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

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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: 000–000.

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 imidazoquinolines; 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.

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Abbreviations used in this article: BAL, bronchoalveolar lavage; cDC, conventional dendritic cell; DC, dendritic cell; DQ-OVA, self-quenched conjugate of OVA; PAS, periodic acid–Schiff; pDC, plasmacytoid dendritic cell; PFA, paraformaldehyde; poly(I:C), polyinosinic-polycytidylic acid; R848, resiquimod; RSV, respiratory syncytial virus; RV, rhinovirus.

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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-ratiopharm; Ratiopharm, Ulm, Germany/Rompun 2%; Bayer, Leverkusen, Germany), and then 50 μ l of the TLR ligands LPS (Calbiochem, Merck, Darmstadt Germany), poly(I:C) (Invitrogen, Eugene, OR), or resiquimod (R848; Alexis Biochemicals, San Diego, CA) in combination with 80 μ g of a fluorescent OVA dye (OVA-Alexa Fluor 647; MoBiTec, Goettingen, Germany) in 40 μ l PBS was applied intranasally. At 4, 24, and 48 h after the application, mice were sacrificed, and tracheal 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) 40 μ g/40 μ l PBS intranasally. At 24 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 endotoxin-free OVA (EndoGrade OVA; Profos, Regensburg, Germany) 80 μ g/50 μ l PBS. Again 24 h 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 μ M CFSE (CFDA; Invitrogen, Eugene, OR), and 10×10^6 cells were injected i.v. into the tail of C57BL/6 recipients on day -1. On day 0, mice were anesthetized with ketamine/rompun and treated intranasally with 50 μ l of either TLR ligands LPS, poly(I:C), or R848, in combination with 80 μ g/40 μ l 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 $\times$ 40 mm cannula. After centrifugation, RBCs were removed by osmotic lysis. After washing, cell counts were determined and adjusted. Lymph nodes were mechanically minced. After washing, cell counts were determined.

After preparation of single-cell suspension, cell numbers were adjusted to 2×10^7 per ml FACS washing buffer. Unspecific binding was blocked by incubating the cells with Fc-receptor blocking Abs (α CD16/CD32; BD Pharmingen, Heidelberg Germany).

To analyze conventional DCs, cells were stained with FITC-labeled anti-MHC class II (eBioscience, NatuTec, Frankfurt, Germany), PE-labeled anti-CD11c, and PerCP-Cy5.5-labeled anti-B220 (BD Pharmingen). To analyze T cell proliferation, cells were stained with PE-labeled anti-CD69, PerCP-Cy5.5-labeled anti-CD3, allophycocyanin-labeled anti-CD4, and Alexa 647-labeled anti-CD8 (all BD Pharmingen), respectively. For assessment of DC activation, cells were stained with FITC-labeled anti-MHC class II, PerCP-Cy5.5-labeled anti-B220, allophycocyanin-labeled anti-CD11c (all BD Pharmingen), and PE-labeled Abs against CD86, CD80, CD40 (all BD Pharmingen), and CCR7 (eBioscience, NatuTec). Cells were additionally stained with PE-labeled CD103, PerCP-Cy5.5-labeled CD11b, PeCy7-labeled CD11c, and V450-labeled Ly6c (all BD Biosciences). After incubation, cells were washed and finally resuspended in FACS fixation buffer (4% paraformaldehyde [PFA]). FACS measurement

was performed on a FACSCalibur cytometer (BD Biosciences) or a FACSCanto II (BD Biosciences) using CellQuest software. Final analysis of the FACS data and graphics were achieved using FlowJo software (Tree Star, Ashland, OR).

Assessment of intracellular cytokine staining and regulatory T cells. Single-cell suspensions of lymph nodes and lungs were made under sterile conditions according to the surface staining. To analyze intracellular 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. Four to six hours after incubation, cells were washed twice, and intracellular staining was performed according to the manufacturer's protocol (BD Biosciences Cytofix/Cytoperm). In short, unspecific surface binding areas were blocked by adding 1 μ l Fc-receptor blocking Abs (α CD16/CD32; BD Pharmingen, Heidelberg, Germany) for 10 min at 4°C. Cells were stained with FITC-labeled V α 2 (BD Pharmingen), PerCP-Cy5.5-labeled CD3 (BD Pharmingen), and PeCy7-labeled CD4 (BD Pharmingen). Abs were incubated for 20 min at 4°C in the dark. After washing in WB, cells were resuspended in 150 μ l Cytofix/Cytoperm (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 allophycocyanin-labeled IFN- γ (BD Pharmingen), PE-labeled IL-17A (BD Pharmingen), and PE-labeled IL-5 (BD Pharmingen). Abs were incubated at 4°C for 30–40 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 PE-labeled CD25 (all BD Pharmingen) as described. Foxp3 staining was performed following the advice of the manufacturer of the fixation/permeabilization set (eBioscience). Briefly, after surface staining and washing in WB, cells were resuspended in 100 μ l Cytofix/Cytoperm (eBioscience) and incubated for 30 min at 4°C in the dark. Cells were washed in Perm/Wash (Perm-buffer) twice, and subsequently allophycocyanin-labeled Foxp3 was added. After 30–40 min incubation in the dark at 4°C, cells were washed twice 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 made under sterile conditions. Cells were adjusted to 1×10^7 cells per milliliter test medium [IMDM (PAA) plus 10% FCS (PAA), 1% Pen/Strep (Sigma)]. Cells were incubated for 72 h with or without 250 μ g/ml OVA (grade V; Sigma) at 37°C. Subsequently, supernatants were harvested and for further analysis stored at -20°C.

Sensitization and rechallenge protocol. Immunization and challenge was performed as described by Nigo et al. (18). In brief, animals were anesthetized with ketamine/rompun and subsequently treated with 10 μ g of endotoxin-free OVA (EndoGrade OVA) and 1 μ g LPS, 1 μ g poly(I:C), or 25 μ g R848, respectively, on days 0, 1, and 2. Anesthetized mice were challenged on days 14, 15, 18, and 19 by an intranasal application of 25 μ g endotoxin-free OVA in 50 μ l PBS, and blood, bronchoalveolar lavage (BAL) fluid, and lung tissue were analyzed 48 h after the last challenge.

After application of an overdose of Narcoren (Merical, Halbergmoos, Germany), lungs were lavaged via the tracheal tube with PBS (1 ml). Cell numbers were assessed as previously described (19), and cytopins were prepared by using a cytocentrifuge. Cellular composition was analyzed after fixation and staining of the spins using a Microscopy Hemacolor Set (Merck, Darmstadt, Germany). Percentage and absolute numbers of each cell type were calculated.

After BAL, lungs were fixed by inflation (1 ml) and immersion in 4% formalin and subsequently embedded in paraffin. Tissue sections were prepared and stained with H&E, periodic acid-Schiff (PAS), and toluidine. Slides were examined in a blinded fashion by two experienced observers as previously described (20). Inflammation was scored in a semiquantitative fashion on H&E slides. Number of goblet cells was analyzed using an imaging software (analysis; Soft Imaging Systems, Stuttgart, Germany) on PAS-stained slides and expressed as positive cells per millimeter basement membrane.

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.

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 serially 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 analyzed. 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/ μ l, and 1 μ l was used in 25 μ l reaction volume. By using a SYBR Green mix (Thermo Scientific), the differences of amplification efficiency between the housekeeping gene (TBP) 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'-GGA CCA GAA CAA CAG CCT TC-3'; TBP-as, 5'-CCG TAA GGC ATC ATT GGA CT-3'. IL-4: IL-4-s, 5'-TCA ACC CCC AGC TAG TTG TC-3'; IL-4-as, 5'-TGT TCT TCG TTG CTG TGA GG-3'. IL-10: IL-10-s, 5'-CCA AGC CTT ATC GGA AAT GA-3'; IL-10-as, 5'-TTT TCA CAG GGG AGA AAT CG-3'. RANTES: RANTES-s, 5'-CTG CTG CTT TGC CTA CCT CT-3'; RANTES-as, 5'-ACT TCT TCT CTG GGT TGG CAC AC-3'. IFN- γ : IFN- γ -s, 5'-TCA AGT GGC ATA GAT GTG GAA GAA-3'; IFN- γ -as, 5'-TGG CTC TGC AGG ATT TTC AT-3'. IFN- α : IFN- α -s, 5'-GCC ATC CCT GTG CTG CGA GA-3'; IFN- α -as, 5'-CAG CCA GCA GGG CGT CTT CC-3'. IFN- β : IFN- β -s, 5'-ACG CCT GGA TGG TGG TCC GA-3'; IFN- β -as, 5'-ACC AGT CCC AGA GTC CGC CT-3'. KC: KC-s, 5'-GCA CCC AAA CCG AAG TCA TAG C-3'; KC-as, 5'-CGT TAC TTG GGG ACA CCT TTT AGC-3'. IL-12p35: IL-12p35-s, 5'-ATG ACC CTG TGC CTT GGT AG-3'; IL-12p35-as, 5'-TCT CCC ACA GGA GGT TTC TG-3'. IL-12p40: IL-12p40-s, 5'-CAT CTG CTG CTC CAC AAG AA-3'; IL-12p40-as, 5'-CGC CAT TCC ACA TGT CAC TGC-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 initially analyzed by non-parametric ANOVA (Kruskal–Wallis test) for overall differences. In case of significant results, the Mann–Whitney *U* 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 $\pm$ 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 few 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 showed 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

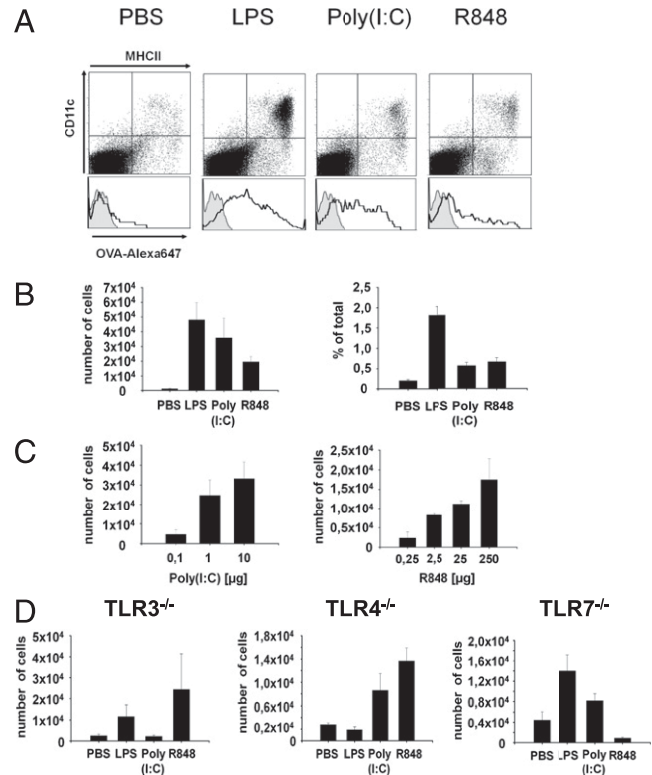


FIGURE 1. Application of bacterial and viral TLR ligands induces enhanced migration of Ag-presenting cDCs (CD11c⁺MHCII⁺B220⁺) from the lung to the draining lymph nodes. **(A)** FACS plots show the CD11c⁺MHCII⁺B220⁺ cells in the draining lymph nodes of animals that received OVA–Alexa 647 with either PBS (PBS/OVA), LPS (LPS/OVA), poly(I:C) [poly(I:C)/OVA], or R848 (R848/OVA). The histograms show the fluorescence among these cells (gray shaded, untreated; black line, current group). **(B)** Total number of OVA–Alexa 647-positive cDCs 24 h after application in mice that also received PBS, LPS, poly(I:C), or R848; *n* = 12 in each group from three independent experiments. Relative numbers of OVA–Alexa 647-positive cDCs among total lymph node cells. Mean $\pm$ SEM is given. **(C)** Graphs show the dose-responses of poly(I:C) and R848, respectively. Increasing doses of each TLR ligand induce growing numbers of Ag-positive cDCs in the draining lymph nodes (*n* = 4 mice per dose). Mean $\pm$ SEM is given. **(D)** Migration of Ag-laden cDCs depends on the expression of specific TLR. Genetically deficient mice that lack either TLR3 (TLR3^{-/-}; *n* = 4 mice), TLR4 (TLR4^{-/-}; *n* = 6 mice), or TLR7 (TLR7^{-/-}; *n* = 6 mice) were treated as described above. Data from two independent experiments. Mean $\pm$ SEM is given.

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 CD11b⁺ and CD103⁺ cells (Supplemental Fig. 1). Ly6c expression was not detectable on cDCs in the regional lymph node (data not shown). To investigate whether these observed effects were specific, mice with genetic deficiency in either TLR3, TLR4, or TLR7 were treated with OVA–Alexa 647 and either poly(I:C), LPS, or R848. As shown in Fig. 1D, deficiency for a given TLR selectively abrogates DC migration upon treatment with the respective TLR ligand only.

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

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).

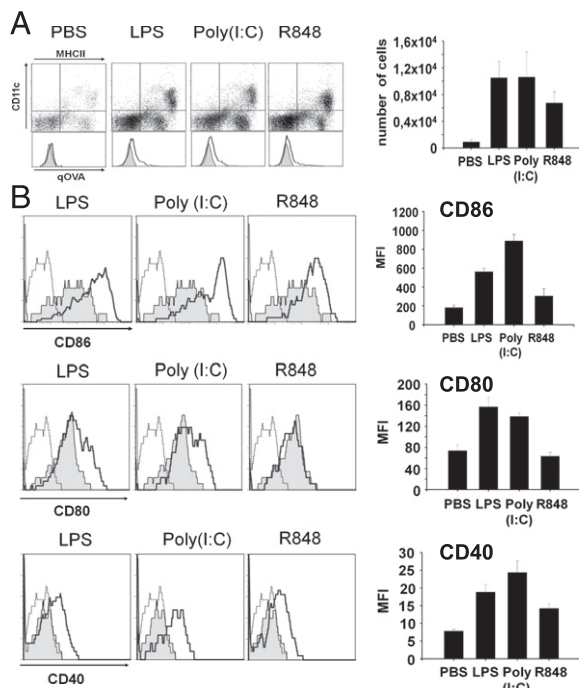


FIGURE 2. After application of viral or bacterial ligands, Ag-laden DCs demonstrate an enhanced Ag processing. **(A)** Using the fluorescent dye DQ-OVA, numbers of fluorescent Ag-processing cells in the draining lymph node were analyzed in mice that received qOVA with either PBS (PBS/OVA), LPS (LPS/OVA), 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 number of DQ-OVA⁺ cDCs in the draining lymph node 24 h after application. Mean $\pm$ 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 $\pm$ 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).

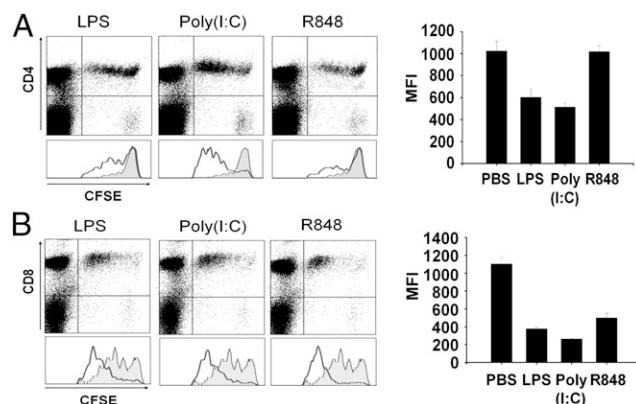


FIGURE 3. Viral and bacterial TLR ligands induce the proliferation of Ag-specific T cells after inhalational coadministration with the Ag. CFSE-labeled splenocytes of OTII and OTI animals, respectively, were injected into the tail veins. Twenty-four hours later, OVA was applied intranasally either in 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 $\pm$ SEM is given.

Exposure to TLR3 or TLR7/8 ligands in combination with allergen results in increased production of allergen-specific 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(I:C)-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 rechallenger

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 rechallenger 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 naive 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

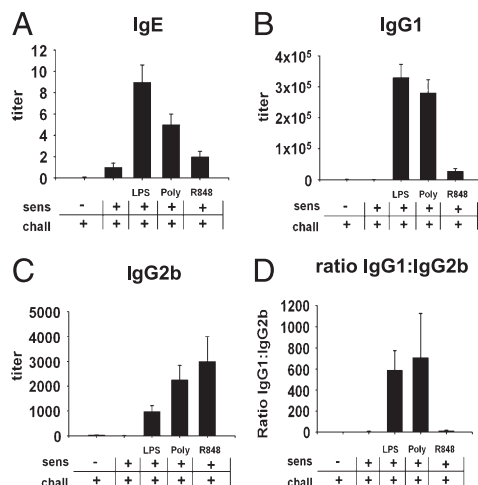
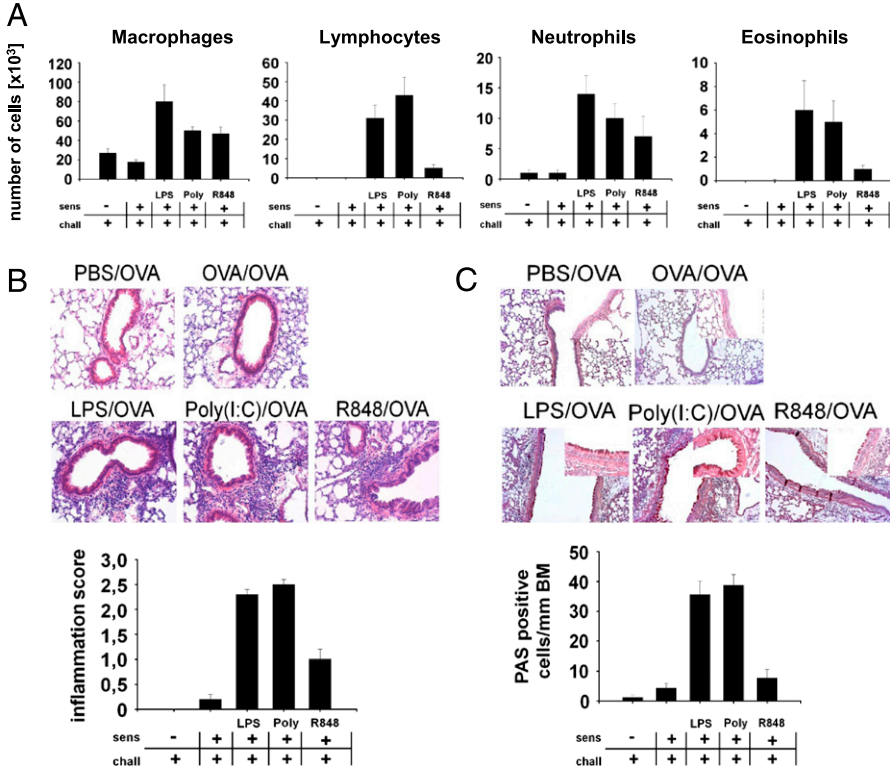


FIGURE 4. TLR3 and TLR7/8 ligation elevates Ag-specific Ig levels in serum. After intranasal application of OVA (sens) in combination with either LPS, poly(I:C), or R848 and subsequent challenge with OVA (chall), Ag-specific Igs were assessed. Bar diagrams demonstrate Ag-specific Ig titers of IgE (A), IgG1 (B), and IgG2b (C). (D) The ratio of OVA-specific IgG1 to IgG2b. Mean $\pm$ SEM is given; $n = 12$ to 18 mice per group from four independent experiments.

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 $\pm$ SEM is given; $n = 12$ to 18 mice per group from four independent experiments. **(B)** H&E staining of lung slices (original magnification $\times 100$) 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 $\pm$ SEM is given. **(C)** PAS staining of lung slices (original magnification $\times 100/\times 400$) in the same groups as those in (B). Bar graph shows PAS⁺ cells per millimeter basement membrane (BM). Mean $\pm$ SEM is given. For (A)–(C), $n = 12$ to 18 mice per group from four independent experiments.



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

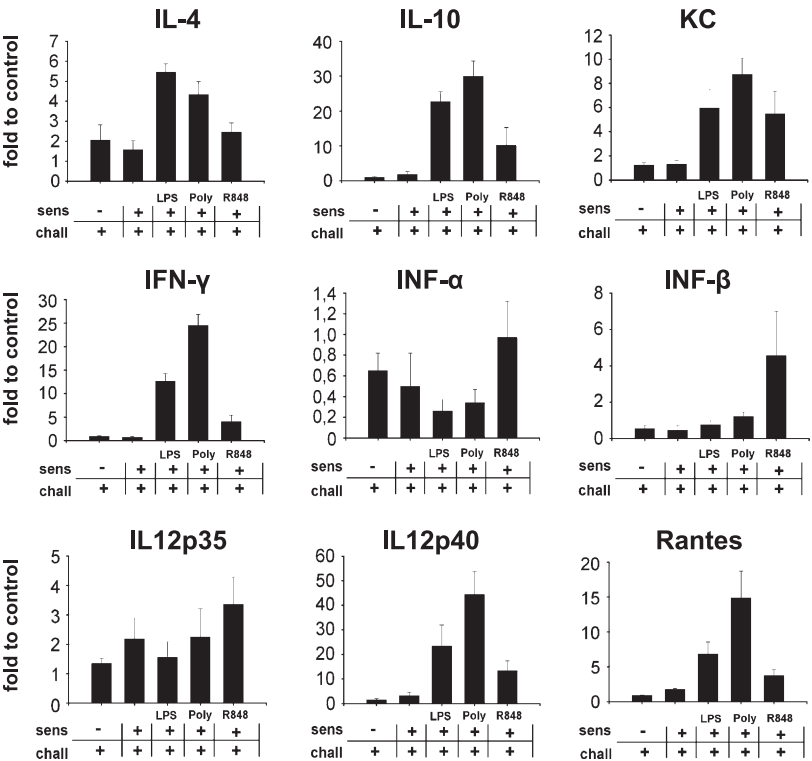


FIGURE 6. Cytokine production in the lungs after Ag rechallenge. Cytokine/chemokine mRNA expression of lungs prepared after sensitization via inhalation and subsequent challenge. Expression of IL-4, IL-10, RANTES, IFN-γ, IFN-α, IFN-β, IL-12p35, IL-12p40, and KC in comparison with unsensitized but OVA-challenged controls is shown. Mean $\pm$ 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 airway disease (39).

In contrast, it is well described that LPS can induce different effector T cell responses against additionally inhaled harmless allergen (8, 40). Administration of poly(I:C) or R848 in addition to allergen have both been described to induce allergen-specific CD8⁺ T cells and Th1 cytokine production when applied via the skin (41, 42). However, in the current model only administration of TLR3 ligand into the lung resulted in an inflammatory response with increased numbers of lymphocytes and eosinophils in the airways and increased expression of cytokines and chemokines. Furthermore, increased serum levels of allergen-specific IgE and high titers of IgG1 to low titers of IgG2b were detected in TLR3 ligand-treated animals, which suggests the development of a predominant Th2 reaction. In contrast, administration of R848 did not induce a specific IgE response, and also OVA-specific IgG1 production was low and comparable to the specific IgG2b titers. Lymphocyte and eosinophil numbers in the BAL after rechallenge with allergen were clearly reduced in these animals in comparison with those in LPS-treated and poly(I:C)-treated animals. In addition to Ag-specific Abs, animals sensitized in the presence of LPS and poly(I:C) developed goblet cell metaplasia and lung inflammation after rechallenge with allergen. The induction of mucus-producing cells is a two-step mechanism. While apoptosis of ciliated cells is avoided by phosphorylation of EGFR, the Th2 cytokine IL-13 induces the differentiation to a mucus-producing goblet cell (43). Again, application with R848 did not result in relevant mucus cell metaplasia. Indeed, our results are in line with findings demonstrating that application of R848 does not induce Th2 response in weanlings; however, if given to neonates, a Th2-type phenotype including mucus cell metaplasia can be induced (44). Analyzing the expression of cytokine-specific RNA in the lung after sensitization by inhalation revealed that LPS-treated as well as poly(I:C)-treated, OVA-exposed animals express more mRNA for Th2 (IL-4)-specific but also Th1 (IFN- γ)-specific cytokines in comparison with that in animals that received R848. IL-4 is important for the differentiation of naive T cells to Th2 cells via STAT6 and GATA3 (45), and many asthmatic features are directly linked to the expression of IL-4 (46). In contrast, the role of IFN- γ in asthma is more controversial as IFN- γ has been used to inhibit Th2-induced asthmatic responses in mice and rats (47). However, depending on time point, number, and location, Th1 cells and IFN- γ seem to support and even enhance Th2-specific immune responses (48). Likewise also RANTES, an important

chemokine for the migration of eosinophils into the lung (49), has been shown necessary for the development of an acute asthmatic response (50). These data confirm that poly(I:C), similar to LPS, can induce sensitization to an allergen, which, after re-exposure, leads to an inflammatory environment supporting the development of allergic airway disease. Moreover, they confirm and expand the observations of Kim and colleagues (51), which demonstrated a Th2-inducing role of low doses of intranasally applied poly(I:C) on the development of an allergic airway disease. Additionally, we show that application of R848 failed to induce an inflammatory cytokine profile.

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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Disclosures

The authors have no financial conflicts of interest.

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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 $\pm$ 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 IFN γ .

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 V α 2 positive cells were analysed for the expression of IL-17A and IFN γ (A). Following 4 hours of stimulation with ionomycin, PMA and brefeldin A. Intracellular expression of IL-17A and IFN γ 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 γ expressing cells among OVA transgenic T cells of these cells in the lung. Mean $\pm$ SEM are given; n=4 per group. E) Lung cells were incubated 72 hours with or without antigen and IL-5 and IFN γ was subsequently measured in the supernatants. The graph bar shows the ratio of OVA specific produced IL-5 to IFN γ in the lung supernatants of the different treated animals. Mean $\pm$ 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 $\pm$ SEM are given; n=3-4.

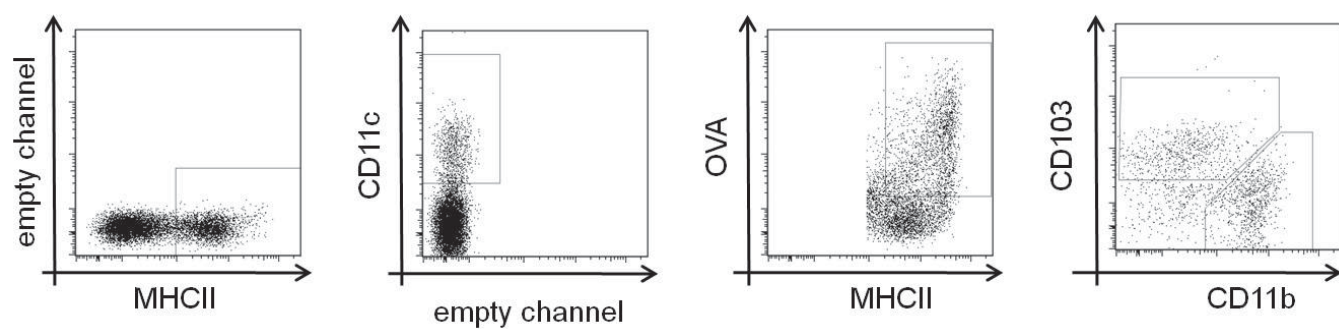
Supplemental Figure 4: Treg frequencies are comparable following inhalative sensitization and challenge with antigen and the different TLR ligands..

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

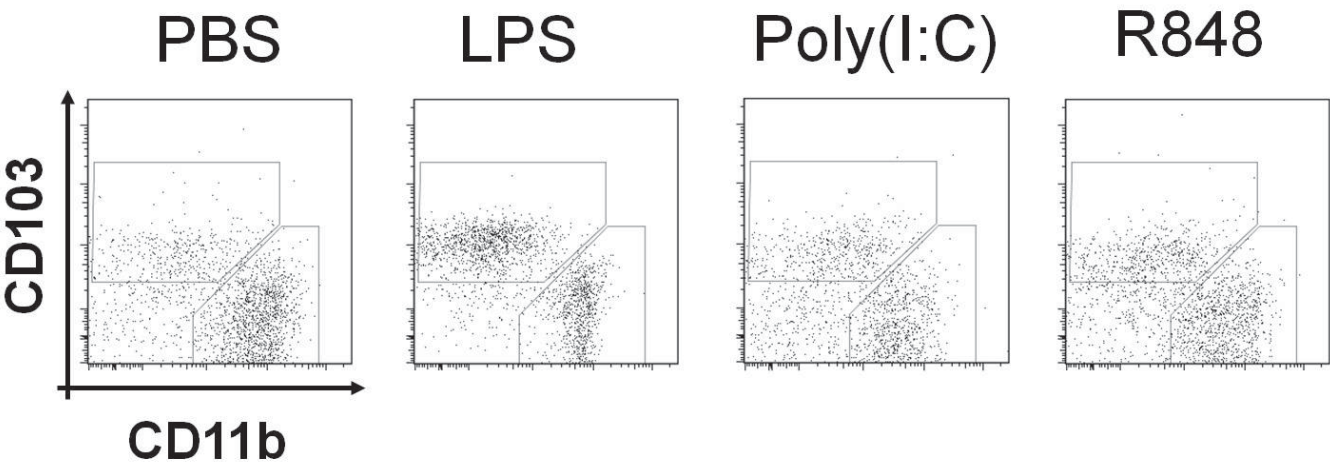
performed and analyzed for regulatory T cells ($CD4^{+}/CD25^{+}/FoxP3^{+}$). Dot blots show CD4, FoxP3 and CD25 positive cells in tLN (A) and lung (B) of the not sensitized but challenged (PBS/OVA) or sensitized by inhalation of OVA in either PBS (OVA/OVA), LPS (LPS/OVA), Poly(I:C) (Poly (I:C)/OVA) 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 $\pm$ SEM are given; n=3-4.

Supplemental Figure 1

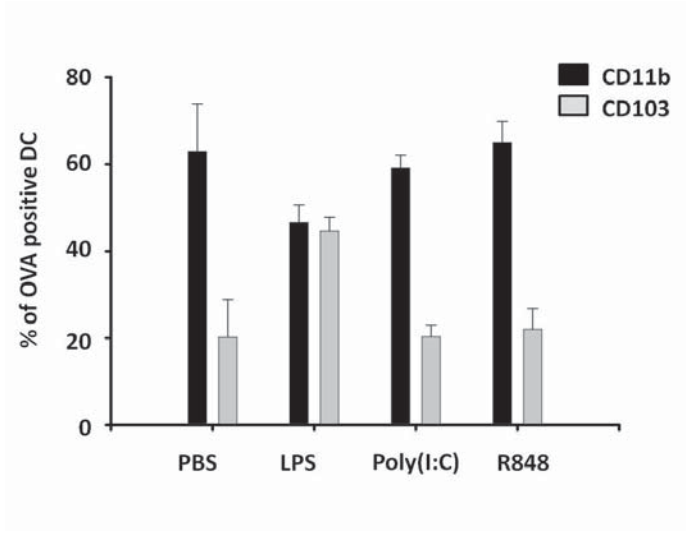
A



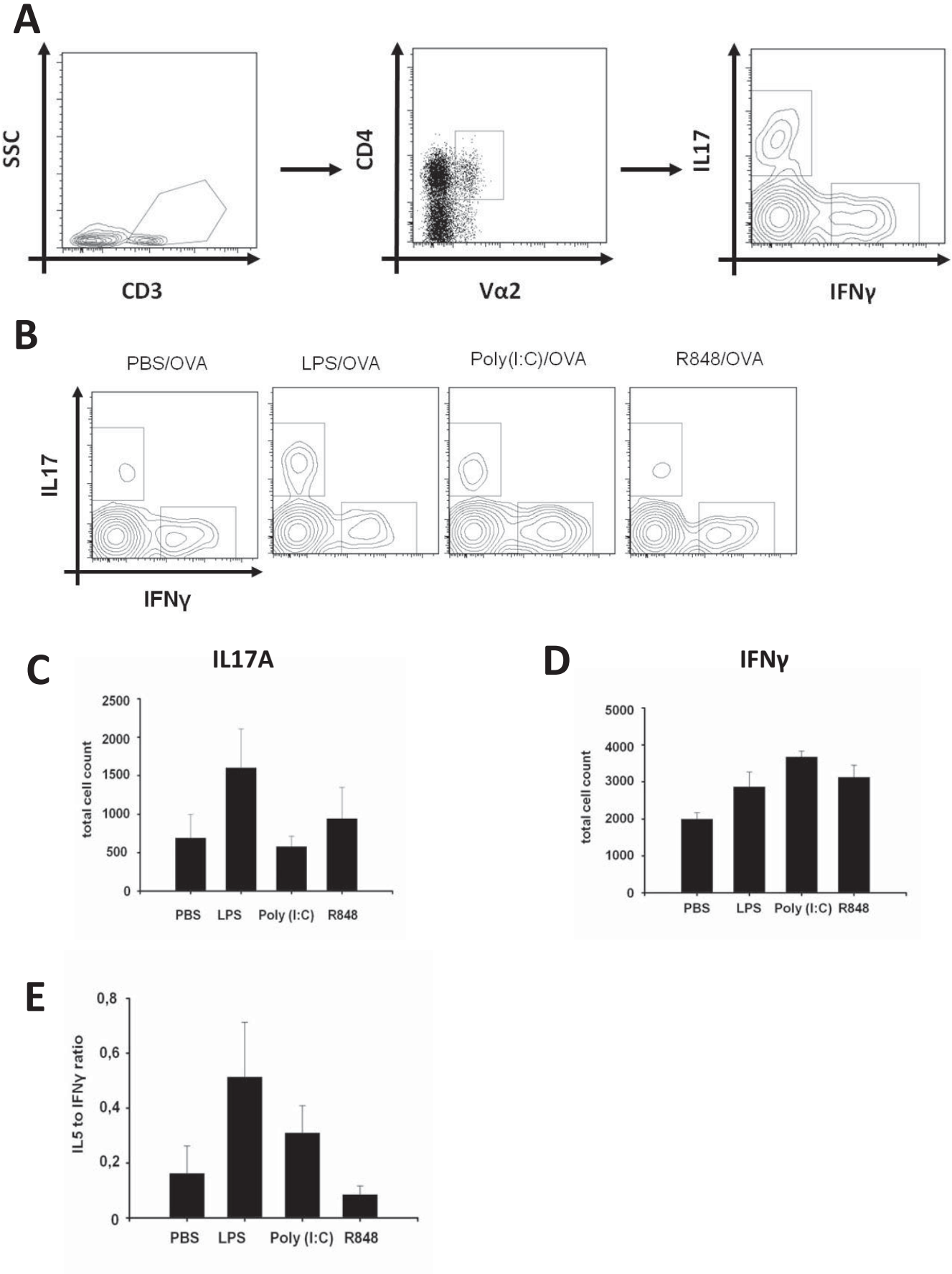
B



C

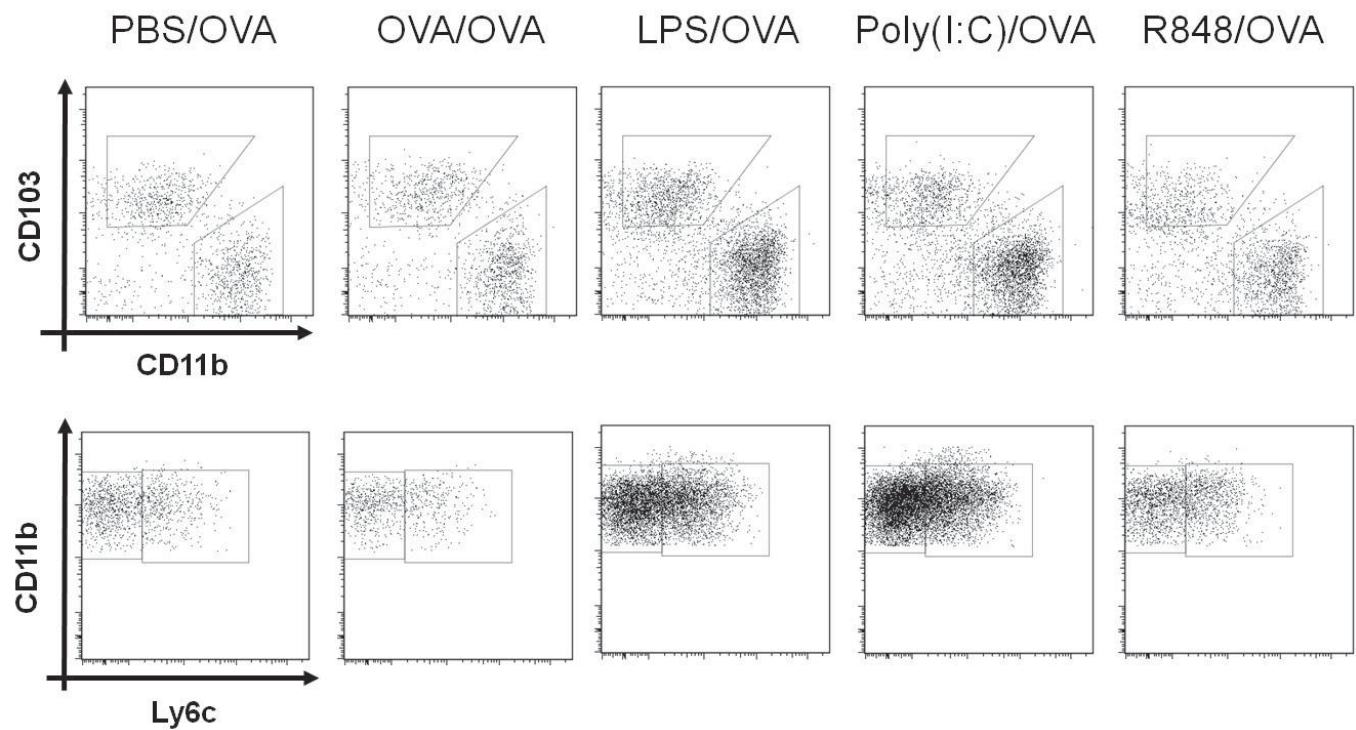


Supplemental Figure 2

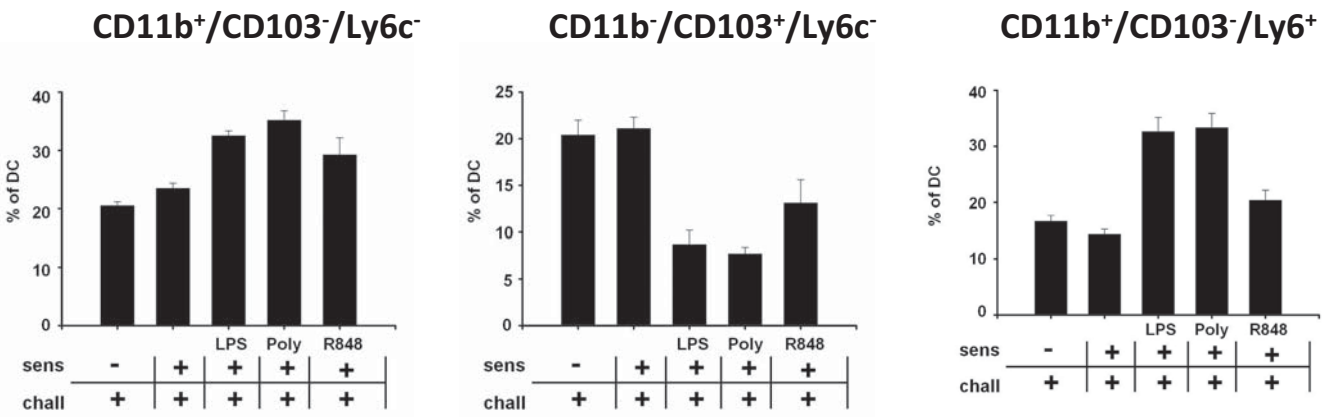


Supplemental Figure 3

A

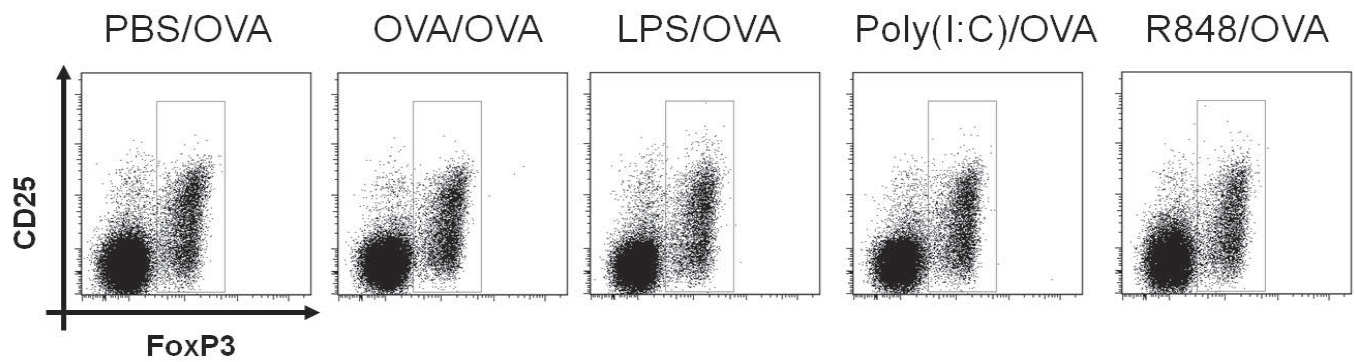


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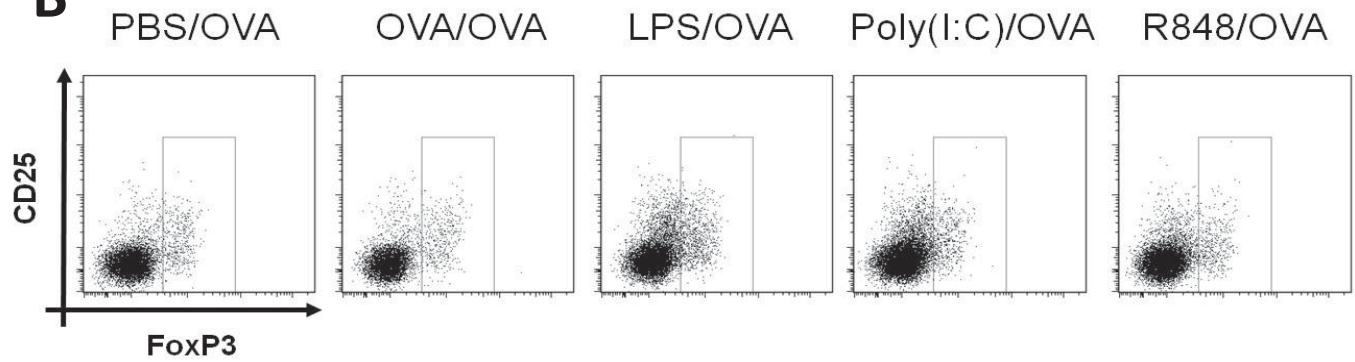


Supplemental Figure 4

A

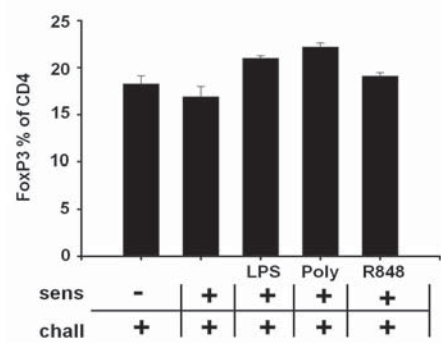


B



C

tracheal lymph node



D

lung

