

Resiquimod, a new immune response modifier from the family of imidazoquinolinamines, inhibits allergen-induced Th2 responses, airway inflammation and airway hyper-reactivity in mice

D. Quarcoo*, S. Weixler*, R.A. Joachim†, P. Stock*, T. Kallinich, B. Ahrens* and E. Hamelmann

Departments of *Pediatric Pneumology and Immunology and †Internal Medicine, Charité- Humboldt University, Berlin, Germany

Summary

Background Allergen-induced sensitization and airway disease are the results of adverse immune reactions against environmental antigens that may be prevented or inhibited by immune modifying strategies.

Objective To investigate the effects of the novel immune response modifier resiquimod (R-848), from the family of imidazol-derivates, in a murine model of allergen-mediated Th2-immune responses and concomitant airway inflammation and airway hyper-reactivity.

Methods BALB/c mice were systemically sensitized with ovalbumin (OVA) on days 1 and 14 and challenged with OVA aerosol on days 28 and 29. R-848 was applied intranasally to sensitized animals once prior to the first allergen airway challenge, on day 27.

Results A single application of R-848 significantly reduced numbers of eosinophils and lymphocytes in bronchoalveolar lavage fluid and inhibited mucus gland hyperplasia, compared with sensitized and challenged controls. Associated with the decrease in airway inflammation, single intranasal treatment with R-848 abolished the development of airway hyper-reactivity after allergen sensitization and airway challenges. Additionally, Th2-cytokine production in lung tissues from sensitized and R-848-treated animals was reduced, whereas IL-12 and IFN- γ production was increased, compared with non-treated sensitized mice.

Conclusion These data indicate that R-848 effectively inhibits allergen-induced airway inflammation and hyper-reactivity by modulation of increased Th2-immune responses.

Keywords allergy, asthma, cytokine, eosinophils, immune response modifier, inflammation, lung, mice, ovalbumin

Submitted 13 October 2003; revised 14 February 2004; accepted 11 May 2004

Introduction

A key feature of many immunological disorders is the expression of a distinct cytokine pattern by T helper (Th) cells. Allergic diseases, like bronchial asthma, have been found to be associated with a humoral (Th2-type) immune response after sensitization with environmental antigens, such as pollen [1–3]. This observation has been supported by numerous studies showing increased production of Th2-cytokines, or Th2-cytokine mRNA in tissues with allergic inflammation [4, 5]. Thus, counterbalancing this excess of Th2-cytokine production by supporting Th1 skewing of T lymphocytes seems to be an appropriate means to treat or prevent the development of allergen-mediated diseases. Different strategies have been employed using various agents to directly enhance Th1 skewing. Application of microbial products, such as bacterial lipopolysaccharide (LPS) [6] or

microbial DNA containing immune stimulatory CpG oligonucleotide motifs [7, 8], to sensitized animals resulted in increased production of IL-12 by dendritic cells (DC) and IFN- γ by T cells [9, 10]. However, promising data derived from animal studies directly applying Th1 cytokines [11, 12] proved less effective or even toxic in human studies [13, 14].

Recently, a new group of low-weight immune response modifiers has emerged, which, similarly to the above-mentioned bacterial products, bind to cell receptors of the innate immune system, the Toll-like receptors (TLR), and induce increased Th1-cytokine production [15]. From the family of imidazoquinolines, imiquimod has been shown to be effective in the treatment of viral infections and neoplastic diseases by enhancing Th1-immune responses [16]. Much of this biological activity has been attributed to the capacity of these compounds to induce significant production of IFNs [17] that are increased in animals and human individuals after topical or systemical treatment with imiquimod [18, 19]. Increased levels of IFN subsequently induce the production of other cytokines [20, 21], such as IL-1, IL-6 and IL-8. Main cellular target of imidazoquinolines are macrophages and DC

Correspondence: Eckard Hamelmann, Department of Pediatric Pneumology and Immunology, Charité Humboldt University, Augustenburger Platz 1, 13353 Berlin, Germany. E-mail: eckard.hamelmann@charite.de

[22], but other cells that are involved in the allergic immune reaction, such as B cells, may also be influenced by the compound [23, 24].

In the current study, we used a new member of the imidazoquinoline family, the imiquimod derivative R-848, to investigate its effects in a murine model of allergen-induced sensitization and airway inflammation. In comparison to imiquimod, R-848 was substituted on the imidazol ring by an ethoxymethyl rest. Additionally, a hydroxy group was added to the methylpropyl of the imidazol molecule. This new molecule exerts enhanced immune stimulatory properties as compared to imiquimod, reflected by a 1000 time stronger antiviral and cytokine inducing capacity [25].

Here, we show for the first time that a single application of R-848 to the airways of sensitized animals prior to airway allergen challenges resulted in the near-complete inhibition of airway inflammation and development of *in vivo* airway hyper-responsiveness (AHR), in association with a shift of the local immune response towards a predominant Th1-cytokine production.

Methods

Animals

All experiments were approved by the animal research ethical institution (LAGetSi, Germany). Female BALB/c mice, 20–23 g (BgVV, Berlin, Germany) arrived pathogen free and were maintained under controlled conditions.

Sensitization and treatment

Animals were systemically sensitized by intraperitoneal injection of 20 µg ovalbumin (OVA) (Sigma Aldrich, Steinheim, Germany, OVA grade VI) emulsified in 2 mg ALOH₃ on days 1 and 14. Negative controls were sham sensitized with phosphate buffered saline (PBS) following the same protocol. All animals were challenged via the airways with aerosolized OVA (1% in PBS) or PBS (negative controls: PBS–PBS) for 20 min, on days 28 and 29, as previously described [26]. Sensitized animals that were sham challenged with PBS did not show any sign of airway inflammation or mucus gland hyperplasia and had similar lung function compared to sham-sensitized, sham-challenged mice (data not shown). Additionally, some mice from the treatment groups received a single intranasal application of resiquimod, R-848 (4-amino-2-ethoxymethyl- α,α -dimethyl-1*H*-imidazo-(4, 5-*c*) quinoline-1-ethanol, kindly provided by 3M Medica, Neuss, Germany), at doses of 25, 250 or 500 µg (OVA–R-848), 24 h before the first allergen airway challenge (day 27). Positive controls were sensitized animals receiving PBS intranasally (*i.n.*) sham treatment prior to OVA airway challenges (OVA–PBS). All animals were killed and lavaged 24 h after the last airway challenge as previously described [26]. For quantitative mRNA studies, sensitized mice were challenged once *i.n.* with 50 µg OVA in 50 µL of PBS (day 28) and analysed 6 h later.

Cell culture

Spleen mononuclear cells (MNC) from OVA-sensitized mice were plated in 96-well round-bottom plates at 1×10^6 cells/

well and pre-incubated with R-848 for 4 h, before addition of OVA (grade V, 25 and 100 µg/mL), or PBS. Lung MNC were extracted by lung digestion as previously described [27]. Briefly, lungs were perfused via the right ventricle using PBS. Lungs were then removed and cut into 300 µm pieces. Four millilitres of RPMI containing 175 U/mL collagenase (type Ia, Sigma Aldrich), 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 µg/mL streptomycin were added to the minced lungs and incubated for 60 min in an orbital shaker at 37 °C. The digested lungs were filtered through 15 µm filters. Obtained cells were resuspended, counted and plated to 96 well plates and stimulated for 24 h with phorbol-12-myristat-13-acetate and ionomycin (PI). After allergen stimulation, cell-free supernatant was harvested at 48 h (for IFN- γ and IL-12), or at 96 h (for IL-4 and IL-5). Following mitogen stimulation, supernatant was harvested after 24 h.

Determination of cytokines from bronchoalveolar lavage fluid (BALF) and culture supernatants

Cytokine levels were determined by ELISA (Pharmingen, Hamburg, Germany), as previously described [6]. Detection levels were 10 pg/mL for IL-4, 7 pg/mL for IL-5, 15 pg/mL for IFN- γ and 8 pg/mL for IL-12.

Histochemistry

Lung tissue was fixed in 4% formalin, dehydrated, mounted in paraffin, sectioned, and stained with periodic acid-Schiff (PAS) reaction using a standard protocol.

Determination of *in vivo* airway reactivity

Allergic rhinitis (AR) was determined in non-anaesthetized, conscious mice by barometric whole-body plethysmography (Buxco, Wilmington, NC, USA), as previously described [26]. Mice were challenged with aerosolized PBS and increasing doses of methacholine (MCh, 6–50 mg/mL) for 3 min each. Data are expressed as fold increase of baseline values after PBS, using the dimensionless parameter 'enhanced pause' (P_{enh}).

Quantitative mRNA analysis

Total RNA was extracted from whole lung tissue and reversely transcribed (Superscript II RT, Perkin Elmer, Weiterstadt, Germany). For quantification of specific RNA, Taqman technique was used. Sequences have been taken from a publication by Overbergh [28]. PCR primers and fluorogenic probes for target genes were purchased from Tib Molbiol (Berlin, Germany). PCR reactions were performed in the ABI-prism 7700 sequence detector, which contains a Gene-Amp PCR system 9600 (Perkin Elmer). Following conditions were used for PCR amplifications: 10 min at 94 °C, followed by a total of 40 two-temperature cycles of 15 s at 94 °C and 1 min at 60 °C. As negative controls, RNA samples and H₂O were processed in order to evaluate putative genomic DNA contamination. For each sample, 'threshold cycle' (C_T) was determined. As endogenous reference for target gene mRNA, the constitutive expressed protein hypoxanthine guanine phosphoribosyl transferase was determined. Each group consisted of eight animals, two separate studies were done.

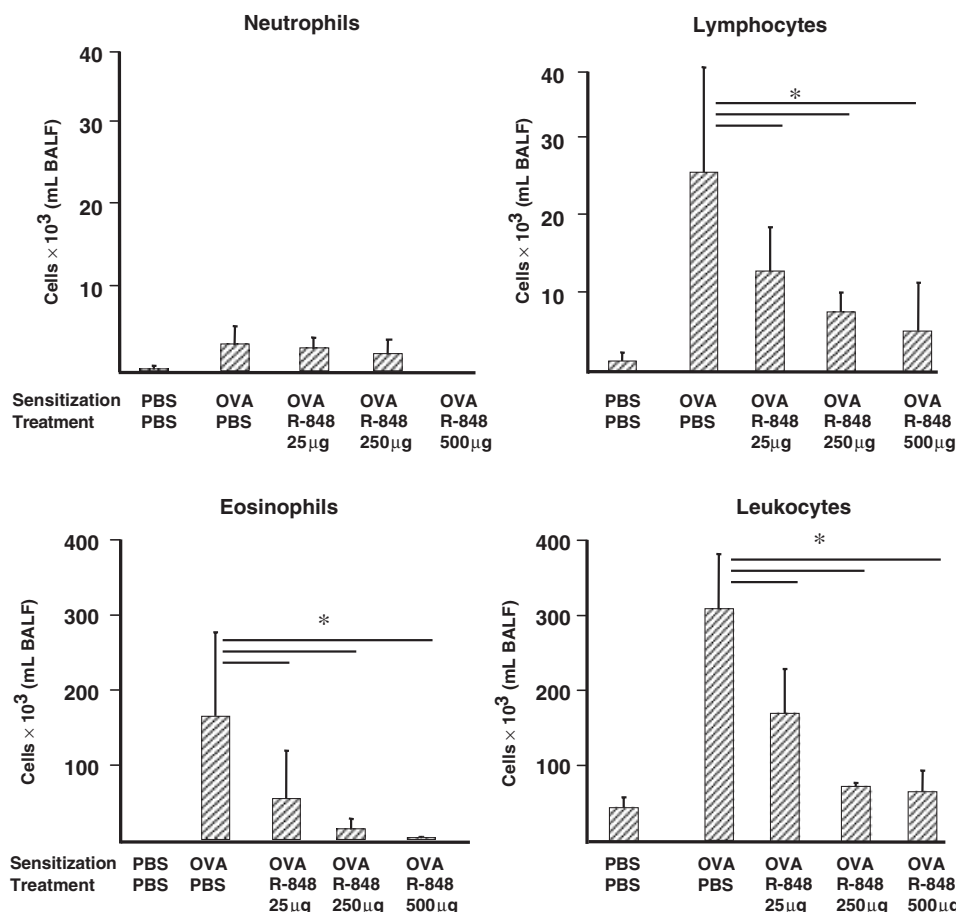


Fig. 1. Cell differentiation in bronchoalveolar lavage fluid (BALF). Neutrophils, lymphocytes, eosinophils and total leukocytes in BALF of sham- or ovalbumin (OVA)-sensitized mice were differentiated according to morphologic criteria 24 h after the last OVA aerosol challenge. Results are expressed as mean $\pm$ SEM. A total of 10 mice from three independent experiments were examined.

Statistical analysis

Results are presented as mean values $\pm$ SEM. Means were compared by using Student's *t*-test, SPSS, Version 7.5.2 G (SPSS Inc., Chicago, IL, USA). *P*-values < 0.05 were regarded as significant differences.

Results

Differential cell count in BALF

The presence and amount of immune cells in the BALF of OVA-sensitized and challenged animals were determined. Following sensitization and airway allergen challenges (OVA-PBS), numbers of total leukocytes, as well as numbers of lymphocytes and eosinophils were significantly increased compared to sham-sensitized animals (PBS-PBS) (Fig. 1). Single local treatment with R-848 (OVA-R-848) significantly reduced numbers of all cell types in the BALF in a dose-dependent manner (Fig. 1). Treatment with the highest dose of R-848 nearly completely abolished leukocytic and eosinophilic airway infiltration.

Assessment of local mucus production

The level of airway metaplasia and mucus production was assessed by PAS staining of centrally located lung tissues and

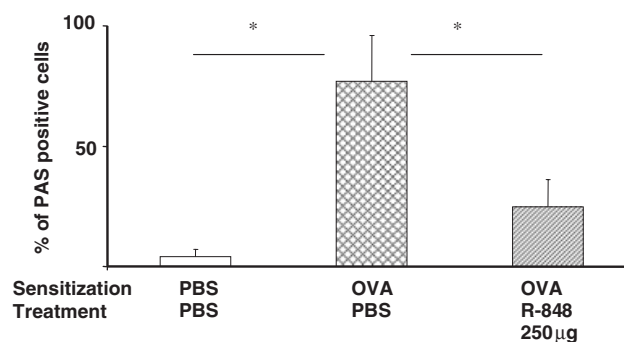


Fig. 2. Lung mucus production. Lungs were harvested, cut and stained with periodic acid Schiff (PAS) 24 h after the last aerosol challenge, PAS positive cells were counted in the large airways and expressed as percentage of total epithelial cells. Results are expressed as mean $\pm$ SEM of two independent experiments ($n = 8$).

the percentage of PAS positive of total epithelial cells was determined. Following sensitization and airway allergen challenges, the percentage of mucus producing goblet cells were increased, as compared to sham-sensitized mice (Fig. 2). In contrast, R-848-treated sensitized and challenged animals displayed a significant reduction in numbers of goblet cells in the airway epithelium to about 30% as compared with sensitized and challenged PBS-treated mice (Fig. 2).

Determination of *in vivo* airway reactivity

In vivo airway reactivity to aerosolized MCh in spontaneously breathing, conscious mice was assessed by barometric whole-body plethysmography [26]. This method reliably detects increases of *in vivo* AR after allergen sensitization and airway challenge in BALB/c mice, and results obtained with this technique correlate with results of invasive measurements of intrapleural pressure and airway resistance [26]. Following OVA sensitization and airway allergen challenges, airway reactivity after provocation with increasing doses of MCh was significantly increased, compared to negative controls. In contrast, airway reactivity of sensitized challenged mice treated once *i.n.* with 250 µg of R-848 prior to allergen challenges was significantly decreased, compared with positive controls (Fig. 3).

Effects of R-848 on Th1-/Th2-cytokine production

To assess the means by which R-848 may induce these anti-inflammatory effects, we studied the effect of R-848 on Th1-/

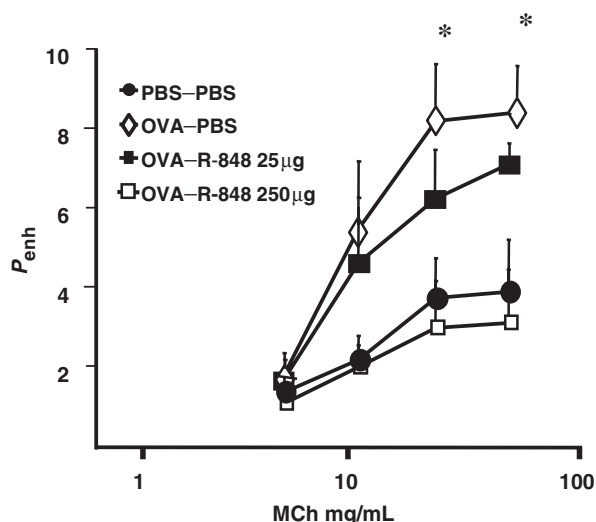


Fig. 3. Airway reactivity. Airway reactivity after challenge with aerosolized metacholin (MCh) was measured in unrestrained, conscious mice by whole-body plethysmography. Expressed are the mean $\pm$ SEM of fold increases in P_{enh} following rising concentrations of MCh compared to P_{enh} after PBS of three independent experiments ($n = 10$). (* $P < 0.05$ for positive control vs. R-848 250 µg.)

Th2-cytokine production *in vivo* and *in vitro*. First, cytokine production by lung MNC of positive controls and R-848-treated sensitized and challenged animals were determined *ex vivo*. Lung cells from sensitized and challenged mice produced significant amounts of Th2-cytokines (IL-4 and IL-5) and only little IFN- γ after PI stimulation (Fig. 4), as compared to negative controls. The single application of R-848 to sensitized mice prior to allergen airway challenges resulted in a significant reduction in Th2-cytokine production and a relative increase in IFN- γ production, compared to positive controls (Fig. 4). Corresponding to these data, increased IL-5 protein levels were found in the BALF of sensitized and challenged animals, compared with negative controls. In animals that were treated with high doses of R-848, IL-5 protein levels were significantly reduced in BALF compared to positive controls (data not shown).

Next, the effects of R-848 treatment on cytokine production were assessed directly in the airways on a molecular level. Therefore whole lung mRNA expression was determined 6 h after single allergen airway challenge using quantitative RNA analysis (Fig. 5). This time point was chosen to evaluate early regulatory changes in the lung tissues. In sham-sensitized mice (PBS-PBS), only little amounts of IL-4 mRNA were detected, and they significantly increased following allergen sensitization and airway challenge (OVA-PBS) (Fig. 5a). In contrast, sensitized animals treated once *i.n.* with R-848 prior to allergen airway challenge showed significantly less expression of IL-4 mRNA compared to positive controls (Fig. 5a). While no significant production of IL-12 mRNA in negative or in positive control animals was detectable, R-848-treated sensitized and challenged animals produced significantly higher amounts of IL-12 mRNA in lung tissues (Fig. 5b).

Finally, the effects of R-848 on *in vitro* cytokine production by cells from sensitized mice were assessed. After allergen-specific stimulation of spleen MNC, *in vitro* IL-4 production was increased nearly ninefold from 47 to 400 pg/mL, and IL-5 production was increased from 110 to 8000 pg/mL, compared to non-stimulated cells, whereas IL-12 and IFN- γ production were hardly detectable in supernatants of both non-stimulated or antigen stimulated cells. The addition of 1 µg/mL of R-848 (a concentration that had proven effective in pre-studies) to cell cultures 4 h prior to allergen stimulation resulted in 75% reduction in IL-4- and nearly 95% reduction in IL-5 production, compared to allergen stimulated cell cultures. The amount of IL-12 after addition of R-848 was

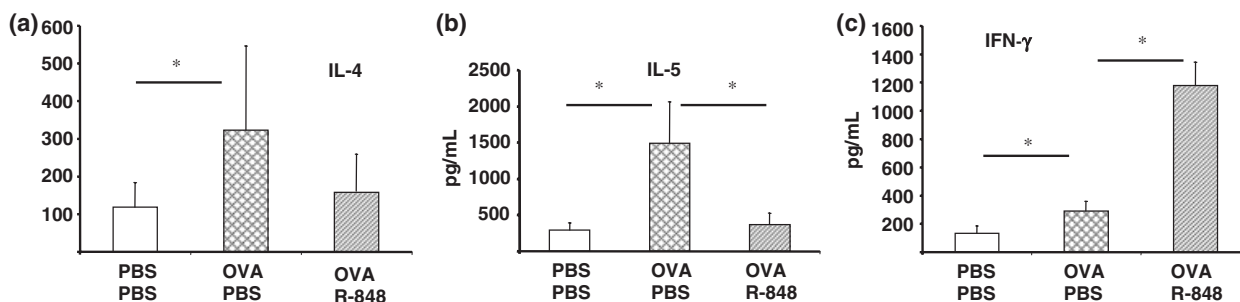


Fig. 4. Cytokine production in lung digests. Lung mononuclear cell (MNC) were stimulated with phorbol-12-myristat-13-acetate and ionomycin (PI). Cytokine levels of IL-4 (a), IL-5 (b) and IFN- γ (c) in the culture supernatant were measured by ELISA. Results are expressed as mean $\pm$ SEM of two independent experiments ($n = 8$). (* $P < 0.05$ for OVA-PBS vs. PBS-PBS or OVA R-848.)

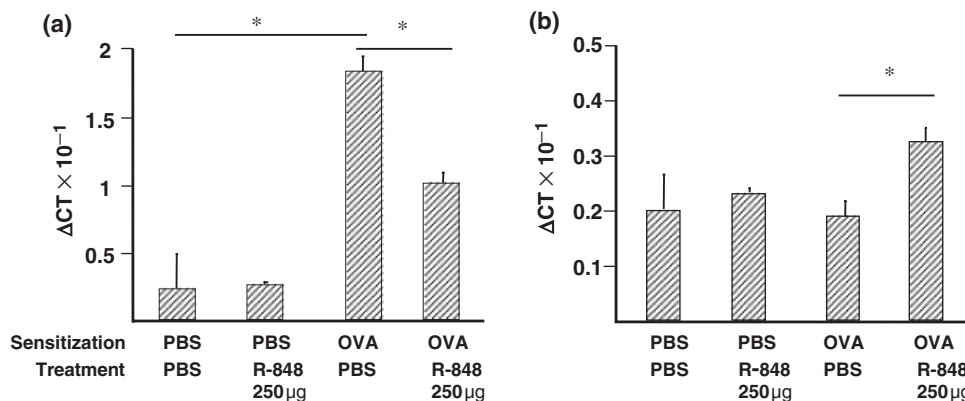


Fig. 5. Cytokine mRNA expression in lungs. RNA levels of IL-4 (a) and IL-12 (b) mRNA in whole lung extracts were measured by real-time RT-PCR and expressed as $\Delta\text{CT} \times 10^{-1}$. High $\Delta\text{CT} \times 10^{-1}$ values correspond to high mRNA amounts. Results are expressed as mean $\pm$ SEM of two independent experiments ($n = 8$). (* $P < 0.05$ for OVA-PBS vs. PBS-PBS or OVA R-848.)

significantly increased fivefold as compared with allergen stimulation, and detectable amounts of IFN- γ were present in the supernatant (data not shown).

Taken together, we detected a switch of *in vivo* and *in vitro* cytokine expression from a prevalent Th2 towards a predominant Th1-cytokine profile in response to R-848 treatment, suggesting this as a possible mechanism for the effects of R-848 on airway inflammation and reactivity.

Discussion

In the present paper, we show in a murine model of allergen-induced airway disease that a single, local administration of the imidazolquinoline derivative R-848 24 h before allergen airway challenge attenuated the development of airway inflammation and concomitant *in vivo* airway hyper-reactivity in a significant and dose-dependent manner. Although a number of studies already have indicated that members of the imidazolquinoline family have the capacity to improve pathological findings in disease models that require sustained cellular immune responses [29, 30], we describe here for the first time the effects of an imiquimod derivative in an *in vivo* model of allergen-induced airway inflammation.

We observed profound changes in the lungs of R-848-treated sensitized animals. Whereas allergen airway challenge of sensitized, but not of sham-sensitized, mice resulted in a robust inflammatory response with predominant eosinophilic infiltration and mucus gland hyperplasia, histological changes that depend on and occur as early as 24–48 h after local allergen challenge [31], application of R-848 to sensitized animals prior to local allergen exposure nearly completely abolished these phenomena. Associated with these changes were alterations in the pulmonary cytokine patterns, leading to an increase in Th1 cytokines, the likely basis for the immune modifying effects of R-848 on the inflammatory response. We detected a significant reduction of *in vivo* IL-5 production in the BALF and reduced capacity to produce IL-4 and IL-5 and enhanced production of IFN- γ by lung MNC of R-848-treated sensitized and challenged animals. Furthermore, we utilized RNA quantification to evaluate *in vivo* cytokine expression in lung tissues and demonstrated that R-848 pre-treatment was associated with significantly

reduced IL-4 and increased IL-12 mRNA expression 6 h after induction of airway inflammation by local allergen challenge.

This significant reduction of IL-4 production may have influenced the subsequent modulation of the inflammatory response by several ways. IL-4 has been shown to enhance the expression of vascular cell adhesion molecule-1 (VCAM-1) [32], an endothelial receptor required for targeted migration and tissue homing of leukocytes, especially eosinophils [33]. Accordingly, we noted in R-848-treated animals a profound reduction of lymphocyte and eosinophil numbers in the airways. In addition, IL-4 sustains differentiation of T cells towards a predominant Th2-type, thus promoting an ongoing Th2-response with further production of IL-4, IL-5 and IL-13 [34]. This link is supported by our data showing that IL-5 production in the BALF of R-848-treated animals was significantly smaller than in untreated sensitized and challenged animals. IL-5, on the other hand, enhances the maturation, terminal differentiation and activation of eosinophils and prevents apoptosis of eosinophils in tissues [35–38]. Finally, mucus gland hyperplasia depends on a Th2-biased airway inflammation [39]. A direct enhancing effect of IL-4 on IL-13-mediated goblet cell hyperplasia and increased mucus formation in the airways of sensitized, challenged mice has recently been reported [40]. Therefore, it appears very likely that the reduced mucus formation in R-848-treated animals observed in our study may be a direct effect of reduced Th2-cytokine expression.

Besides decreased Th2-cytokine formation, we observed increased *in vivo* expression of IL-12 mRNA in R-848-treated animals. IL-12, a major Th1 cytokine, has already been shown to directly reduce development of AHR and airway inflammation, when administered either systemically [11] or locally to the airways [41] of sensitized mice prior to allergen airway challenges. The data of increased IL-12 mRNA production together with the phenotypic findings of reduced eosinophilic airway inflammation and inhibition of *in vivo* AHR greatly support the assumption that R-848 modifies the allergic immune responses by counterbalancing increased Th2-cytokine production with increased Th1 responses. In contrast to direct application of IL-12, treatment with imiquimod derivatives is likely to be less toxic.

There is an ongoing controversy on discussion regarding the value of treatment strategies aiming at direct inhibition of

enhanced Th2-cytokine production in Th2 biased diseases. Some authors have demonstrated that the reduction of increased Th2-cytokine production by treatment with Th1 cytokines was associated with a marked symptomatic relief [11, 12]. On the other hand, it has been shown that allergen-specific Th1 cells, after transfer into airways of naïve mice prior to allergen airway challenges, did not reduce development of Th2-mediated AHR and inflammation, but in contrast caused severe airway inflammation themselves [42, 43]. In accordance to the former observations, we found in the current study nearly complete attenuation of airway inflammation following treatment with R-848, rather supporting a preventive or therapeutic effect of immune response modifiers with Th1-augmenting properties. Moreover, complete inhibition of allergen-induced development of *in vivo* AHR by single local treatment with R-848 strongly supports this concept.

Our findings suggest that the imidazoquinoline derivative interacts with the allergen-induced immune cascade at a stage, when the direction towards a predominant cellular or humoral immune response still is open and may be influenced. This implies that R-848 affects the innate immune system, which processes immunological threats at a very early stage. Indeed, *in vitro* data showed that imiquimod-triggered induction of Th1 cytokines is provided by activation of macrophages [22], the main source of IL-12 in early inflammation. In addition, recent findings by Hemmi et al. [44] have revealed that activation of monocytes by imidazoquinolines requires interaction with TCR-7 and intracellular signalling via MyD88. This puts R-848 in a decisive row with other immune modifiers like CpG and LPS, both bacterial compounds enhancing Th1-like immune responses [6, 45] through interaction with TLR on macrophages.

This notion may help to clarify the above-mentioned apparent controversy regarding the role of Th1 cells/Th1 cytokines in counterbalancing or rather augmenting allergen-mediated airway inflammation. Activation of allergen-specific and previously differentiated Th1 cells by subsequent allergen contact via the airways (e.g. allergen challenge following specific Th1 cell transfer) may induce further production of pro-inflammatory (Th1) mediators, thus further enhancing airway infiltration and development of altered airway function. In contrast, induction of a 'danger signal' prior to first allergen contact via the airways (e.g. local application of R-848 prior to allergen airway challenges) may enhance the responses of the unspecific, innate immune system, thus inhibiting the development of (Th2-mediated) allergen-induced airway inflammation and AHR. Nevertheless, it cannot be ruled out that R-848 exerts a direct effect on T cells.

In conclusion, our data indicate that *in vivo* treatment with R-848 effectively inhibits allergen-induced airway inflammation and development of *in vivo* AHR, presumably due to counterbalancing increased Th2-cytokine production in response to allergen sensitization and airway challenges.

Acknowledgements

This work was supported by 3M Medica and the DFG (Ha2162/2-1). We thank Christine Seib and Kathrin Borgwald for excellent technical assistance.

References

- 1 Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; 7:145–73.
- 2 Robinson DS, Hamid Q, Ying S et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992; 326:298–304.
- 3 Huang SK, Xiao HQ, Kleine-Tebbe J et al. IL-13 expression at the sites of allergen challenge in patients with asthma. *J Immunol* 1995; 155:2688–94.
- 4 Hamid Q, Azzawi M, Ying S et al. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J Clin Invest* 1991; 87:1541–6.
- 5 Hamelmann E, Oshiba A, Schwarze J et al. Allergen-specific IgE and IL-5 are essential for the development of airway hyperresponsiveness. *Am J Respir Cell Mol Biol* 1997; 16:674–82.
- 6 Gerhold K, Blumchen K, Bock A et al. Endotoxins prevent murine IgE production, TH2 immune responses, and development of airway eosinophilia but not airway hyperreactivity. *J Allergy Clin Immunol* 2002; 110:110–6.
- 7 Kline JN, Waldschmidt TJ, Businga TR et al. Modulation of airway inflammation by CpG oligodeoxynucleotides in a murine model of asthma. *J Immunol* 1998; 160:2555–9.
- 8 Sur S, Wild JS, Choudhury BK, Sur N, Alam R, Klinman DM. Long term prevention of allergic lung inflammation in a mouse model of asthma by CpG oligodeoxynucleotides. *J Immunol* 1999; 162:6284–93.
- 9 Mingari MC, Maggi E, Cambiaggi A et al. Development *in vitro* of human CD4+ thymocytes into functionally mature Th2 cells. Exogenous interleukin-12 is required for priming thymocytes to produce both Th1 cytokines and interleukin-10. *Eur J Immunol* 1996; 26:1083–7.
- 10 Manetti R, Gerosa F, Giudizi MG et al. Interleukin 12 induces stable priming for interferon gamma (IFN-gamma) production during differentiation of human T helper (Th) cells and transient IFN-gamma production in established Th2 cell clones. *J Exp Med* 1994; 179:1273–83.
- 11 Gavett SH, O'Hearn DJ, Li X, Huang SK, Finkelman FD, Wills-Karp M. Interleukin 12 inhibits antigen-induced airway hyperresponsiveness, inflammation, and Th2 cytokine expression in mice. *J Exp Med* 1995; 182:1527–36.
- 12 Lack G, Bradley KL, Hamelmann E et al. Nebulized IFN-gamma inhibits the development of secondary allergic responses in mice. *J Immunol* 1996; 157:1432–9.
- 13 Leonard JP, Sherman ML, Fisher GL et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood* 1997; 90:2541–8.
- 14 van der Pouw Kraan TC, Boeije LC, de Groot ER et al. Reduced production of IL-12 and IL-12-dependent IFN-gamma release in patients with allergic asthma. *J Immunol* 1997; 158:5560–5.
- 15 Vasilakos JP, Smith RM, Gibson SJ et al. Adjuvant activities of immune response modifier R-848: comparison with CpG ODN. *Cell Immunol* 2000; 204:64–74.
- 16 Moore RA, Edwards JE, Hopwood J, Hicks D. Imiquimod for the treatment of genital warts: a quantitative systematic review. *BMC Infect Dis* 2001; 1:3.
- 17 Frotscher B, Anton K, Worm M. Inhibition of IgE production by the Imidazoquinoline Resiquimod in nonallergic and allergic donors. *J Invest Dermatol* 2002; 119:1059–64.
- 18 Reiter MJ, Testerman TL, Miller RL, Weeks CE, Tomai MA. Cytokine induction in mice by the immunomodulator imiquimod. *J Leukoc Biol* 1994; 55:234–40.
- 19 Weeks CE, Gibson SJ. Induction of interferon and other cytokines by imiquimod and its hydroxylated metabolite R-842 in human blood cells *in vitro*. *J Interferon Res* 1994; 14:81–5.

- 20 Imbertson LM, Beaurline JM, Couture AM et al. Cytokine induction in hairless mouse and rat skin after topical application of the immune response modifiers imiquimod and S-28463. *J Invest Dermatol* 1998; 110:734–9.
- 21 Wagner TL, Ahonen CL, Couture AM et al. Modulation of TH1 and TH2 cytokine production with the immune response modifiers, R-848 and imiquimod. *Cell Immunol* 1999; 191:10–9.
- 22 Gibson SJ, Imbertson LM, Wagner TL et al. Cellular requirements for cytokine production in response to the immunomodulators imiquimod and S-27609. *J Interferon Cytokine Res* 1995; 15: 537–45.
- 23 Bishop GA, Hsing Y, Hostager BS, Jalukar SV, Ramirez LM, Tomai MA. Molecular mechanisms of B lymphocyte activation by the immune response modifier R-848. *J Immunol* 2000; 165:5552–7.
- 24 Bishop GA, Ramirez LM, Baccam M, Busch LK, Pederson LK, Tomai MA. The immune response modifier resiquimod mimics CD40-induced B cell activation. *Cell Immunol* 2001; 208:9–17.
- 25 Tomai MA, Gibson SJ, Imbertson LM et al. Immunomodulating and antiviral activities of the imidazoquinoline S-28463. *Antiviral Res* 1995; 28:253–64.
- 26 Hamelmann E, Schwarze J, Takeda K et al. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 1997; 156:766–75.
- 27 Hamelmann E, Oshiba A, Loader J et al. Antiinterleukin-5 antibody prevents airway hyperresponsiveness in a murine model of airway sensitization. *Am J Respir Crit Care Med* 1997; 155:819–25.
- 28 Overbergh L, Valckx D, Waer M et al. Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine* 1999; 11:305–12.
- 29 Buates S, Matlashewski G. Treatment of experimental leishmaniasis with the immunomodulators imiquimod and S-28463: efficacy and mode of action. *J Infect Dis* 1999; 179:1485–94.
- 30 Martin J, Quiroga JA, Navas S, Pardo M, Carreno V. Modulation by biologic response modifiers of hepatitis C virus antigen-independent cytokine secretion in blood mononuclear cells. *Cytokine* 1999; 11:267–73.
- 31 Tomkinson A, Cieslewicz G, Duez C, Larson KA, Lee JJ, Gelfand EW. Temporal association between airway hyperresponsiveness and airway eosinophilia in ovalbumin-sensitized mice. *Am J Respir Crit Care Med* 2001; 163:721–30.
- 32 Chin JE, Hatfield CA, Winterrowd GE et al. Airway recruitment of leukocytes in mice is dependent on alpha4-integrins and vascular cell adhesion molecule-1. *Am J Physiol* 1997; 272:L219–29.
- 33 Fukuda T, Fukushima Y, Numao T et al. Role of interleukin-4 and vascular cell adhesion molecule-1 in selective eosinophil migration into the airways in allergic asthma. *Am J Respir Cell Mol Biol* 1996; 14:84–94.
- 34 Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. *J Immunol* 1990; 145:3796–806.
- 35 Van Oosterhout AJ, Fattah D, Van Ark I, Hofman G, Buckley TL, Nijkamp FP. Eosinophil infiltration precedes development of airway hyperreactivity and mucosal exudation after intranasal administration of interleukin-5 to mice. *J Allergy Clin Immunol* 1995; 96:104–12.
- 36 Lee JJ, McGarry MP, Farmer SC et al. Interleukin-5 expression in the lung epithelium of transgenic mice leads to pulmonary changes pathognomonic of asthma. *J Exp Med* 1997; 185:2143–56.
- 37 Hamelmann E, Takeda K, Vella AT, Irvin CG, Gelfand EW. Development of eosinophilic airway inflammation and airway hyperresponsiveness requires IL-5 but not IgE or B-cells. *Am J Respir Crit Care Med* 1999; 21:480–9.
- 38 Simon HU. Molecular mechanisms of defective eosinophil apoptosis in diseases associated with eosinophilia. *Int Arch Allergy Immunol* 1997; 113:206–8.
- 39 Taube C, Duez C, Cui ZH et al. The role of IL-13 in established allergic airway disease. *J Immunol* 2002; 169:6482–9.
- 40 Dabbagh K, Takeyama K, Lee HM, Ueki IF, Lausier JA, Nadel JA. IL-4 induces mucin gene expression and goblet cell metaplasia *in vitro* and *in vivo*. *J Immunol* 1999; 162:6233–7.
- 41 Schwarze J, Hamelmann E, Cieslewicz G et al. Local treatment with IL-12 is an effective inhibitor of airway hyperresponsiveness and lung eosinophilia after airway challenge in sensitized mice. *J Allergy Clin Immunol* 1998; 102:86–93.
- 42 Hansen G, Berry G, DeKruyff RH, Umetsu DT. Allergen-specific Th1 cells fail to counterbalance Th2 cell-induced airway hyper-reactivity but cause severe airway inflammation. *J Clin Invest* 1999; 103:175–83.
- 43 Randolph DA, Carruthers CJ, Szabo SJ, Murphy KM, Chaplin DD. Modulation of airway inflammation by passive transfer of allergen-specific Th1 and Th2 cells in a mouse model of asthma. *J Immunol* 1999; 162:2375–83.
- 44 Hemmi H, Kaisho T, Takeuchi O et al. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat Immunol* 2002; 3:196–200.
- 45 Broide DH, Stachnick G, Castaneda D et al. Systemic administration of immunostimulatory DNA sequences mediates reversible inhibition of Th2 responses in a mouse model of asthma. *J Clin Immunol* 2001; 21:175–82.