhR-deficient mice, Gonzalez and colleagues (33) reported a decreased accumulation of lymphocytes in the spleen and the lymph nodes, suggesting the requirement for AhR in the development of the immune system. In contrast, Kerkvliet and colleagues (34) reported that AhR-deficient mice generate normal immune responses in Ag-stimulation models. The apparent discrepancies between these results may result from differences in either the mouse strains or the methods used. More extensive analyses using a variety of immunological parameters and analytical methods should be performed to clarify these apparent discrepancies.

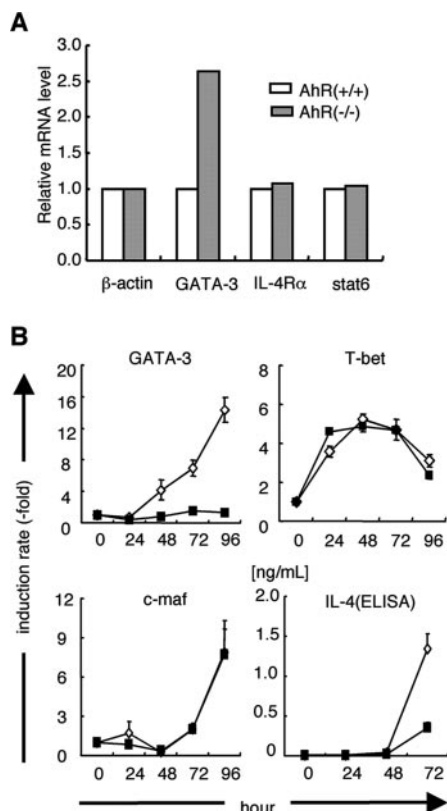


**FIGURE 7.** Effects of AhR signaling and M50354 treatment on *GATA-3* expression. *A*, Naive Th cells prepared from *AhR*<sup>-/-</sup> or wt mice were stimulated with immobilized anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) mAbs. After 24 h, cells were harvested and analyzed for gene expression by quantitative RT-PCR using an ABI7000 analyzer. Data are normalized to the expression levels of each gene in *AhR*<sup>+/+</sup> mice. *B*, Naive Th cells prepared from C57BL/6 mice were stimulated with immobilized anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) mAbs in the presence of either vehicle alone (diamonds) or M50354 (squares). After 72 h of culture, cells were expanded 6-fold in fresh medium, then cultured for an additional day. After 24, 48, 72, and 96 h of culture, the harvested cells were analyzed for gene expression by quantitative RT-PCR. Data are normalized to *β-actin* mRNA expression level. The means ± SD of triplicate determinations are shown.

Concerning the role of AhR signaling in the human immune system, Weisglas-Kuperus et al. (35) have reported that perinatal exposure to polychlorinated biphenyls (PCBs) and dioxins is associated with a lower prevalence of allergic diseases in preschool age children. These authors suggested that the lower prevalence is due to the susceptibility to infectious diseases in infants. Taken together with our results, the combined effects of infectious diseases and Th1-dominated conditions caused by exposure to PCBs and dioxins may act synergistically to prevent allergic diseases. Recently, Li et al. (36, 37) have reported the pharmacological actions of MSSM-002, a Chinese herbal formula used as an anti-asthma drug. The effects of MSSM-002 on animal models of allergy are similar to those observed for M50367, exhibiting IgE production inhibition and Th1-dominant differentiation with suppression of *GATA-3* expression. As MSSM-002 is a plant derivative that is abundant in a broad range of flavonoids, also probable AhR ligands, the antiallergic effects of this drug are likely mediated by AhR signaling.

We have demonstrated that M50367 is an AhR ligand of AhR that modulates the Th1/Th2 balance by influencing the differentiation of naive Th cells into Th1 dominance. This effect is probably mediated by down-regulation of *GATA-3* expression, resulting in

antiallergic activity. These results suggest that AhR signaling plays a significant role in normal immune responses, making AhR signaling a promising target for chemo therapeutic agents for the treatment of allergic diseases.

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## Disclosures

The authors have no financial conflict of interest.

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