

Toll-like Receptor 7–triggered Immune Response in the Lung Mediates Acute and Long-Lasting Suppression of Experimental Asthma

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Rationale: Toll-like receptor (TLR) 7/8 ligands are promising candidate drugs for the treatment of allergic asthma and rhinitis. Although their clinical application depends on the development of strategies for topical administration to the lung, this has not been explored in preclinical disease models.

Objectives: To examine the therapeutic effectiveness, persistence of effect, and mode of action of intranasal TLR7 ligand administration in allergic airway disease.

Methods: Wild-type, IFN- α receptor (IFN- α R)^{-/-}, IFN- γ ^{-/-}, CD8^{-/-}, TLR7^{-/-}, and radiation-induced chimeric mice deficient in hematopoietic TLR7 expression were subjected to an established model of allergic airway disease. R-848, a specific TLR7 agonist in mice, was administered prophylactically or therapeutically and effects of treatment on helper T-cell type 2 (Th2) responses, eosinophilia, goblet cell metaplasia, and airway hyperresponsiveness were assessed.

Measurements and Main Results: Intranasal R-848 administration induced a transient immune response characterized by type I interferon production and infiltration of innate immune cells into the lung. This conferred long-term suppression of allergic airway disease via two complementary molecular processes, one mediated by type I interferons and providing acute protection by directly inhibiting effector Th2 responses, and one mediated by immunoregulatory CD8⁺ T cells and inducing long-lasting protection by suppressing Th2 responses in an IFN- γ -dependent manner.

Conclusions: Intranasal R-848 administration is an effective treatment for allergic airway disease. It hijacks an otherwise proinflammatory immune process triggered by TLR7 to mediate long-lasting disease suppression. This provides important insight into the efficacy and mode of action of TLR7 ligands in murine models of allergic airway disease and paves the way for their clinical application in humans.

Keywords: asthma; Toll-like receptor; immunomodulation; helper T-cell type 2

Immunomodulation constitutes a major therapeutic approach that is being investigated for the treatment of allergic asthma. Evidence from experimental models of asthma and human allergen-specific immunotherapy indicates that it is possible to

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Toll-like receptor (TLR) 7/8 ligands are candidate new drugs for the treatment of allergic asthma and rhinitis in humans. However, their clinical application is dependent on the development of effective administration strategies to the lung and the unwinding of their mechanism of action.

What This Study Adds to the Field

Intranasal administration of a TLR7 ligand confers acute and long-lasting suppression of experimental allergic airway disease through the induction of type I interferons and IFN- γ -producing CD8⁺ T cells. This supports the further development of TLR7 ligands in the clinic.

reeducate the immune system to develop reduced helper T-cell type 2 (Th2) responses to inhaled allergens, resulting in decreased airway inflammation, airway hyperresponsiveness, and airway obstruction, critical features of the asthmatic phenotype.

Central to current immunomodulatory approaches for the treatment of allergic disease are Toll-like receptors (TLRs), key components of the innate immune system that mediate recognition and response to various microbial and viral pathogens and their ligands including endotoxin (TLR4), lipoproteins (TLR2 and TLR6), viral RNA (TLR3 and TLR7/8), and bacterial CpG-containing DNA (TLR9) (1). In preclinical models of asthma, triggering of TLRs through the administration of various TLR ligands has been shown to potentially suppress the allergic response by inhibiting Th2 cytokine production and reducing airway eosinophilia and bronchial hyperreactivity (reviewed in Reference 2). For CpG oligodeoxynucleotides (CpG-ODNs), the best studied TLR ligands and the most advanced in the clinic, benefit has been shown to be long-lasting and to depend on multiple mechanisms triggered by TLR9 activation including direct suppression of Th2 effector function (3, 4), induction of Th1 cytokines such as IL-12 and IFN- γ (5), and the generation of regulatory responses involving indoleamine 2,3-dioxygenase up-regulation (6, 7), IL-10 secretion, and CD4⁺CD25⁺ regulatory T-cell expansion (8, 9). In the clinic, however, first results have been ambivalent, with CpG-ODN showing limited efficacy when administered to patients with asthma before allergen challenge (10) but significant benefit when conjugated to ragweed allergen and administered to patients with allergic rhinitis (11, 12).

TLR7 and TLR8 are by homology the closest evolutionary relatives of TLR9 (13). Both TLR7 and TLR8 have affinity for viral single-stranded RNA and activate a similar signaling cascade but differ somehow in their expression pattern. TLR7

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is found most abundantly on plasmacytoid dendritic cells (DCs) although myeloid DCs and epithelial cells also express significant levels (14), whereas TLR8 is found on monocytes, myeloid DCs, and neutrophils (15–17). In mice TLR8 is not functional. TLR7 and TLR8 polymorphisms have been strongly linked to asthma and related atopic disorders in humans (18) and systemic administration of TLR7/8 ligands has been shown to suppress both acute inflammation and airway remodeling in rodent models of allergic airway disease (19, 20). However, systemic administration of TLR7/8 ligands is toxic in humans, and topical rather than systemic treatment strategies seem imperative for further development of TLR7/8 ligands in the clinic (21, 22). Still, topical administration of TLR7/8 ligands in the lung has not been explored.

In this study, we investigated the therapeutic effectiveness, persistence of effect, and mechanism of action of intranasal TLR7 ligand administration in mice. We show that administration of R-848 through the intranasal route not only results in potent suppression of allergic airway disease but also induces long-lasting suppression and memory. We also unwind two distinct immunomodulatory mechanisms involved, one mediated by type I interferons and providing acute suppression and one mediated by immunoregulatory CD8⁺ T cells and IFN- γ and conferring long-term protection. These findings provide new insight into the therapeutic application of TLR7 ligands for the treatment of allergic asthma and rhinitis in humans.

METHODS

Mice

C57BL/6 wild-type (WT), C57BL/6-CD45.1 congenic, CD8^{-/-}, and IFN- γ ^{-/-} mice were purchased from Jackson Laboratory (Bar Harbor, ME). TLR7^{-/-} mice have been described previously (23). All mice were housed in individually ventilated cages under specific pathogen-free conditions, in full compliance with FELASA (Federation of Laboratory Animal Science Associations) recommendations, at the Animal House Facility of the Foundation for Biomedical Research of the Academy of Athens (Athens, Greece).

Generation of Chimeric Mice

Bone marrow (BM) cells were harvested from TLR7^{-/-} and WT mice and transplanted into WT C57BL/6-CD45.1 congenic mice lethally irradiated with 6 Gy (Gammacell 40 Exactor; MDS Nordion, Ottawa, ON, Canada). Two combinations of BM chimeras were created: WT $\rightarrow$ WT (donor BM $\rightarrow$ lethally irradiated recipient) and TLR7 $\rightarrow$ WT. Eight weeks after BM reconstitution, congenicity with donor-derived CD45.2⁺ cells was confirmed to be greater than 90% of the peripheral blood leukocytes. These mice were then used for experiments.

Sensitization and Challenge Protocols and Treatments

An established model of allergic airway disease in mice was used (24). A detailed description of the protocol and slight modifications for prophylactic and therapeutic studies are described in the online supplement. R-848 (resiquimod; Alexis, Lausen, Switzerland) treatment was administered intranasally at 200 μ g/mouse (unless otherwise indicated) according to the experimental protocol. Recombinant IFN- α (PBL Laboratories, Paskataway, NJ) was administered at 10,000 U/mouse. One day after the last challenge mice were examined for airway hyperresponsiveness; bronchoalveolar lavage (BAL) was performed and tissues (mediastinal lymph nodes, spleen, and lung) were collected for analysis. BAL, differential cell counting, histological scoring, and morphometry were performed as described in the online supplement.

Flow Cytometry Analysis of Lung Single-Cell Suspensions

Single-cell suspensions from total lung, prepared as described in the online supplement, were stained with cell type-specific antibodies

(eBioscience, San Diego, CA) and numbers of myeloid DCs (CD11c⁺CD11b⁺), plasmacytoid DCs (CD11c⁺CD11b⁻B220⁺), natural killer cells (NK1.1⁺CD3⁻), and granulocytes (Gr1⁺SSC^{hi}) were determined.

Measurement of Airway Hyperresponsiveness

Anesthetized mice were mechanically ventilated (flexiVent; SciReq, Montreal, PQ, Canada) and aerosolized methacholine was delivered in-line through the inhalation port for 10 seconds (25). Lung resistance was determined as the highest of 12 measurements obtained for each concentration of methacholine and is reported as the percent increase from baseline.

Draining Lymph Nodes

Draining lymph nodes were removed, single-cell suspensions were prepared, and cells were cultured at 5×10^5 per well in 96-well plates in the presence or absence of ovalbumin (100 μ g/ml). After 48 hours supernatants were collected and examined by ELISA for the presence of Th1 and Th2 cytokines.

Cytokine Measurements

Cytokines were analyzed in BAL supernatant by ELISA. Kits for IFN- γ , IL-5, IL-10, and IL-13 were from eBioscience. IFN- α and IFN- β were analyzed in whole lung by real-time polymerase chain reaction. The following mouse primer sequences were designed with Applied Biosystems (Foster City, CA) Primer Express software: *ifna*-F, 5'-TCTGATGCAGCAGGTGGG; *ifna*-R, 5'-AGGGCTCTCCA GACTTCTGCTCTG; *ifnb*-F, 5'-GCACTGGGTGGAATGAGACT; and *ifnb*-R, 5'-AGTGGAGAGCAGTTGAGGACA.

Intracellular Flow Cytometry Staining

Intracellular flow cytometry staining of mediastinal lymph node (MLN) cells was performed with a fixation/permeabilization solution and antibodies against CD8, IFN- γ , and isotype controls from eBioscience. Before staining, cells were stimulated for 6 hours with phorbol 12-myristate 13-acetate (50 ng/ml; Sigma, St. Louis, MO) and ionomycin (500 ng/ml; Sigma). Two hours after stimulation, brefeldin A (10 μ g/ml; eBioscience) was also added.

Statistical Analysis

Statistical significance of differences was assessed by Student *t* test for parametric data and by Mann-Whitney *U* test for nonparametric data. In all cases, treatment was compared with that of vehicle-treated controls and statistical significance was accepted when *P* < 0.05.

RESULTS

R-848 Administration through the Intranasal Route Suppresses Allergic Airway Inflammation

For the clinical application of TLR ligands in allergic airway disease local rather than systemic administration is the preferred if not the only treatment option. Therefore, we sought to determine the effectiveness of the TLR7/8 ligand R-848 in experimental asthma after intranasal delivery. Using an established model of allergic airway disease in mice (Figure 1A), we found that intranasal R-848 treatment profoundly suppressed allergic airway inflammation and hyperresponsiveness in response to inhaled allergen challenge. This was dose dependent, with maximal therapeutic efficacy achieved at 200 μ g/mouse (Figure 1B). A higher dose of 400 μ g/mouse was not more effective than the 200- μ g/mouse dose at suppressing disease whereas lower doses of 100 or 50 μ g/mouse were less effective or not effective at all.

At 200 μ g/mouse, R-848 treatment profoundly reduced eosinophil and mononuclear leukocyte numbers in the BAL (Figure 1C). It also inhibited the production of Th2 cytokines such as IL-5 and IL-13 in both MLNs (Figure 1D) and lung (see

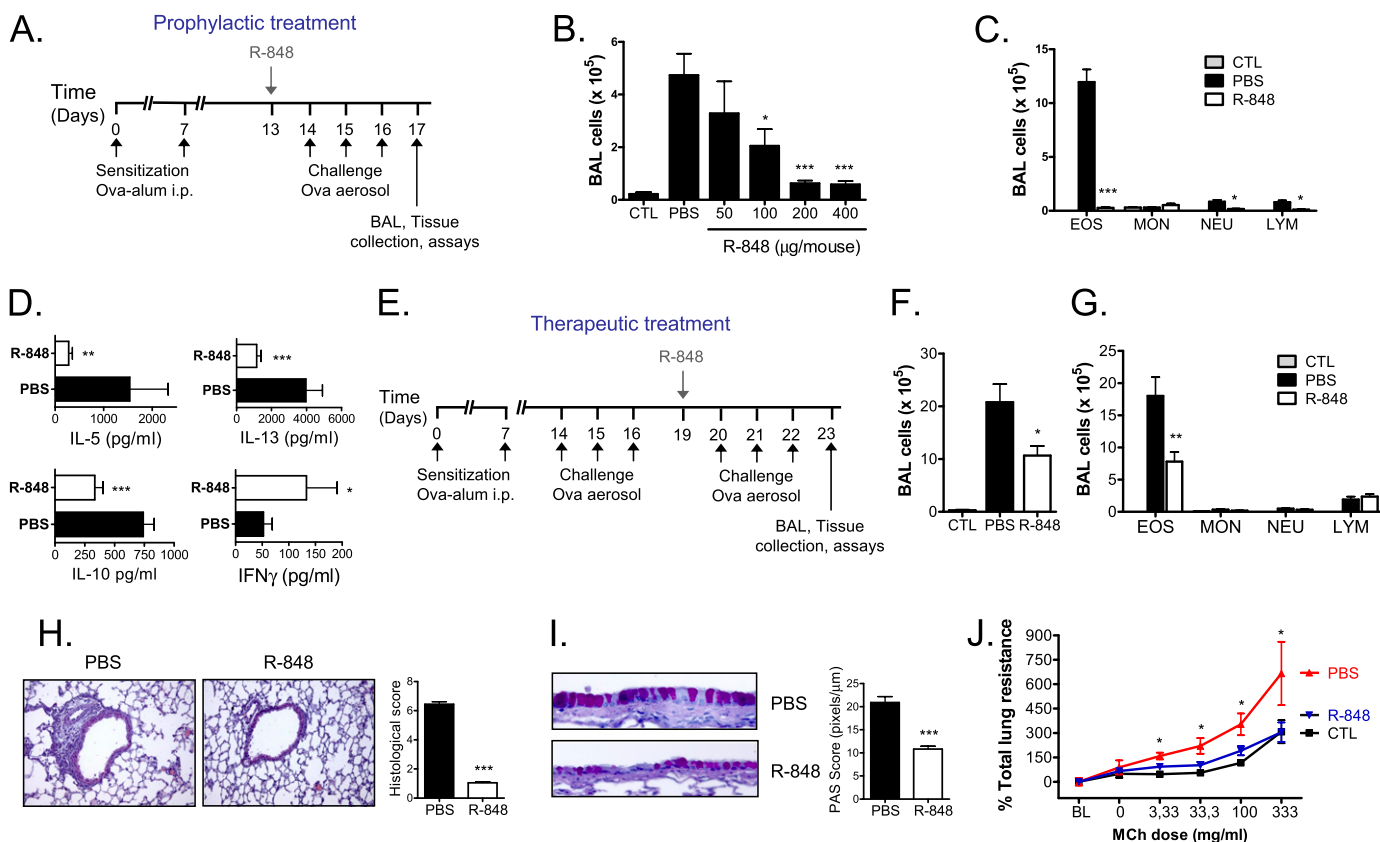


Figure 1. Intranasal R-848 administration treats allergic airway disease in mice. (A) Experimental protocol for prophylactic administration. C57BL/6 mice were sensitized twice with ovalbumin (OVA)-alum before receiving intranasal R-848 at various doses or vehicle control (phosphate-buffered saline [PBS]) 1 day before OVA challenge. Control mice (CTL) were challenged with saline. (B) Total bronchoalveolar lavage (BAL) counts are expressed as means $\pm$ SEM of five or six mice per group; data from one of two independent experiments are shown. (C) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) of mice treated with R-848 (200 μ g) are expressed as means $\pm$ SEM of 15 mice per group from three independent experiments. (D) OVA-specific T-cell responses after R-848 treatment at 200 μ g/mouse. Levels of IL-5, IL-13, IL-10, and IFN- γ are expressed as means $\pm$ SEM in supernatants of OVA-stimulated mediastinal lymph nodes of 14 mice per group, pooled from three independent experiments. (E) Experimental protocol for therapeutic administration. C57BL/6 mice were sensitized twice with OVA-alum, challenged with OVA, treated intranasally with 200 μ g of R-848 or vehicle control (PBS), and rechallenged with OVA. Control mice (CTL) were challenged with saline. (F) Total BAL counts are expressed as means $\pm$ SEM of seven mice per group; one from three independent experiments. (G) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) are expressed as means $\pm$ SEM of seven mice per group; data from one of three independent experiments are shown. (H) Histological assessment of lung inflammation of mice treated with 200 μ g of R-848 or vehicle control (PBS). Hematoxylin and eosin-stained lung sections and histological scoring, expressed as mean values $\pm$ SEM from six mice per group, are shown. (I) Histological assessment of mucus secretion of mice treated with 200 μ g of R-848 or vehicle control (PBS). Periodic acid-Schiff (PAS)-stained sections and morphometric analysis, expressed as mean values $\pm$ SEM from nine mice per group, are shown. (J) Airway hyperresponsiveness of OVA-sensitized and challenged mice treated with 200 μ g of R-848 or vehicle control (PBS), or healthy controls (CTL). Methacholine (MCh)-induced increases in total lung resistance in mechanically ventilated mice are shown. Data are expressed as mean values of percentage increase from baseline of the total lung resistance $\pm$ SEM from six mice per group. *** P < 0.001; ** P < 0.01; * P < 0.05. i.p. = intraperitoneal.

Figure E1 in the online supplement) whereas it up-regulated IFN- γ (Figure 1D). Similarly, at this dose regimen R-848 administration to mice with already established allergic airway inflammation induced with three ovalbumin (OVA) challenges (Figure 1E) markedly suppressed disease deterioration provoked by three further challenges with inhaled allergen (Figures 1F and 1G). In agreement with these observations, histopathological analysis of lung tissue revealed significantly decreased leukocyte infiltration in the perivascular and peribronchial areas of lung tissue (Figure 1H) and significantly decreased goblet cell metaplasia and hypersecretion of airway epithelial mucus in lung tissue from R-848-treated mice compared with their control counterparts (Figure 1I). Functionally, R-848 treatment reduced airway hyperresponsiveness defined as methacholine-induced increases in total lung resistance in mechanically ventilated mice (Figure 1J). These findings demonstrate that intranasal R-848

administration at 200 μ g/mouse effectively suppresses all the principal features of allergic airway disease in mice.

R-848-mediated Suppression Is Long-Lived

Subsequently, we examined the persistence of the effect of intranasal R-848 administration, as this is an important parameter when therapeutic application of this treatment is considered. To our surprise, we found that TLR7-mediated suppression of allergic airway disease persisted long after R-848 administration. Mice treated intranasally with R-848 and challenged 2 or 4 weeks later with inhaled allergen (Figure 2A) demonstrated significantly suppressed allergic airway inflammation. There was significantly inhibited inflammatory cell infiltration in the BAL characterized by decreased eosinophil numbers (Figures 2B and 2C), and significantly reduced allergen-induced

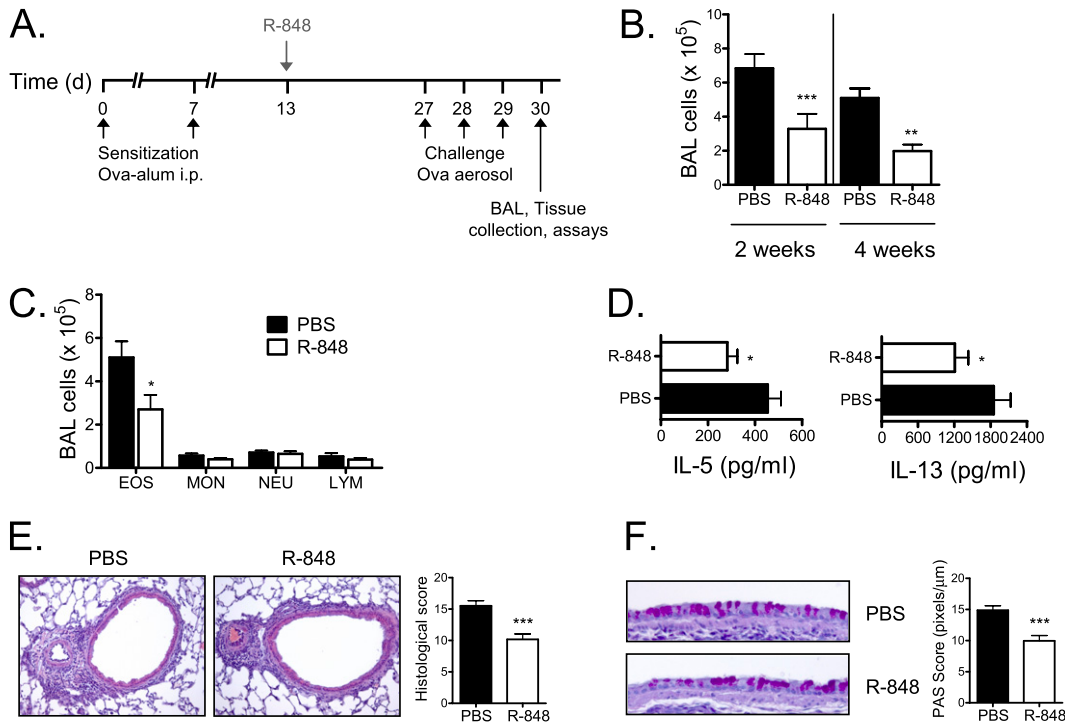


Figure 2. Intranasal R-848 administration provides long-term protection from allergic airway disease. (A) Experimental protocol for long-term efficacy. C57BL/6 mice were sensitized twice with ovalbumin (OVA)-alum before receiving intranasally 200 μ g of R-848 or vehicle control (phosphate-buffered saline [PBS]) 2 or 4 weeks before OVA challenge. (B) Total bronchoalveolar lavage (BAL) counts are expressed as means $\pm$ SEM of six mice per group; data from one of two independent experiments are shown. (C) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) are expressed as means $\pm$ SEM of 12 mice per group from two independent experiments. (D) OVA-specific T-cell responses after R-848 treat-

ment. Levels of IL-5 and IL-13 are expressed as means $\pm$ SEM in supernatants of OVA-stimulated mediastinal lymph nodes of 12 mice per group from two independent experiments. (E) Histological assessment of lung inflammation. Hematoxylin and eosin-stained lung sections and histological scoring, expressed as mean values $\pm$ SEM from 6 to 10 mice per group, are shown. (F) Histological assessment of mucus secretion. Periodic acid-Schiff (PAS)-stained sections and morphometric analysis, expressed as mean values $\pm$ SEM from 10 to 12 mice per group, are shown. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$. i.p. = intraperitoneal.

IL-5 and to a lesser extent IL-13 production in MLNs of R-848-treated mice as compared with vehicle controls (Figure 2D). In the lung interstitium infiltration of leukocytes was suppressed (Figure 2E), as was goblet cell metaplasia of the airway epithelium (Figure 2F), although the extent of suppression was always less than that seen in mice challenged immediately after treatment. These results demonstrate that R-848 treatment exerts a long-lasting suppressive effect in allergic airway disease.

R-848-mediated Suppression of Allergic Airway Disease Requires TLR7 Signaling on Hematopoietic Cells

To determine whether R-848 acts on immune cells to mediate suppression of allergic airway disease, we generated radiation-induced chimeric mice (Figure 3A). Chimerism with donor-derived CD45.2⁺ cells was confirmed by flow cytometry to be greater than 90% 8 weeks after BM reconstitution (data not shown). We found that in WT $\rightarrow$ WT mice (in which wild-type BM was used to reconstitute lethally irradiated wild-type recipients), R-848 treatment still conferred suppression of allergic airway disease, as expected. However, in TLR7^{-/-} $\rightarrow$ WT mice (in which TLR7^{-/-} BM was used to reconstitute lethally irradiated wild-type recipients) the suppressive effect of R-848 treatment was completely lost. There was a complete reversal of the eosinophil and total inflammatory cell numbers in the BAL (Figures 3B and 3C), and in the extent of leukocytic cell infiltration in perivascular and peribronchial areas of lung tissue as determined by histopathological analysis (Figure 3D). In addition, goblet cell metaplasia in the lung tissue of R-848-treated mice was not inhibited in TLR7^{-/-} $\rightarrow$ WT mice, in sharp contrast to their WT $\rightarrow$ WT counterparts (Figure 3E). These observations suggest that TLR7 signaling in hematopoietic cells is essential for mediating suppression of allergic airway disease.

R-848 Administration in Naive Mice Triggers Type I IFN Production and Infiltration of Innate Immune Cells into the Lung

We next reasoned that mapping the early innate immune events triggered by TLR7 activation would provide important insight into the mechanisms involved in TLR7 immunomodulation. We found that one of the earliest effects of intranasal R-848 administration in naive mice was the induction of type I interferons. IFN- α and IFN- β were rapidly up-regulated in the lung, reaching peak mRNA levels 2 hours after treatment and declining to basal levels thereafter (Figure 4A). This was accompanied by the influx of major innate immune cell populations. Differential cell count analysis of BAL revealed a significant infiltration of neutrophils and macrophage/DC-like cells after R-848 treatment (Figure 4B), whereas flow cytometric analysis of lung digests, using specific cell markers, demonstrated a large influx of natural killer cells and granulocytes as early as 12 hours after treatment accompanied by an increase in the number of myeloid and plasmacytoid DCs (Figure 4C). In sharp contrast to the inflammatory response after OVA challenge, no eosinophils were observed in either the BAL or lung digests (data not shown). Inflammation, however, was transient and declined to basal levels 48 hours after treatment. These data indicate that treatment of mice with R-848 triggers a "classical" innate immune response characterized by rapid production of type I IFNs and transient recruitment of major innate immune cell populations.

TLR7-induced Type I IFNs Mediate Acute Suppression by Directly Inhibiting Allergen-specific Th2 Responses and Allergic Airway Disease

Type I IFNs are major regulators of the immune response by controlling T-cell activation, DC antigen presentation, and

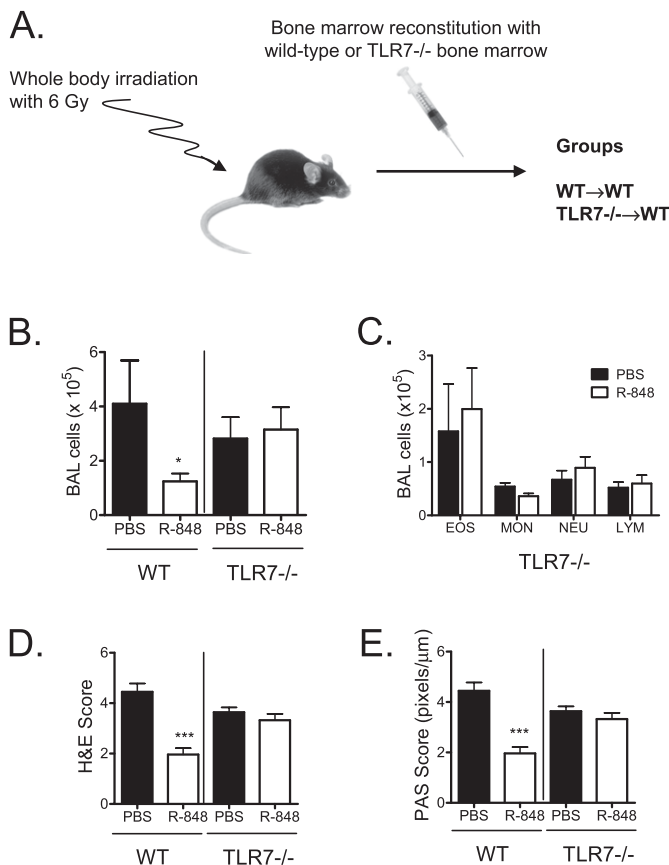


Figure 3. R-848-mediated suppression of allergic airway disease requires Toll-like receptor (TLR) 7 signaling on hematopoietic cells. (A) Experimental protocol for radiation-induced chimerism. C57BL/6 mice were irradiated and then reconstituted with wild-type (WT) or TLR7^{-/-} bone marrow. After 8 weeks, mice were sensitized twice with ovalbumin (OVA)-alum before receiving 200 μg of R-848 or vehicle control (phosphate-buffered saline [PBS]) intranasally 1 day before OVA challenge. (B) Total bronchoalveolar lavage (BAL) counts are expressed as means $\pm$ SEM of eight mice per group from two independent experiments. (C) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) are expressed as means $\pm$ SEM of eight mice per group from two independent experiments. (D) Histological assessment of lung inflammation. Hematoxylin and eosin (H&E)-stained lung sections and histological scoring, expressed as mean values $\pm$ SEM from eight or nine mice per group, are shown. (E) Histological assessment of mucus secretion. Periodic acid-Schiff (PAS)-stained sections and morphometric analysis, expressed as mean values $\pm$ SEM from eight or nine mice per group, are shown. *** $P < 0.001$; * $P < 0.05$.

inflammatory cytokine production (26). We therefore examined whether type I IFNs induced after TLR7 activation are involved in the immunosuppressive effects of R-848 treatment by using IFN- α R^{-/-} mice, in which signaling of type I IFNs is defective. Notably, we found that R-848-mediated acute suppression of allergic airway disease was largely lost in mice deficient in IFN- α receptor (IFN- α R^{-/-}) mice. In the “prophylactic” setting (Figure 1A), R-848-mediated inhibition of total inflammatory cell and eosinophil numbers in the BAL of IFN- α R^{-/-} mice was severely reduced compared with wild-type animals (31 vs. 77% suppression) (Figures 5A and 5B) whereas allergen-specific Th2 responses in MLNs were normalized (Figure 5C). In the “therapeutic” setting (Figure 1E), reversal of the inhibitory effect of R-848 treatment was even more pronounced, with no differences observed in inflammatory

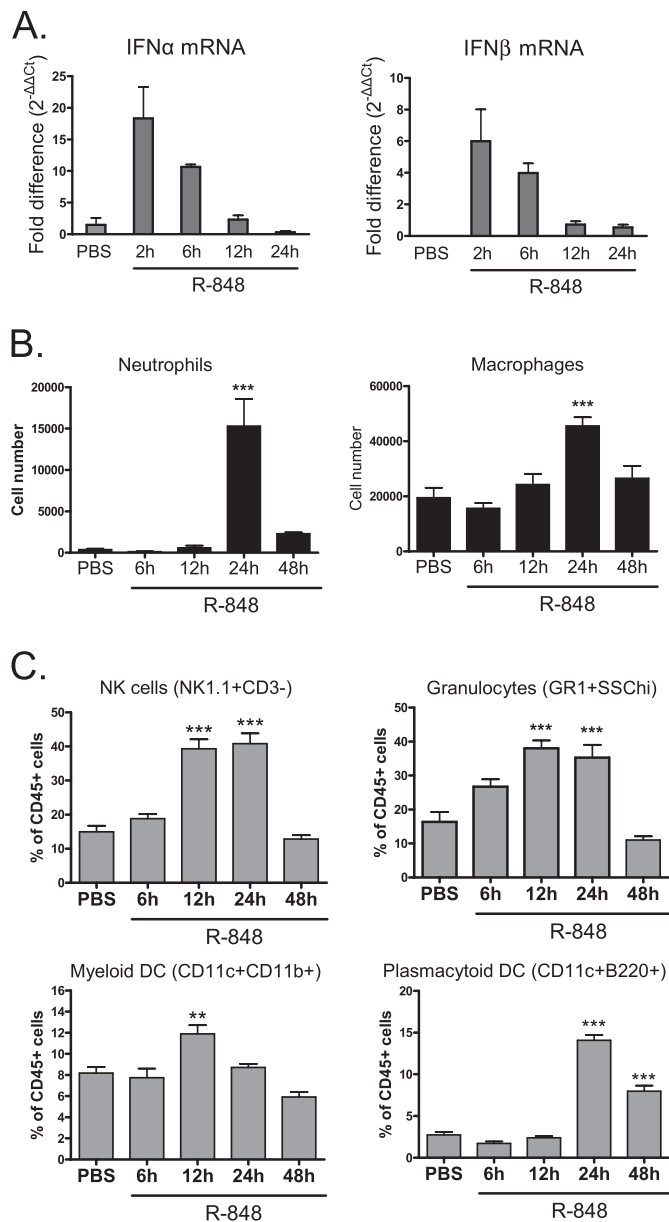


Figure 4. Intranasal R-848 administration triggers a transient innate antiviral immune response characterized by high type I IFN production. (A) IFN- α and IFN- β expression after administration of 200 μg of R-848 in the lung. Results are expressed as fold induction compared with vehicle (phosphate-buffered saline [PBS])-treated controls. (B) Bronchoalveolar lavage differential counts of neutrophils and macrophages after R-848 administration are expressed as means $\pm$ SEM of five mice per group; data from one of two independent experiments are shown. (C) Flow cytometric analysis of lung digests from mice treated with R-848 intranasally. Results are expressed as mean $\pm$ SEM percentages of CD45⁺ cells from five mice per group from two independent experiments. *** $P < 0.001$; ** $P < 0.01$. DC = dendritic cells; NK = natural killer cells.

cell and eosinophil numbers in the BAL (Figures 5D and 5E) or Th2 responses in MLNs (data not shown) between R-848-treated and control mice. In line with these findings, R-848-mediated inhibition of leukocytic cell infiltration in the lung tissue (Figure 5F) and goblet cell metaplasia in the airways was also largely lost (Figure 5G).

Inhibition of Th2 responses and Th2-mediated inflammation after R-848 treatment was directly mediated by type I IFNs, as we

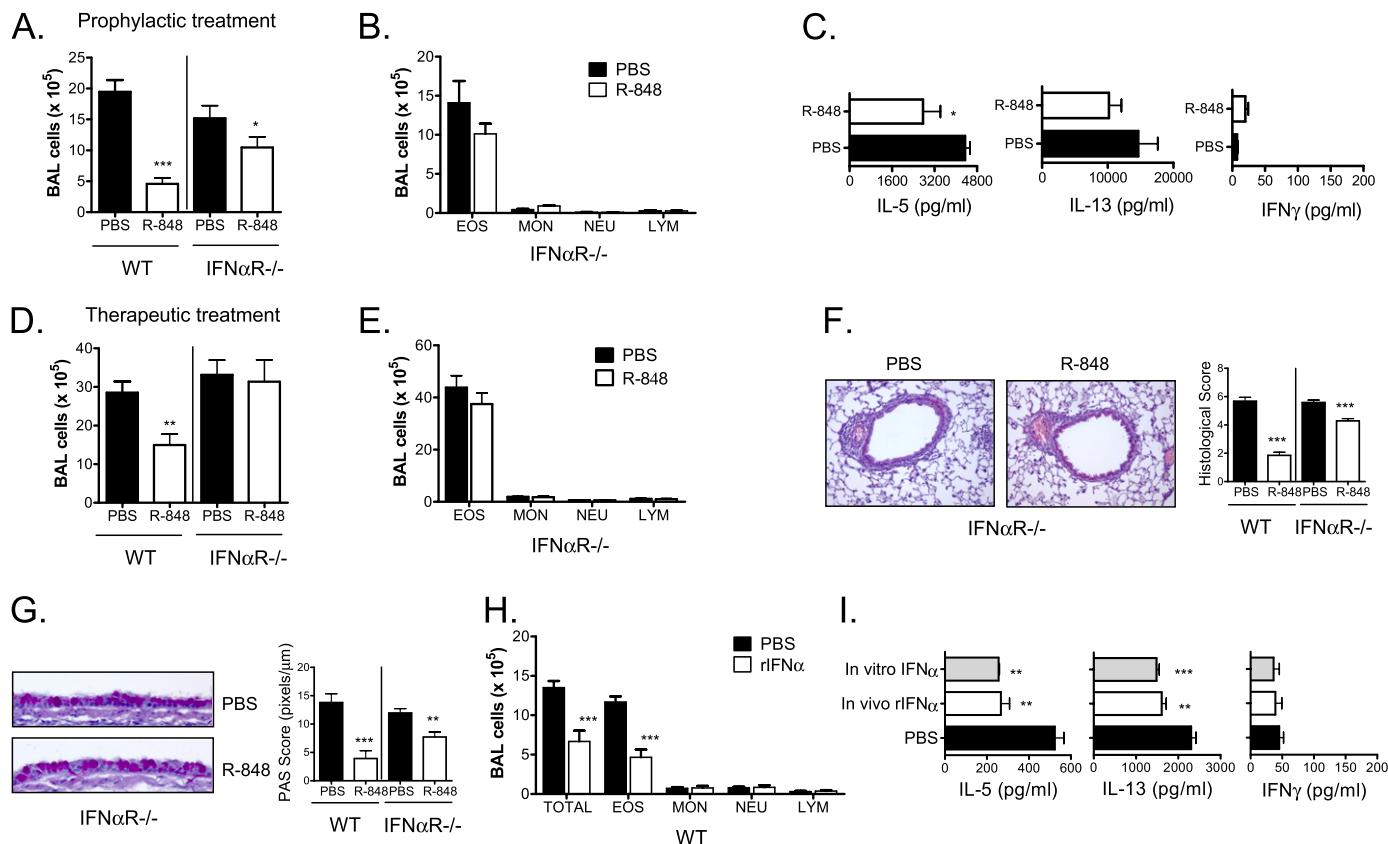


Figure 5. Toll-like receptor (TLR) 7-induced type I IFNs mediate acute suppression by directly inhibiting allergen-specific helper T cell type 2 (Th2) responses and allergic airway disease. (A) Total bronchoalveolar lavage (BAL) counts of wild-type (WT) versus IFN- α receptor (IFN- α R) $^{-/-}$ mice treated prophylactically, expressed as means $\pm$ SEM of 11–15 mice per group from three independent experiments, are shown. PBS = phosphate-buffered saline. (B) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) from IFN- α R $^{-/-}$ mice, expressed as means $\pm$ SEM of 10 mice per group from two independent experiments, are shown. (C) Ovalbumin (OVA)-specific T-cell responses after R-848 treatment. Levels of IL-5 and IL-13 are expressed as means $\pm$ SEM in supernatants of OVA-stimulated mediastinal lymph nodes (MLNs) of six mice per group; data from one of two independent experiments are shown. (D) Total BAL counts of WT versus IFN- α R $^{-/-}$ mice treated therapeutically, are expressed as means $\pm$ SEM of eight mice per group; data from one of three independent experiments are shown. (E) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) from IFN- α R $^{-/-}$ mice are expressed as means $\pm$ SEM of eight mice per group; data from one of three independent experiments are shown. (F) Histological assessment of lung inflammation. Hematoxylin and eosin-stained lung sections and histological scoring, expressed as mean values $\pm$ SEM from 9 to 11 mice per group, are shown. (G) Histological assessment of mucus secretion. Periodic acid–Schiff (PAS)-stained sections and morphometric analysis, expressed as mean values $\pm$ SEM from 9 to 11 mice per group, are shown. (H) Total and differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) of mice treated with rIFN- α are expressed as means $\pm$ SEM of nine mice per group from two independent experiments. (I) OVA-specific T-cell responses after rIFN- α treatment *in vivo* or addition *in vitro*. Levels of IL-5 and IL-13 are expressed as means $\pm$ SEM in supernatants of OVA-stimulated MLNs of five mice per group; data from one of two independent experiments are shown. *** P < 0.001; ** P < 0.01; * P < 0.05.

found that administration of recombinant IFN- α to WT mice was sufficient to suppress allergic airway inflammation (Figure 5H) and Th2-mediated allergic responses in MLNs (Figure 5I). Moreover, addition of IFN- α to MLN cultures of WT mice was capable of directly suppressing Th2 responses (Figure 5I), suggesting that IFN- α and R-848 treatment acts at the level of T-cell activation to suppress inflammation *in vivo*. Collectively, these findings suggest that type I IFNs up-regulated by TLR7 activation play a major role in mediating acute suppression of allergic airway disease.

CD8 $^{+}$ T Cells Secreting IFN- γ Are Essential for Long-Lasting Suppression of Allergic Airway Disease

A consistent effect observed after intranasal R-848 administration was the induction of CD8 $^{+}$ T cells secreting IFN- γ (Figure 6A). As CD8 $^{+}$ T cells secreting IFN- γ have been previously shown to exert immunomodulatory effects in experimental models of allergic airway disease, we examined their involve-

ment in the mechanism of action of R-848 treatment. Notably, we found that neither CD8 $^{+}$ T cells nor IFN- γ was required for acute suppression of allergic responses mediated by TLR7 activation (Figures 6B and 6D). R-848 treatment in CD8 $^{-/-}$ or IFN- γ $^{-/-}$ mice was still effective in suppressing allergic airway inflammation in mice challenged immediately after treatment with inhaled allergen, although the suppressive activity of R-848 as compared with that in wild-type mice was slightly reduced. Interestingly, although IL-5 and IL-13 production in MLNs was inhibited in CD8 $^{-/-}$ mice (Figure 6E) as in WT mice, R-848 treatment did not result in up-regulation of IFN- γ (Figure 6E), further confirming that CD8 $^{+}$ T cells are the main source of IFN- γ *in vivo*. In sharp contrast to these findings, the long-term suppression induced by R-848 was highly dependent on both CD8 $^{+}$ cells and IFN- γ (Figure 6F). Inhibition of total inflammatory and eosinophil cell numbers in the BAL (Figures 6G and 6H) and Th2 responses in MLNs (Figure 6I) was completely reversed

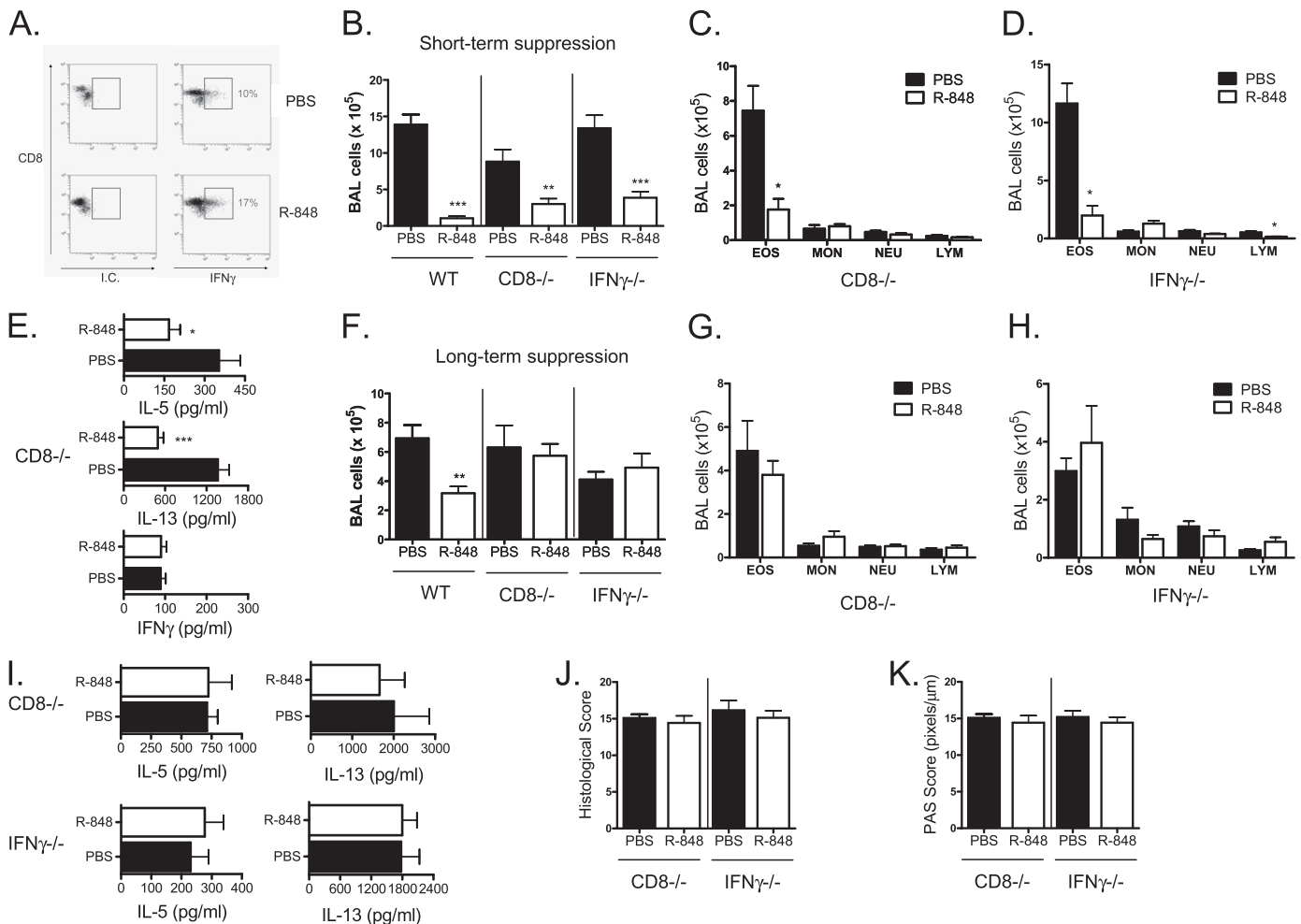


Figure 6. CD8⁺ T cells secreting IFN-γ are essential for long-lasting suppression of allergic airway disease. (A) Intracellular fluorescence-activated flow cytometric staining of CD8⁺ T cells for IFN-γ expression. Dot-plot graphs of mediastinal lymph node (MLN) cells from vehicle (phosphate-buffered saline [PBS]) versus R-848 treated mice stained with an anti-IFN-γ or isotype control (I.C.) antibody are shown. These are representative of three independent experiments. (B) Total bronchoalveolar lavage (BAL) counts of wild-type (WT), CD8^{-/-}, and IFN-γ^{-/-} mice are expressed as means ± SEM of 11–15 mice per group from three independent experiments. (C) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) from CD8^{-/-} mice are expressed as means ± SEM of 11 mice per group from two independent experiments. (D) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) from IFN-γ^{-/-} mice are expressed as means ± SEM of 10–12 mice per group from two independent experiments. (E) Ovalbumin (OVA)-specific T-cell responses after R-848 treatment of CD8^{-/-} mice. Levels of IL-5, IL-13, and IFN-γ are expressed as means ± SEM in supernatants of OVA-stimulated MLNs of 10 or 11 mice per group from two independent experiments. (F) Total BAL counts of WT, CD8^{-/-}, and IFN-γ^{-/-} mice are expressed as means ± SEM of 10–12 mice per group from two independent experiments. (G) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) from CD8^{-/-} mice are expressed as means ± SEM of 10 mice per group from two independent experiments. (H) BAL differential counts of eosinophils (EOS), mononuclear phagocytes (MON), neutrophils (NEU), and lymphocytes (LYM) from IFN-γ^{-/-} mice are expressed as means ± SEM of 12 mice per group from two independent experiments. (I) OVA-specific T-cell responses after R-848 treatment. Levels of IL-5 and IL-13 are expressed as means ± SEM in supernatants of OVA-stimulated MLNs of 8–10 mice per group from two independent experiments. (J) Histological assessment of lung inflammation. Hematoxylin and eosin-stained lung sections and histological scoring, expressed as mean values ± SEM from eight or nine mice per group, are shown. (K) Histological assessment of mucus secretion. Periodic acid–Schiff (PAS)-stained sections and morphometric analysis, expressed as mean values ± SEM from eight or nine mice per group, are shown. ****p* < 0.001; ***p* < 0.01; **p* < 0.05.

in CD8^{-/-} and IFN-γ^{-/-} mice, as was perivascular and peribronchial cell infiltration (Figure 6J) and goblet cell metaplasia (Figure 6K) in lung tissue examined by histopathological analysis. These data suggest that CD8⁺ T cells secreting IFN-γ are part of an adaptive immune response triggered by TLR7 activation that ensures long-lasting suppression of allergic airway disease.

DISCUSSION

The innate immune system has become a critical area of research for investigators of asthma pathogenesis. There has been growing

appreciation that microbial exposures significantly affect the development of the immune system in a complex manner that can either reduce or increase the risk for allergic disease, and growing interest in elements of microbial or viral pathogens that may exert immunomodulatory activities and thus be useful therapeutically (27). Although most research has focused on CpG-ODNs that trigger TLR9-mediated innate immune responses (9), other molecular structures that activate TLR3-, TLR4-, or TLR7/8-mediated processes have also shown promise for the suppression of allergic responses *in vitro* and/or *in vivo* (2). Among them the

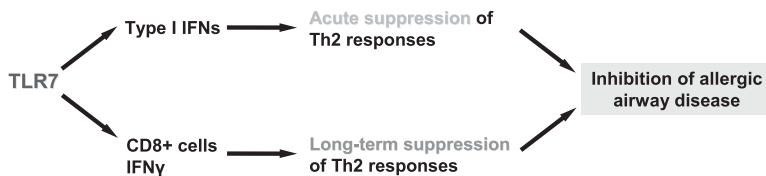


Figure 7. Mode of action of Toll-like receptor (TLR) 7-mediated suppression. Intranasal R-848 administration induces type I IFNs, which provide acute protection from allergic airway inflammation, and CD8⁺ T cells, which provide long-term protection by suppressing helper T cell type 2 (Th2) responses in an IFN- γ -dependent manner.

TLR7/8 agonist R-848, specific for TLR7 only in mice, has been particularly effective when administered systemically to mouse models, but its clinical utility in humans has been limited by toxicity. In a phase IIa clinical trial involving R-848 administration for the treatment of chronic hepatitis C infection, severe adverse events including fever, headache, shivering, and lymphopenia were reported (21). However, more recent trials indicate that it is possible to administer TLR7/8 ligands with safety and efficacy by employing topical rather than systemic approaches (28) and probably specific TLR7 ligands to avoid TLR8-mediated induction of tumor necrosis factor (29) and activation of peripheral blood neutrophils (30). Despite that, topical administration of TLR7 or TLR7/8 ligands in the lung has not yet been explored in preclinical models of allergic airway disease.

In this study, we show that administration of the TLR7 ligand R-848 to the lung through the intranasal route constitutes an alternative and highly effective immunotherapeutic strategy for the treatment of experimental allergic airway disease in mice. We demonstrate that intranasal R-848 treatment at 200 μ g/mouse rapidly suppresses the development of allergic airway responses and ensuing airway inflammation and hyperresponsiveness. It profoundly reduces total inflammatory cell infiltration and eosinophilia in the BAL and lung tissue, goblet cell metaplasia in the airways, Th2 cell development in MLNs, and Th2 cytokine production in the lung whereas it up-regulates IFN- γ . Notably, R-848 treatment is also effective when administered “therapeutically” to mice with full-blown disease that have already been exposed for three consecutive days to inhaled allergen. In this setting, R-848 markedly suppresses disease deterioration by inhibiting further eosinophilic cell infiltration in the lung and Th2 cell expansion in the lung-draining MLNs. These findings demonstrate that local R-848 treatment to the lung is a highly effective approach to suppress allergic airway disease while avoiding systemic delivery, which has been associated with severe toxicity in humans.

We further show that TLR7-mediated suppression of allergic airway responses is long-lived. We found that intranasal R-848 administration was capable of suppressing allergic airway inflammation triggered by inhaled allergen challenge 2 and even 4 weeks after treatment. There was generalized suppression of all parameters of disease assessed, although the extent of suppression was lower than that seen in mice challenged immediately after treatment. Accordingly, total inflammatory and eosinophilic cell infiltration in the BAL or lung tissue, Th2 cytokine responses in MLNs, and goblet cell metaplasia in the airways were all significantly inhibited in R-848-treated mice for at least 4 weeks. This was unexpected, but not without precedent. Long-term persistence of treatment has been described previously for CpG-ODNs, which were shown to inhibit the development of allergic airway responses for up to 6 weeks after treatment (31). Thus, it is possible to harness TLR7 function to achieve long-lasting protection from allergic airway disease.

The effectiveness of intranasal R-848 administration is reminiscent of previous studies on systemic administration. In all these reports, profound suppression of allergic airway disease was observed after TLR7 ligand treatment, although this was assessed only in a prophylactic setting (19). In addition, in one of these reports in C57BL/6 mice, a dose-response profile similar to that seen after intranasal administration was described and was

associated with a marked skewing to a Th1 cytokine profile (32). This was in contrast to two other reports on C57BL/6 and A/J mice, or Brown Norway rats, in which no apparent Th1 skewing was noted but rather a generalized reduction of both Th1 and Th2 cytokines (19, 20). In our study, we consistently observed a Th1 cytokine switching in the lung (Figure E1) and MLNs draining the lung in both prophylactic and therapeutic settings. Whether this reflects a more focused and effective delivery strategy or simply slight differences in experimental design is not clear at this stage.

In this study, we took a step forward and systematically investigated the molecular and cellular mechanisms involved. By constructing radiation-induced chimeric mice that lack TLR7 expression in their hematopoietic compartment, we found that TLR7 signaling on immune cells is essential for R-848-mediated suppression. Although this does not exclude a role for epithelial TLR7 in this process, as bronchial epithelial cells also express significant levels of TLR7 (see Figure E2 in the online supplement), it clearly demonstrates how critical R-848-induced activation of immune cells is in this process. Moreover, by defining the early immune events triggered by R-848 administration we found that suppression was dependent on elements of a transient innate immune response initiated by TLR7. Thus, type I IFNs were key for mediating acute suppression of allergic airway disease after R-848 treatment before or after allergen challenge. Absence of type I IFN signaling in IFN- α - γ mice largely reversed the inhibitory effect of R-848 treatment whereas administration of recombinant IFN- α was sufficient to suppress allergic airway disease in the absence of additional treatment. Acute suppression occurred at the level of the DC/T-cell interphase. R-848 administration *in vivo* led to severely impaired Th2 responses to allergen in the lung-draining MLNs whereas *in vitro* addition of either R-848 or IFN- α to MLN cultures of untreated OVA-primed and challenged mice potently reproduced the suppressive effect of the *in vivo* treatment. Moreover, absence of type I IFN signaling in IFN- α - γ mice reversed the inhibitory effect of *in vivo* R-848 treatment on allergen-specific Th2 responses.

In contrast to acute suppression, long-term suppression of allergic airway disease was key to the induction of CD8⁺ T cells and IFN- γ . The generation of cytotoxic CD8⁺ T-cell responses and IFN- γ is a well-known effect of R-848 treatment currently exploited for the development of improved antiviral or anti-cancer strategies. It is well established that R-848, through the engagement of TLR7 and the production of type I IFNs, constitutes a powerful adjuvant for the generation of effector and memory CD8⁺ T-cell responses *in vivo* (33). Here, we found that intranasal R-848 treatment also induces CD8⁺ T-cell responses secreting IFN- γ , which are critical for conferring long-lasting suppression of allergic airway disease. Although CD8⁺ T cells and IFN- γ are not required for acute suppression of allergic airway disease, they are essential for long-term suppression mediated in response to R-848 treatment. Thus, in CD8^{-/-} or IFN- γ - γ mice R-848 suppression is completely reversed. CD8⁺ T cells with immunoregulatory properties in allergic airway disease have been described previously. It has been shown that CD8⁺ T cells induced by respiratory viral infection and secreting IFN- γ potently inhibit allergic airway

inflammation and hyperresponsiveness in mice (34–36) and correlate with a better disease outcome in humans (37). Moreover, induction of CD8⁺ T cells secreting IFN- γ has been shown to be key to potent immunotherapeutic approaches developed for the treatment of experimental allergic airway disease in mice (38, 39). Why respiratory viruses that often trigger TLR7 and activate CD8⁺ T cells exacerbate rather than ameliorate airway inflammation in patients with asthma is not yet clear. It may be that respiratory viruses trigger multiple other processes, the net effect of which aggravates airway inflammation. Alternatively, patients with asthma may exhibit defective induction of CD8⁺ responses (40) due to deficient production of type I and type III interferons (40, 41) and preexisting Th2 inflammation in the airways, which rapidly switches CD8⁺ T cells to a cytotoxic T cell type 2 phenotype (42). Finally, defective clearance of respiratory viruses observed in patients with atopic asthma compared with healthy individuals may further contribute to disease exacerbation in these patients (43).

On the basis of these data we propose the following model for the mode of action of the TLR7 ligand R-848 in allergic airway disease (Figure 7). Intranasal R-848 administration induces the expression of type I IFNs, which rapidly suppress effector Th2 responses in the lung in an IFN- γ -independent manner and provide acute protection from allergic airway disease. This occurs when R-848 is administered “prophylactically” before allergen challenge but also “therapeutically” after three consecutive allergen challenges and well-established disease. In parallel, intranasal R-848 administration induces a CD8⁺ T-cell response with immunoregulatory properties that suppresses effector Th2 responses in an IFN- γ -dependent manner. This response is long-lived and provides protection from allergic airway disease even 1 month after treatment. Therefore, through these two distinct mechanisms R-848 treatment achieves optimal and long-lasting suppression of allergic airway disease.

Our findings are clinically relevant. Clinical studies in humans have shown that it is possible to use TLR ligands to treat allergic responses with safety and efficacy. In two controlled trials of ragweed allergy, subcutaneous injections of ragweed antigen conjugated to CpG-ODN (AIC, Amb a1-immunostimulatory DNA complex) increased the IFN- γ /IL-4 cell ratio and improved peak season rhinitis and nasal symptom scores (11, 12). These effects were also seen in the following ragweed season with no further treatment, supporting the use of CpG-ODN for long-term treatment of allergic rhinitis. In another controlled study of patients with mild asthma, four weekly doses of nebulized CpG-ODN were a safe and pharmacologically active treatment inducing both IFN- γ and IFN-inducible genes, even though at this dose regimen no significant differences in allergen-induced sputum eosinophilia and FEV₁ reduction were demonstrated (10). Our data now suggest that intranasal TLR7 ligand administration may constitute a potent alternative to CpG-ODN for the suppression of allergic airway disease that needs to be further explored. The development and/or selection of optimal TLR7-specific ligands (as R-848 and other TLR7/8 ligands are likely to raise toxicity issues in humans) and their safety and effectiveness in relevant experimental models including larger animals, will ultimately determine whether this highly promising experimental approach can eventually be evaluated in the clinic.

Conflict of Interest Statement: C.X. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; O.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; A.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; V.A. does not have a financial relationship with a commercial entity that has an interest in the

subject of this manuscript; P.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.A. received more than \$100,001 from NOVOSOM AG in industry-sponsored grants (collaborative title: Amphoteric liposomes for systemic oligonucleotide delivery).

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TLR7-triggered immune response in the lung mediates acute and long-lasting
suppression of experimental asthma

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Thanassopoulou, Vassilis Aidinis, Paschalis Sideras, Evangelos Andreakos**

Online Data Supplement

Supplemental Information

Materials and Methods

Mice

All procedures had received prior approval from the Institutions and Regional Ethical Review Boards and were in accordance with the US National Institutes of Health Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals (#A5736-01) and with the European Union Directive 86/609/EEC for animal research.

Sensitization and challenge protocols

Three distinct experimental protocols were used as indicated in the manuscript. At all cases mice were sensitized with two i.p. injections of 7.5 µg OVA (Grade V; Sigma) and 1 mg aluminum hydroxide as adjuvant (Sigma) and challenged for three consecutive days with 5% (wt/vol) OVA for 30 min. In the 'prophylactic' protocol treatment was administered one day before three consecutive allergen challenges. In the 'therapeutic' protocol treatment was administered after three consecutive allergen challenges but before three additional ones. In the protocol of long-term studies, treatment was administered two and four weeks before three consecutive allergen challenges.

Bronchoalveolar lavage and differential cell counts

Bronchoalveolar lavage was obtained by lavaging the airways of mice two times with 0.5 ml PBS via a tracheal cannula. Cells were counted, pelleted onto glass slides and differential cell counts for eosinophils, lymphocyte/mononuclear cells, neutrophils, and macrophages performed on May-Grünwald stained cytopins.

Histological analysis, scoring and morphometry

Mouse lungs were fixed in 10% (vol/vol) buffered formalin and embedded in paraffin for staining with hematoxylin and eosin (H&E) and with periodic acid–Schiff (PAS) reagent (Sigma). Histopathological analysis of inflammatory cells in H&E stained lung sections from at least six mice was performed in a blinded fashion using a semiquantitative scoring system as previously described {Tong, 2006 #137}. Both peribronchiolar and perivascular inflammation were scored giving a maximum score of 8 as follows: 0, normal; 1, few cells; 2, a ring of inflammatory cells one cell layer deep; 3, a ring of inflammatory cells two to four cells deep; and 4, a ring of inflammatory cells of more than four cells deep. Morphometric analysis of PAS stained sections was performed by quantifying PAS pixels per µm length distance of bronchial epithelium of central airways using the Image J software. At least six areas from similar sections per mouse and at least six mice were assessed blindly.

Lung cell isolation

Total pulmonary leukocytes were recovered by cutting lungs into small pieces, and placing them in complete media with collagenase IV for 60 min at 37°C. Single-cell suspensions were then obtained by homogenization and filtered through a 40-µm nylon filter. Viability of the cells determined by Trypan blue exclusion was >95%.

Measurement of airway hyper-responsiveness

For the measurement of airway responsiveness to metacholine, a computer-controlled small animal ventilator was used (flexiVent, SCIREQ Montreal, PQ) (1,2,3). 24 h after the last OVA aerosol exposure, the mice were anesthetized with intra-peritoneal injection of 0.051 ml/10 g bodyweight of a mixture of ketamine (20 mg/ml, Merial SAS, Lyon, France) and xylazine (1.3 mg/ml, Bayer AG, Leverkusen, Germany). Once surgical anaesthesia had been established the trachea was exposed and cannulated with a blunted metal 18-gauge needle. The mice were placed on a 37°C heating pad to maintain body temperature during the experiment and ventilated with a tidal volume of 8 ml/kg, at a normal respiratory rate (150 breaths/minute). A positive end-expiratory pressure (PEEP) of 0.2 kPa was established by placing the expiratory line in a water trap. (4). Each animal was paralyzed with intra-peritoneal injection of 0.05 ml/10 g bodyweight of rocuronium bromide (0.01 mg/ml, N.V. Organon, PO Box 20, 5340 BH Oss, The Netherlands) to suppress spontaneous breathing and allowed to equilibrate on the ventilator (5 minutes). The lung volume history of the mice was standardised prior to measurement of lung mechanics using three deep inflations at total lung capacity (TLC). Evolvement of the mechanical parts of the ventilator and the tracheal cannula to the measurements were eliminated using dynamic calibration as previously described (5, 6). An aerosol of PBS was given as a control, followed by increasing concentrations (3.33, 33.3, 100, and 333 mg/ml) of metacholine aerosol (Aldrich Chemical, Milwaukee, WI) delivered into the trachea.

The Total Lung Resistance (R), which reflects the level of constriction in the lungs, measured by fitting the linear single-compartment model using Multiple Linear Regression, and the average of three replicate measurements before the PBS administration represented the individual baseline value. For each metacholine dose, twelve measurements were collected for analysis and the peak of the response was determined from real-time measurements of (R), and is reported as the percent increase from baseline.

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