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TLR3 but Not TLR7/8 Ligand Induces Allergic Sensitization to Inhaled Allergen

Sebastian Reuter,* Nina Dehzad,* Helen Martin,* Livia Böhm, † Marc Becker, † Roland Buhl,* Michael Stassen, † and Christian Taube*‡

Epidemiological studies suggest that viral infections during childhood are a risk factor for the development of asthma. However, the role of virus-specific pattern recognition receptors in this process is not well defined. In the current study, we compare the effects of the inhaled viral TLR ligands polyinosinic-polycytidylic acid (TLR3) and resiquimod (TLR7/8) on sensitization to a model allergen (OVA) in a murine model. Both compounds enhance the migration, activation, and Ag-processing of myeloid dendritic cells from the lung to the draining lymph nodes comparable to the effects of LPS. Application of polyinosinic-polycytidylic acid [poly(I:C)] or LPS induces production of allergen-specific IgE and IgG1, whereas resiquimod (R848) had no effect. In addition, rechallenge of mice with OVA resulted in airway inflammation and mucus production in animals that received either poly(I:C) or LPS but not after application of R848. In summary, these results show that activation of TLR3 in combination with inhaled allergen results in induction of dendritic cell activation and migration similar to the effects of LPS. This leads to the development of allergic airway disease after allergen rechallenge, whereas mice treated with R848 did not develop allergic airway disease. These findings give further insight into the effects of stimulation of different TLRs on the development of asthma. The Journal of Immunology, 2012, 188: 5123–5131.

Asthma is a chronic airway disease including many different clinical phenotypes, characterized by inflammation, airway hyperresponsiveness, and airway obstruction (1). The prevalence of this disease is high and still increasing worldwide, making asthma an important health care burden and socioeconomic factor (2). The origin of asthma is multifactorial, and genetic predisposition and environmental conditions contribute to increased risk of development of asthma (3). One important risk factor for the development of asthma is sensitization to a harmless allergen, as patients with atopy have an increased risk of development of asthma (4). Viral infections have been associated with the development of acute exacerbations of asthma (5), however studies also suggest that viral infections in childhood are important risk factors for the development of atopy, sensitization to harmless allergen (6), and asthma (7).

Animal models have helped to understand the pathophysiological mechanisms underlying the development of sensitization to inhaled allergens. Studies in murine models have shown that additional exposure to bacteria or bacterial compounds is necessary to induce sensitization toward allergen (8). Pivotal for the induction of an adaptive immune response are dendritic cells (DCs). Under steady-state conditions, DCs sample Ag in the lung, migrate to the draining lymph node as immature or semimature cells, and present Ags to T cells resulting in the induction of tolerance (9). In the presence of an additional endogenous or exogenous stimulus acting as a danger signal, DCs mature and induce effective adaptive immune responses. Indeed, application of bacterial LPS combined with exposure to a harmless Ag leads to Ag-specific sensitization and development of an allergic airway disease after inhaled re-exposure to Ag alone (8). Necessary for these mechanisms is the recognition of LPS via TLR4. Furthermore, viruses and viral compounds are also recognized by TLRs, mainly by endosomally located TLR3, TLR7, and TLR8. TLR3 detects dsRNA, which appears during viral replication processes and is expressed in placenta, pancreas, lung, liver, and heart (10), and especially in myeloid DCs and epithelial cells. In contrast, TLR7 and TLR8 recognize ssRNA (11). In humans, TLR7 and TLR8 are predominantly expressed by plasmacytoid dendritic cells (pDCs) and B cells (12, 13) but have been found in mice in many different cell types, such as DCs, B cells, monocytes, NK cells, and T cells (13, 14). Synthetic ligands exist that allow specific activation of these TLRs. TLR3 can be activated by the synthetic polyinosinic-polycytidylic acid [poly(I:C)] (15), whereas TLR7 and TLR8 can be activated using imidazoquinines; for example, resiquimod (16). However, the roles of these TLRs during the development of allergen sensitization are not well described.

In the current study, we therefore compare the effects of exposure to inhaled TLR3 or TLR7/8 ligands poly(I:C) and R848 on sensitization toward the model Ag OVA. To this end, in vivo Ag uptake, processing, migration, and activation of DCs were assessed, and Ag-specific T cell responses were analyzed. Furthermore, the development of allergic airway disease was investigated using an allergen rechallenge model. The current study shows that application of a TLR3 ligand can induce allergic airway disease comparable to that by a TLR4 ligand, whereas a TLR7/8 ligand fails to induce allergic airway disease.
Materials and Methods

Animals

C57BL/6J mice, B6 OTI and OTII mice, and C57BL/6J mice deficient in TLR4 (TLR4−/−) or TLR7 (TLR7−/−) were obtained from the Zentrale Tierzuchtanstalt of the Johannes-Gutenberg-University Medical Center. C57BL/6J mice deficient in TLR3 (TLR3−/−) were kindly provided by Sebastian Zimmer (Universitätsklinikum, Bonn, Germany).

All mice were used at the age of 8–12 wk. Animal procedures were conducted in accordance with current federal, state, and institutional guidelines, and all experiments were approved by the local regulatory authorities.

Experimental protocols

Experimental groups consist of three to five animals per group, and each experiment was performed at least twice. To assess the migration of Ag-laden DCs, mice were anesthetized on day 0 by i.p. injection of ketamine/rompun (Ketamin-ratipherm; Ratipherm, Ulm, Germany,/ Rompun 2%; Bayer, Leverkusen, Germany), and then 50 µl of the TLR ligands LPS (Calbiochem, Merck, Darmstadt, Germany), poly(IC) (Invitrogen, Eugene, OR), or resiquimod (R848; Alexis Biochemicals, San Diego, CA) in combination with 80 µg of a fluorescent OVA dye (OVA–Alexa Fluor 647; MoBiTec, Gottingen, Germany) in 40 µl PBS was applied intranasally. At 4, 24, and 48 h after the application, mice were sacriﬁced, thoracic lymph nodes, inguinal lymph nodes, and lungs were isolated and single-cell suspensions prepared. The cellular composition of the organs was analyzed via FACS measurement.

To assess Ag processing, mice were treated the same way as described in the migration protocol. However, instead of OVA–Alexa 647, mice were treated with a self-quenched conjugate of OVA (DQ-OVA) that exhibits bright green ﬂuorescence upon proteolytic degradation (Invitrogen Molecular Probes, Eugene, OR) at 40 µg/ml PBS intranasally. At 1 h after application, single-cell suspensions of isolated draining lymph nodes, inguinal lymph nodes, and lungs were prepared, and Ag-processing cells were investigated via FACS analysis.

To assess surface expression of costimulatory molecules on DCs, mice received intranasally TLR ligands as described earlier. In addition, animals received a self-quenched OVA (EndoGrade OVA). Three days after this treatment, single-cell suspensions of tracheal lymph nodes or lung cells were adjusted and surface stained for PeCy7-labeled CD4 PeCy7 and PeCy7-labeled CD25 (all BD Pharmingen) as described. Flow cytometry was performed following the advice of the manufacturer of the immunofluorescence/permeabilization set (eBioscience). Brieﬂy, after surface staining and washing in WB, cells were resuspended in 100 µl Cytox/Cytperm (eBioscience) and incubated for 30 min at 4˚C in the dark. Cells were washed in Perm/Wash (Perm-buffer) twice, and subsequently allopurinol-labeled Foxp3 was added. After 30–40 min incubation in the dark at 4˚C, cells were washed in Perm-buffer and ﬁnally resuspended in FACs ﬁxation buffer (4% PFA).

Ex vivo restimulation of lung cells. As already described, single-cell suspensions of lung cells were resuspended in RPMI (Gibco/ThermoFisher, Germany) 80 µg/50 µl PBS. After 24 h again after the application, single-cell suspensions of the isolated organs were analyzed via FACS for the expression of costimulatory molecules.

In vivo analysis of speciﬁc T cell responses. Single-cell suspensions from spleens of animals with a transgenic OVA-speciﬁc TCR (OTI and OTII) were prepared. Cells were labeled with 5 µM CFSE (CFDA; Invitrogen, Eugene, OR), and 10 × 10^6 cells were injected i.v. into the tail of C57BL/6J recipients. One day later, mice were anesthetized with ketamine/rompun and treated intranasally with 50 µl of either TLR ligands LPS, poly(IC), or R848, in combination with 80 µg/40 µl endotoxin-free OVA (EndoGrade OVA). Three days after this treatment, single-cell suspensions of isolated draining lymph nodes, inguinal lymph nodes, and lungs were prepared and analyzed via FACS.

Preparation of single-cell suspension and FACS analysis. Lungs were disrupted mechanically and enzymatically digested by incubation in collagenase type I (0.5 mg/ml PBS) for 1 h at 37˚C in a water bath. Single-cell suspensions were generated by resuspending the digested tissue through an 0.9 × 40 mm cannula. After centrifugation, RBCs were removed by osmotic lysis. After washing, cell counts were determined and adjusted. Lymph node and lung tissues were mechanically minced. After washing, cell counts were determined.

Ag-specific ELISA. Serum levels of OVA-specific IgE, IgG1, and IgG2b were assessed as previously described (19) by ELISA (BD Pharmingen). The Ab titer was defined as the reciprocal serum dilution yielding an absorbance reading of OD = 0.2 after linear regression analysis. Inflammation was scored in a semiquantitative fashion on H&E slides. Number of goblet cells was analyzed using an image analysis software (analySIS, Soft Imaging Systems, Stuttgart, Germany) on PAS-stained slides and expressed as positive cells per millimeter basement membrane.

Cytokine ELISA Set; BD Pharmingen, Heidelberg, Germany). For the measurement of cytokines, lung cells were treated as already described (17). In brief, single-cell suspensions were incubated with PMA (Sigma, St. Louis, MO), ionomycin (Sigma), and brefeldin A (Sigma) at 37˚C for 4 to 6 hours after incubation. After staining, cells were washed twice, and intracellular staining was performed according to the manufacturer’s protocol (BD Biosciences Cytoclix/ Cytperm). In short, unspeciﬁc surface binding areas were blocked by adding 1 µl Fc-receptor blocking Abs (oCD16/CD32; BD Pharmingen, Heidelberg, Germany) for 10 min at 4˚C. Cells were stained with FITC-labeled Vα2 (BD Pharmingen), PerCP-Cy5.5-labeled CD8 (BD Pharmingen) and PerCy7-labeled CD4 (BD Pharmingen). Cells were washed in WB, cells were resuspended in 100 µl Cytox/Cytperm (BD Pharmingen) and incubated for 20 min at 4˚C in the dark. Cells were washed in Perm/Wash (BD Pharmingen) twice, and subsequently intracellular antibody mix was added. For intracellular cytokine analysis, we used allopurinol-labeled IFN-γ (BD Pharmingen), PE-labeled IL-17A (BD Pharmingen), and PE-labeled IL-17F (eBioscience, NatuTec). Cells were incubated at 37˚C for 20–40 min in the dark. Afterwards, cells were washed twice in Perm/Wash and ﬁnally resuspended in FACs ﬁxation buffer (4% PFA). To analyze regulatory T cells (CD4+/CD25+/Foxp3+), single-cell suspensions of tracheal lymph nodes or lung cells were adjusted and surface stained for PeCy7-labeled CD4 PeCy7 and PeCy7-labeled CD25 (all BD Pharmingen) as described. Foxp3 staining was performed following the advice of the manufacturer of the immunofluorescence/permeabilization set (eBioscience). Brieﬂy, after surface staining and washing in WB, cells were resuspended in 100 µl Cytox/Cytperm (eBioscience) and incubated for 30 min at 4˚C in the dark. Cells were washed in Perm/Wash (Perm-buffer) twice, and subsequently allopurinol-labeled Foxp3 was added. After 30–40 min incubation in the dark at 4˚C, cells were washed in Perm-buffer and ﬁnally resuspended in FACs ﬁxation buffer (4% PFA).

Measurement of cytokines. Cytokines were measured by ELISA following the manufacturer’s protocol (IL-5 and IFN-γ Cytokine ELISA Set; BD Pharmingen). Briefly, plates were coated overnight according to the cytokine-specific coating buffer. Plates were washed and blocked. Afterwards, supernatants of OVA-stimulated and nonstimulated cells as well as
cytokine standard were coated to the plate and serial diluted. After an incubation time of 2 h at room temperature, plates were washed, and the detection Ab and HRP were added to the plate. After 1-h incubation, plates were washed again, and substrate was added. Reaction was stopped by adding acid, and absorbance was measured. A linear regression analysis standard curve was compared with samples and their concentrations determined.

**Real-time PCR.** Lung cytokine expression was determined via real-time PCR. RNA was isolated via TRizol reagent (Invitrogen, Carlsbad, CA) using the manufacturer's extraction protocol. RNA was reverse transcribed into cDNA using a mix of M-MuLV reverse transcriptase (Thermo Scientific), anchored oligonucleotides (Thermo Scientific), random hexamers (Thermo Scientific), RNase inhibitor (Thermo Scientific), and deoxyribonucleotide triphosphate (Thermo Scientific). cDNA concentration and purity were analyzed using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE). cDNA concentrations were adjusted to 200 ng/μl, and 1 μl was used in 25 μl reaction volume. By using an SYBR Green mix (Thermo Scientific), the differences of amplification efficiency between the housekeeping gene (TPB) and the gene of interest were determined and compared between a calibrator (PBS/OVA) and the different other treatment groups. The real-time PCR was performed on an ABI7300 (Applied Biosystem).

The following primers were used for analysis. TBP: TBP-s, 5′-GGG ACA CCT TCC TTT AGC-3′; TBP-as, 5′-CTG TCT TGG TGG TCC-3′. IL-10: IL-10-s, 5′-TCA GGC AAT GAT GTA GAA GAA-3′; IL-10-as, 5′-TCT TGT GGT GGT TGC-3′. RANTES: RANTES-s, 5′-CTG CTG TCT TCG TCA CCT CT-3′; RANTES-as, 5′-ACT TCT TCT CGT GTG TGG CAC AC-3′. IFN-γ: IFN-γ-s, 5′-TCA AGT GGA AGT ATT TGC AT-3′; IFN-γ-as, 5′-GGG GGG ACA CCT TCC-TTT AGC-3′. IL-4: IL-4-s, 5′-CTG TCT TGG GGG ACA CCT TTT AGC-3′; IL-4-as, 5′-CAG GCA GGG CTT CCT CC-3′. IFN-β: IFN-β-s, 5′-ACC AGT CTT AGG TGG TCC GC C-3′; IFN-β-as, 5′-AGC CCC ACCA AGA AAT TGC CTA C-3′. KC: KC-s, 5′-CGA CCC AAA CGG GGG AGG ACA CCT TTG GGG ACA CCT TTT AGC-3′; IL-12p40: IL-12p40-s, 5′-AGC ACC TCT TGC CAC ACC AAG AA-3′; IL-12p40-as, 5′-GGC CAT TCC ACA TCT GAC TAC C-3′.

**Statistical analysis**

ANOVA was used to determine the levels of difference between all groups. Number of eosinophils, histology score, and number of PAS+ cells were determined. In case of significant results, the Mann–Whitney test was used to elucidate which specific differences were statistically significant. The p values for significance were set at 0.05. Values for all measurements are expressed as the mean ± SEM.

**Results**

**Bacterial as well as viral TLR ligands enhance the migration of DCs from lungs to regional lymph nodes**

To investigate the effect of different TLR ligands on the migratory behavior of APCs, animals were treated with fluorescent-labeled OVA in combination with either a TLR3, TLR4, or TLR7/8 ligand. Animals treated with OVA alone showed only low conventional DCs (cDCs, characterized as CD11c+MHCII+B220+ cells) in the mediastinal lymph nodes, and these cells were mainly OVA–Alexa 647 negative (Fig. 1A, 1B). Animals that additionally received TLR3, TLR4, or TLR7/8 ligands increased cell numbers in regional lymph nodes. Indeed, relative and absolute numbers of cDCs and of fluorescent cDCs were increased (Fig. 1A, 1B). This effect was dose dependent (Fig. 1C). The highest number of fluorescent cDCs in the draining lymph nodes was detectable 24 h after OVA application. In addition, fluorescent cells were only detected in the draining tracheal lymph nodes but not in mandibular and inguinal lymph nodes. Coadministration of LPS leads to the strongest emigration of cDCs from the lung to the draining lymph nodes. Application of either poly(I:C) or R848 resulted in comparable numbers of cDCs in the regional lymph nodes; however, the numbers of fluorescent cDCs were lower compared with those after LPS (Fig. 1A, 1B). Migratory lung cDCs consist at least of two subsets, CD103+ and CD11b+ cDCs (21). OVA+ cDCs in the draining lymph node were analyzed for the expression of these two markers. In animals exposed to OVA–Alexa 647 alone, ~60% of cDCs were CD11b+ whereas ~20% were CD103+. This ratio was similar in mice exposed to the viral TLR ligands poly(I:C) or R848. In contrast, mice exposed to LPS showed an even proportion of cDCs were CD11b+ whereas ~50% were CD103+ and ~50% were CD11b+. Administration of TLR ligands enhances Ag processing and activation of cDCs

To assess the Ag-processing capacity of DCs after application of TLR ligands, animals were treated with DQ-OVA, a quenched peptide of OVA, and the ability of cDCs to process and present OVA with the DQ-rich peptide was assessed. The percentage of DQ-OVA+ cDCs in the mediastinal lymph nodes was determined by flow cytometry. Animals treated with DQ-OVA alone showed only few cDCs expressing DQ-OVA. In contrast, animals treated with DQ-OVA and a TLR ligand showed an increased number of DQ-OVA+ cDCs in the mediastinal lymph nodes. This effect was dose dependent and did not occur in mice lacking either TLR3, TLR4, or TLR7/8.

**FIGURE 1.** Application of bacterial and viral TLR ligands induces enhanced migration of Ag-presenting cDCs (CD11c+MHCII+B220+) from the lung to the draining lymph nodes. (A) FACS plots show the CD11c+MHCII+B220+ cells in the draining lymph node were analyzed for the expression of these two subsets, CD103+ and CD11b+ cDCs (21). OVA+ cDCs in the draining lymph node were analyzed for the expression of these two markers. In animals exposed to OVA–Alexa 647 alone, ~60% of cDCs were CD11b+ whereas ~20% were CD103+. This ratio was similar in mice exposed to the viral TLR ligands poly(I:C) or R848. In contrast, mice exposed to LPS showed an even proportion of cDCs were CD11b+ whereas ~50% were CD103+ and ~50% were CD11b+. Administration of TLR ligands enhances Ag processing and activation of cDCs

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fluorescent dye that starts to fluoresce after its intracellular degradation. Twenty-four hours after application of DQ-OVA alone or in combination with the different TLR ligands, draining lymph nodes were analyzed. Only few fluorescent cDCs were detectable in animals treated with DQ-OVA alone. Additional exposure to either TLR3, TLR4, or TLR7/8 ligand increased the relative and total numbers of fluorescent DCs in the draining lymph nodes (Fig. 2A).

To assess the expression of costimulatory molecules, CD40, CD80, and CD86 were determined on the surface of cDCs in the draining lymph node after application of OVA alone or in combination with either TLR3, TLR4, or TLR7/8 ligand. Application of either TLR3 or TLR4 ligand led to an increased surface expression of CD40, CD80, and CD86, whereas exposure to TLR7/8 ligand resulted in increased expression of CD40, but only slight increase in CD86 and no effect on expression of CD80 (Fig. 2B). In the lung, the expression pattern of costimulatory molecules was comparable to that seen in lymph nodes. CD80 and CD86 was increasingly expressed in poly(I:C)-treated and LPS-treated animals, whereas R848-treated animals showed lower expression (data not shown).

**FIGURE 2.** After application of viral or bacterial ligands, Ag-laden DCs demonstrate an enhanced Ag processing. (A) Using the fluorescent dye DQ-OVA, numbers of fluorescent Ag-processing cells in the draining lymph node were analyzed in mice that received qOVA with either PBS (PBS/OVA), LPS (LPS/OVA), poly(I:C) [poly(I:C)/OVA], or R848 (R848/OVA). Dot plots show CD11c+MHCII+ cells; gray-shaded histograms demonstrate DQ-OVA fluorescence of a negative control; black line displays the fluorescence of the corresponding group. Graph shows the total numbers of DQ-OVA+ cDCs in the draining lymph node 24 h after application. Mean ± SEM is given; n = 10 mice per group from three independent experiments. (B) Intranasal treatment leads to increased activation of cDCs. Histograms demonstrate expression of the costimulatory molecules CD86, CD80, and CD40, respectively, on the surface of cDCs in the draining lymph node 24 h after treatment. Dotted line, isotype control; gray shaded, PBS/OVA; black line, the corresponding treated group. n = 4 mice per group from two independent experiments. The bar graphs demonstrate the mean fluorescence of the corresponding costimulatory molecule. Mean ± SEM is given.

**FIGURE 3.** Viral and bacterial TLR ligands induce the proliferation of Ag-specific T cells after inhalational coadministration with the Ag. CFSE-labeled splenocytes of OTII and OTI animals, respectively, were injected into the tail veins. Twenty-four hours later, OVA was applied intranasally either in PBS or in combination with LPS (LPS/OVA), poly(I:C) [poly(I:C)/OVA], or R848 (R848/OVA). Seventy-two hours after treatment, draining lymph nodes were prepared, and the T cell proliferation was analyzed via FACS. Dot plots demonstrate CFSE+ CD4+ T cells (A) and CD8+ T cells (B). Histograms show CFSE expression of OVA-treated-only animals (gray shaded) in comparison with additionally TLR ligand-treated animals (black line). Bar graphs show the mean fluorescence of CFSE+ CD4+ T cells (A) (n = 11 animals per group from two independent experiments) and CD8+ T cells (B) (n = 6 animals per group from two independent experiments). Mean ± SEM is given.

Exposure of Ag and TLR ligand increases proliferation of Ag-specific T cells in regional lymph nodes

To assess whether enhanced migration, activation, and expression of costimulatory molecules on DCs also affects Ag-specific T cells in vivo, either CD4+ or CD8+ T cells with transgenic TCR specific for OVA epitopes were CFSE-labeled in vitro and injected into the animals. Twenty-four hours later, animals were treated with OVA alone or in combination with the different TLR ligands. Seventy-two hours after the Ag administration, draining lymph nodes were harvested and analyzed. Treatment with OVA alone resulted in weak proliferation of either Ag-specific CD4+ T cells or CD8+ T cells, and application of either PBS, LPS, poly(I:C), or R848 alone without OVA resulted in no proliferation (data not shown). However, additional application of LPS and poly(I:C) led to an increased proliferation of CD4+ and CD8+ T cells in comparison with the animals treated with OVA alone (Fig. 3). Treatment with R848 in combination with OVA resulted in weak proliferation of CD4+ with no significant differences compared with that in OVA-treated animals. However, the proliferation of CD8+ T cells was increased compared with that in animals that were only treated with allergen and was comparable to that in mice that received allergen and either LPS or poly(I:C) (Fig. 3). To analyze which cytokines were produced by the transferred OTII cells, lung cells were analyzed 72 h after treatment for their expression of IL-5, IL-17A, and IFN-γ. In lung, expression of IL-5 was not detectable in any group. In contrast, IL-17A+ and IFN-γ+ T cells were detected. Mice that were treated with LPS showed more IL-17A– and IFN-γ–producing cells, whereas mice that received poly(I:C) or R848 showed only an increase in IFN-γ– but not IL-17A–producing cells (Supplemental Fig. 2). Notably, analysis of cytokines in supernatant after Ag-specific restimulation with OVA showed an increased IL-5 to IFN-γ ratio in mice that received LPS or poly(I:C) compared with R848 (Supplemental Fig. 2).
Exposure to TLR3 or TLR7/8 ligands in combination with allergen results in increased production of allergen-specific Ig Abs

To assess whether TLR3 or TLR7/8 ligands induce allergen-specific Igs, animals were treated with OVA alone or in combination with LPS, poly(I:C), or R848 on three consecutive days. On days 14, 15, and 18, 19 animals were challenged with OVA intranasal application, and 48 h after the last exposure, Ig production and airway inflammation were assessed.

Similar to previous reports, in animals that received LPS in combination with allergen increased levels of allergen-specific IgE and IgG1 were detectable. Similarly, levels of allergen-specific Igs were also elevated in mice that received poly(I:C) in combination with allergen. In contrast, in animals that received R848, only low amounts of OVA-specific IgG1 and IgE were detectable (Fig. 4A, 4B). OVA-specific IgG2b was produced by all animals that received a treatment with OVA plus TLR ligand (Fig. 4C). Regarding the ratio of IgG1 to IgG2b, LPS-treated as well as poly-IC-treated animals demonstrated high IgG1 to IgG2b ratio, whereas R848-treated animals demonstrated nearly comparable proportions of IgG1 and IgG2b (Fig. 4D).

Exposure to TLR3 or TLR4 ligand in combination with allergen results in allergic airway disease after allergen rechallenge

To assess the development of allergic airway inflammation, BAL fluid and lung tissue were analyzed 48 h after the last airway exposure. In animals sensitized to allergen in combination with either LPS or poly(I:C), increases in total cell counts and numbers of eosinophils, lymphocytes, and neutrophils were detectable after rechallenge with the allergen. In contrast, animals that received R848 displayed only a small increase in eosinophils and lymphocytes (Fig. 5A).

Similar to findings in the BAL fluid, either LPS or poly(I:C) exposure resulted in increased peribronchial airway inflammation 48 h after the last airway challenge (Fig. 5B). Again, animals that had been sensitized with allergen and R848 showed only weak infiltration of inflammatory cells into the lung tissue (Fig. 5B). Enhanced production of mucus is another feature of allergic airway disease. Numbers of goblet cells in airway epithelium were measured using PAS staining. Indeed, poly(I:C) as well as LPS treatment during sensitization phase led to more PAS+ cells (Fig. 5C). After treatment with R848, only few PAS+ cells were detectable. Analysis of cytokine expression showed that treatment with poly(I:C) or LPS in combination with OVA induced the expression of Th2- and Th1-specific cytokine RNAs. In comparison with nontreated controls and R848-exposed animals, expression of IL-4, IL-10, and IFN-γ was significantly increased. Moreover, the eosinophil-attracting chemokine CCL5 (RANTES) was upregulated in LPS-treated or poly(I:C)-treated animals but not upon R848 treatment. The neutrophil-attracting chemokine CXCL1 (KC) was increased in all animals that were treated with TLR ligands during sensitization. The type I IFN IFN-α was not altered between control mice and animals treated with bacterial or viral TLR ligands during the sensitization process. However, IFN-β was upregulated in the lungs of animals treated with R848 during sensitization in comparison with that in the other groups (Fig. 6). In addition, DC subpopulations were also analyzed in the lung. LPS-treated and poly(I:C)-treated mice demonstrate an increase of CD11c+MHCII+CD11b+/Ly6c+ cells in the lung compared with that in all other groups (Supplemental Fig. 3). Analysis of regulatory T cells in the draining lymph nodes was comparable between all groups, and in the lung an increase in regulatory T cells was only detectable in mice that received LPS (Supplemental Fig. 4).

Discussion

DCs are the major APCs of the body necessary for the initial activation of naïve T cells (22, 23). In the lung, several subtypes of DCs have been described, and cDCs have been found to be important for development of sensitization (24, 25). Indeed, cDCs form a network in and beneath the airway epithelium (26), and under steady-state conditions cDCs migrate to the draining lymph nodes and induce tolerance (9). Exposure to an exogenous or endogenous stimulus or danger signal results in maturation of DCs while migrating to the draining lymph node, and these professional APCs are able to induce specific adaptive immune responses. In this context, many studies have shown that LPS acts as a danger signal, and coexposure to LPS and allergen results in development of sensitization. In the current study, we show that a ligand [poly(I:C)] for the TLR3 receptor, which recognizes ssRNA, or a ligand for TLR7/8 receptor (R848), which recognizes dsRNA, also induces enhanced migration of Ag-exposed cDCs from the lung to the draining lymph nodes in comparison with that in animals only treated with Ag. Similar to previous studies, the highest number of fluorescent DCs in the draining lymph node were detectable 24 h after allergen application (27). Increased DC migration has been described in animals exposed to influenza virus, which also leads to immune responses to simultaneously inhaled allergen (8, 28). Therefore, on the basis of increased DC migration after TLR3 or TLR7/8 exposure, we speculated that this also would result in the development of specific immune response.

To elucidate further the Ag processing of cDCs, we used the fluorescent dye DQ-OVA after exposure to TLRs. Again, application of either R848 or poly(I:C) led to more OVA-processing cDCs in the draining lymph node comparable to the effect of LPS. To induce efficient adaptive immune responses, cDCs have to mature, which is characterized by an upregulation of costimulatory molecules such as CD40, CD80, and CD86 on the surface of the DC (29, 30). Poly(I:C) induces an increased expression of CD80, CD86, and CD40 comparable to that in LPS-exposed animals. This is similar to splenic cDCs, which upregulate CD86, CD80, and CD40 after i.p. injection of the TLR3 ligand (31). In addition, proliferation of Ag-specific T cells was assessed. Poly(I:C) has
been described as an inducer for CD8+ T cell proliferation via cross-presentation of extracellular Ag by DCs (32), and poly(I:C) can activate CD8+ memory cells in absence of CD4+ T cells (33). In the current study, application of poly(I:C) resulted in increased proliferation of Ag-specific CD4+ T cells and CD8+ T cells, comparable to that in animals exposed to LPS and OVA. In contrast, application of R848 did not result in increased expression of CD80 or CD86 or augmented proliferation of CD4+ T cells. Still, proliferation of CD8+ T cells was increased after R848 exposure similar to effects seen after LPS or poly(I:C) administration. To date, the effects of R848 on T cell proliferation have mainly been investigated in the human system. R848 is able to induce T cell proliferation and activation of human CD4+ T cells (34); however, in MLRs, T cell proliferation was reduced after R848 application (35). In our in vivo system, proliferation of Ag-specific CD4+ T cells was likewise reduced suggesting that despite increased

FIGURE 5. Ag rechallenge leads to lung inflammation and to the development of a goblet cell metaplasia. (A) After intranasal application of OVA (sens) in combination with either LPS, poly(I:C), or R848 and subsequent challenge with OVA (chall), cellular composition of the BAL was assessed. Bar charts show the total number of macrophages, lymphocytes, neutrophils, and eosinophils. Mean ± SEM is given; n = 12 to 18 mice per group from four independent experiments. (B) H&E staining of lung slices (original magnification ×100) of mice that were not sensitized but challenged (PBS/OVA) or sensitized by inhalation of OVA in either PBS (OVA/OVA), LPS (LPS/OVA), poly(I:C) [poly(I:C)/OVA], or R848 (R848/OVA) and subsequently challenged with OVA. H&E-stained lung sections were scored for inflammatory infiltrations; bar graphs demonstrate peribronchial and perivascular score. Mean ± SEM is given. (C) PAS staining of lung slices (original magnification ×100/×400) in the same groups as those in (B). Bar graph shows PAS+ cells per millimeter basement membrane (BM). Mean ± SEM is given. For (A)–(C), n = 12 to 18 mice per group from four independent experiments.

FIGURE 6. Cytokine production in the lungs after Ag rechallenge. Cytokine/chemokine mRNA expression of lungs prepared after sensitization via inhalation and subsequent challenge. Expression of IL-4, IL-10, RANTES, IFN-γ, IFN-α, IFN-β, IL-12p35, IL-12p40, and KC in comparison with unsensitized but OVA-challenged controls is shown. Mean ± SEM is given; n = 9 mice per group from three independent experiments.
migration of cDCs, no effect could be detected on CD4 proliferation.

In addition, we analyzed the immune response after sensitization and subsequent Ag challenge via the airways. Similar to LPS, initial application of poly(I:C) and allergen leads to the development of allergic airway disease after re-exposure to the allergen. In contrast, exposure to R848 did not result in the development of allergic airway disease. Most studies have investigated the effects of bacterial or viral compounds on allergic diseases when administered either during systemic sensitization or to already sensitized animals prior to allergen challenge. Systemic application of R848 or poly(I:C) during allergen sensitization results in reduction of allergic airway disease after allergen challenge (36). In addition, systemic treatment of sensitized animals during primary challenge (37) or during secondary Ag challenge (36) again reduced allergic airway disease. Application of TLR ligands into the lung prior to airway challenge efficiently prevents induction of allergic diseases probably by a mechanism depending on CD8+ T cells (38). Moreover, viral TLR ligands given prior to and during allergen challenge seem to be able to modulate already established Ig responses and also the development of the developing allergic challenge. We have shown that viral TLR ligands given to naive animals that were sensitized with R848. Depending on the environmental situation, IFN-β is described as an anti-inflammatory factor (54) and could so be responsible for the reduced inflammatory phenotype of the animals intranasally sensitized with R848.

As we could not detect differences in the proportion of regulatory T cells, a significant effect of R848 on this cell type is unlikely in this model. Another explanation of the reduced effects of R848 could be the increased activation of pDCs. This cell type is described as a regulatory counterpart to cDCs (52). After activation with ssRNA/dsRNA, pDCs are able to produce high amounts of IFN-α and are also able to secrete IFN-β (53). However, IFN-α was not detectable in the lungs after sensitization and challenge, but we could detect increased amounts of IFN-β in the lungs of animals that were sensitized with R848. Depending on the environmental situation, IFN-β is described as an anti-inflammatory factor (54) and could so be responsible for the reduced inflammatory phenotype of the animals intranasally sensitized with R848.

All these data support observations of human studies investigating the role of viral infections in the exacerbation and/or development of asthma. RNA viruses like respiratory syncytial virus (RSV) or rhinovirus (RV) demonstrate a relationship with the exacerbation of asthma (55, 56). In addition, recent studies underline that mainly RV but not RSV infections contribute to a sensitization to a harmless Ag and development of asthma (57, 58). Notably, RV is recognized by TLR3 during its replication process where ssRNA forms a double-stranded intermediate (59, 60). The detection and signaling of RSV is more complex and is mainly mediated by TLR2, TLR4, and TLR7 (61).

In summary, viral TLR3 ligands similar to bacterial TLR ligands lead to an activation of APCs, resulting in proliferation of Ag-specific CD4+ and CD8+ T cells. Moreover, animals that inhale a harmless Ag in combination with a viral TLR ligand develop Ag-specific adaptive immune responses. However, this depends on the TLR ligand involved. Indeed, a TLR3 ligand induces allergic airway disease, similar to low doses of the bacterial TLR4 ligand LPS. These findings further support the concept that viral infection can influence the development of an allergic airway disease. This fact has to be considered when choosing TLR ligands as immune response modulators for treatment of diseases.

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References
Toll-like receptors (TLR) are a family of transmembrane proteins that recognize pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) and initiate an innate immune response. This response is critical for the induction of adaptive immunity and for the regulation of inflammation. The TLR family consists of 13 members in humans, each with a unique ligand specificity and biological function. The activation of TLRs leads to the induction of interferons (IFNs) and the production of pro-inflammatory cytokines, which are crucial for the initiation of an adaptive immune response. TLR activation also contributes to the induction of regulatory T cells (Tregs) by promoting the production of interleukin-6 (IL-6) and transforming growth factor-beta (TGF-β), which are required for the differentiation of Tregs. The role of TLR signaling in allergic sensitization is highlighted by studies showing that inhibition of TLR signaling can prevent the development of allergic asthma and rhinitis. Therefore, targeting TLRs may represent a novel therapeutic strategy for allergic diseases.

References:

Supplemental Material

Supplemental Figure 1: CD11b but also CD103 positive OVA laden DC migrating into the draining lymph node following inhaled application of antigen and TLR ligand

At 24 hours following application of the fluorescence labeled antigen (OVA-Alexa647) in combination with PBS (PBS/OVA) or the TLR ligands LPS (LPS/OVA), Poly(I:C) (Poly(I:C)/OVA) or R848 (R848/OVA), Alexa647-positive DC in the draining lymph nodes were analysed for the expression of CD11b and CD103. MHCII and CD11c positive cells were assessed for their expression of Alexa647. Among these cells the proportion of CD103 to CD11b positive cells was determined (A). Dot plots demonstrate the CD11c+/MHCII+/OVA+ cells which express either CD103 or CD11b in the different treated groups (B). The bar chart shows the proportion of the CD11b (black bar) and CD103 (grey bar) positive cells among the OVA positive cDC in the draining lymph node. Mean ± SEM are given; n=4 per group.

Supplemental Figure 2: Following application of antigen and TLR ligand OVA transgenic Th2 cells produce mainly IL-17A and IFNγ.

Splenocytes of OTII mice were injected into the tail veins. 24 hours later OVA was applied intranasally either in PBS (PBS) or in combination with LPS (LPS/OVA), Poly(I:C) (Poly(I:C)/OVA) or R848 (R848/OVA). 72 hours following treatment lungs were processed and cytokine production of the OVA positive T cells was analyzed via FACS (A-D) or by ELISA (E). Dot Plots demonstrate the gating strategy, among the CD3 positive cells the CD4 and Vα2 positive cells were analysed for the expression of IL-17A and IFNγ (A). Following 4 hours of stimulation with ionomycin, PMA and brefeldin A. Intracellular expression of IL-17A and IFNγ was analyzed in the OVA transgenic Th2 T cells. Contour plots demonstrate exemplary the expression of the two cytokines in the different treated groups (B). C) Graph
plots demonstrate the total number of IL-17A expressing cells among OVA transgenic T cells in the lung. D) Graph plots demonstrate the total of IFN\(\gamma\) expressing cells among OVA transgenic T cells of these cells in the lung. Mean ± SEM are given; n=4 per group. E) Lung cells were incubated 72 hours with or without antigen and IL-5 and IFN\(\gamma\) was subsequently measured in the supernatants. The graph bar shows the ratio of OVA specific produced IL-5 to IFN\(\gamma\) in the lung supernatants of the different treated animals. Mean ± SEM are given; n=4.

**Supplemental Figure 3:** Following inhalative sensitization and challenge with antigen and TLR ligand different subtypes of DC are detectable in the lung.

Following i.n. application of OVA (sens) either in combination with LPS, Poly(I:C) or R848 and subsequent challenge with OVA (chall) single cell suspensions of lungs were performed and analyzed for different DC subsets. DC were characterized as non fluorescent cells which were positive for CD11c and MHCII. Lung DC were further subdivided in Ly6c negative cells which express CD11b (regional DC) or which express CD103 (cross priming DC). Inflammatory DCs were characterized as CD11c and MHCII positive cells which additionally are CD11b and Ly6c positive. A) Dot blots in the upper row demonstrate exemplary the CD11b or CD103 positive lung DC of the not sensitized but challenged (PBS/OVA) or sensitized by inhalation of OVA in either PBS (OVA/OVA), LPS (LPS/OVA), Poly(I:C) (Poly (I:C)/OVA) or R848 (R848/OVA) and subsequently challenged with OVA animals. B) Graph bars demonstrate the proportion of the different DC subsets among the DCs in the lung. Mean ± SEM are given; n=3-4.

**Supplemental Figure 4:** Treg frequencies are comparable following inhalative sensitization and challenge with antigen and the different TLR ligands.

Following i.n. application of OVA (sens) either in combination with LPS, Poly(I:C) or R848 and subsequent challenge with OVA (chall) single cell suspensions of lungs and tLN were
performed and analyzed for regulatory T cells (CD4+/CD25+/FoxP3+). Dot blots show CD4, FoxP3 and CD25 positive cells in tLN (A) and lung (B) of the not sensitized but challenged (PBS/OVA) or sensitized by inhalation of OVA in either PBS (OVA/OVA), LPS (LPS/OVA), Poly(I:C) (Poly (I:C)/OVA) or R848 (R848/OVA) and subsequently challenged with OVA animals. Graph bars demonstrate the proportion of FoxP3 positive cells among CD4 T cells in the tLN (C) or the lung (D). Mean ± SEM are given; n=3-4.
Supplemental Figure 1

A

empty channel

MHCII

CD11c

empty channel

MHCII

OVA

CD103

CD11b

B

PB

LPS

Poly(I:C)

R848

CD103

CD11b

C

% of OVA positive DC

PBS

LPS

Poly(I:C)

R848

CD11b

CD103
Supplemental Figure 3

A

PBS/OVA  OVA/OVA  LPS/OVA  Poly(I:C)/OVA  R848/OVA

CD11b  CD103  Ly6c

B

CD11b+/CD103−/Ly6c−  CD11b−/CD103+/Ly6c−  CD11b+/CD103−/Ly6c+

% of DC

sens - + + + +
chall + + + + +
Supplemental Figure 4

A

PBS/OVA  OVA/OVA  LPS/OVA  Poly(I:C)/OVA  R848/OVA

CD25 vs. FoxP3

B

PBS/OVA  OVA/OVA  LPS/OVA  Poly(I:C)/OVA  R848/OVA

CD25 vs. FoxP3

C

tracheal lymph node

FoxP3 % of CD4

sens - + + + +
chall + + + + +

D

lung

FoxP3 % of CD4

sens - + + + +
chall + + + + +