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Immunomodulatory Effects of Viral TLR Ligands on Experimental Asthma Depend on the Additive Effects of IL-12 and IL-10

Serdar Sel,2* Michael Wegmann,2,3* Sarper Sel,† Stefan Bauer,† Holger Garn,* Gottfried Alber,‡ and Harald Renz*

Based on epidemiological data, the hygiene hypothesis associates poor hygienic living conditions during childhood with a lower risk for the development of allergic diseases such as bronchial asthma. The role of viral infections, and especially of viral TLR ligands, in the context of asthma remains to be clarified. Viral TLR ligands involve dsRNA and ssRNA which are recognized by TLR-3 or TLR-7, respectively. In this study, we evaluated the impact of TLR-3 or TLR-7 activation on experimental asthma in mice. Systemic application of the synthetic TLR-3 or TLR-7 ligands polycytidylic-polyinosinic acid (p(I:C)) or R-848, respectively, during the sensitization phase prevented the production of OVA-specific IgE and IgG1 Abs and subsequently abolished all features of experimental asthma including airway hyperresponsiveness and allergic airway inflammation. Furthermore, administration of p(I:C) or R-848 to animals with already established primary allergic responses revealed a markedly reduced secondary response following allergen aerosol rechallenges. In contrast to wild-type animals, application of p(I:C) or R-848 to IL-12p35−/− mice had no effect on airway inflammation, goblet cell hyperplasia, and airway hyperresponsiveness. However, in the absence of IL-12, the numbers of eosinophils and lymphocytes in bronchoalveolar lavage fluids were still significantly reduced. These partial effects could also be abolished by neutralizing anti-IL-10 Abs in IL-12p35−/− mice. These data indicate that TLR-3 or TLR-7 activation by viral TLR ligands has both preventive as well as suppressive effects on experimental asthma which is mediated by the additive effects of IL-12 and IL-10. The Journal of Immunology, 2007, 178: 7805–7813.

A

Allergic bronchial asthma is a complex inflammatory disease of the airways that is associated with bronchial hyperreactivity, airway obstruction, and increased mucus production (1). The imbalance between TH1 and TH2 lymphocytes with a predominant TH2-type immune response is a central component in the regulation and perpetuation of the asthma pathology (2). The release of TH2 cytokines including IL-4, IL-5, IL-13, and GM-CSF in response to allergen challenge induces recruitment, activation, and chemotaxis of eosinophils, mast cell activation, and the switch to IgE production by B cells in asthmatic patients (3).

The inverse relationship between microbial load in childhood and later development of allergic diseases has led to the hygiene hypothesis (4). According to this hypothesis, frequent exposure to microbial products results in a predominant TH1 phenotype, whereas lack of such interactions could promote TH2-driven allergic diseases. In this regard, it has been shown that TH1 cells are able to directly or indirectly counteract TH2 immunity. One mode of action is via IFN-γ (5).

Therefore, creating a milieu which prevents or suppresses the development of TH2 cells could be an approach toward modulating the allergic immune response. The development of T effector responses is under close control of cells of the innate immune system. A critical role for the Th cell development is described for dendritic cells (DCs).4 On the one hand, production of IL-4 and IL-10 by DCs biases Th cell development to TH2 cells. On the other hand, IL-12-producing DCs act as strong TH1 inducers (6). A molecular basis for DC activation has been provided with the identification of the TLR which recognize various conserved microbial structures termed pathogen-associated molecular patterns. Various bacterial TLR ligands such as unmethylated CpG motif-containing DNA and LPS have been shown to stimulate production of IL-12 in host cells and consequently down-regulate TH2 responses in animal models of allergic airway inflammation (7, 8).

A critical question still left unanswered is the capacity of viral TLR ligands to interfere with allergic sensitization or already established allergic airway inflammation. dsRNAs or ssRNAs are produced as intermediates during viral replication and are recognized by TLR-3 and TLR-7, respectively (9, 10). Because epidemiological studies reported an inverse relation between systemic infections with hepatitis A virus, measles virus, or others (11–14),

4 Abbreviations used in this paper: DC, dendritic cell; p(I:C), polycytidylic-polyinosinic acid; MNC, mononuclear cell; BAL, bronchoalveolar lavage; PL, peritoneal lavage; MCh, metacholine; PAS, periodic-acid Schiff; CBA, cytometric bead array; AHR, airway hyperresponsiveness; wt, wild type.

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it was the aim of this study to evaluate the effect of systemic TLR-3 or TLR-7 ligand application on allergic sensitization and on allergic airway inflammation in a mouse model of experimental asthma.

Materials and Methods

Animals, sensitization, and treatment protocols

Pathogen-free 6- to 8-wk-old female BALB/c mice were obtained from Harlan Winkelman. IL-12p35<sup>−/−</sup> mice were generated by mating TLR-3 with TLR-7-deficient BALB/c mice backcrossed into BALB/c background (15, 16). TLR-3<sup>−/−</sup> double-deficient mice were generated by mating TLR-3 with TLR-7-deficient BALB/c mice (17, 18). All animals were kept under standard housing conditions. All animal studies were performed under the approval of the local authorities.

Mice were sensitized to OVA (OVA grade VI; Sigma-Aldrich) by three i.p. injections of 10 μg of OVA adsorbed to 150 μg of aluminum hydroxide (inject alum; Pierce) on days 1, 15, and 22 followed by four challenges with 1% (w/v) OVA aerosol on days 26–29 (protocol 1) as previously described (19). The endotoxin concentration of the used OVA solutions was under detection level as assessed by Limulus lysate assay (Pyrochrome). To investigate the effect of TLR-3 and TLR-7 activation during sensitization polycytidylic-polynosinic acid (p(I:C); 200 μg of p(I:C) dissolved in 200 μl of PBS) or R-848 (50 μg of R-848 dissolved in 100 μl of PBS) were delivered by i.p. or s.c. injections, respectively, given at least 24 h before each of the OVA i.p. injections. A second protocol was used to assess the effect of TLR activation on already established experimental asthma in mice (protocol 2). BALB/c mice were sensitized to OVA as described above and allergic airway inflammation was induced by four OVA aerosol challenges on days 26–29. OVA aerosol rechallenges were performed on days 47 and 48. p(I:C) or R-848 were administered by i.v. or s.c. injections on days 41, 43, 45, 47, and 48, respectively. To investigate the role of IL-12 in mediating the effects of either R-848 or p(I:C) protocol 2 was performed using IL-12p35<sup>−/−</sup> mice. Additionally, IL-10-blocking Abs (anti-IL-10 Ab; MAB417; R&D Systems) were applied into IL-12p35<sup>−/−</sup> mice by two i.p. injections (125 μg of Ab dissolved in 100 μl of sterile PBS) on days 39 and 44. A rat IgG1 isotype (125 μg) Ab (MAB005; R&D Systems) was used as control. All analyses were performed 24 h after the last OVA aerosol challenge.

![FIGURE 1.](http://www.jimmunol.org.Downloaded from http://www.jimmunol.org.org by guest on April 14, 2017)
as described for protocol 2 in Materials and Methods. Animals received p(i:C) (n = 8) or R-848 (n = 8) during OVA aerosol rechallenge. Analyses were performed 24 h after the last OVA aerosol challenge. Lungs were fixed and inflated by in situ intratracheal instillation of 1 ml of formalin solution. Three-micrometer sections were paraffin embedded and PAS stained. Representative cross-sections of intermediate airways are shown. BAL levels of IL-5 (D(OVA, n/H9262 cells were counted in PAS-stained lung sections using light microscopy. Numbers of goblet cells is expressed as cells per 100-μm basement membrane.

Preparation and cell culture of splenic mononuclear cells (MNCs)

MNCs were isolated from spleens of OVA-sensitized mice. A total of 2 × 10^7 cells/well were incubated with only OVA (50 μg/ml) or together with R-848 (1 μg/ml) or p(i:C) (100 μg/ml) for 72 h as described previously (20). Cell-culture supernatants were collected for cytokine detection.

Assessment of leukocyte distribution in bronchoalveolar lavage (BAL) and peritoneal lavage (PL) fluids

BAL was performed as described previously (19). For PL collection, 12 h after i.p. injection of p(i:C) or R-848, animals were sacrificed and 5 ml of ice-cold PBS was injected in the abdominal cave. After 2 min, PL fluids were collected. Total number of leukocytes was determined by using Diff-Quick (Dade Diagnostics). Cell-free lavage fluids were stored at −20°C for further cytokine analysis.

Lung histology

Lungs were fixed and periodic-acid Schiff (PAS) was stained for light microscopy as described previously (19). Goblet cell hyperplasia was assessed in PAS-stained lung sections and expressed as cells per 100-μm basement membrane according to Foster et al. (21).

Assessment of airway responsiveness to metacholine (MCh)

The airway responsiveness to MCh (acetyl-β-methylecholine chloride; Sigma-Aldrich) was measured on the basis of the alteration of the midexpiratory airflow in response to increasing doses of inhaled MCh as described previously (22). Midexpiratory airflow (EF50) was measured using headout body plethysmography. Briefly, the system consists of a glass-made head-out body plethysmograph which is attached to an aerosol exposure chamber (Forschungsstatten; Medical School Hannover, Hannover, Germany). The mouse was positioned in the headout body plethysmograph while the head of the animal protruded through a neck collar (9-mm ID, dental latex dam; Roeko) into the aerosol exposure chamber, which was ventilated by continuous airflow of 200 ml/min.

<table>
<thead>
<tr>
<th></th>
<th>IgE (LU/ml)</th>
<th>IgG1 (ng/ml)</th>
<th>IgG2a (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>107 ± 10</td>
<td>13.7 ± 13.7</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>OVA</td>
<td>1804 ± 281</td>
<td>614.9 ± 41.7</td>
<td>461 ± 56</td>
</tr>
<tr>
<td>R-848</td>
<td>880 ± 152*</td>
<td>372.7 ± 46.9*</td>
<td>1528 ± 192**</td>
</tr>
<tr>
<td>p(i:C)</td>
<td>620 ± 55**</td>
<td>395.5 ± 48.2*</td>
<td>312 ± 75</td>
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</table>

* BALB/c mice were sham sensitized (PBS; n = 8) or sensitized to OVA (n = 8; p(i:C); R-848) followed by OVA aerosol challenge. Animals received p(i:C) (n = 8) or R-848 (n = 8) during the sensitization phase as described for protocol 1 in Materials and Methods. Blood samples were collected 24 h after the last OVA aerosol challenge. Titers of OVA-specific IgE, IgG1, and IgG2a Abs were determined by ELISA. Mean ± SD is presented for each group. Statistical significance is indicated by asterisks with *p < 0.05 (×) and †p < 0.01 (***), respectively.
For airflow measurement, a calibrated pneumotachograph (PTM 378/1.2; Hugo Sachs Elektronic) and a differential pressure transducer (8T-2; Gaeltec) coupled to an amplifier (HSE-IA; Hugo Sachs Elektronic) were attached to the top port of each plethysmograph. For each animal, the amplified analog signal from the pressure transducer was digitized via an A/D converter (DT301 PCI; Data Translation; Marlboro) at a sampling rate of 2000/sec. Notocord hem 3.5 was used for data calculation.

Mice were exposed to MCh aerosols with gradient concentrations (12.5, 25, 50, 75, 100, 125 mg/ml) while EF50 was assessed continuously. The MCh concentration that caused a 50% reduction in baseline EF50 (MCH50) is used as a parameter for airway constriction.

Measurements of cytokine levels in BAL and PL fluids

In cell-free lavage fluids, IL-5 was measured by ELISA (BD Biosciences) as described previously (22). IL-4, IL-2, IFN-γ/H9253, IL-10, and IL-12p70 were measured using cytometric bead arrays (CBA; BD Biosciences) according to manufacturer’s protocol.

Measurements of OVA-specific Ig titers in serum samples

OVA-specific IgE, IgG1, and IgG2a concentrations were determined by ELISA technique as described previously (23).

Statistics

Results are presented as mean values ± SEM unless stated otherwise. ANOVA test was performed to determine the level of significance between the different animal groups.

Results

p(I:C) or R-848 prevent development of experimental asthma

BALB/c mice were sensitized to and aerosol-challenged with OVA as described in Materials and Methods. This protocol resulted in high levels of OVA-specific IgE and IgG1 Abs and slightly enhanced production of OVA-specific IgG2a paralleled by

| Cytokine concentration in PL fluids after local application of p(I:C) and R-848a |
|-----------------------------|-----------------------------|-----------------------------|
| pg/ml                       | PBS                        | p(I:C)                     |
|                             | PBS                        | R-848                      |
|                             | PBS                        | p(I:C)                     | TLR-3/-7/7                  |
|                             | PBS                        | R-848                      |
| IL-10                       | 7.8 ± 1.2                  | 1860.5 ± 485.9**           | 74.7 ± 27.2*               | 12.2 ± 3.5                  |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
| IL-12p70                    | 14.9 ± 4.0                 | 62.6 ± 9.8*                | 28.4 ± 3.7*                | 13.3 ± 8.6                  |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
| IFN-γ                       | 352 ± 31                   | 539 ± 56**                 | 315 ± 59*                  | <DL                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |
|                             | 1.2                        | 1.2                        | 1.2                        | 1.2                        |

a wt or TLR-3/-7 double knockout (TLR-3/-7/7) BALB/c mice (n = 4) received p(I:C), R-848, or sterile PBS by i.p. injection as described in Materials and Methods. Twelve hours later, animals were sacrificed and the peritoneal cavity was lavaged with 5 ml of ice-cold PBS. Cytokine concentrations were determined by using the CBA technique. Means ± SD are shown. Significant differences in comparison to the PBS group are indicated by asterisks with p < 0.05 (+) and p < 0.01 (**), significant differences between WT and TLR-3/-7/7 mice are indicated with p < 0.05 (○) and p < 0.01 with (○○), respectively. DL, Detection limit.

FIGURE 3. Cytokine concentration in cell-culture supernatants of splenic MNCs after Ag-specific restimulation in presence of p(I:C) and R-848. BALB/c mice (n = 4) were sensitized to OVA. Splenic MNC cultures were restimulated with OVA alone, with OVA together with 100 μg/ml p(I:C) or 1 μg/ml R-848 or with p(I:C) or R-848 alone for 72 h as described in Materials and Methods. Supernatant concentrations of IL-2, IL-10, IL-4, IL-12, IL-5, and IFN-γ were determined using the CBA technique. Means ± SEM are shown; significant differences compared with the OVA group are indicated by asterisks with p < 0.05 (+) and p < 0.01 (**), respectively. DL, Detection limit.
Table IV. Effects of p(I:C) and R-848 treatment of IL-12p35−/− mice during secondary challenge on the development of OVA-specific Ab isotypes: immune-suppressive effects of R-848 and p(I:C) on established experimental asthma require IL-12a

<table>
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<th>IgE (LU/ml)</th>
<th>IgG1 (ng/ml)</th>
<th>IgG2a (ng/ml)</th>
</tr>
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<tbody>
<tr>
<td>PBS</td>
<td>35.6 ± 25.3</td>
<td>&lt;DL</td>
<td>&lt;DL</td>
</tr>
<tr>
<td>OVA</td>
<td>2053.4 ± 678.5</td>
<td>883.4 ± 211.9</td>
<td>77 ± 62</td>
</tr>
<tr>
<td>R-848</td>
<td>893 ± 333.1</td>
<td>951.7 ± 159.4</td>
<td>428 ± 204*</td>
</tr>
<tr>
<td>p(I:C)</td>
<td>145.8 ± 115.3**</td>
<td>788.7 ± 123.1</td>
<td>31 ± 27</td>
</tr>
</tbody>
</table>

* IL-12p35−/− BALB/c mice were sham sensitized (PBS; n = 8) or sensitized to OVA (n = 8; p(I:C); R-848) followed by OVA aerosol challenge. Animals received p(I:C) (n = 8) or R-848 (n = 8) during the sensitization phase as described for protocol 2 in Materials and Methods. Blood samples were collected 24 h after the last OVA aerosol challenge. Titers of OVA-specific IgE, IgG1, and IgG2a Abs were determined by ELISA. Mean ± SD is presented for each group. Statistical significance is indicated by asterisks with p < 0.05 (+) and p < 0.01 (***), respectively. DL, Detection limit.

The analysis of cytokine concentrations in BAL fluids revealed high levels of IL-5 in samples of the OVA group, reflecting an ongoing TH2 immune response in the airways. In contrast, after p(I:C) as well as after R-848 application IL-5 remained undetectable in BAL fluids (Fig. 1B). This effect was accompanied by the absence of eosinophils as well as lymphocytes in BAL fluids and in lung tissues (Fig. 1, A, C, and D). Furthermore, goblet cell hyperplasia was completely absent in the airways of these animals (Fig. 1E). Airway hyperresponsiveness (AHR) to MCh was present in the OVA group after repeated OVA aerosol challenges as compared with sham-sensitized mice. In contrast, animals did not exhibit AHR after application of R-848 or p(I:C) (Fig. 1F).

p(I:C) or R-848 suppress established experimental asthma

The next experiments addressed the question whether these marked effects would also be observed in a model of experimental asthma with an already established pulmonary pathology. After the first set of allergen challenges, a secondary inflammatory response was induced by repeated OVA aerosol rechallenges. One day before and at the day of the secondary challenges, p(I:C) or R-848 decreased after R-848 application but, in contrast to p(I:C), IgG2a levels were increased as compared with the OVA group (Table I).

FIGURE 4. Immune-suppressive effects of R-848 and p(I:C) on established experimental asthma require IL-12. IL-12p35−/− BALB/c mice were sham-sensitized (PBS; n = 8) or sensitized to OVA (n = 8; p(I:C); R-848) followed by OVA aerosol challenge. Animals received p(I:C) (n = 8) or R-848 (n = 8) during the sensitization phase as described for protocol 2 in Materials and Methods. Analyses were performed 24 h after the last OVA aerosol challenge. Titers of OVA-specific IgE, IgG1, and IgG2a Abs were determined by ELISA. Mean ± SD is presented for each group. Statistical significance is indicated by asterisks with p < 0.05 (+) and p < 0.01 (***), respectively. The level of significance between the PBS and the OVA group is indicated by circles with p < 0.01 (○).
were administered. The secondary response was characterized by high numbers of eosinophils and lymphocytes in BAL fluids and in bronchial tissues. This was accompanied by the development of AHR and increased mucus production as indicated by goblet cell hyperplasia of the airway epithelium (Fig. 2). In contrast, the inflammatory response was markedly reduced in p(I:C)- or R-848-treated animals as shown by lower numbers of eosinophils and lymphocytes in BAL fluids and lung tissues (Fig. 2, A, D, and E). Strikingly, goblet cell hyperplasia was completely absent in these mice (Fig. 2F). The reduction in eosinophils was paralleled by markedly suppressed levels of IL-5 in BAL fluids (Fig. 2B). Concentrations of IL-5 (B) and the total number of eosinophils (C) and lymphocytes (D) were determined in the BAL fluid. Numbers of goblet cell per 100 μm of basement membrane were counted in PAS-stained lung sections (E). Airway responsiveness to MCh was measured using headout body plethysmography. F, Airway constriction was measured by changes in midexpiratory airflow (EF50) during consecutive exposures to aerosolized MCH solutions with increasing concentrations. The concentration of MCh that induces a 50% reduction of baseline EF50 is defined as MCh50. Mean ± SEM is presented for each group. Statistical significance between the OVA group and TLR ligand-treated groups is indicated by asterisks with \( p < 0.05 \) (+) and \( p < 0.001 \) (**), respectively.

**FIGURE 5.** Immune-modulatory effects of R-848 and p(I:C) on established experimental asthma are mediated by IL-12 and IL-10. BALB/c mice were sensitized and challenged as described for protocol 2 in Materials and Methods. R-848 or p(I:C) were administered during OVA aerosol rechallenge. The anti-IL-10 Ab or control Ab was applied 6 h before TLR-ligand administration. Representative cross-sections of intermediate airways were PAS stained (A). Levels of IL-5 (B) and the total number of eosinophils (C) and lymphocytes (D) were determined in the BAL fluid. Numbers of goblet cells per 100 μm of basement membrane were counted in PAS-stained lung sections (E). Airway responsiveness to MCh was measured using headout body plethysmography. F, Airway constriction was measured by changes in midexpiratory airflow (EF50) during consecutive exposures to aerosolized MCH solutions with increasing concentrations. The concentration of MCh that induces a 50% reduction of baseline EF50 is defined as MCh50. Mean ± SEM is presented for each group. Statistical significance between the OVA group and TLR ligand-treated groups is indicated by asterisks with \( p < 0.05 \) (+) and \( p < 0.001 \) (**), respectively.

Differential effects of R-848 or p(I:C) on cytokine production related to T cell effector responses

The i.p. injection of either p(I:C) or R-848 resulted in enhanced production of IL-12p70 and even higher levels of IFN-γ in PL fluids of wild-type (wt) BALB/c mice (Table III). p(I:C) had a significantly stronger effect on IL-10 production than R-848. The induction of these cytokines appeared to be specific for TLR-3 or TLR-7 activation, respectively, because neither administration of p(I:C) nor R-848 stimulated production of IL-12, or IFN-γ in PL fluids of TLR-3/-7 double-deficient animals (Table III). The results from these experiments suggest that R-848 and p(I:C) might affect T cell responses through different pathways. To further delineate these phenotypes, we analyzed the cytokine production of MNC after in vitro OVA restimulation. Briefly, MNC cultures from OVA-sensitized BALB/c mice were stimulated with OVA alone in combination with R-848 or p(I:C). Seventy-two hours later, levels of IFN-γ, IL-2, IL-4, IL-5, IL-10, and IL-12p70 were analyzed in cell-culture supernatants. Dose-response experiments revealed concentrations of 1 μg/ml R-848 and 100 μg/ml p(I:C) to be the most effective (data not shown). Stimulation of splenic MNCs with OVA alone resulted, as expected, in high levels of IL-2, IL-10, IL-4, and IL-5 in parallel to relatively low levels...
of IFN-γ and IL-12p70. In the presence of R-848 alone, the levels of IL-10, IL-12, and IFN-γ increased, whereas p(lC) alone had no effect on any cytokine production. Coapplication of OVA and R-848 or p(lC) inhibited the production of IL-2, IL-4, and IL-5, but continued to trigger IL-10. Furthermore, concentrations of IL-12p70 and IFN-γ were significantly increased after coapplication of OVA and R-848. Thus, R-848 suppressed TH2 cell responses and induced a TH1-like cytokine response. p(lC) showed similar results on the suppression of TH2 cytokines, but TH1 cytokines were not stimulated (Fig. 3).

**Immunosuppressive activities of R-848 and p(lC) on established experimental asthma require IL-12 and IL-10**

It has been demonstrated that some TLR ligands trigger the production of IL-12 in APCs, thereby mediating the suppression of TH2 responses. To test the contribution of IL-12 to the immunomodulatory effects of R-848 and p(lC) on established experimental asthma, IL-12p35−/− mice were studied. IL-12p35−/− mice sensitized to OVA developed the typical asthma pathology with allergic airway inflammation as indicated by a marked influx of eosinophils and lymphocytes into the airways together with pronounced inflammation of the airway walls dominantly consisting of eosinophils. In contrast to wt animals, eosinophils and lymphocytes appeared in lower numbers in BAL fluids. Nevertheless, OVA-sensitized and -challenged IL-12p35−/− mice demonstrated development of goblet cell hyperplasia and they were more sensitive to MCh than nonsensitized IL-12p35−/− animals, reflecting development of AHR.

R-848 and p(lC) application had differential effects on the phenotype of experimental asthma in IL-12p35−/− animals that were OVA sensitized and challenged following protocol 2 of Materials and Methods. Both TLR ligands markedly prevented the strong increase in OVA-specific IgE Abs, but had no effect on OVA-specific IgG1 Ab titers (Table IV). This was accompanied by a strong increase in OVA-specific IgG2a Abs following R-848 administration but not in the p(lC)-treated group. In terms of airway inflammation, most of the inflammation related parameters such as tissue inflammation (as revealed by histology), presence of goblet cells, numbers of BAL lymphocytes, BAL IL-5 levels, and airway hyperreactivity were still observed in R-848- or p(lC)-treated IL-12p35−/− mice. An exception was a marked reduction of the influx of eosinophils into the airway lumen which was still observed after TLR ligand administration. To address this question, IL-12p35−/− mice were treated with a neutralizing anti-IL-10 mAbs. IL-10 neutralization resulted in enhanced numbers of eosinophils and concentration of IL-5 in BAL fluids. However, airway wall inflammation (Fig. 5A), lymphocyte cell counts (Fig. SD), and goblet cell hyperplasia (Fig. 5, A and E) remained unchanged. Furthermore, neutralizing IL-10 had no effect on AHR (Fig. 5F). These findings suggest that diminishment of eosinophils and IL-5 in BAL fluids depends on IL-10.

**Discussion**

Although viral infections are mostly implicated in the development or exacerbation of allergic asthma, few viral products represent TLR ligands and, therefore, could be able to skew TH2 responses toward a TH1-dominated phenotype by activating the innate immune system. One mode of recognition of dsRNA is mediated by TLR-3 and leads to the activation of dendritic cells and macrophages (24). Viral ssRNA requires TLR-7 for activation of APCs (9). Both, TLR-3 and TLR-7 ligands may induce production of type I IFNs and of proinflammatory and regulatory cytokines. In this study, we used p(lC) and R-848 as synthetic TLR-3 or TLR-7 ligands that are known to activate APCs such as DCs and macrophages and to promote the production of IL-12 and IFN-γ (25–27).

In the present study, we demonstrated that 1) systemic application of TLR-3 as well as of TLR-7 ligands during the sensitization phase almost completely protected BALB/c mice from allergic sensitization and, subsequently, from the development of experimental asthma. 2) Administration of p(lC) as well as of R-848 following elicitation of a primary inflammatory response markedly prevented the development of a secondary response, which 3) depend on the production of IL-12 and IL-10.

In response to both p(lC) and R-848, we observed the production of IL-10 which has potent anti-inflammatory effects as also described for TLR-2 activation by Candida albicans (28). Furthermore, increased concentrations of IL-12 and IFN-γ were also detected in peritoneal lavage fluids after p(lC) or R-848 administration. We found a similar cytokine profile when MNCs from OVA-sensitized animals were in vitro restimulated with the allergen in presence of p(lC) or R-848. IL-12 is an important inducer of TH1 responses and is predominantly produced by DCs, monocytes, and macrophages (29–31). DCs stimulated with various TLR ligands such as LPS or CpG readily produce IL-12 (32). We observed IL-12 production by peritoneal macrophages in response to i.p. injection of p(lC) or R-848 suggesting that certain effects of TLR-ligands on the secondary immune response to inhaled allergen are at least partly mediated by IL-12. This conclusion is supported by experiments with mice genetically deficient for the IL-12p35 subunit and, therefore, being unable to produce bioactive IL-12p70. In these animals, p(lC) as well as R-848 application were ineffective in preventing/suppressing allergic airway inflammation and goblet cell hyperplasia. IL-12 has been shown to drive the development of naive CD4-positive T cells toward a TH1 phenotype and it promotes the production of IFN-γ by CD4-positive T cells (33). Therefore, induction of a TH1 response that counterbalances the TH2-type allergic immune response could be responsible for the observed effects of R-848 and p(lC).

p(lC) and R-848 had different effects on OVA-specific Ab production. Both, p(lC) as well as R-848 markedly reduced the production of OVA-specific IgE and IgG1. But in contrast to p(lC), administration of R-848 did not result in diminished, but did result in enhanced production of OVA-specific IgG2a indicating the induction of an OVA-specific TH1 response. It has been shown that IL-12 increases serum IgG2a levels and suppresses IgG1 and IgE response and IL-4 switches the Ab production toward IgG1 and IgE in mice and IgE in humans (34, 35). An allergen-specific TH1 response could have been induced IL-12 following R-848 application and may counteract the allergen-specific TH2 response via induction of IFN-γ production (36). The different effects of p(lC) and R-848 on the production of OVA-specific Abs as well as on cytokine production in cell culture may be based on the differential expression of TLR-3 and TLR-7 on T cells and APCs (37, 38). Because murine T cells express TLR-7 but not TLR-3, R-848 could have direct effects on T cells, while p(lC) has not. These could include a direct shift from IL-4-producing TH2 cells into IFN-γ-producing T cells as it has been reported for R-848 and human T cells in vitro (39).
After p(I:C) as well as R-848 application, airway eosinophilia persisted in IL-12p35−/− mice and the production of IL-10 was enhanced. Therefore, we hypothesized that IL-10 participates in the development of the beneficial effects in addition to IL-12. Indeed, neutralizing anti-IL-10 Abs abolished the inhibition of airway eosinophilia in IL-12p35−/− mice. IL-10 is a potent anti-inflammatory cytokine that inhibits the production of several inflammatory proteins. IL-10 inhibits survival of eosinophils and the activation of TH2 cells (40). Furthermore, IL-10 suppresses innate immune responses by dampening responses to many microbial stimuli including those recognized by certain TLRs (41). In addition, IL-10 is also a potent inhibitor of adaptive T cell-mediated immune responses (42). The protective as well as suppressive effects of TLR-3 or TLR-7 ligand application on allergic TH2-type immune responses may be based on the anti-inflammatory effects of IL-10.

Both the anti-inflammatory effects of IL-10 and an Ag-specific TH1 response may have inhibiting effects on the allergen-specific TH2 response. Thus, by reducing IL-4 and IL-5 production in T cells, differentiation of allergen-specific TH2 cells, influx of eosinophils into the airways, as well as production of allergen-specific IgE and IgG1 could be suppressed. In accordance with Moisan et al. (43) and Quarcoc et al. (44), we also observed protective effects of TLR-7 activation against the development of experimental asthma. In extension of these observations, we demonstrated that activation of TLR-7 further has the potential to significantly improve already established experimental asthma and that IL-12 as well as IL-10 are required for the mediation of these effects. Recently published data point toward a novel pathway which could explain the production of high levels of IL-12 and IL-10 (and IFN-γ) in the absence of measurable TH1 activities and any TH1-related lung pathology. These effects have been related to Th type 1-like regulatory cells developing in the presence of IL-10 and IL-12. Because these TH1-type regulatory cells are able to suppress allergic airway inflammation as well as AHR in a mouse model of allergic asthma by producing IL-10 as well as IFN-γ (45), we cannot exclude a regulatory role of them in our model.

In conclusion, systemic application of TLR-3 as well as of TLR-7 ligands revealed effects that not only protected from the development of experimental asthma but also reduced asthma parameters, we also observed protective effects of TLR-7 activation against the development of experimental asthma. In extension of these observations, we demonstrated that activation of TLR-7 further has the potential to significantly improve already established experimental asthma and that IL-12 as well as IL-10 are required for the mediation of these effects. Recently published data point toward a novel pathway which could explain the production of high levels of IL-12 and IL-10 (and IFN-γ) in the absence of measurable TH1 activities and any TH1-related lung pathology. These effects have been related to Th type 1-like regulatory cells developing in the presence of IL-10 and IL-12. Because these TH1-type regulatory cells are able to suppress allergic airway inflammation as well as AHR in a mouse model of allergic asthma by producing IL-10 as well as IFN-γ (45), we cannot exclude a regulatory role of them in our model.

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**Disclosures**

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