

CUTTING EDGE

Cutting Edge: Inhibition of the Retinoid X Receptor (RXR) Blocks T Helper 2 Differentiation and Prevents Allergic Lung Inflammation

Roland Grenningloh,^{1*‡} Andrea Gho,^{*} Pietro di Lucia,^{*} Michael Klaus,^{2†} Werner Bollag,^{3†} I-Cheng Ho,[‡] Francesco Sinigaglia,^{*} and Paola Panina-Bordignon^{*}

Among the many factors regulating Th cell differentiation, some nuclear hormone receptors are emerging as important players. The retinoid X receptor (RXR) functions as heterodimerization partner for a variety of nuclear hormone receptors. We show in this study that RXR is critical for Th2-mediated immunity. An RXR antagonist inhibited Th2 differentiation, resulting in reduced production of IL-4, IL-10, and IL-13, whereas IFN- γ production was enhanced. This effect was dependent on the presence of APCs. In addition, IL-5 production was blocked directly in Th cells. In vivo, inhibition of RXR prevented experimentally induced allergic lung inflammation. Th1-mediated inflammation was not affected. Its specific role in Th2-mediated inflammation makes RXR a promising target for the development of therapies against diseases such as allergic asthma and atopic dermatitis. The Journal of Immunology, 2006, 176: 5161–5171.

Th cells play a critical role in immune regulation. Th1 cells produce IL-2 and IFN- γ , whereas Th2 cells secrete mostly IL-4, IL-5, and IL-13. Th1 and Th2 cells arise from a common precursor cell. Many factors control Th differentiation, including the cytokine milieu, type of APC, and mode of costimulation (1, 2).

It is becoming increasingly evident that certain nuclear hormone receptors profoundly affect the immune response. The retinoid X receptor (RXR)⁴ is activated by 9-*cis*-retinoic acid (9-*cis*-RA), a metabolite of vitamin A. Activation of RXR has been shown to inhibit IL-12 production by macrophages and to promote Th2 differentiation in vitro (3, 4). In vivo, vitamin A-deficient mice are unable to mount an effective Th2-mediated immune response and launch an excessive Th1-response instead (5). RXR serves as a heterodimerization partner for a variety of other nuclear hormone receptors (6). Among these

are the retinoic acid receptor (RAR) and the vitamin D receptor (VDR). Ligands for both receptors have been shown to inhibit IL-12 production and to promote Th2 differentiation (3, 7, 8).

The development of allergic asthma is favored by an excessive Th2 response marked by high levels of IL-4 and IL-5, which augment IgE synthesis and eosinophil survival. Because RXR alone and in combination with other nuclear hormone receptors promotes Th2 differentiation, we wondered whether RXR could be exploited to block Th2 differentiation and associated disease. We show in this study that a pan-RXR-specific antagonist efficiently inhibited Th2 differentiation while promoting Th1 differentiation in vitro. Furthermore, the development of allergic lung inflammation was suppressed in vivo. This demonstrates that RXR might be a valuable target for interfering with the development of Th2-driven diseases such as allergic asthma.

Materials and Methods

In vitro differentiation of mouse Th cells

CD4⁺ Th cells were isolated from the spleen and lymph nodes of DO11.10 mice using anti-CD4-coupled magnetic beads (Miltenyi Biotec). Purified Th cells were stimulated with 0.3 μ M OVA_{323–339} peptide and irradiated (2500 rad) BALB/c splenocytes. The pan-RXR antagonist Ro 26-5405 or 9-*cis*-RA (synthesized at F. Hoffmann-La Roche) were added at the indicated concentrations and were present throughout the culture period. Stock solutions for both compounds were prepared in DMSO. The final concentration of DMSO in the cultures was 0.1% in all conditions. To some cultures, cytokines and Abs were added as indicated. After 72 h, 100 U/ml human rIL-2 was added. The cells were expanded for 1 wk, and growing cells were split with medium containing fresh antagonist and IL-2. In some experiments, the Th cells were restimulated once more under the same conditions. Cytokine production was measured by either ELISA after restimulation with OVA and APC for 48 h or by intracellular cytokine staining after restimulation with PMA and ionomycin for 4 h (9).

Mice and in vivo procedures

Mice were housed under specific pathogen-free conditions, and all experiments were done in accordance with the institutional guidelines for animal care at the DIBIT (Milan, Italy). BALB/c and C57BL/6^{p40} (B6.129S1-IL12b^{tm1jm}/J) mice were purchased from Charles River Laboratories and The Jackson

*Bioxell SpA, Milan, Italy; †Basilea Pharmaceutica AG, Basel, Switzerland; and ‡Department of Medicine, Division of Rheumatology, Immunology, and Allergy, Brigham and Women's Hospital, Boston MA 02115

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¹ Address correspondence and reprint requests to Dr. Roland Grenningloh, Brigham and Women's Hospital, One Jimmy Fund Way, Smith Building, Room 524, Boston, MA 02115. E-mail address: rgrenningloh@rics.bwh.harvard.edu

² Current address: Am Hellenrain 6, D-79576 Weil a/Rh, Germany.

³ Current address: Birmannsgasse 14a, 4055 Basel, Switzerland.

⁴ Abbreviations used in this paper: RXR, retinoid X receptor; 9-*cis*-RA, 9-*cis*-retinoic acid; BAL, bronchoalveolar lavage; PBLN, peribronchial lymph node; VDR, vitamin D receptor.

Laboratory, respectively. DO11.10 (C.Cg-Tg(DO11.10)10Dlo/J) and BALB/c $IFN\gamma^{-/-}$ (C.129S7(B6)-Ifng^{tm1Ts}/J) mice were bred in our animal facilities. Female mice aged 8–10 wk were used for all experiments. To induce allergic lung inflammation, mice were sensitized by injecting 10 μ g of OVA adsorbed to 200 μ l of alum (Serva) i.p. on days 0 and 7. Additionally, some mice were injected i.p. with 2 mg (100 mg/kg) of the RXR antagonist Ro26-5405 suspended in 50 μ l of arachis oil on days 0, 1, 7, and 8. Control animals received arachis oil only. On day 14, the mice were placed in a Plexiglas box and exposed to an aerosol of 5% OVA solution in PBS generated by a Pari Star nebulizer (Pari GmbH) for 20 min. Seventy-two hours after this aerosol challenge, bronchoalveolar lavage (BAL) was performed. For differential cell counts, cytopins were prepared and stained with Diff-Quik (Dade Behring). Frozen sections of lung tissue were stained with H&E. OVA-specific serum IgE was measured using a kit (BD Pharmingen) and OVA (0.5 μ g/ml) as Ag.

Results and Discussion

An RXR antagonist blocks Th2 differentiation *in vitro*

To examine the effect of RXR blockade on Th2 differentiation, we stimulated purified Th cells with APCs and OVA_{323–339} in the presence of different doses of the pan-RXR antagonist Ro26-5405 (Fig. 1*g*). This compound binds RXR α and γ without activating them. It does not bind to any of the RAR isoforms (Table I). Th cells primed in the absence of Ro26-5405 predominantly produced IL-4 but very little IFN- γ (Fig. 1*a*). Treatment with the RXR antagonist reduced IL-4 and enhanced IFN- γ production in a dose-dependent manner. To characterize the effect of RXR blockade on a single-cell level, we performed intracellular cytokine staining. As shown in Fig. 1*b*, DMSO-treated Th cells were predominantly of the Th2 phenotype, as judged by the high percentages of IL-4⁺ (54%), IL-5⁺ (57%), IL-10⁺ (80%), and IL-13⁺ (90%) producing cells. Treatment with the RXR antagonist nearly completely inhibited

the development of IL-4⁺, IL-10⁺, and IL-5⁺ producing cells and reduced the number of IL-13⁺ cells to 50%. Instead, the number of IFN- γ -producing cells increased from 7 to 29%. Therefore, inhibition of RXR *in vitro* impairs Th2 differentiation while promoting Th1 differentiation.

IL-12 drives Th1 differentiation (2), and its expression by monocytes can be inhibited by some nuclear hormone receptor ligands (8, 10), providing a possible explanation for the reduced Th2 differentiation that we observed. However, neutralization of IL-12 did not influence the effect of the RXR antagonist on Th differentiation at all (Fig. 1*c*, *left panel*). In addition to IL-12, IFN- γ is a very potent Th1-inducing cytokine (2). In the presence of neutralizing Abs against IFN- γ , the RXR antagonist only partially inhibited IL-4 and IL-10 production and failed to reduce IL-13 or enhance IFN- γ production. Interestingly, production of IL-5 was still completely blocked (Fig. 1*c*, *middle panel*).

IL-4 is a very potent Th2-driving cytokine. Its reduced expression (Fig. 1*a*) might account for the inhibition of other Th2 cytokines. We therefore tested whether addition of IL-4 would overcome the inhibition of Th2 differentiation by RXR blockade. As shown in Fig. 1*c* (*right panel*), stimulation of Th cells under Th2-skewing conditions (IL-4 plus anti-IL-12) further increased the percentage of IL-4-producing cells, compared with nonskewing conditions. The addition of the RXR antagonist did not significantly reduce the production of IL-4, IL-10, and IL-13. However, to our surprise, the development of IL-5-producing Th cells was still completely inhibited by Ro26-5405.

FIGURE 1. An RXR antagonist inhibits Th2 differentiation. *a*, Purified Th cells derived from DO11.10 mice were stimulated with OVA_{323–339} and irradiated BALB/c spleen cells. The RXR antagonist Ro 26-5405 was added in the indicated concentrations. After 1 wk of culture, the Th cells were restimulated with Ag and fresh APC, without addition of the RXR antagonist. Cytokine production was measured by ELISA 48 h later. *b*, Th cells were stimulated as described in *a* in the presence of DMSO or 1 μ M Ro26-5405. The cells were expanded for 7 days and underwent a second round of stimulation. The additional round of stimulation was necessary to induce high-level expression of IL-5. After 1 wk, the cells were stimulated with PMA/ionomycin, and the cytokine production was measured by flow cytometry. KJ1-26-positive cells were gated. *c*, Th cells underwent two rounds of stimulation as described in *b*. Anti-IL-12, anti-IFN- γ (both 10 μ g/ml), and IL-4 (10 ng/ml) were added during both stimulations as indicated. Bar graphs show the percentage of cells positive for the indicated cytokine after stimulation with PMA/ionomycin. (□, Ro26-5405; ■, DMSO). *d*, In the absence of APC, only IL-5 production is inhibited. Purified DO11.10 Th cells were stimulated with anti-CD3 and anti-CD28 in the presence of 1 μ M Ro26-5405 (□) or DMSO (■). After two rounds of stimulation, cytokine production was determined as in *b*. *e*, Expression of GATA-3 and T-bet. Th cells were stimulated as described in *b* and *d*. After two rounds of stimulation, the cells were stimulated with PMA/ionomycin for 4 h. Total cell lysates were subjected to Western blot analysis using Abs against the indicated proteins. *f*, Band intensities from *e* were quantified using ImageJ software (National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>) and normalized to Hsp90. *a–d*, Data representative of at least three experiments are shown. *g*, Structure of 9-*cis*-RA and Ro26-5405.

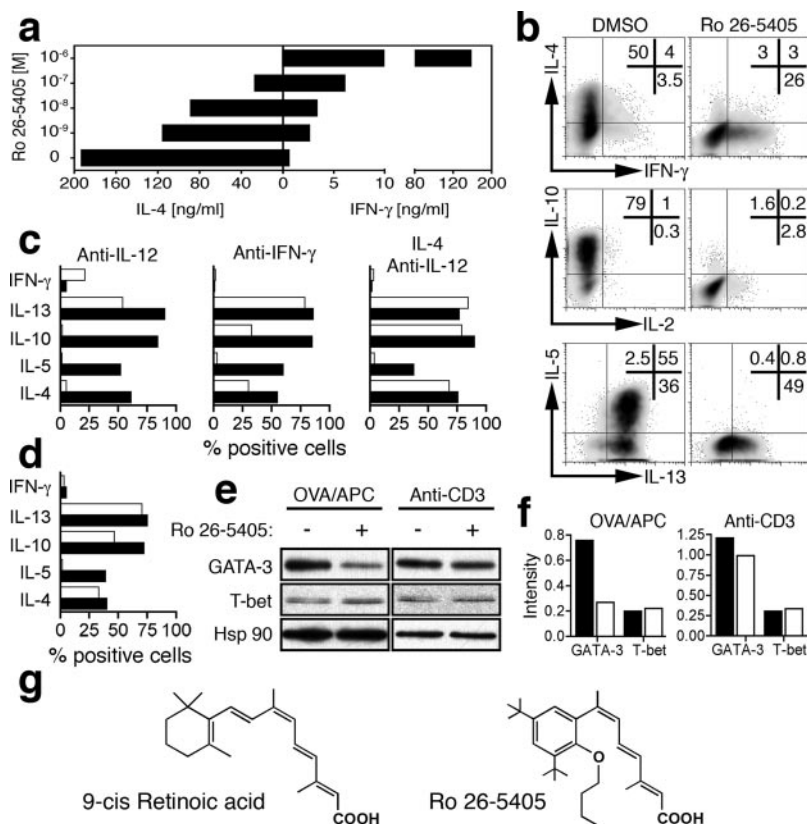


Table I. Ro26-5405 binds to human RXR α and γ without activating them

	RAR α	RAR β	RAR γ	RXR α	RXR β	RXR γ
Binding (IC ₅₀ ^a nM)	4,200	>10,000	2,700	30	ND ^c	47
Activation (EC ₅₀ ^b nM)	10,000	10,000	10,000	10,000	ND	10,000

^a IC₅₀, Concentration required to inhibit 50% of specific retinoic acid binding. Values were determined as described in Ref. 19.^b EC₅₀, Concentration required to achieve 50% of maximal receptor activation.^c ND, Not done.

The RXR antagonist blocks Th2 differentiation via APC and IL-5 production directly in T cells

The inhibition of Th2 differentiation by Ro26-5405 was partly mediated by IFN- γ . In our culture conditions, IFN- γ could be derived from several cell types contained in the splenic APC, but also from the Th cells themselves. To test whether Ro 26-5405 inhibited Th2 differentiation by enhancing Th cell-derived IFN- γ , we stimulated purified Th cells with anti-CD3 and anti-CD28 in the presence or absence of Ro26-5405. As shown in Fig. 1*d*, the Th cells developed mainly into Th2 cells, and cytokine production was comparable to cells stimulated with Ag and APC. Addition of Ro26-5405 did not alter the production of IL-4 and IL-13 and only moderately reduced IL-10 production. Therefore, RXR blockade most likely enhances IFN- γ production in non-T cells. In fact, dendritic cells have been demonstrated to produce significant amounts of IFN- γ (11). An early spike in IFN- γ could then induce IFN- γ production by Th cells (2) and thus contribute to the inhibition of Th2 cytokine production. In contrast, the production of IL-5 was completely abolished by RXR blockade even in the absence of APC. This indicated that the RXR antagonist inhibited Th2 differentiation on multiple levels: inhibition of IL-4, -10, and -13 was mediated by APC, whereas IL-5 production was directly inhibited in Th cells.

Th1 and Th2 differentiation are largely controlled by the two transcription factors, T-bet and GATA-3, respectively (2). Both vitamin D and vitamin A have been described to induce GATA-3 (4, 7). Consistent with their Th2-like phenotype, Th cells stimulated under neutral conditions expressed GATA-3 but very little T-bet, regardless of whether the cells had been stimulated with OVA and APC or with anti-CD3 and anti-CD28 (Fig. 1*e*, lanes 1 and 3, and Fig. 1*f*). Treatment with Ro 26-5405 reduced GATA-3 expression in OVA/APC-stimulated Th cells but not in anti-CD3-stimulated Th cells. This argues against a direct regulation of GATA-3 by RXR and in-

dicates that the lower GATA-3 level was a secondary effect of impaired Th2 differentiation. However, it cannot be excluded that APC might serve as a source for RXR agonists (12) that might be counteracted by Ro26-5405.

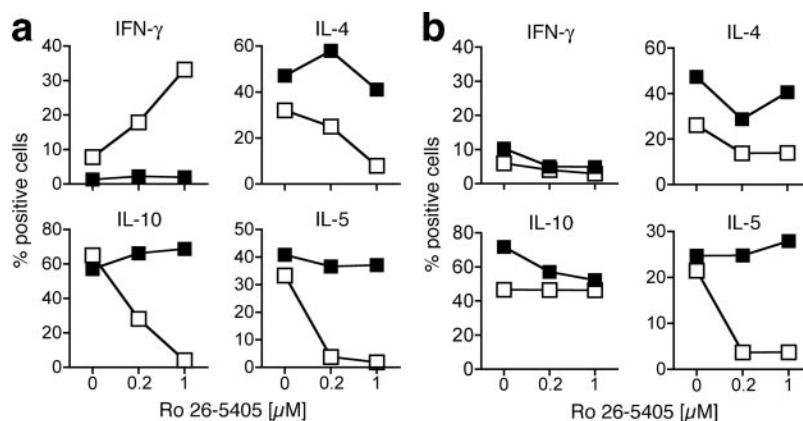
Ro26-5405 inhibits Th2 differentiation via RXR

Both retinoids and vitamin D have been described to directly enhance IL-4 production by Th cells (4, 7). In contrast, we found that inhibition of most Th2 cytokines by the RXR antagonist required the presence of APC, whereas IL-5 was inhibited directly in Th cells. To exclude the possibility that Ro 26-5405 might inhibit Th2 differentiation via a mechanism not involving RXR or its binding partners, we tested whether activation of RXR would revert all effects of the RXR antagonist. Addition of 9-*cis*-RA, a ligand for both RAR and RXR, completely restored Th2 differentiation in the APC/OVA system (Fig. 2*a*) and IL-5 production in anti-CD3/anti-CD28-stimulated Th cells (Fig. 2*b*). This suggests that Ro26-5405 acts on RXR homodimers or on RXR-containing heterodimers.

RXR blockade reduces allergic lung inflammation in vivo

BALB/c mice were sensitized by two i.p. injections with OVA adsorbed to alum. Additionally, mice received either 2 mg of Ro26-5405 or vehicle alone. Fourteen days after the first immunization, the mice were challenged with an OVA aerosol, and lung inflammation was measured 72 h hours later by BAL. As shown in Fig. 3*a*, the BAL of challenged, untreated mice contained high numbers of eosinophils at this time point. Mice that had received the RXR antagonist during sensitization contained significantly lower numbers of eosinophils and lymphocytes in their airways, whereas the numbers of macrophages and neutrophils were unaffected. Lung sections stained with H&E revealed a strongly reduced inflammatory response in treated vs mock-treated mice (Fig. 3*d*). Quantitative analysis of the number of eosinophils found in the lung parenchyma near venules

FIGURE 2. Ro26-5405 inhibits Th2 cytokine production specifically via RXR. DO11.10 Th cells were stimulated with OVA/APC (*a*) or anti-CD3 and anti-CD28 (*b*) in the presence of the indicated amounts of Ro26-5405 alone ($\square$) or together with 1 μ M 9-*cis*-RA ($\blacksquare$). After two rounds of stimulation, the cells were restimulated with PMA/ionomycin, and cytokine production was measured by flow cytometry. The graphs show the percentage of cells positive for the indicated cytokine.



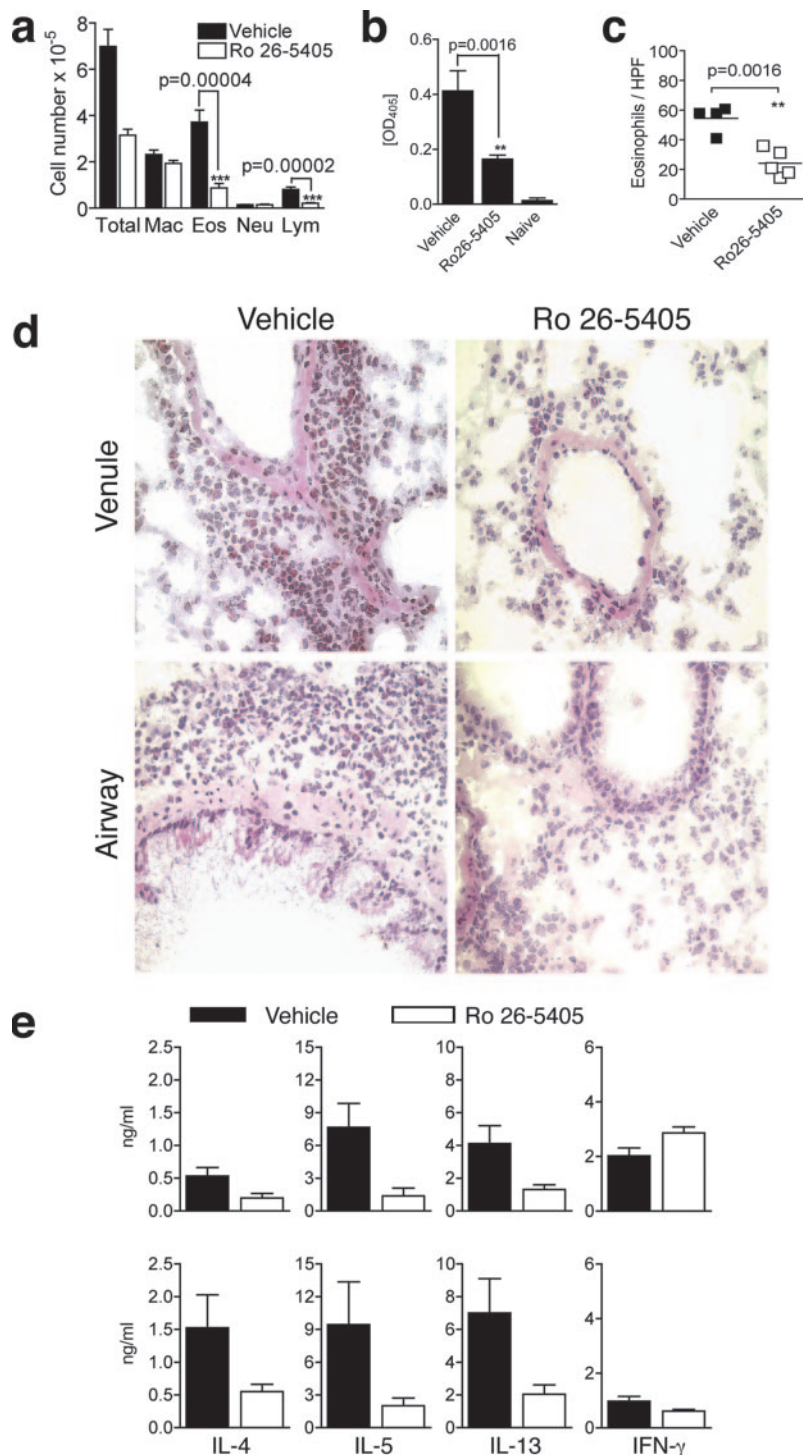


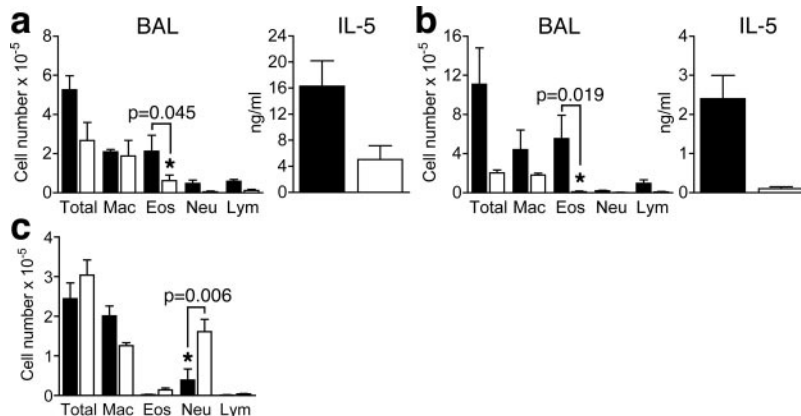
FIGURE 3. Inhibition of RXR prevents experimentally induced allergic lung inflammation. BALB/c mice were treated with the RXR antagonist Ro26-5405 or vehicle alone during the sensitization phase. Seventy-two hours after challenge with aerosolized Ag, the mice were sacrificed. *a*, BAL cell numbers. Mean values and SE were calculated from the combined results of six independent experiments, each with five mice per group. Values of *p* were calculated using an unpaired *t* test. *b*, OVA-specific serum IgE levels in treated, mock-treated, and naive mice. *c* and *d*, Leukocyte accumulation in the lung tissue of treated vs untreated mice. *d*, Sections of frozen lung tissue were stained with H&E. The severity of eosinophilia was determined by counting the numbers of eosinophils per high-power field in several different sections for each individual animal (*c*). Mean values for single mice are shown. Results are representative for two independent experiments. *e*, Spleen (*upper panels*) or PBLN (*lower panels*) were harvested 72 h after the OVA aerosol challenge and were restimulated *in vitro* with 10 μ g/ml OVA and 20 U/ml IL-2. After 48 h, cytokine production was measured by ELISA. The results are representative of three independent experiments. No cytokines were detected in cultures without OVA.

or airways reflected the reduction of eosinophil numbers already observed in BAL (Fig. 3*c*). Along with eosinophilia, the levels of OVA-specific IgE were reduced significantly in treated mice (Fig. 3*b*). The numbers of eosinophils and the level of Ag-specific IgE are strongly dependent on IL-5 and IL-4, respectively. We therefore examined the cytokine production by spleen and peribronchial lymph node (PBLN) cells isolated from treated vs mock-treated animals. Both spleen and LN cells from treated mice produced 3-fold less IL-4, IL-5, and IL-13 after restimulation than cells obtained from control animals. The production of IFN- γ was unchanged (Fig. 3*e*).

Inhibition of Th2-mediated inflammation in vivo is not dependent on IL-12 or IFN- γ

Our *in vitro* results showed that blockade of RXR resulted in reduced Th2 and enhanced Th1 differentiation. This effect was independent of IL-12 but partially dependent on IFN- γ . However, *in vivo*, up-regulation of IL-12 by treatment with Ro26-5405 might still occur and might contribute to the prevention of allergic inflammation. To test whether this was the case, we used IL-12 (p40)-deficient mice in the model of allergic lung inflammation. In IL-12-deficient mice, treatment with the

FIGURE 4. Protection against allergic lung inflammation by Ro26-5405 in vivo does not depend on IL-12 or IFN- γ . IL-12-deficient (*a*) or IFN- γ -deficient (*b*) mice were treated with Ro26-5405 (□) or vehicle alone (■) during sensitization and then challenged with aerosolized Ag. BAL cell numbers were determined 72 h after aerosol challenge. PBLN cells were restimulated with 10 μ g/ml OVA in vitro. Seventy-two hours later, the IL-5 production was measured by ELISA. *c*, Th1-driven inflammation was enhanced by Ro26-5405 treatment. BALB/c mice were immunized with OVA emulsified in CFA and treated with Ro26-5405 as described before. Thirty-six hours after the OVA aerosol challenge, BAL cells were quantified. In all experiments, five mice per group were used. Values of *p* were calculated using an unpaired, one-tailed *t* test.



RXR antagonist reduced the eosinophil influx into the airways to the same extent as it did in wild-type animals. In addition, PBLN cells isolated from treated mice produced less IL-5 than those derived from control mice (Fig. 4*a*). Therefore, IL-12 did not play a role in the inhibition of allergic inflammation by Ro26-5405.

In vitro, neutralization of IFN- γ partly prevented the switch from Th2 to Th1 differentiation caused by RXR blockade, although it did not restore IL-5 production. Likewise, treatment with Ro26-5405 in vivo might cause increased IFN- γ production and thereby prevent the development of allergic inflammation. However, when we used IFN- γ -deficient mice, treatment with the RXR antagonist still completely prevented lung eosinophilia and IL-5 production by PBLN cells (Fig. 4*b*). To rule out the possibility that Ro26-5405 simply inhibits the immune response in general, we examined how treatment with the RXR antagonist would affect a Th1-driven immune response. To this aim, we modified the asthma model such that BALB/c mice were immunized with OVA emulsified in CFA instead of alum. As shown in Fig. 4*c*, this protocol induced a Th1 response manifesting in a modest influx of neutrophils instead of eosinophils into the bronchi. The number of neutrophils increased significantly in mice that had been treated with the RXR antagonist during the sensitization phase.

Our data demonstrate that blockade of RXR did not induce a counterregulatory Th1 response, but rather specifically inhibited Th2-mediated inflammation. In fact, others have shown that Th1 cells do not counteract Th2 inflammation in this model, but rather exacerbate disease (13). It might be argued that the RXR antagonist might be toxic for certain cell types and thereby inhibit the immune response. We believe this not to be the case for several reasons. First, as described before, a Th1-driven inflammation was not inhibited. Second, a toxic effect on eosinophils is unlikely because we found the same number of eosinophil-like cells in the bone marrow of treated and untreated mice, and the RXR antagonist did not inhibit IL-5-driven eosinophil differentiation from bone marrow cells in vitro (data not shown). Third, the proliferation of Th cells stimulated with anti-CD3 and anti-CD28 in vitro was not affected by Ro26-5405 (data not shown). However, we did observe somewhat reduced proliferation in the OVA/APC system. This would indicate that Ag presentation or costimulation by APC might be affected. This, in turn, might influence Th differentiation. For example, B7/CD28 interactions appear to be required for Th2 differentiation (14, 15). In contrast, LFA-1/

ICAM-1 interactions have been reported to favor Th1 over Th2 differentiation (15–17), and the signal strength appears to influence T cell differentiation as well (2).

Because RXR serves as a heterodimerization partner for several different nuclear hormone receptors such as VDR, RAR, and peroxisome proliferator-activated receptor, it is possible that treatment with an RXR antagonist might inhibit signaling by these receptors as well. In this context, it is noteworthy that VDR-deficient mice have been reported to be resistant against development of experimentally induced allergic asthma (18).

Taken together, we have shown for the first time that, by blocking the nuclear hormone receptor RXR, the differentiation of Th2 cells can be inhibited in vitro, and that the development of allergic disease is efficiently prevented in vivo. Modulation of nuclear hormone receptor signaling might prove to be a powerful tool to treat Th2-mediated diseases such as allergic asthma or atopic dermatitis.

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Disclosures

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