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Chronic Asthma-induced Airway Remodeling Is Prevented by Toll-like Receptor-7/8 Ligand S28463

P. Camateros, M. Tamaoka, M. Hassan, R. Marino, J. Moisan, D. Marion, M.-C. Guiot, J. G. Martin and D. Radzioch
Am. J. Respir. Crit. Care Med., June 15, 2007; 175 (12): 1241-1249.

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TLR7 ligand prevents allergen-induced airway hyperresponsiveness and eosinophilia in allergic asthma by a MYD88-dependent and MK2-independent pathway

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Toll-like receptors; asthma; eosinophils; lung; inflammation; mitogen-activated protein kinase-activated protein-2

ALLERGIC ASTHMA IS A COMPLEX and heterogeneous respiratory and immune disorder. It is a leading cause of childhood hospitalization, whose prevalence is increasing in westernized countries (5). Asthma is generally defined as a chronic inflammation of the bronchial airways, characterized by intermittent episodes of reversible airway obstruction, excessive mucus production, and infiltration of the airway submucosa by acti-

vated lymphocytes, monocytes, neutrophils, and especially eosinophils (8).

One of the most important pathological features of asthma is airway (or bronchial) hyperresponsiveness (AHR). AHR is an increased sensitivity, exhibited by asthmatic subjects, to a variety of stimuli including allergens, pollutants, cold air, exercise, distilled water, and methacholine that cause bronchoconstriction (34). The AHR seen in human asthmatic patients is associated with airway remodeling characterized by the deposition of excess collagen in the subepithelial layer of the airway mucosa due to chronic inflammation of the bronchial airway wall and airway smooth muscle hyperplasia (6, 29).

Attempts at controlling the inflammatory reaction have mainly consisted of approaches targeting the effector inflammatory symptoms present in asthmatic patients. Present medications that require chronic dosing are not fully effective and have been reported to have some side effects. The majority of treatments do not target the early upstream events in the pathways of allergic inflammation and bronchospasm, which has prompted the search for new therapeutic targets (3). One of these new strategies involves modulation of the immune response through Toll-like receptor (TLR) activation. Most of the ligands defined for these receptors are pathogen-derived products. TLR4 is important for the response to LPS from gram-negative bacteria (16). TLR2 transduces signals for lipoprotein and cell wall products from gram-positive bacteria as well as peptidoglycan and lipoarabinomannan from mycobacteria (26, 35). TLR9 has been found to be responsible for the response to unmethylated CpG oligodeoxynucleotides in both mice and humans (4, 14). S-28463, a member of the imidazoquinoline family also called resiquimod, has been shown to be a TLR7/8 ligand in humans (18). The imidazoquinolines are potent inducers of a whole spectrum of cytokines, such as IL-1, IL-6, IL-8, and TNF- α , in a number of animal species such as the mice, guinea pigs, and monkeys (39). TLR7 and -8 have also been implicated in recognizing single-stranded RNA of viral origin (9, 12). Although mice express both TLR7 and TLR8, only TLR7 appears to be functional (18). Furthermore, although TLR4 signal transduction can occur through an MYD88-dependent and -independent pathway, TLR7-mediated immunomodulatory effects are solely dependent on the

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Table 1. Primer sequence for real-time quantitative PCR

Gene	Sense 5' to 3'	Antisense 5' to 3'	Product Size,
CCL5	TCTGCCCCACGTCAAGGAGTATTT	TCTTCTCTGGGTGGGCACACACTT	107
CCL11	TATTCTGTGCTCAGGTCACCTT	TCTCTTTGCCCAACCTGGTCTTGA	192
CCL17	AGGGATGCCATCGTGTCTGACT	ATGTTGAAACCATGGACAGCAGCC	162
CCL24	TTGACGCTTACCAGGCTGCTTTG	ATGCTTGACGCTCACTCAGGTTCT	128
GAPDH	ATGTGTCCGTCGTGGATCTGA	TTGAAGTCGCAGGAGACAACCT	145

MYD88 pathway (13). TLR stimulation can affect various aspects of the asthmatic phenotype. Unmethylated CpG oligodeoxynucleotides have been shown to reverse allergen-induced inflammation and prevent airway remodeling and AHR in response to allergen in a mouse model of asthma (7, 21). Inoculation of mice with *Mycobacterium bovis* bacillus Calmette-Guérin and *Mycobacterium vaccae*, whose cell wall components are TLR2 ligands, has also been shown to reduce AHR in response to allergens (15, 33).

Using mice sensitized and challenged with ovalbumin, we demonstrate that imidazoquinoline treatment prevents increased lung resistance and elastance in mice that exhibit low

(C57BL/6) and high (A/J) naive lung AHR. TLR7 ligand administration also led to a decrease in serum IgE levels. TLR7 activation prevented lung cellular infiltration, completely abrogating lung eosinophil recruitment. We also demonstrate that the protective effect of TLR7 ligands is dependent on MYD88, but independent of MAPKAP-2 (MK2). Furthermore, complete inhibition of T_H2 , as well as T_H1 , cytokines following imidazoquinoline treatment in ovalbumin-challenged animals suggests that TLR7 triggering in the context of allergic asthma plays an anti-inflammatory role.

MATERIALS AND METHODS

Mice. Eight- to ten-week-old male A/J and C57BL/6 mice were purchased from Harlan (Indianapolis, IN). C57BL/6 MK2 knockout (KO) mice previously generated by Kotlyarov et al. (22) were bred at the Research Institute of the McGill University Health Center. MYD88^{-/-} mice were generated in C57BL/6 × 129/SvJ (1) and subsequently backcrossed six times to the C57BL/6 background at the Weill Medical College of Cornell University. All animals were specific pathogen free, and all procedures were reviewed and approved by the Animal Care Committee of the McGill University Health Center in accordance with the Canadian Council on Animal Care guidelines.

Challenge protocol and respiratory system physiology. Mice were sensitized once a week for three consecutive weeks by intraperitoneal injections of 100 µg of ovalbumin adsorbed to 1.5 mg of aluminum

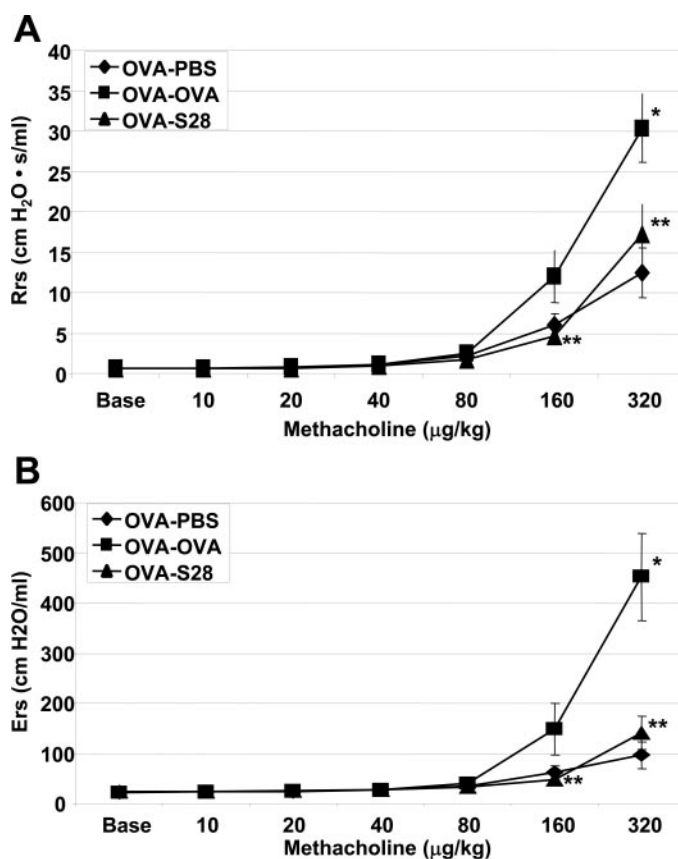


Fig. 1. Treatment with S-28463 abrogates allergen-induced airway hyperresponsiveness (AHR) in response to methacholine (MCh). A/J mice were sensitized with ovalbumin (OVA) and challenged by aerosol exposure to PBS (OVA-PBS) or OVA (OVA-OVA), as described in MATERIALS AND METHODS. One group of mice received 100 µg of S-28463 intraperitoneally 1 day before each OVA challenge (OVA-S28). Respiratory system resistance (Rrs, A) and elastance (Ers, B) in response to increasing doses of MCh were measured 48 h after the last PBS or OVA aerosol challenge. Results are presented as means ± SE from 3 independent experiments ($n = 10$). * $P < 0.05$ between OVA-PBS and OVA-OVA group; ** $P < 0.05$ between OVA-OVA and OVA-S28 group.

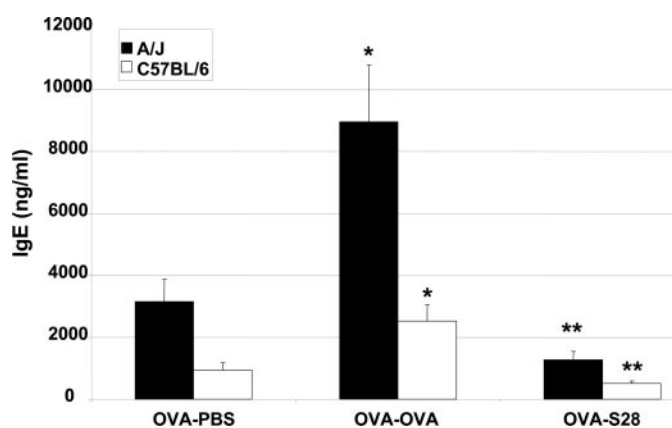


Fig. 2. IgE levels in OVA-challenged A/J and C57BL/6 mice. Mice were sensitized with OVA and challenged with PBS (OVA-PBS) or OVA (OVA-OVA), and serum was collected as described in MATERIALS AND METHODS. One group of mice received 100 µg of S28 intraperitoneally 1 day before each OVA challenge (OVA-S28). Total serum IgE levels were determined by ELISA 48 h after PBS or OVA challenge. OVA challenge in A/J mice led to a significant increase in IgE levels ($n = 13$) compared with PBS challenge ($n = 11$). S28 treatment led to a dramatic reduction in serum IgE levels ($n = 12$). A similar pattern was observed in C57BL/6 mice for OVA-PBS ($n = 24$), OVA-OVA ($n = 26$), and OVA-S28 ($n = 25$) group. Results are presented as means ± SE from 5 independent experiments. * $P < 0.05$ between OVA-PBS and OVA-OVA group; ** $P < 0.05$ between OVA-OVA and OVA-S28 group.

hydroxide (Imject Alum, Pierce, Rockford, IL) in a total volume of 0.2 ml of sterile PBS. Seven days following the final sensitization mice were challenged on 3 consecutive days by aerosol exposure to either a 1% ovalbumin solution (OVA-OVA and OVA-S28 group) or PBS alone (OVA-PBS group) for 30 min. One group of mice was injected intraperitoneally with 100 μ g of S-28463 (generously provided by Dr. R. Miller; 3M Pharmaceuticals, St. Paul, MN) on 3 consecutive days starting 1 day before the first ovalbumin challenge (OVA-S28 group). Measurements of respiratory system resistance and elastance were measured using a Flexivent small animal ventilator (Scireq, Montreal, PQ, Canada). In brief, 48 h after final challenge, animals were anaesthetized, tracheotomized, and connected to the ventilator. Mechanical ventilation was carried out, and peak resistance and elastance were measured after intrajugular administration of increasing doses of methacholine (0–320 μ g/kg).

Histology. Immediately following death of the mouse, the lungs were removed, inflated with 10% buffered formalin, dehydrated, mounted in paraffin, and sectioned. Deparaffinized and hydrated sections were stained with hematoxylin and eosin (H&E) and Congo red stains by standard procedure. For quantitation, 300 infiltrating cells in the lungs were counted by two blinded observers, and the number of Congo red-positive cells was quantitated.

Bronchoalveolar lavage fluid analysis. After the final antigen challenge, mice were killed, and the bronchoalveolar lavage (BAL)

analysis was performed. After insertion of a catheter into the trachea, the lungs of the animal were lavaged with 1.2 ml of Hanks balanced salt solution (Invitrogen), and the samples were kept on ice for further processing. The lavage fluid was centrifuged at 250 g, and the cell pellet was resuspended in RPMI (Invitrogen). Cells were then loaded onto a cytospin machine and spun onto slides at 350 rpm. The slides were subsequently stained with a Diff-Quik stain set (Dade Behring, Newark, DE), and a differential cell count was performed to determine the cellular makeup of the inflammatory cells.

Cytokine and IgE measurements. Immediately following death of the mouse, the lungs of the animal were removed and placed in PBS containing protease inhibitors (Complete inhibitor; Roche Diagnostics, Laval, PQ, Canada). Shortly thereafter, the lungs were homogenized with a Polytron, and the homogenates were stored at -80°C . Cytokine production was measured 3 and 6 h after the last ovalbumin challenge. IL-4, IL-5, IL-6, CCL5 [regulated on activation, normal T cell expressed, and presumably secreted (RANTES)], and IL-10 levels were quantitated by Lincoplex murine cytokine analysis (Linco Research, St. Charles, MO). IL-12p70, IL-13, and IFN- γ production was measured with Beadlyte cytokine kits (Upstate, Charlottesville, VA). Serum IgE levels were measured 48 h following the last challenge with the BD OptEIA ELISA kit (BD Biosciences, Mississauga, ON, Canada) according to the manufacturer's protocol.

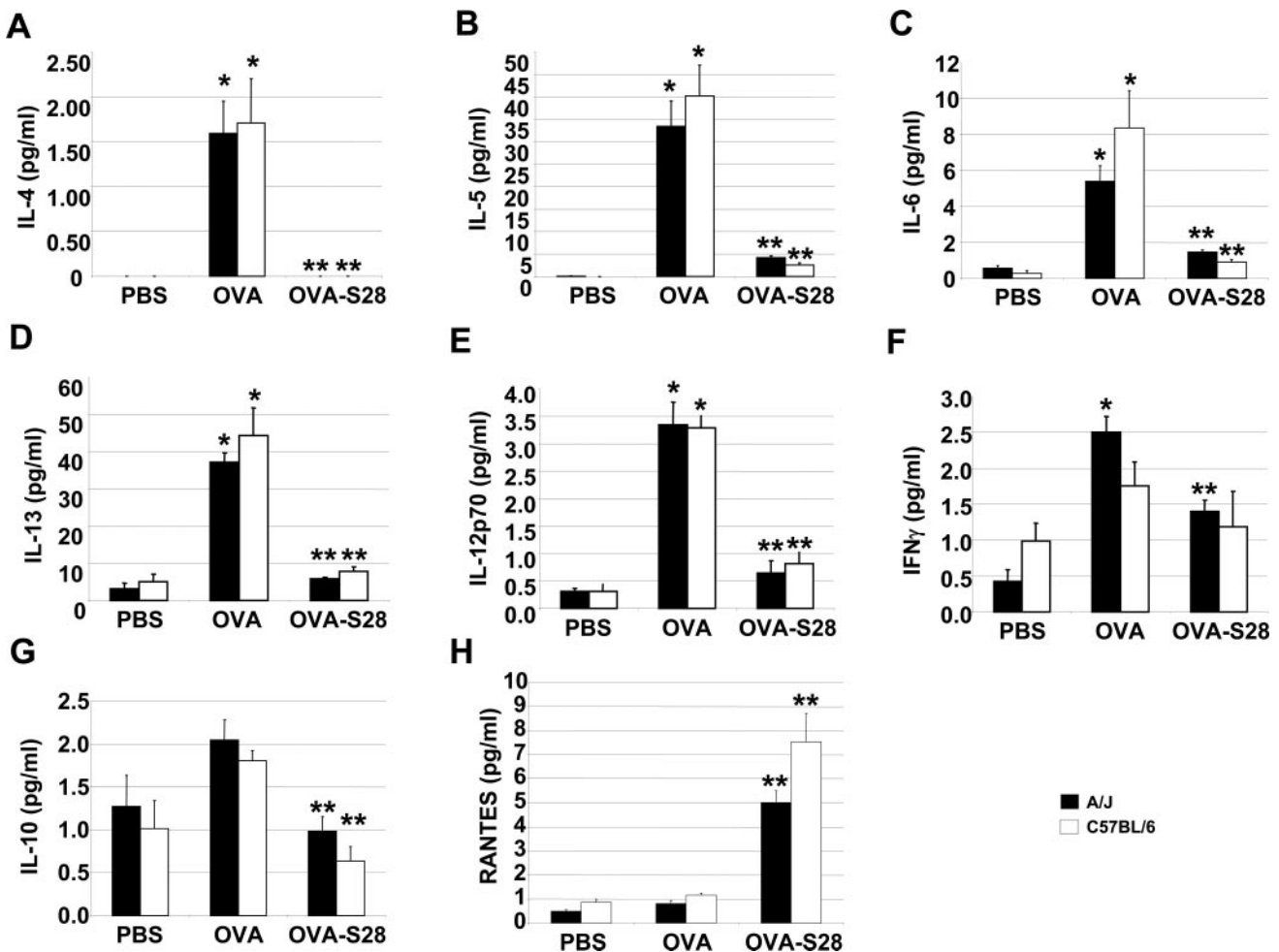


Fig. 3. Cytokine levels following OVA challenge. After sensitization and challenge with OVA or PBS, whole lung tissue homogenates from A/J (solid bars) and C57BL/6 (open bars) were collected as described in MATERIALS AND METHODS. Lung IL-4 (A); IL-5 (B); IL-6 (C); IL-10 (G); and CCL5 (RANTES, H) levels were determined by Lincoplex murine cytokine array 6 h after PBS or OVA challenge. IL-13 (D), IL-12p70 (E), and IFN- γ (F) levels were quantitated with Beadlyte cytokine kits. Results are represented as means $\pm$ SE from 4 animals. *P < 0.05 between OVA-PBS (PBS) and OVA-OVA (OVA) group; **P < 0.05 between OVA-OVA and OVA-S28 group.

RNA analysis. Total lung RNA was purified with TRIzol reagent (Invitrogen). After DNase digestion of 4 μ g of total RNA (DNA-free, Ambion, Austin, TX), reverse transcription was performed with random primers (Stratascript First Strand cDNA synthesis kit; Stratagene, La Jolla, CA). CCL5, CCL11, CCL17, and CCL24 mRNA levels were determined by real-time PCR analysis performed using the Brilliant SYBR Green QPCR kit according to the manufacturer's protocol (Stratagene) on a Stratagene MX-4000 apparatus under the following cycling conditions: denaturation at 95°C for 30 s, annealing at 56°C for 60 s, and extension at 72°C for 30 s. Primer sequences employed in this study are presented in Table 1. Levels of chemokine mRNA were adjusted for differences in GAPDH expression and normalized to the levels in the A/J OVA-PBS group.

Statistical analysis. A Mann-Whitney nonparametric test was performed using the SigmaStat software (SPSS, Chicago, IL) to calculate statistical significance for all analyses except gene expression where an unpaired *t*-test with Welch correction was applied (Prism 4; GraphPad Software, San Diego, CA). Differences among treatments are considered significant if $P \leq 0.05$.

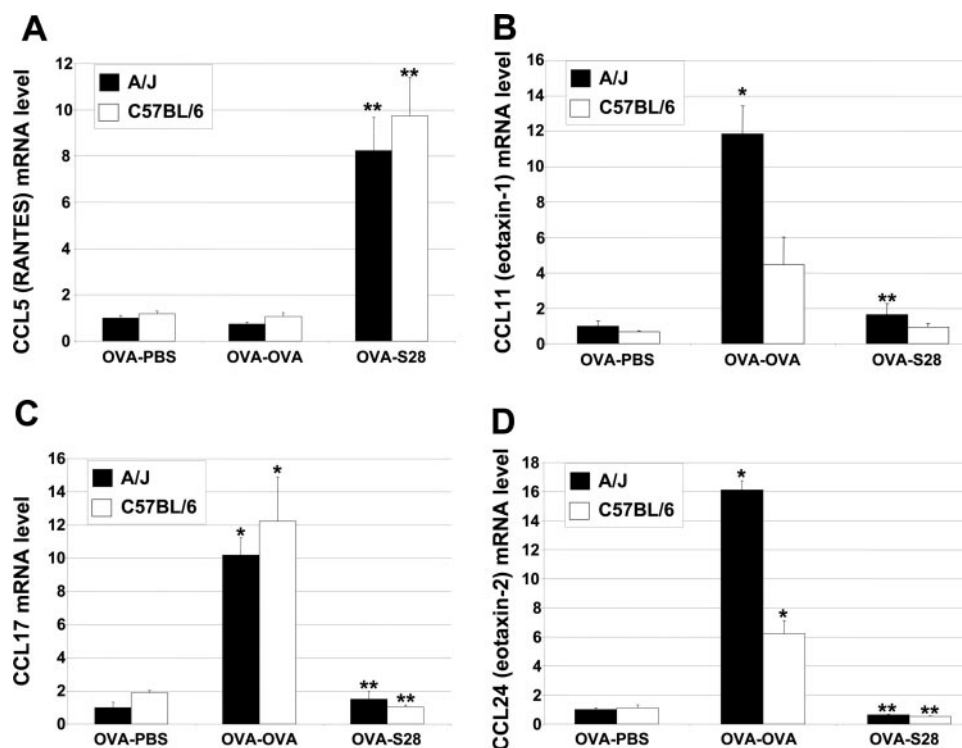
RESULTS

Imidazoquinoline treatment prevents ovalbumin-induced increases in AHR. Previous studies have indicated the potential of TLR signaling in modulating allergen-induced AHR. TLR4 and -9 ligands have been shown to downregulate allergic immune responses in murine models of asthma (17, 21, 30, 32). In this study, the potential of TLR7 ligands to regulate allergic diseases was investigated in A/J and C57BL/6 mice. All animals were sensitized once a week for 3 consecutive weeks. Mice were then separated into three groups receiving a challenge with PBS, 1% ovalbumin, or treatment with S-28463 followed by challenge with ovalbumin. Treatment with ovalbumin induced an increase in lung responsiveness that could be prevented by TLR7 ligand treatment (Fig. 1A). There was a statistically significant increase in lung resistance values in

mice receiving ovalbumin challenge vs. PBS at 320 μ g/kg of methacholine (30.4 ± 4.2 and 12.5 ± 3.1 $\text{cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}$, respectively; $P < 0.05$). Interestingly, the animals receiving S-28463 treatment before ovalbumin challenge had significantly lower lung resistance than the ovalbumin-challenged group at the same methacholine dose (30.4 ± 4.2 for OVA-OVA and 17.3 ± 3.6 $\text{cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}$ for the OVA-S28 group, $P < 0.05$). This reduction in lung resistance by S-28463 could also be observed at the 160 μ g/kg methacholine dose (12.1 ± 3.2 for the OVA-OVA group and 4.6 ± 0.7 $\text{cmH}_2\text{O} \cdot \text{ml}^{-1} \cdot \text{s}^{-1}$ for the OVA-S28 group, $P < 0.05$). Similar effects were observed in C57BL/6 mice treated with TLR7 ligand and challenged with ovalbumin (data not shown). Concurrently with modulation of airway resistance, ovalbumin challenge also increased lung elastance, which could also be prevented by S-28463 treatment (Fig. 1B). These data suggest that TLR7 signaling is able to prevent AHR following allergen challenge in a murine model of asthma.

TLR7 signaling abrogates increases in serum IgE levels following ovalbumin challenge. One of the hallmarks of asthma and other atopic diseases is the presence of increased levels of IgE (25). A/J mice sensitized and challenged with ovalbumin displayed increased levels of serum IgE compared with naïve animals (Fig. 2). Challenge with ovalbumin resulted in an increase in the levels of IgE compared with the group receiving PBS challenge ($8,959 \pm 1,837$ ng/ml vs. $3,161 \pm 710$ ng/ml, respectively). Imidazoquinoline treatment resulted in a dramatic reduction in IgE levels compared with the ovalbumin-challenged group ($1,266 \pm 282$ and $8,959 \pm 1,837$ ng/ml, respectively), bringing the levels into the range of the PBS-challenged group. Interestingly, both the A/J and C57BL/6 strains display a similar pattern of response, except that ovalbumin challenge induced four to five times more IgE

Fig. 4. Chemokine mRNA expression following OVA challenge. Total lung RNA from A/J (solid bars) and C57BL/6 (open bars) mice was purified with TRIzol reagent. Real-time PCR analysis was performed with the Stratagene MX-4000 apparatus. Levels of CCL5 (A), CCL11 (B), CCL17 (C), and CCL24 (D) mRNA were adjusted for differences in GAPDH expression and normalized to the levels of the A/J OVA-PBS group. Results are represented as means $\pm$ SE from 3 animals for each group, * $P < 0.05$ between OVA-PBS and OVA-OVA group; ** $P < 0.05$ between OVA-OVA and OVA-S28 group.



in A/J compared with C57BL/6 mice. These data demonstrate that TLR7 signaling can reduce the increase in IgE levels induced by ovalbumin challenge even in a genetically predisposed strain of mice such as A/J.

Treatment with TLR7 ligand prevents induction of both T_H2 and T_H1 cytokines following ovalbumin sensitization and challenge. Cytokine profiling in asthmatic patients has pointed to a role for T_H2 cytokines such as IL-4, -5, and -13 in the pathology of this disease (38). Studies with TLR ligands have shown a tendency to skew the asthmatic response from a T_H2 to a T_H1 immune response, resulting in prevention of the asthmatic phenotype in animal models. We assessed the ability of TLR7 ligands to modulate cytokine production in animals sensitized and challenged with OVA. IL-4, -5, -6, and -13 were all strongly induced 6 h following OVA challenge in both A/J and C57BL/6 mice (Fig. 3, A–D). PBS challenge did not lead to any increase in these cytokines. Treatment with S-28463 completely prevented induction of these cytokines following OVA challenge. TLR7 activation did not lead to induction of T_H1 cytokines as both IFN- γ and IL-12p70 levels were reduced by S-28463 treatment (Fig. 3, E and F). Levels of IL-10 were only slightly elevated following ovalbumin challenge and returned to PBS levels in animals treated with imidazoquinolines (Fig. 3G). Imidazoquinoline treatment also led to a significant induction of CCL5 (RANTES) protein levels in the lungs of challenged animals (Fig. 3H), suggesting that the mode of action of these compounds is not through induction of a general immunosuppressed state. Furthermore, mRNA expression analysis in lungs from A/J and C57BL/6 mice revealed an upregulation by TLR7 ligand treatment of CCL5 (RANTES) mRNA (Fig. 4A), as well as chemokines normally associated with a T_H1 response (CCL4, data not shown). Moreover, we found that imidazoquinoline treatment led to inhibition in the mRNA levels of chemokines normally involved in eosinophil recruitment and T_H2 cell infiltration (CCL11, CCL17, and CCL24) following ovalbumin challenge (Fig. 4, B, C, and D, respectively). Together, our data demonstrate that TLR7 ligand treatment results in an almost complete prevention of the inflammatory reaction following ovalbumin sensitization and challenge characterized by an inhibition of induction of several T_H2 and T_H1 cytokines in the lungs.

TLR7 ligand treatment prevents airway inflammation. Proinflammatory cytokines such as IL-5 are known to be important in the recruitment of inflammatory cells to the lung (23). The inhibition of cytokine induction by ovalbumin challenge in imidazoquinoline-treated animals suggested that this might lead to a change in the cellular influx to the lungs of antigen-challenged animals. Ovalbumin challenge induced a significant increase in the amount of cells in the BAL. Total BAL cell numbers went from $28,295 \pm 18,942$ in the PBS group to $115,682 \pm 35,825$ for ovalbumin-challenged A/J mice and from $13,766 \pm 1,995$ to $283,807 \pm 138,121$ in C57BL/6 (Fig. 5A). Treatment with imidazoquinolines completely prevented cellular infiltration in the BAL of both strains of mice. Analysis of cellular composition revealed that ovalbumin challenge induced a significant increase in eosinophil composition of the BAL representing $31 \pm 7\%$ in A/J (Fig. 5B) and $30 \pm 8\%$ in C57BL/6 mice (Fig. 5C). Treatment with a TLR7 ligand completely prevented eosinophil recruitment in both strains of mice. Ovalbumin challenge also increased the relative amounts of lymphocytes in the BAL of both strains of mice that was not

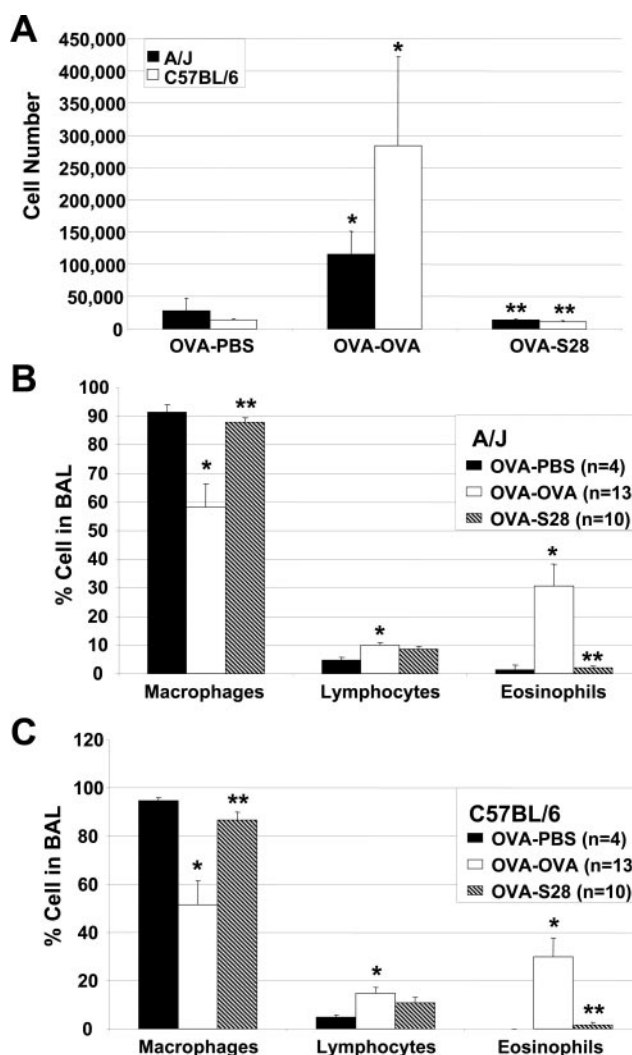


Fig. 5. Bronchoalveolar lavage (BAL) analysis. Analysis of inflammatory cells found in BAL following OVA challenge. Total number of cells was increased in both A/J and C57BL/6 following OVA challenge (A). Treatment with imidazoquinoline significantly inhibited influx of inflammatory cells to the lungs. Analysis of cell types in BAL from A/J (B) and C57BL/6 (C) revealed influx of eosinophils following OVA challenge, which was prevented by TLR7 ligand treatment. Results for total BAL cell count are represented as means $\pm$ SE from 3 independent experiments ($n = 8$ for all groups). * $P < 0.05$ between OVA-PBS and OVA-OVA group; ** $P < 0.05$ between OVA-OVA and OVA-S28 group.

prevented by imidazoquinoline treatment. We did not observe any significant modulation of neutrophil levels by any of the treatments (data not shown). These results demonstrate the ability of imidazoquinolines to prevent influx of inflammatory cells, mainly eosinophils, to the lumen of airways in both A/J and C57BL/6 mice.

Imidazoquinoline treatment prevents lung inflammatory cell recruitment in ovalbumin-challenged mice. Paraffin-embedded lungs from animals sensitized and challenged with ovalbumin displayed increased cell infiltration in both strains of mice as shown by H&E staining (Fig. 6). The recruited cells tended to accumulate around the blood vessels close to airways (Fig. 6, B and E). Imidazoquinoline treatment almost completely abrogated the recruitment of cells to the lungs around both blood vessels and the surrounding airways (Fig. 6, C and F). Chal-

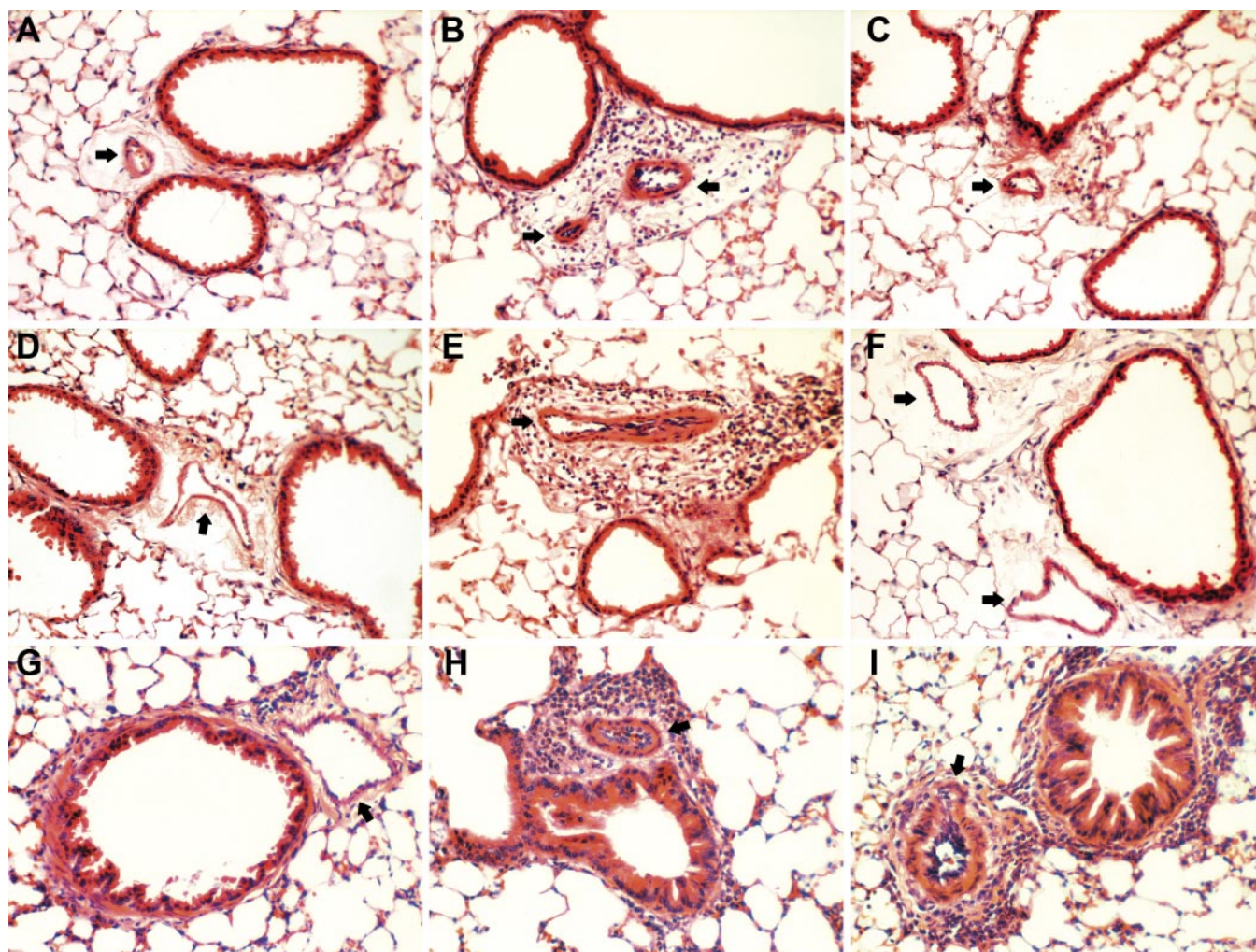


Fig. 6. Histological analysis of lungs from OVA-challenged mice. Pictures illustrate representative hematoxylin- and eosin- (H&E)-stained lung sections prepared from mice sensitized and challenged as described in MATERIALS AND METHODS. The 3 *top* panels are from A/J mice, the *middle* panels represent lungs from C57BL/6, and the *bottom* panels represent lungs from MYD88 knockout (KO) mice. The panels are representative lung sections of H&E stains ($\times 200$ magnification) from OVA-PBS (A, D, and G), OVA-OVA (B, E, and H), and OVA-S28 (C, F, and I) mice 48 h after challenge. OVA-challenged A/J and C57BL/6 mice displayed a markedly increased cellular infiltration around blood vessels (arrows) and airways compared with both OVA-PBS and S28 treated lungs. Lungs from MYD88^{-/-} mice displayed a marked cellular infiltration following OVA challenge that could not be reversed by S28 treatment.

lenge with PBS alone did not induce any significant cellular recruitment to the lungs (Fig. 6, A and D). Flow cytometry analysis of digested lungs revealed an increased influx of CD45-positive cells following ovalbumin challenge that could be prevented by imidazoquinoline treatment (data not shown), further confirming these observations. In addition, MYD88^{-/-} mice displayed significant cellular infiltration to the lungs following ovalbumin challenge that was not prevented with imidazoquinoline treatment. These findings further confirm the involvement of TLRs in the mechanism of action of imidazoquinolines in the treatment of asthma (Fig. 6, H and I, respectively). Interestingly, although very different in their lung AHR, A/J and C57BL/6 mice exhibit very similar cellular infiltration following ovalbumin challenge.

Eosinophil infiltration is abrogated following TLR7 ligand treatment. Eosinophilic infiltration has been implicated in many of the pathological features of asthma. They release mediators such as leukotriene C₄, major basic protein, as well

as many cytokines that contribute to the asthmatic phenotype (23). Histological analysis of lungs following ovalbumin challenge revealed a significant increase in eosinophilic infiltration (Fig. 7). Eosinophils represented $42.2 \pm 5.7\%$ of infiltrating cells in the lungs of A/J mice and $39.8 \pm 4.7\%$ in C57BL/6. Treatment with S-28463 completely prevented eosinophil infiltration into ovalbumin-challenged lungs in both strains of mice. MYD88^{-/-} mice sensitized and challenged with ovalbumin displayed a similar increase in lung eosinophilia that could not be prevented by imidazoquinoline treatment (Fig. 7). Interestingly, MK2 KO mice responded normally to S-28463 treatment as shown by a reduction in airway eosinophilia (Fig. 7) as well as AHR (data not shown). PBS challenge did not induce any detectable levels of eosinophils in any of the strains tested. These data demonstrate that TLR7 ligand treatment prevents eosinophil recruitment to the lungs leading to attenuated AHR levels in both A/J and C57BL/6 mice, which is dependent on MYD88, but independent of MK2.

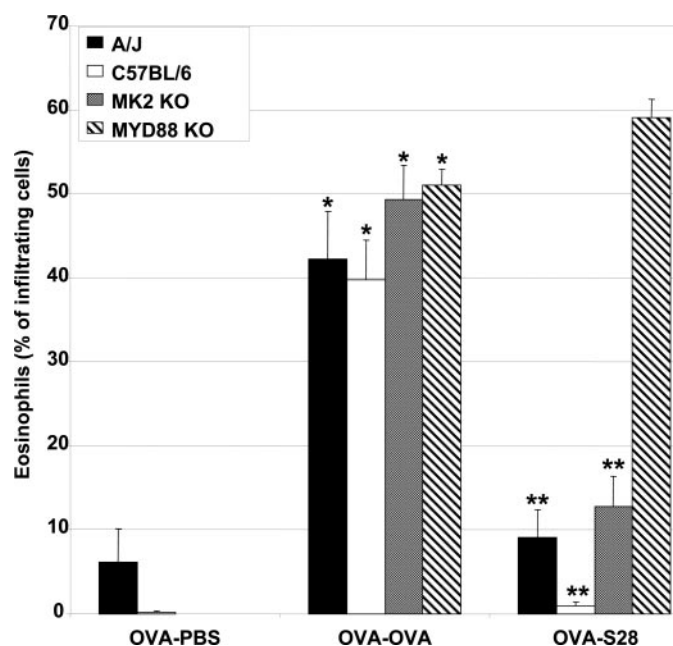


Fig. 7. Lung eosinophilia following OVA challenge is inhibited by imidazoquinoline treatment. Imidazoquinoline treatment resulted in a dramatic reduction in eosinophil level following OVA challenge. Quantitation of Congo red-positive cells revealed a similar amount of eosinophil infiltration in both A/J ($n = 8$ for PBS, $n = 9$ for OVA and $n = 7$ for OVA-S28) and C57BL/6 ($n = 9$ for PBS, $n = 8$ for OVA and $n = 9$ for OVA-S28) mice following OVA challenge that was prevented by S28 treatment. MYD88^{-/-} mice also displayed similar eosinophil infiltration following OVA challenge that could not be prevented by imidazoquinoline challenge ($n = 3$ for all groups). MAPKAP-2 (MK2) KO mice responded normally to S28 treatment resulting in reduction of airway eosinophilia following OVA challenge ($n = 3$ for PBS, $n = 3$ for OVA, and $n = 8$ for OVA-S28). Data for A/J and C57BL/6 are represented as means $\pm$ SE from 3 experiments ($n = 8$ for all groups). * $P < 0.05$ between OVA-PBS and OVA-OVA group; ** $P < 0.05$ between OVA-OVA and OVA-S28 group.

DISCUSSION

The results presented in this study demonstrate that TLR7 ligand treatment in the context of atopic allergic asthma leads to a reversal of the acute inflammatory state. The inhibition of both T_H2 (IL-4, -5, -6, and -13) and T_H1 (IL12p70 and IFN- γ) cytokines prevents infiltration of inflammatory cells, especially eosinophils, to the lungs of animals sensitized and challenged with ovalbumin. The inhibition of early inflammatory events associated with ovalbumin challenge led to a reduction in serum IgE levels as well as a reduced lung response to methacholine challenge in both A/J and C57BL/6 mice. Furthermore, the protective effect of imidazoquinolines was found to be dependent on the presence of MYD88, but not on the MK2 pathway.

In both A/J and C57BL/6 strains, we did not observe any induction of T_H1 cytokines following imidazoquinoline treatment. A previous study looking at resiquimod treatment of allergic asthma in BALB/c mice has shown a decrease in enhanced pause (Penh) induction following ovalbumin challenge associated with a shift in cytokine profile from T_H2 to T_H1 (28). In the previous study, responsiveness to methacholine was measured by the whole body plethysmography method, which does not directly measure any lung parameter but rather measures differences in respiratory pattern. This

method of measurement has been found to behave in a strain-specific manner and to correlate poorly with changes in lung properties (2, 31). To illustrate the drawbacks of relying on the Penh value to approximate lung parameters, we have observed that, although lung resistance and elastance are increased following ovalbumin challenge in A/J mice, Penh values for these animals were actually decreased following challenge (data not shown). This observation in A/J mice has also been reported by another laboratory (37). Furthermore, the possible discrepancy in cytokine profile can be attributed to the fact that cytokine production was measured from in vitro cultured lung cells in the previous study rather than direct lung measurement as presented here. It is of great clinical importance whether TLR7 ligand treatment induces a shift in cytokine production or a complete block of the inflammatory reaction. We have used two different mouse strains, directly measured lung parameters, as well as measured cytokines in vivo, which we believe gives a better approximation of the physiological effect of TLR7 ligand in the lungs.

Both TLR4 and TLR9 activation have been associated with positive outcomes in allergic asthma models (17, 30). Initial observations had suggested that the mechanism of action involved a shift in cytokine production, although further analysis revealed that T_H1 cytokines could not account for the observed beneficial effect associated with both LPS and CpG treatment of allergic asthma. This suggests that T_H2 cytokine inhibition and induction of regulatory mechanism account for the observed amelioration (17, 30). IL-10 has been shown to be crucial in mediating CpG efficacy in preventing allergic asthma (20). We saw very little modulation in the levels of this cytokine suggesting an alternative mechanism for TLR7 ligand action in allergic asthma treatment. The fact that imidazoquinolines are currently employed in humans for the treatment of human papilloma virus-induced warts and the observation that human lungs have tenfold higher mRNA levels for TLR7 than TLR9 suggests a greater clinical applicability of imidazoquinolines for asthma treatment (41).

Although A/J and C57BL/6 mice displayed comparable inflammatory responses following ovalbumin sensitization and challenge, serum IgE levels were found to be differentially induced in these animals. Ovalbumin sensitization induced five times more serum IgE in A/J mice compared with C57BL/6. Ovalbumin challenge maintained the difference in serum IgE between the two strains. Levels of serum IgE are also associated with differences in airway resistance and elastance between A/J and C57BL/6 following ovalbumin challenge. Imidazoquinoline treatment prevented increases in IgE levels as well as improved the lung physiology in both strains of mice. Using S-28463 as an adjuvant in conjunction with Alum, investigators have reported that TLR7 ligands tended to favor IgG2a production, which led to reduced IgE production in a murine model (36). Human peripheral blood mononuclear cells stimulated in vitro with CD40 and IL-4 also produced significantly less IgE in the presence of TLR7 ligands (11). We demonstrate that administration of TLR7 ligands, even after sensitization, leads to a dramatic reduction in serum IgE levels induced following ovalbumin challenge.

Ovalbumin challenge induced a dramatic influx of cells both in the BAL and in the interstitial lung environment. The main recruited cell types were eosinophils and macrophages. Ovalbumin challenge also led to a significant increase in lung

responsiveness to methacholine in A/J mice following ovalbumin challenge. Both effects were greatly diminished by imidazoquinoline treatment. This was also associated with a reduction in CD45-positive lung cells in both A/J and C57BL/6 animals (data not shown). The complete lack of eosinophils as well as IL-5 and IL-13 can account for the observed reduction in lung AHR levels (23, 40). Because of their role in airway remodeling and lung inflammation (19), inhibition of eosinophilia through lack of cytokine (IL-5) and chemokine (CCL11, CCL17, and CCL24) induction following TLR7 ligand treatment provides a plausible mechanism for the protective effect of imidazoquinolines in the treatment of allergic asthma (24).

The mechanism of action of imidazoquinoline treatment in allergic asthma was also addressed in mice lacking important signal transduction components that have been previously shown to play a major role in immunomodulatory effects induced by various TLR. MYD88 is a crucial adapter molecule linking the TLR with other signal transduction components. TLR7 has been previously shown to be dependent on the presence of MYD88 for signal transduction to be initiated following imidazoquinoline stimulation (13). We demonstrate that in the context of atopic allergic asthma, the protective effect of imidazoquinoline on lung cellular infiltration and eosinophilia is dependent on the presence of a functional MYD88. The p38 MAPK substrate, MK2, has previously been shown to be important in cytokine induction by TLR (22). Using MK2 KO mice, we demonstrate that this kinase was not required for the protective effect of imidazoquinoline in allergic asthma as measured by both lung eosinophilia and AHR. A recent study has demonstrated a role for p38 MAPK in the induction of allergic asthma (10). MK2 KO mice displayed normal increases in lung resistance following OVA challenge (data not shown). Furthermore, MK2 KO mice exhibited increased lung eosinophilia following ovalbumin challenge. This suggests that p38 MAPK, but not MK2, is important for allergic asthma induction in mice. This is the first study describing the role of various signal transduction molecule in the protective effect of TLR7 ligands in the treatment of allergic asthma.

Previous studies in our laboratory have pointed to a role of NRAMP1 (natural resistance-associated macrophage protein 1 or SLC11A1) in modulating responsiveness to TLR7 ligands in mycobacterial infections (27). Mice carrying the wild-type (or resistant) allele of *Nramp1* responded more to imidazoquinoline treatment than mice carrying the susceptible allele of this gene. NRAMP1 expression in mice is restricted to macrophages. In the present study, both A/J and C57BL/6 mice responded equally to S-28463 treatment even though they carry different *Nramp1* alleles. This might suggest that the response of macrophages to TLR7 ligands in the lungs might not be critical for its protective effect against allergic asthma.

We demonstrate that the ability of TLR7 to prevent acute atopic allergic asthma through inhibition of cytokine production, as well as cellular infiltration leading to a reduction in airway hyperresponsiveness, is dependent on MYD88 but does not require functional MK2. The data presented in this study warrant further clinical investigation of the potential for TLR7 ligands in the treatment of allergic asthma.

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