

Treatment with the TLR7 agonist R848 induces regulatory T-cell-mediated suppression of established asthma symptoms

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The evolution of allergic asthma is tightly controlled by effector and regulatory cells, as well as cytokines such as IL-10 and/or TGF- β , and it is widely acknowledged that environmental exposure to allergens and infectious agents can influence these processes. In this context, the recognition of pathogen-associated motifs, which trigger TLR activation pathways, plays a critical role with important consequences for disease progression and outcome. We addressed the question whether the TLR7 ligand resiquimod (R848), which has been shown to be protective in several experimental allergic asthma protocols, can also suppress typical asthma symptoms once the disease is established. To this end, we used an OVA-induced experimental model of murine allergic asthma in which R848 was injected after a series of challenges with aerosolized OVA. We found that the treatment attenuated allergic symptoms through a mechanism that required Tregs, as assessed by the expansion of this population in the lungs of mice having received R848, and the loss of R848-mediated suppression of allergic responses after *in vivo* Treg depletion. IL-10 provided only a minor contribution to this suppressive effect that was largely mediated through a TGF- β -dependent pathway, a finding that opens new therapeutic opportunities for the pharmacological targeting of Tregs.

Key words: Asthma • TLR7 agonist • Tregs

Introduction

Epidemiological studies have established that in recent decades the prevalence of allergic asthma has significantly increased in developed countries. Emerging evidence attests that early life

events, including exposure to allergens and infections, are critical in programming effective regulatory pathways to maintain pulmonary homeostasis. Toll-like receptor (TLR) signaling contributes prominently to CD4⁺ T-cell activation by connecting innate and acquired immunity. Mouse models of allergic asthma have long been used to dissect the immunological mechanisms leading to asthma, and despite the heterogeneity of experimental protocols in terms of strain differences, type and dose of antigen, time and route of administration, most

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studies have provided evidence for protective effects of natural or synthetic TLR2, TLR4, or TLR9 ligands in allergen-induced lung inflammation [1–5]. Similarly, the synthetic ligand of TLR7, R848 (or resiquimod), has been reported to prevent typical respiratory syndromes (airway hyperresponsiveness) and allergic inflammation (recruitment of eosinophils to the lung, production of IgE Abs, and Th2-driven cytokine production) [5–8] in treated mice.

R848 was initially developed as a potential antiviral agent and exerts its immunostimulatory function via mouse TLR7 as well as human TLR7 and TLR8 [9, 10]. TLR7 stimulation activates APCs through the MyD88 pathway leading to the induction of pro-inflammatory cytokines, chemokines, and the release of large amounts of type I IFNs together with upregulation of costimulatory molecules [11]. In the rat, R848 has been shown to inhibit the inflammatory reaction and to abrogate airway remodeling [12]. These protective effects were observed after administration of R848 during the sensitization phase, whereas in mice having already mounted a primary allergic response, the treatment resulted in a marked reduction of secondary reactions following repeated allergen aerosol challenges mediated through IL-12 and IL-10 [8, 12]. Although IFN- γ -producing NK cells have been held responsible for R848-induced asthma protection, it is now clear that they cannot solely account for the protection that is abolished neither by their depletion nor in IFN- γ -deficient mice [13, 14].

Major cellular components driving asthmatic reactions include eosinophils and CD4⁺ Th2 cells, which generate a number of soluble mediators [15]. Furthermore, several Treg populations contribute to the maintenance of lung homeostasis, particularly CD4⁺CD25⁺ Tregs [16]. Naturally occurring cells of this lineage develop in the thymus, whereas their induced counterpart originates from conventional T cells in peripheral lymphoid tissues, comprising several subsets with distinct suppressive functions [17–19]. Knowing that Foxp3⁺ Tregs increase following microbial stimulation and inhibit allergen-induced lung pathologies in a manner that is not antigen-specific, it can be assumed that they take part in the process that leads to reduced asthma and allergy development upon infection. These suppressive functions are mediated through cell-to-cell contact as well as cytokines [20], such as IL-10 and TGF- β , which are both produced by Tregs and widely acknowledged for their role in allergic inflammation. Indeed, IL-10 is an essential suppressive factor during allergic responses in the lung [21], whereas TGF- β ensures pulmonary homeostasis by inhibiting both Th2 and Th1 responses and has been implicated in the control of allergic airway inflammation [22, 23]. This does not exclude the contribution of other cell populations, such as the CD8⁺ T cells, IL-17-producing cells, or natural killer T cells (NKT), which have likewise been documented [20].

In the present study, we investigated the effect of the TLR7 agonist R848 in a murine experimental model of established allergic asthma. We provide evidence that this compound targets pulmonary Tregs, enabling them to exert a protective effect mediated mainly through TGF- β production.

Results

R848 treatment suppresses ongoing established allergic inflammation

We examined the effect of the TLR7 agonist R848 on established allergic symptoms, by injecting the compound at the end of a series of antigen challenges. Hyperresponsiveness was drastically reduced when R848 was injected during the last aerosol administration on D11 and D12 in C57BL/6 mice (Fig. 1A), whether it was evaluated by plethysmography or lung resistance and compliance measurements. Total cell and eosinophil recruitment into BALF was likewise decreased in mice having received R848, as were the concentrations in lung homogenates of Th2-type cytokines and chemokines, such as IL-4, IL-5, IL-13, and eotaxin (Fig. 1B and C). Furthermore, IL-10 and IL-17 dropped to control levels, whereas IL-12 and IFN- γ were increased (Fig. 1C).

Treg counts increase in the lung of R848-treated mice and mediate the suppression of asthma symptoms

Tregs being the most likely mediators of R848-induced asthma suppression, we evaluated whether their incidence was modified in the lung of treated mice relative to saline controls. We observed a significant increase in the percentage of both CD4⁺CD25⁺ and CD4⁺Foxp3⁺ T cells in response to R848 in C57BL/6 mice (Fig. 2A), indicating that this population was recruited upon activation. To assess its contribution to the anti-allergic effect of R848, we depleted CD25⁺ T cells from mice having developed allergic asthma before receiving R848. The efficiency of CD25⁺ cell depletion depended on the strain and the dose of mAb, which in our protocol amounted to 1 mg administered twice i.p. Preliminary experiments revealed that the PC61 mAb depleted CD4⁺CD25^{high} Tregs from C57BL/6 mice rather inefficiently, even at this high dose (data not shown), whereas in the NOD strain an almost complete depletion was achieved 2 days after the second injection of 200 μ g of PC61. Inasmuch as NOD mice develop OVA-induced asthma symptoms more promptly than C57BL/6 mice and respond at higher enhanced pause (Penh) values in the same protocol, we assessed the suppressive effect of R848 in this strain using the same protocol.

OVA-sensitized and -challenged NOD mice were depleted for CD25⁺ cells by i.p. injection of PC61 (200 μ g) at days 9 and 11 before treatment with R848 or saline. As shown in Fig. 2B, 10% of pulmonary CD4⁺CD25^{high} T cells remained detectable in these conditions. R848-induced suppression of asthmatic symptoms after OVA challenge was fully reversed in the absence of Tregs that had no aggravating effect per se, whether in terms of airway hyperreactivity (AHR) (Fig. 3A), total cell recruitment into BALF, eosinophilia (Fig. 3B), or increased levels of Th2-type cytokine and chemokine production in the lung (Fig. 3C). Note that a less consistent reversal occurred also in C57BL/6 mice, although the percentage of CD25 depletion was too low to attain statistically significant levels (data not shown). It can therefore be concluded

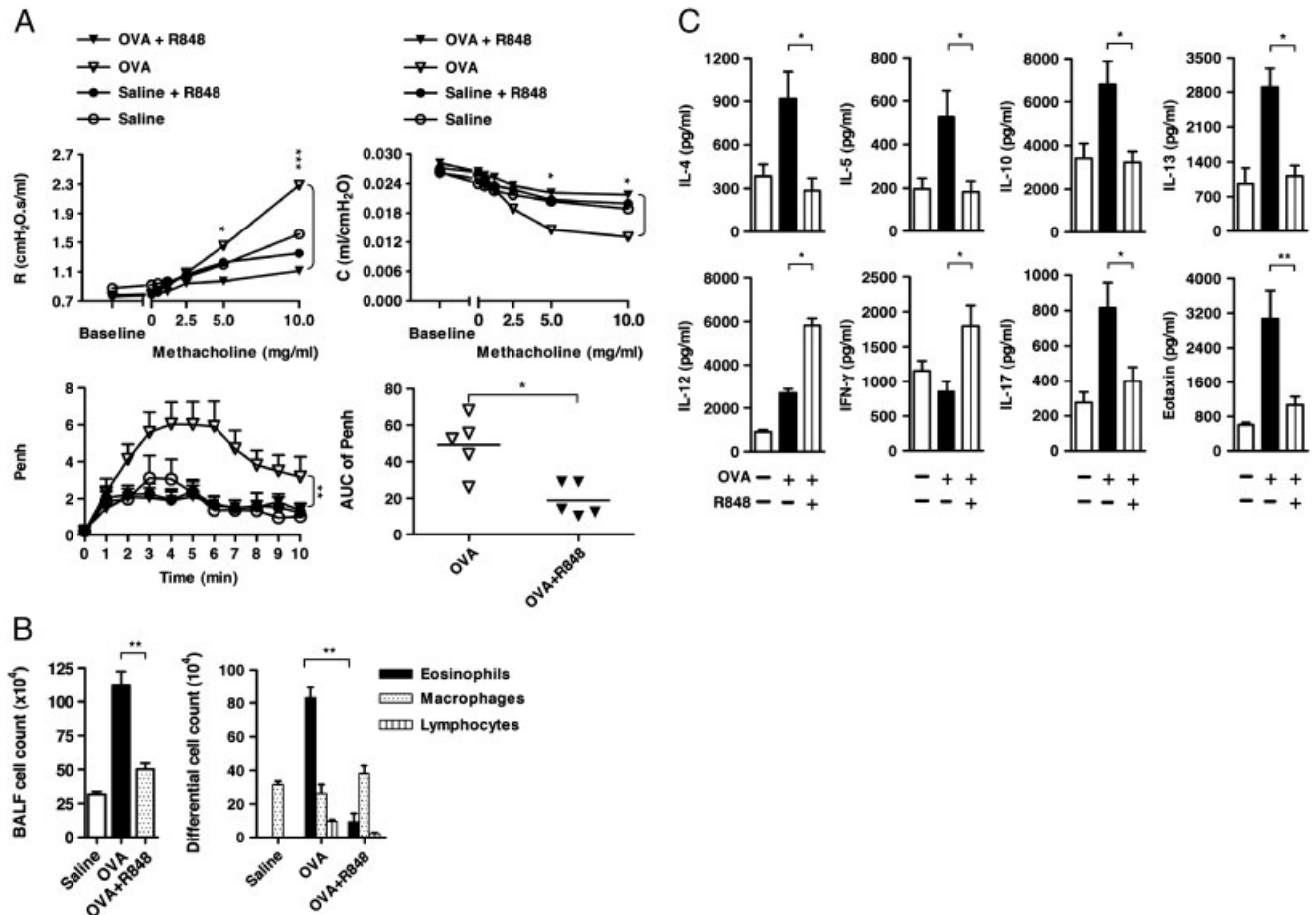


Figure 1. R848 treatment suppresses allergic responses in mice. (A) Methacholine-induced lung resistance (R) and compliance (C), and whole body plethysmography (Penh and AUC of Penh) were determined in OVA-sensitized C57BL/6 mice treated or not with R848 and in saline controls (see induction of experimental asthma and in vivo treatment with R848 in the *Materials and methods*). In the AUC of Penh graph, the symbols represent individual mice. (B) BALF total and differential cell count and (C) levels of the indicated cytokines and chemokines in lung homogenates in the groups detailed in (A). For AHR data, the variance test was used to compare differences between groups, followed by Bonferroni's correction within each set of comparisons. For the remainder, the data are the mean $\pm$ SEM from four to five mice/group and the statistical significance was established by the Mann-Whitney nonparametric test. Results are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

that Tregs are critical for the suppression of allergic asthma induced by R848.

The suppression of asthma symptoms in mice treated with R848 is TGF- β dependent

Both IL-10 and TGF- β are recognized as important mediators of Treg-suppressive functions, prompting us to examine their role in our model. To this purpose, C57BL/6 mice were injected i.p. with neutralizing mAbs directed against TGF- β (2.5 mg per mouse) or IL-10 receptor (500 μ g per mouse) at days 11 and 12, 1 h after saline or OVA challenge. It turned out that the suppressive effect of R848 treatment assessed by decreased AHR (Fig. 4A) was completely abolished upon injection of neutralizing anti-TGF- β mAbs, whereas immunological parameters such as total cell recruitment in BALF, eosinophilia (Fig. 4B), and Th2-type pulmonary cytokine and chemokine production (Fig. 4C) were

only partially restored. In comparison, the blocking anti-IL-10R mAbs failed to reverse R848-induced suppression of hyper-reactivity and eosinophilia completely, and increased IL-4 and IL-13 production in the lung only slightly. However, the generation of other cytokines and chemokines, such as eotaxin, IL-5, IL-10, and IFN- γ , differed significantly from controls, suggesting that IL-10 may be involved to some extent, although the major contribution in terms of AHR is undoubtedly provided by TGF- β .

Discussion

In the present study, we demonstrate that treatment with the TLR7 agonist R848 suppresses the symptoms of established asthma through a mechanism that involves TGF- β . R848 has been previously reported to be able to prevent disease onset when given before allergen challenge [8, 13]. However, taking into account that original exposure to the sensitizing allergen remains

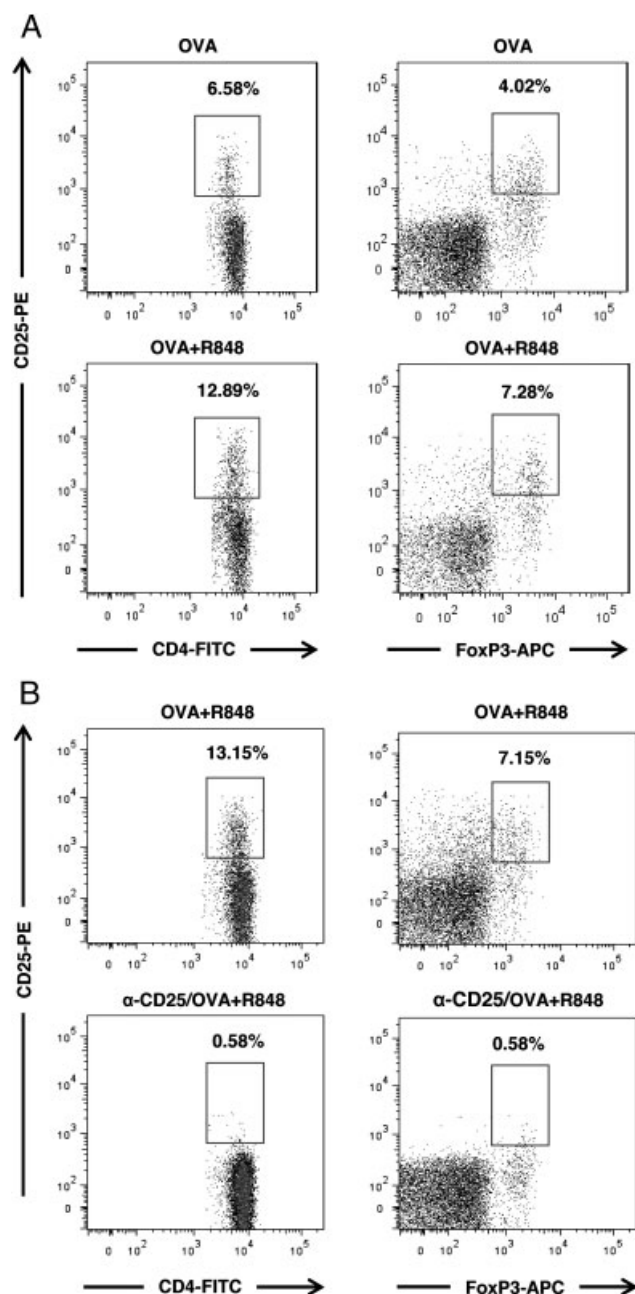


Figure 2. Suppression of asthma by R848 is associated with pulmonary Treg expansion. (A) Following R848 or saline injection, cells infiltrating the lungs were isolated and stained. The percentage of Tregs infiltrating the lung identified as CD4⁺CD25^{high} and CD25^{high}Foxp3⁺ T cells (among gated CD4⁺ T cells) was analyzed by flow cytometry. Representative plots from each group are shown. (B) OVA-treated NOD mice received an anti-CD25 Ab injection and pulmonary CD25⁺Foxp3⁺ T cells were isolated and labeled as described previously. Representative dot plots of two experiments, each performed with four to six mice per group. Data are from analysis of cells from individual and representative mice.

undefined and consecutive challenge is usually unpredictable, the prevention of the initial event that leads to sensitization and/or suppression of established asthma would be more valuable from a therapeutic point of view. We found that administration of R848

before immunization did not protect against disease onset (personal unpublished data). Hence, we set up an acute asthma model with well-established allergic hallmarks to assess whether TLR7 stimulation could suppress as well as prevent these symptoms. Our protocol differed from those currently used for asthma prevention, insofar as R848 was given at the end rather than at the beginning of a series of OVA challenges, once disease symptoms were manifest [7, 8, 12]. Other investigators have reported the suppression of established asthma after in vivo treatment with the TLR7 agonist between a first and a second series of allergen challenges at an interval of 3–4 wk [8]. In a recent study, the protective treatment was given 1 day before a series of three challenges [13]. In this setting, the authors observed asthma suppression when mice received a repeated treatment with antigen 1 wk before the second series of challenges.

Tregs being the most obvious candidates for asthma suppression, we found that their depletion effectively restored the allergic symptoms alleviated by the injection of R848. The removal of this cell population did not significantly aggravate the disease in the asthma-prone NOD mice per se, indicating that it is not inherently suppressive in this type of disorder. In agreement with this result, similar studies performed with other murine strains susceptible to asthma, such as A/J and BALB/c mice also showed that Treg depletion resulted only in a modest increase of airway eosinophilia without enhancement of AHR or Th2-type cytokine production [24, 25].

Thus, treatment with R848 rendered Tregs functionally competent to counteract disease symptoms. This observation is in accordance with the concept that regulatory responses can be induced by TLR stimulation, which seems to be a natural strategy to prevent an excessive immune response. Since plasmacytoid DCs (pDCs) are also activated through TLR7 stimulation, it can be assumed that they are likewise involved in R848-induced asthma suppression, as recently demonstrated for the suppressive effects induced by *Mycobacterium bovis* Bacillus Calmette-Guérin [18], which depend on both plasmacytoid DCs and Tregs. Increased suppressive activity of Tregs in response to TLR activation has also been demonstrated in response to the TLR5 ligand flagellin, which upregulates Foxp3 expression [26].

It has been reported that R848-induced asthma suppression is IL-12-dependent in terms of AHR but not of eosinophilia [8]. In agreement with this notion, we have recently shown that R848-induced NKT cell activation alleviates asthma symptoms through IFN- γ production in an IL-12-dependent manner [27]. The present study shows that CD25 depletion reverses the effect of R848 on all asthma hallmarks tested, namely AHR, cellular infiltration in BALF and production of Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) as well as chemokines (eotaxin) and the Th17 cytokine IL-17. This result contrasts with earlier evidence for the intervention of a particular cytokine or cell subset to control one or several parameters of R848-induced asthma protection/suppression, suggesting that the TLR7 stimulation alters lung inflammation through a variety of cytokines and immunoregulatory subpopulations.

Tregs can exert their suppressive action through cell-to-cell contact or immunosuppressive cytokines such as IL-10 or TGF- β

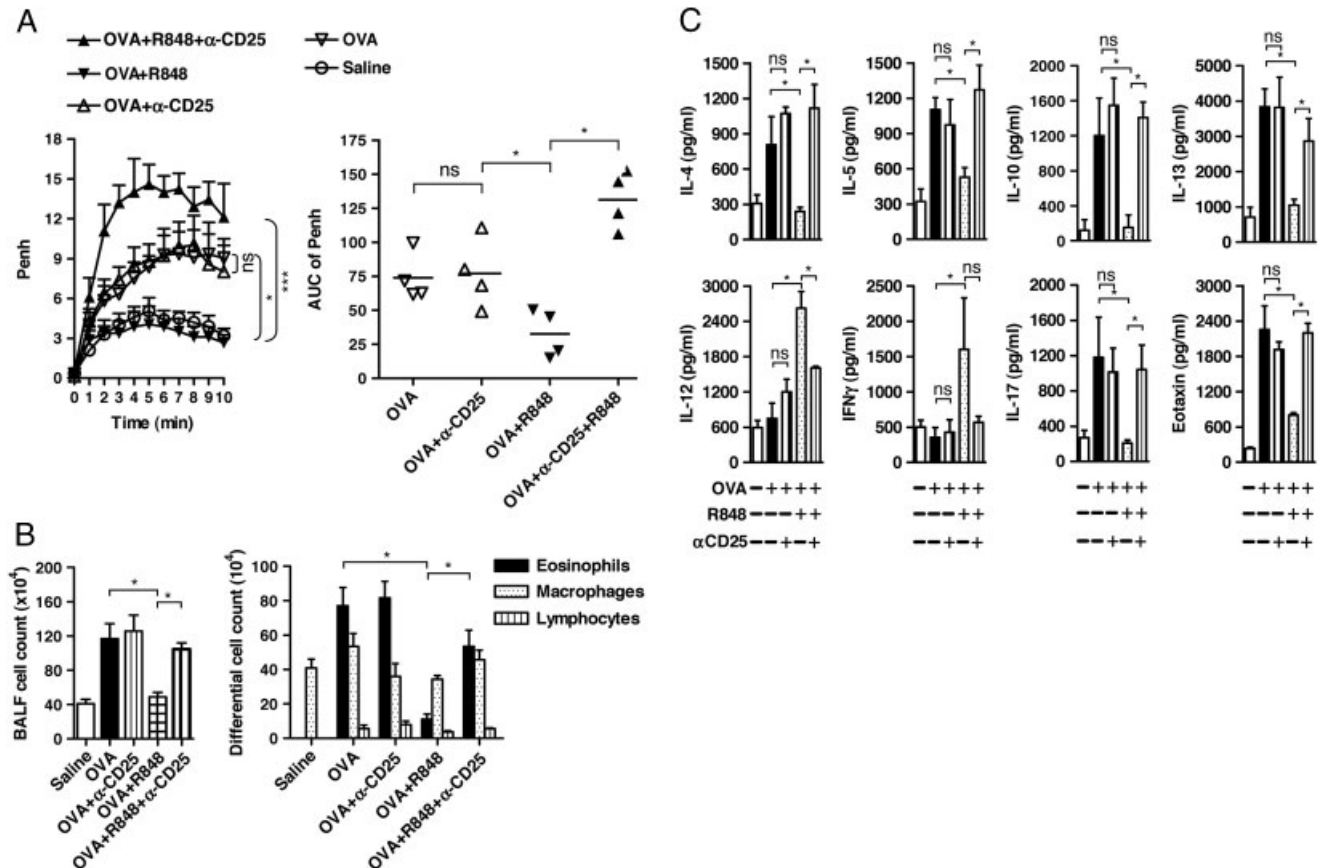


Figure 3. R848-mediated suppression of allergic responses depends on the presence of Tregs. OVA-sensitized NOD mice treated or not with R848 and saline controls were treated with anti-CD25 or PBS. (A) Airway hyperresponsiveness, (B) cell counts in BALF, and (C) cytokine levels in lung homogenates were determined. For AHR data, the variance test was used to compare the differences between groups, followed by Bonferroni's correction within each set of comparisons. For the remainder, the data are the mean+SEM from four to five mice/group and the statistical significance was established by Mann-Whitney nonparametric test. Results are representative of two independent experiments. * $p < 0.05$, and *** $p < 0.001$.

[28]. Most studies have assigned a central role to IL-10 alone or in combination with TGF- β in the control of allergic airway inflammation. In our model, the suppressive effect was predominantly mediated by TGF- β since its neutralization by specific Abs abolished R848-induced asthma suppression completely, whereas IL-10 receptor blockade did not restore the airway hyperresponsiveness. Sel et al. reported that IL-10 plays a limited role in the suppression of IL-5 production and eosinophilia, whereas other inflammatory symptoms (AHR, airway inflammation) remained unchanged [8]. The minor differences between these data and ours can be explained by distinct experimental protocols and the use of IL-12-deficient mice for evaluating the potential role of IL-10 [8]. Yet, in neither study could the suppression be ascribed entirely to IL-10. In humans, IL-10 is produced mainly by subsets of natural and induced Tregs that express low levels of CD25 (IL-10-producing CD4⁺Foxp3⁺ CD25^{low}ICOS⁺ nTreg and induced Tr1 cells). These cells appear to inhibit only T-cell proliferation by blocking the function of APCs and are more prone to apoptosis in an inflammatory environment or in culture compared with other TGF- β -producing subsets. In our experimental conditions, pulmonary IL-10 levels

were consistently lower in mice treated with R848 than in untreated asthmatic controls. In addition to its ability to inhibit effector T-cell proliferation and functions and to induce the release of mediators by APCs and apoptosis in numerous cell types, TGF- β influences the lineage specificity of effector T-cell subsets, depending on the concurrent presence of maturation and polarization factors. It might therefore be hypothesized that the alleviation of asthma symptoms by the TLR7 agonist could result from its capacity to redirect allergen-specific T-cell responses.

It is noteworthy that treatment with R848 normalized the IL-17 levels that were substantially increased in the lung of asthmatic mice relative to saline controls. TGF- β is known to be crucially involved at the interface between Treg and Th17 cell differentiation pathways through its ability to induce their respective signature transcription factor FoxP3 and ROR- γ t. The other proinflammatory cytokines generated in our experimental setting modulate preferentially the Th17 differentiation pathway.

Exposure to pathogens as well as TLR agonists modulates the development of regulatory pathways. Immune reprogramming remains an exciting prospect. Our study has revealed an immune

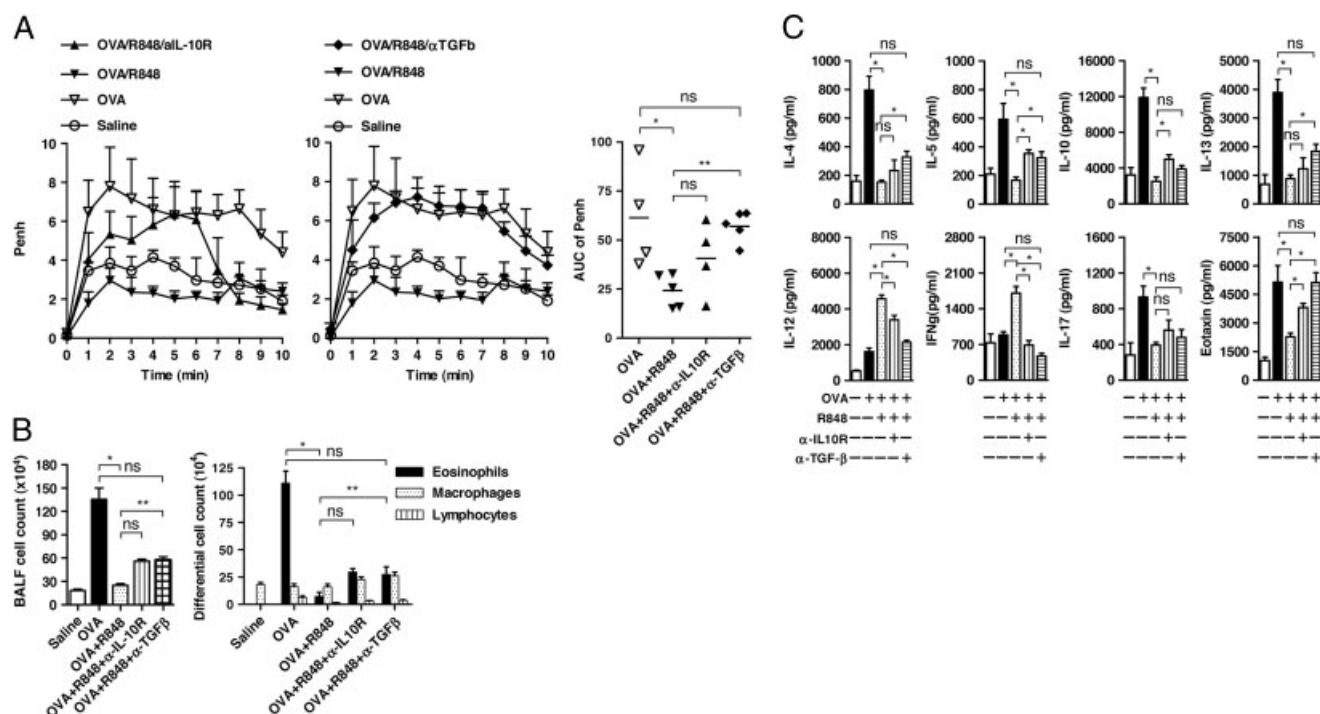


Figure 4. Suppression of OVA-induced allergic responses depends on TGF- β . OVA-sensitized C57BL/6 mice treated or not with R848 and saline controls were injected with blocking α -IL10R mAb or neutralizing α -TGF- β mAb or PBS (saline, OVA and OVA+R848 groups). (A) Methacholine-induced whole body plethysmography. (B) Total and differential cell counts in BALF and (C) levels of the indicated cytokines and chemokines in lung homogenates in each group were determined. The mice in the NaCl-challenged control group were pooled since treatment with R848 or Abs induced no significant modifications. For Penh and AUC of Penh data, the variance test was used to compare the differences between groups, followed by Bonferroni's correction within each set of comparisons. For the remainder, the data are the mean $\pm$ SEM from four to five mice/group and the statistical significance was established by Mann-Whitney nonparametric test. Similar results were obtained in two independent experiments. * $p < 0.05$ and ** $p < 0.01$.

regulatory mechanism in lung homeostasis induced by TLR7 stimulation that can be exploited for improving asthma therapy.

Materials and methods

Animals

C57BL/6J mice were purchased from CERJ (Les Genest St. Isle, France). Ja18^{-/-} mice on a C57BL/6 genetic background (kindly provided by Dr. T. Taniguchi, Japan) and nonobese diabetic (NOD)(K^d, I-A^{g7}, D^b) mice were bred in our animal facility under specific pathogen-free conditions. All experiments were conducted in accordance with European Union Council Directives (86/609/EEC) and according to institutional guidelines (INSERM: Institut National de la Santé et de la Recherche Médicale). The animal facility is accredited by an agreement delivered by the *Préfecture de Police* of Paris, France.

Reagents

R848 was purchased from Alexis Biochemicals (Paris, France). A. O'Garra (DNAX, Palo Alto, CA, USA) kindly provided the rat anti-

IL-10 receptor mAb (1B1 2.1C4 clone) and the mAb against human TGF- β that crossreacts and neutralizes murine TGF- β (2G7 clone) was a gift from C. J. M. Melief (Leiden University Medical Center, Leiden, the Netherlands). These Abs have been reported before for blocking either IL-10R or TGF- β in different protocols [29–31]. The purified anti-CD25 mAb PC61 was used for in vivo depletion of CD25 cells, relative to rat anti-IgG mAb as a negative control.

Induction of experimental asthma and in vivo treatment with R848

Eight- to nine-wk-old male C57BL/6 or NOD mice were immunized by an i.p. injection of 100 μ g of chicken egg OVA (Sigma-Aldrich, Saint Quentin Fallavier, France) adsorbed into 1.6 mg aluminum hydroxide (Merck, Fontenay-sous-Bois, France) in a volume of 0.4 mL. Seven days later, mice were challenged on six consecutive days (D7, D8, D9, D10, D11, and D12) with aerosolized OVA diluted in 0.9% saline solution (5% w/v). R848 was injected i.p. 1 h before the challenge on D11 and D12 at the dose of 100 μ g/injection. Twenty-four hours after the last challenge, AHR was measured, mice were sacrificed, and samples were collected for further analysis.

In some experiments, mice were injected i.p. with 500 μ g of blocking anti-IL10R mAb or 2.5 mg of neutralizing anti-TGF- β mAb on days 11 and 12, 1 h after saline or OVA challenge.

Depletion of Tregs in vivo

Male NOD mice were treated i.p. with 200 µg PC61 at days 9 and 11 (2 h after OVA challenges). Since in preliminary experiments we observed no significant difference between mice having received 200 µg rat IgG1 isotype control of PBS, we limited the control groups to mice injected with PBS.

Measurement of AHR

AHR was evaluated 24 h after the last challenge using both whole body plethysmography (EMKA Technologies, Paris, France) and an invasive forced oscillation technique measuring airway resistance and compliance in anaesthetized and ventilated mice with a FlexiVent system (FlexiVent; SCIREQ, Montreal, Quebec, Canada). Plethysmography measurements were performed with unrestrained, conscious mice receiving an aerosol of methacholine (Sigma-Aldrich) for 1 min at 150 mM (C57BL/6 mice) or 100 mM (NOD mice). The index of airflow obstruction was expressed as Penh, calculated as: $\text{Penh} = \text{Pause} \times (\text{Pef} [\text{peak expiratory flow}]/\text{Pif} [\text{peak inspiratory flow}])$, $\text{Pause} = \text{Te} ([\text{expiratory time}] - \text{Tr} [\text{relaxation time}])/\text{Tr}$. The values of Penh were expressed per minute and are the average of three determinations recorded every 20 s. For simplification, the area under the curve (AUC) was calculated for 10 min. The resulting AUC values representing the quantitative expression of AHR are indicated in the graphs. The data obtained by this technique were confirmed by measuring airway resistance and compliance to methacholine with a FlexiVent system 24 h after the least challenge.

Collection and analysis of BALF

Immediately after AHR measurement, mice were anesthetized by i.p. injection of urethane (2 g/kg body mass, Sigma-Aldrich). Airways were washed with sterile PBS. Total and differential cell counts were carried out in BALFs after May-Grunwald/Giemsa (Merck) staining of cells on cytospin slides.

Isolation of lung mononuclear cells

Intraparenchymal lung mononuclear cells were isolated from minced lung tissue incubated in RPMI 1640 supplemented with 5% FCS, 100 U penicillin/streptomycin, 10 mM HEPES, 50 µM 2-ME, 20 mM L-glutamine containing 20 U/mL collagenase and 1 g/mL DNase type I. After incubation for 60 min at 37°C, remaining tissue fragments were eliminated by passing through a 75-µm filter and cells were collected by centrifugation. They were then suspended in 5 mL of 40% Percoll, layered onto 5 mL of 70% Percoll, centrifuged (2400 rpm, 4°C) for 30 min and collected.

Cytokine and chemokine assays

Both lungs were removed after cannulation of the trachea and airway perfusion, homogenized (in 1 mL PBS) and centrifuged for 10 min at $1600 \times g$ at 4°C. Supernatants were removed and cytokines (IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, and IFN-γ) and chemokines (eotaxin and tarc) were measured using DuoSet ELISA kits (R&D Systems).

Cytometry analysis

Cells were stained with PE-conjugated anti-CD25 and FITC-conjugated anti-CD4 (BD Biosciences, Pont de Claix, France) before fixation and intracellular labeling using a Foxp3 staining kit (BD Biosciences) according to the manufacturer's instructions. Cells were acquired on a FACSCantoII cytometer (BD Biosciences), gated on mononuclear cells according to forward- and side-scatter properties and analyzed using FACS Diva and Flowjo software.

Statistical analysis

Results are expressed as mean values $\pm$ SEM and statistical significance was established by Mann–Whitney test when appropriate (GraphPad Prism Software, La Jolla, CA, USA). The variance test was used to compare the differences between groups for data of AHR followed by Bonferroni's correction within each set of comparisons. Differences were considered significant when $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.005$).

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

- 1 Akdis, C. A., Kussebi, F., Pulendran, B., Akdis, M., Lauener, R. P., Schmidt-Weber, C. B., Klunker, R. P. et al., Inhibition of T helper 2-type responses, IgE production and eosinophilia by synthetic lipopeptides. *Eur. J. Immunol.* 2003. 33: 2717–2726.
- 2 Hessel, E. M., Chu, M., Lizcano, J. O., Chang, B., Herman, N., Kell, S. A., Wills-Karp, M. et al., Immunostimulatory oligonucleotides block allergic airway inflammation by inhibiting Th2 cell activation and IgE-mediated cytokine induction. *J. Exp. Med.* 2005. 202: 1563–1573.

- 3 Patel, M., Xu, D., Kewin, P., Choo-Kang, B., McSharry, C., Thoemson, N. C., and Liew, F. Y. TLR2 agonist ameliorates established allergic airway inflammation by promoting Th1 response and not via regulatory T cells. *J. Immunol.* 2005. **174**: 7558–7563.
- 4 Revets, H., Pynaert, G., Grooten, J. and De Baetselier, P., Lipoprotein I, a TLR2/4 ligand modulates Th2-driven allergic immune responses. *J. Immunol.* 2005. **174**: 1097–1103.
- 5 Aumeunier, A., Grela, F., Ramadan, A., Pham Van, L., Bardel, E., Gomez Alcala, A., Jeannin, P. et al., Systemic Toll-like receptor stimulation suppresses experimental allergic asthma and autoimmune diabetes in NOD mice. *PLoS One* 2010. **5**: e11484.
- 6 Moisan, J., Camateros, P., Thuraingam, T., Marion, D., Koohsari, H., Martin, P., Boghdady, M. L. et al., TLR7 ligand prevents allergen-induced airway hyperresponsiveness and eosinophilia in allergic asthma by a MYD88-dependent and MK2-independent pathway. *Am. J. Physiol. Lung Cell Mol. Physiol.* 2006. **290**: L987–L995.
- 7 Quarcoo, D., Weixler, S., Joachim, R. A., Stock, P., Kallinich, T., Ahrens, B., Hamelmann, E., Resiquimod, a new immune response modifier from the family of imidazoquinolinamines, inhibits allergen-induced Th2 responses, airway inflammation and airway hyper-reactivity in mice. *Clin. Exp. Allergy* 2004. **34**: 1314–1320.
- 8 Sel, S., Wegmann, M., Bauer, S., Bauer, S., Gam, H., Alber, G., Renz, H., Immunomodulatory effects of viral TLR ligands on experimental asthma depend on the additive effects of IL-12 and IL-10. *J. Immunol.* 2007. **178**: 7805–7813.
- 9 Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanio, H., Hoshino, K., Horiuchi, T. et al., Small anti-viral compounds activate immune cells via the TLR7/MyD88-dependent signaling pathway. *Nat. Immunol.* 2002. **3**: 196–200.
- 10 Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A. M., Wagner, H., Lipford, G. and Bauer, S., Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. *Nat. Immunol.* 2002. **3**: 499.
- 11 Kawai, T. and Akira, S., The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat. Immunol.* 2010. **11**: 373–384.
- 12 Camateros, P., Tamaoka, M., Hassan, M., Marino, R., Moisan, J., Marion, D., Guiot, M. C. et al., Chronic asthma-induced airway remodeling is prevented by toll-like receptor-7/8 ligand S28463. *Am. J. Respir. Crit. Care Med.* 2007. **175**: 1241–1249.
- 13 Xirakia, C., Koltsida, O., Stavropoulos, A., Thanassopoulou, A., Aidinis, V., Sideras, P. and Andreacos, E., Toll-like receptor 7-triggered immune response in the lung mediates acute and long-lasting suppression of experimental asthma. *Am. J. Respir. Crit. Care Med.* 2010. **181**: 1207–1216.
- 14 Camateros, P., Kanagaratham, C., Henri, J., Sladek, R., Hudson, T. J. and Radzioch, D., Modulation of the allergic asthma transcriptome following resiquimod treatment. *Physiol. Genomics* 2009. **38**: 303–318.
- 15 Barrett, N. A. and Austen, K. F., Innate cells and T helper 2 cell immunity in airway inflammation. *Immunity* 2009. **31**: 425–437.
- 16 Kearley, J., Barker, J. E., Robinson, D. S. and Lloyd, C. M., Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J. Exp. Med.* 2005. **202**: 1539–1547.
- 17 Akbari, O., Stock, P., DeKruyff, R. H. and Umetsu, D. T., Role of regulatory T cells in allergy and asthma. *Curr. Opin. Immunol.* 2003. **15**: 627–633.
- 18 Lagranderie, M., Abolhassani, M., Vanoirbeek, J. A., Lima, C., Balazuc, A. M., Vargaftig, B. B., Marchal, G., *Mycobacterium bovis* bacillus Calmette-Guerin killed by extended freeze-drying targets plasmacytoid dendritic cells to regulate lung inflammation. *J. Immunol.* 2010. **184**: 1062–1070.
- 19 Zuany-Amorim, C., Sawicka, E., Manlius, C., Le, M. A., Brunet, L. R., Kemeny, D. M. and Bowen, G., Suppression of airway eosinophilia by killed *Mycobacterium vaccae*-induced allergen-specific regulatory T-cells. *Nat. Med.* 2002. **8**: 625–629.
- 20 Lloyd, C. M. and Hawrylowicz, C. M., Regulatory T cells in asthma. *Immunity* 2009. **31**: 438–449.
- 21 Leech, M. D., Benson, R. A., De Vries, A., Fitch, P. M. and Howie, S. E., Resolution of Der p1-induced allergic airway inflammation is dependent on CD4+CD25+Foxp3+ regulatory cells. *J. Immunol.* 2007. **179**: 7050–7088.
- 22 Joetham, A., Takeda, K., Taube, C., Miyahara, N., Matsubara, S., Koya, T., Rha, Y. H. et al., Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta. *J. Immunol.* 2007. **178**: 1433–1442.
- 23 Ostroukhova, M., Seguin-Devaux, C., Oriss, T. B., Dixon-McCarthy, B., Yang, L., Ameredes, B. T., Corcoran, T. E. and Ray, A., Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J. Clin. Invest.* 2004. **114**: 28–38.
- 24 Hadeiba, H. and Locksley, R. M., Lung CD25 CD4 regulatory T cells suppress type 2 immune responses but not bronchial hyperreactivity. *J. Immunol.* 2003. **170**: 5502–5510.
- 25 Jaffar, Z., Sivakuru, T. and Roberts, K., CD4+CD25+ T cells regulate airway eosinophilic inflammation by modulating the Th2 cell phenotype. *J. Immunol.* 2004. **172**: 3842–3849.
- 26 Crellin, N. K., Garcia, R. V., Hadisfar, O., Allan, S. E., Steiner, T. S. and Levings, M. K., Human CD4+ T cells express TLR5 and its ligand flagellin enhances the suppressive capacity and expression of FOXP3 in CD4+CD25+ T regulatory cells. *J. Immunol.* 2005. **175**: 8051–8059.
- 27 Grela, F., Aumeunier, A., Bardel, E., Pham Van, L., Bourgeois, E., Vanoirbeek, J., Leite-de-Moraes, M. et al., The TLR7 agonist R848 alleviates allergic inflammation by targeting invariant NKT cells to produce IFN-gamma. *J. Immunol.* 2011. **186**: 284–290.
- 28 Palomares, O., Yaman, G., Azkur, A. K., Akkoc, T., Akdis, M. and Akdis, C. A., Role of Treg in immune regulation of allergic diseases. *Eur. J. Immunol.* 2010. **40**: 1232–1240.
- 29 Taher, Y. A., van Esch, B. C., Hofman, G. A., Henricks, P. A. and van Oosterhout, A. J., 1Alpha,25-dihydroxyvitamin D3 potentiates the beneficial effects of allergen immunotherapy in a mouse model of allergic asthma: role for IL-10 and TGF-beta. *J. Immunol.* 2008. **180**: 5211–5221.
- 30 Oida, T., Zhang, X., Goto, M., Hachimura, S., Totsuka, M., Kaminogawa, S. and Weiner, H. L., CD4+CD25- T cells that express latency-associated peptide on the surface suppress CD4+CD45RBhigh-induced colitis by a TGF-beta-dependent mechanism. *J. Immunol.* 2003. **170**: 2516–2522.
- 31 Vissers, J. L., van Esch, B. C., Hofman, G. A., Kapsenberg, M. L., Weller, F. R. and Van Oosterhout, A. J., Allergen immunotherapy induces a suppressive memory response mediated by IL-10 in a mouse asthma model. *J. Allergy Clin. Immunol.* 2004. **113**: 1204–1210.

Abbreviations: AHR: airway hyperreactivity · AUC: area under the curve · Penh: enhanced pause · R848: resiquimod

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