

Toll-Like Receptor–2 Agonist–Allergen Coupling Efficiently Redirects Th2 Cell Responses and Inhibits Allergic Airway Eosinophilia

Jayendra Kumar Krishnaswamy¹, Adan Chari Jirno¹, Abdul Mannan Baru², Thomas Ebensen³, Carlos A. Guzmán³, Tim Sparwasser², and Georg M. N. Behrens¹

¹Department for Clinical Immunology and Rheumatology, Hannover Medical School, Hannover, Germany; ²Institute of Infection Immunology, Twincore, Center for Experimental and Clinical Infection Research, Helmholtz Center for Infection Research, Braunschweig, Germany, and the Hannover Medical School, Hannover, Germany; and ³Department of Vaccinology and Applied Microbiology, Helmholtz Center for Infection Research, Braunschweig, Germany

Toll-like receptor (TLR) agonists beneficially modulate allergic airway inflammation. However, the efficiency of TLR agonists varies considerably, and their exact cellular mechanisms (especially of TLR 2/6 agonists) are incompletely understood. We investigated at a cellular level whether the administration of the pharmacologically improved TLR2/6 agonist S-[2,3-bisphalmitoyloxy-(2R)-propyl]-R-cysteinyl-amido-monomethoxy polyethylene glycol (BPP) conjugated to antigenic peptide (BPP-OVA) could divert an existing Th2 response and influence airway eosinophilia. The effects of BPP-OVA on airway inflammation were assessed in a classic murine sensitization/challenge model and an adoptive transfer model, which involved the adoptive transfer of *in vitro* differentiated ovalbumin (OVA)–specific Th2 cells. Functional T-cell stimulation by lung dendritic cells (DCs) was determined both *in vitro* and *in vivo*, combined with a cytokine secretion analysis. A single mucosal application of BPP-OVA efficiently delivered antigen, led to TLR2-mediated DC activation, and resulted in OVA-specific T-cell proliferation via lung DCs *in vivo*. In alternative models of allergic airway disease, a single administration of BPP-OVA before OVA challenge (but not BPP alone) significantly reduced airway eosinophilia, most likely through altered antigen-specific T-cell stimulation via DCs. Analyses of adoptively transferred Th2-biased cells after BPP-OVA administration *in vivo* suggested that BPP-OVA guides antigen-specific Th2 cells to produce significantly higher amounts of IFN- γ upon allergen challenge. In conclusion, our data show for the first time that a single mucosal administration of a TLR 2/6 agonist–allergen conjugate can provoke IFN- γ responses in Th2-biased cells and alleviate allergic airway inflammation.

Keywords: dendritic cells; airway inflammation; antigen presentation/processing; TLR; BPPcysMPEG

Dendritic cells (DCs) play a crucial role in linking the innate and adaptive arms of the immune system (1, 2). DCs recognize pathogens through Toll-like receptors (TLRs) (3), which have been well characterized in terms of cellular expression, specific ligands

(4), and signaling cascades. The role of TLR ligands in modulating DC functions has also been extensively studied (3, 5–7).

TLR2 is expressed by a variety of cells and recognizes lipopeptides. A TLR2/1 heterodimeric complex senses triacylated lipopeptides, whereas the TLR2/6 heterodimeric complex senses diacylated lipopeptides. Macrophage-activating lipopeptide–2 (MALP-2), a component of the cell membrane of *Mycoplasma fermentas*, is a TLR2/6 agonist (8). BPPcysMPEG (S-[2,3-bisphalmitoyloxy-(2R)-propyl]-R-cysteinyl-amido-monomethoxy polyethylene glycol), henceforth referred to as BPP, is a synthetic derivative of MALP-2, which exhibits improved pharmacokinetic properties. Our previous investigation with BPP revealed its potential as an efficient adjuvant for cross-presentation and cross-priming by DCs (6).

Th2 cells, characterized by their signature cytokine profile (e.g., IL-4, IL-5, and IL-13), play a significant role in promoting immunity against multicellular pathogens such as parasitic nematodes and in allergic responses (9–11). To study allergic Th2 responses, murine sensitization/challenge models for airway inflammation are favored (12), given that eosinophil infiltration is one of the hallmarks of Th2-mediated airway inflammation (13). Previous studies using such murine airway allergy models have suggested a role for TLR ligands in modulating airway eosinophilia and other Th2-dependent responses (14–17). The induction of indoleamine 2,3-dioxygenase (18), the impairment of lung DC migration (5), and the induction of regulatory T cells (Tregs) (19) by TLR ligands have been shown to impair allergic airway inflammation.

Specifically for TLR2 agonists, reduction of Th2 cytokines in bronchoalveolar lavage (BAL) and lungs has been proposed. However, simultaneous allergen and IFN- γ administration, as well as repetitive TLR agonist application, hampered a detailed cellular characterization of the mechanisms underlying the reduction of Th2 cytokines. This can probably be attributed to the differences in the models of allergic airway inflammation used in these studies (14, 20, 21).

In earlier studies, we demonstrated that BPP conjugated to antigenic peptides from chicken egg ovalbumin (BPP-OVA) was successful in inducing a robust helper-cell–dependent cytotoxic T lymphocyte response (6). Based on these findings, we aimed to study the effects of a single BPP-OVA application in modulating an existing Th2 response at the cellular level, using different murine models of allergic airway disease.

MATERIALS AND METHODS

Mice

Six- to eight-week-old female BALB/c, C57BL/6 (B6) and DO11.10 mice were purchased from Charles River Laboratories (Sulzfeld, Germany). TLR2-deficient mice were purchased from the Jackson Laboratory (Bar Harbor, ME). MyD88-deficient mice were kindly provided by Professor

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Correspondence and requests for reprints should be addressed to Georg M.N. Behrens, M.D., Department for Clinical Immunology and Rheumatology, Hannover Medical School, Carl-Neuberg Strasse 1, 30625 Hannover, Germany. E-mail: behrens.georg@mh-hannover.de

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Dr. Tim Sparwasser (Twincore, Hannover, Germany). Transgenic mice with ovalbumin-specific T cell receptors on CD⁺ T cells (OT-I mice) have been described previously (7), and were kindly provided by Dr. William Heath of the Walter Eliza Hall Institute of Medical Research (Melbourne, Victoria, Australia). BALB/cA-RAG2KO/IL2-R γ KO mice (recombination-activating gene 2 [RAG2]-deficient) were kindly provided by the Central Institute for Experimental Animals (Kawasaki, Japan). Mice were housed at the animal facility of the Medical School of Hannover (Hannover, Germany) under specific pathogen-free conditions, and all experimental procedures were performed according to a protocol approved by the appropriate governmental authority and ethics committees.

Antibodies and Antigens

CD11c (HL3), MHC II (2G9), CD86 (GL1), CD45RA (14.8), CD24 (ML5), CD172a (P84), CD4 (GK1.5), IFN- γ (XMG 1.2) (all from BD Pharmingen, Heidelberg, Germany), and IL-4 (11B11) (eBioscience, Natutec, Frankfurt am Main, Germany) antibodies were used. Neutralizing antibodies to IFN- γ (XMG 1.2) were purchased from Biolegend (Fell, Germany). Chicken egg ovalbumin (Grades V and VI) was obtained from Sigma (Diesenhofen, Germany), and endograde ovalbumin (endotoxin concentration, < 1 EU/mg) was obtained from Hyglos (Regensburg, Germany).

TLR Ligands

LPS from *Escherichia coli* serotype 026:B6 was purchased from Sigma. BPPcysMPEG (BPP), International Patent Classification A61K 47/48 (2006.01), Pub. No. WO/2007/059931, is a pegylated derivative of MALP-2. BPPcysOvaTMPEG (BPP-OVA) was synthesized at the Hemholtz Center for Infection Research by coupling a synthetic peptide encompassing the major histocompatibility complex (MHC) Class I (OVA_{257–264}) and II (OVA_{323–339})-restricted epitopes from OVA to BPP, as previously described (6). Other BPP constructs used included BPP-Pep I and BPP-Pep II, for which BPP was conjugated only to MHC Class I (OVA_{257–264}) or II (OVA_{323–339})-restricted epitopes from OVA, respectively.

Induction of Allergic Airway Inflammation and TLR Ligand Treatment of Mice

Two different murine models of allergic airway disease were used in this study. As described previously (5, 22), the classic sensitization model involved intraperitoneal injections of OVA (10 μ g, Grade VI) adsorbed to aluminum hydroxide (alum, 1.5 mg; Pierce Biotechnology, Rockford, IL) on Days 0, 14, and 21. On Day 27, mice were treated with BPP (1 μ g per mouse) or BPP-OVA (1 μ g per mouse) via the intranasal route under ketamine/rompun-induced anesthesia, followed by allergen challenge on Days 35 and 36 with aerosolized 1% OVA (Grade V). Control mice were challenged with PBS alone (Figure 1A) (5). For the adoptive transfer model (5, 23), antigen-specific Th2-biased DO11.10 cells were generated *in vitro*. Purified CD4⁺ T cells from DO11.10 mice were cocultured with enriched splenic DCs in complete RPMI medium containing IL-2 (10 U/ml), IL-4 (10 ng/ml), anti-IFN- γ (3 μ g/ml), and OVA_{323–339} peptide (1 μ g/ml; Biosyntan, Berlin, Germany). After 5 days of co-culture, cells were restimulated using the Leukocyte Activation Cocktail with BD GolgiPlug (BD Pharmingen) to analyze samples for the intracellular expression of IL-4 and IFN- γ . We adoptively transferred $3\text{--}5 \times 10^6$ Th2-biased cells intravenously into female BALB/c recipients. Twenty-four hours after the transfer, mice were treated with BPP (1 μ g per mouse) or BPP-OVA constructs (1 μ g per mouse each), as already described. Four days after treatment, mice were challenged twice on consecutive days with OVA dissolved in PBS (25 μ g/mouse, Grade V) via the intranasal route under ketamine/rompun-induced anesthesia. For inhibiting IFN- γ , 3 μ g of anti-IFN- γ antibodies were administered to mice (intraperitoneally) on Days 3 and 4 after the adoptive transfer of Th2-biased cells. Mice were killed with a lethal dose of ketamine/rompun, 24 hours after the final challenge (Figure 1B). BAL from each individual mouse was collected and analyzed.

Differential Cell Count from BAL

The total live cell number from individual BAL samples was estimated using trypan blue exclusion dye. Cells (1×10^4) in 100 μ l PBS were

cytospun, and the frequency of various leukocyte subsets (eosinophils, macrophages, neutrophils, and lymphocytes) was enumerated in BAL samples after staining with a Diff-Quik kit (Medion Diagnostics, Duedingen, Germany). In total, 200 cells were counted from random fields per sample.

Cytokine Analysis

Adoptively transferred Th2-biased cells (CD4⁺ KJ1.26⁺) were FACS-sorted from lungs and restimulated *in vitro* with OVA peptide (1 μ g/ml)-pulsed spleen DCs from BALB/c mice for 72 hours at 37°C. Cell-free supernatants were collected, and concentrations of IL-4, IL-5, IL-13, IL-12, and IFN- γ were estimated using Flowcytomix simplex kits (eBioscience, Vienna, Austria).

Statistical Analysis

All values are expressed as means plus standard errors of the mean (SEMs). One-way ANOVA with the Bonferroni *post hoc* test was performed using GraphPad Prism (version 5.0b, Mac OS X; GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Supplemental Information

For details regarding the generation of bone marrow-derived dendritic cells (BMDCs), the enrichment of DCs from lung tissue, mediastinal lymph nodes (LN), and spleen, *in vitro* and *in vivo* T-cell proliferation assays, and histology analyses, please refer to the online supplement.

RESULTS

BPP-OVA Acts via TLR2/6 on DCs and Leads to Efficient T-Cell Proliferation

Previous work established that the synthetic TLR2/6 agonist BPP exerted a receptor-dependent effect on DCs and led to phenotypic changes suggestive of maturation, such as the up-regulation of MHC Class II and costimulatory molecules (6). We first tested whether the synthetic BPP-OVA construct exerted similar effects on Flt-3-derived BMDCs from B6-deficient and TLR2-deficient mice, which can be discriminated into CD11c⁺ CD45RA⁺ plasmacytoid DCs and CD11c⁺ CD45RA[−] conventional DCs. These populations could be further separated into “CD8⁺ splenic DC equivalent” (CD24^{hi} CD172^{lo}) and “CD8[−] splenic DC equivalent” (CD24^{lo} CD172^{hi}) subsets (24). Flt-3 DC cultures were stimulated overnight with BPP-OVA, LPS, or CpG, and analyzed by FACS. The TLR2-mediated maturation of DCs by BPP-OVA, as determined by the up-regulation of CD86 and MHC Class II, was equivalent to that observed by stimulation with other TLR agonists, suggesting the functional integrity of the compound (Figure 2A). In addition, BPP-OVA stimulated Flt-3 DC secreted inflammatory cytokines, such as IL-6 and TNF- α , in a TLR2-dependent manner (Figure 2B). Similar experiments performed on Flt-3 DCs derived from BALB/c mice and BPP-OVA treatment led to the up-regulation of MHC Class II and CD86, as well as the production of IL-6 and TNF- α , as previously observed (*see* Figure E1 in the online supplement). The intranasal administration of BPP-OVA to B6- and TLR2-deficient mice led to the up-regulation of MHC Class II and CD86 in lung DCs and mediastinal LN DCs within 6 hours in a TLR2-dependent manner (Figure 2C). These results confirm the functional influence of BPP-OVA conjugate on DCs via TLR2.

To better assess the effects of BPP-OVA-treated DCs for their ability to induce T-cell proliferation, lung DCs (Figures 3A and 3B) and splenic DCs (data not shown) were sorted, pulsed with BPP-OVA, OVA plus BPP, or OVA alone, and cocultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled OT-I cells (Figure 3A) or DO11.10 cells (Figure 3B) *in vitro*. BPP-OVA-pulsed lung and splenic DCs from B6 mice induced

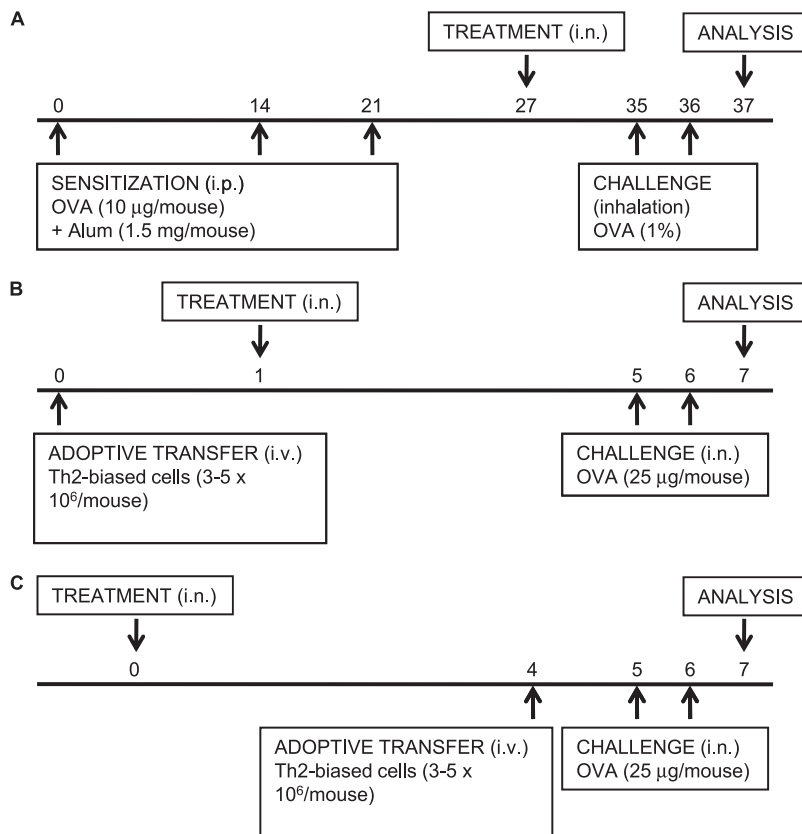


Figure 1. Schematic representation of the study design. (A) BALB/c mice were sensitized on Days 0, 14, and 21 with 10 µg of ovalbumin (OVA) adsorbed to 1.5 mg of alum. On Day 27, mice were treated with a single intranasal (i.n.) dose of (S-[2,3-bisphalmitoyloxy-(2R)-propyl]-R-cysteiny]-amido-monomethoxy polyethylene glycol (BPP)-OVA or BPP (1 µg/mouse). On Days 35 and 36, treated mice were challenged by inhalation with OVA (1%) and analyzed on Day 37. Control mice were left untreated and were challenged with either OVA (positive control) or PBS (negative control). (B) We adoptively transferred $3\text{--}5 \times 10^6$ Th2-biased cells into naive BALB/c mice, which were treated the following day with BPP or BPP conjugates, or a mixture of BPP and antigen. On Days 5 and 6, treated mice were challenged with OVA (25 µg/mouse, i.n.) and analyzed on Day 7. Control mice were left untreated and were challenged with either OVA (positive control) or PBS (negative control). (C) Modified adoptive transfer model to study the impact of BPP-OVA on endogenous cell populations. Naive BALB/c mice were pretreated with BPP-OVA or BPP (1 µg/mouse). Three days after treatment, $3\text{--}5 \times 10^6$ Th2-biased cells were adoptively transferred into treated and control mice. On Days 5 and 6, mice were challenged with OVA (25 µg/mouse, i.n.), and were analyzed on Day 7. Control mice were left untreated and were challenged with either OVA (positive control) or PBS (negative control). i.p., intraperitoneal; i.v., intravenous.

more OT-I cell proliferation, as compared to DCs pulsed with a mixture of OVA and BPP. This increase was markedly reduced when DCs from MyD88-deficient mice were used. Although the proliferation of DO11.10 cells induced by BALB/c splenic DCs was clearly increased by BPP-OVA compared with OVA alone (data not shown), no major differences were observed with lung DCs (Figure 3B). To validate these observations *in vivo*, mice received BPP-OVA or OVA intranasally 1 day after the adoptive transfer of 3×10^6 CFSE-labeled OT-I cells (B6 recipients) or DO11.10 cells (BALB/c recipients). Flow cytometric analysis showed that BPP-OVA treatment enhanced OT-I (Figure 3C) and DO11.10 (Figure 3D) cell proliferation in the mediastinal LN. BPP-OVA administration (intranasal) to TLR2-deficient mice induced significantly less OT-I proliferation in mediastinal LN as compared to B6 mice (Figure E2). We conclude that BPP-OVA can be used to deliver antigen and maturation signals to lung DCs in a TLR2-dependent manner, thereby inducing robust antigen-specific T-cell proliferation.

BPP-OVA Reduced Eosinophilic Airway Inflammation

To investigate the effects of the combined administration of a TLR-2 agonist and antigen on allergic airway inflammation, mice were sensitized to OVA in the presence of alum on Days 0, 14, and 21 (Figure 1A). Before the inhalative OVA challenge, mice were treated intranasally with either BPP-OVA or BPP alone. The analysis of BAL fluid revealed a significant reduction in the total cellular infiltration and in eosinophil numbers in mice treated with BPP-OVA (Figure 4A). Treatment with BPP alone exerted no effect in alleviating airway eosinophilia.

To elucidate potential mechanisms, we used a primarily T cell-dependent model of allergic airway disease (Figure 1B). We used *in vitro*-generated OVA-specific Th2-biased cells that expressed IL-4 but no IFN-γ, confirming their Th2-biased phenotype (Figure E3). The adoptive transfer (intravenous) of

these cells, followed by an intranasal challenge with OVA, led to eosinophilic airway inflammation in recipient mice, as observed in the sensitization model (5). One day after the transfer of Th2-biased cells, mice were treated intranasally with BPP plus OVA protein, BPP plus OVA_{257–264} and OVA_{323–339} peptides, or BPP-OVA. Four days after treatment, mice were subjected to intranasal allergen challenges for 2 consecutive days. BAL analysis revealed a significant reduction in airway eosinophilia after BPP-OVA treatment (Figure 4B), as observed in the sensitization model. Treatment with BPP plus OVA protein showed a partial reduction in airway eosinophil numbers, with a concomitant enhancement of lymphocytes in the BAL (Figure 4B), which is in line with previous investigations (14). Furthermore, histologic analyses of the lungs revealed that the administration of BPP-OVA in both the sensitization (Figure 5A) and the adoptive transfer (Figure 5B) models led to a reduction of total cellular infiltration. Taken together, our results suggest that treatment with BPP-OVA directly affects the phenotype and/or function of the Th2-biased cells, and thereby ameliorates allergic airway eosinophilia.

Effect of BPP-OVA on Allergen-Specific Th2-Biased Cells

In a T cell-dependent transfer model, the *in vivo* administration of BPP-OVA could induce an immune response from endogenous cell populations of recipient mice (e.g., CD8⁺ T cells and Tregs), which in turn could influence the response of transferred Th2-biased cells. To address this, we modified the adoptive transfer model (Figure 1C) to distinguish between the effects of BPP-OVA on either the endogenous cell populations or the transferred Th2-biased cells. For this, BALB/c mice were pretreated intranasally with either BPP-OVA or BPP alone. Three days after treatment, Th2-biased cells were adoptively transferred intravenously into these mice, followed by allergen challenge. The BAL analysis revealed no reduction in airway eosinophilia upon BPP-OVA pretreatment (Figure 6A). These results strongly suggest

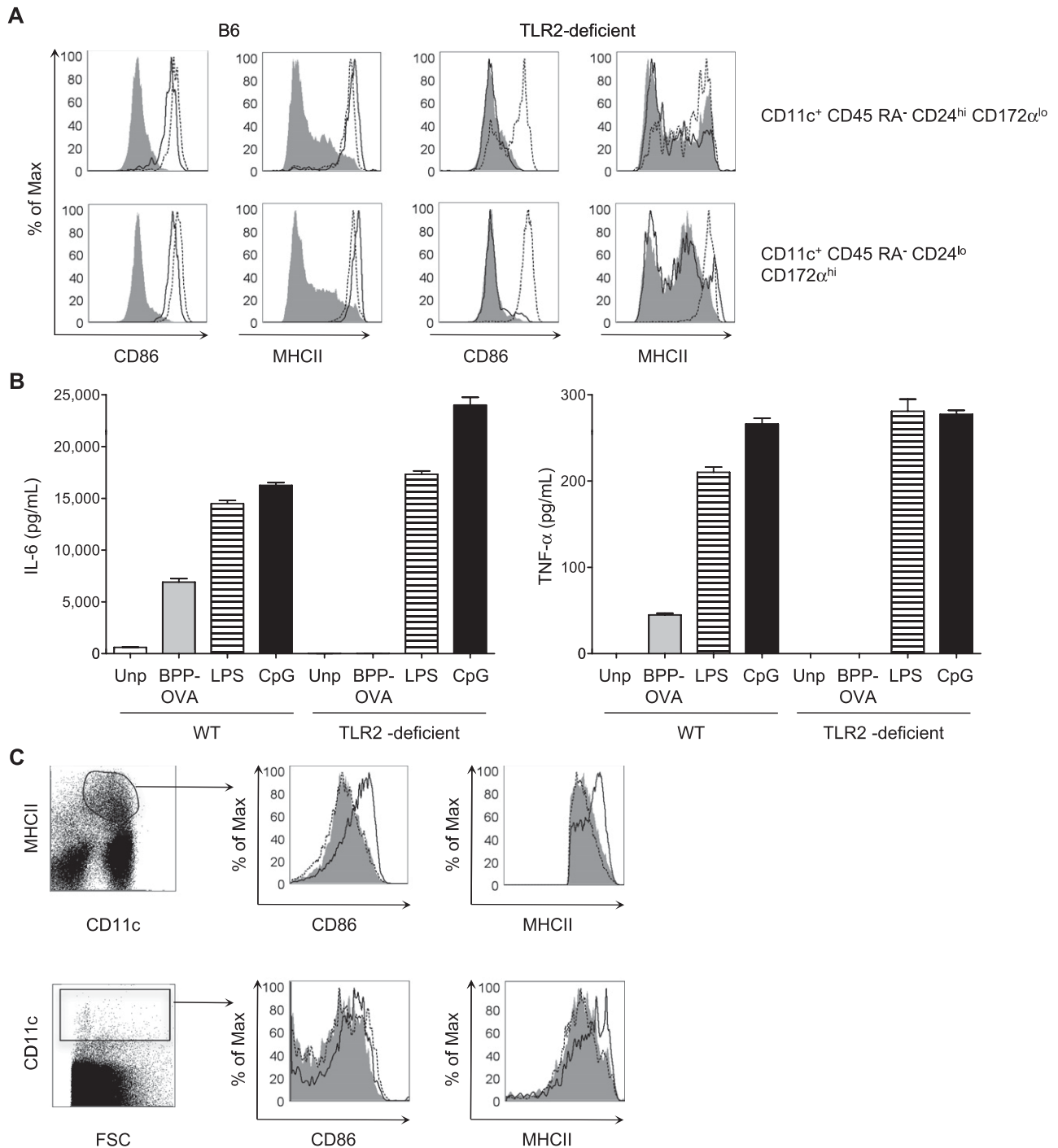


Figure 2. Maturation of bone marrow-derived dendritic cells (BMDCs) and lung dendritic cells (DCs) after Toll-like receptor (TLR) stimulation. (A) Surface expression of CD86 and major histocompatibility complex (MHC) Class II on Flt-3-derived BMDCs from B6-deficient and TLR2-deficient mice, after overnight treatment with BPP-OVA (solid lines, 1 μ g/ml) or LPS (dotted lines, 1 μ g/ml). Untreated Flt-3-derived BMDCs served as negative controls (gray histograms). Upper panels represent the CD8⁺ splenic DC equivalent (CD11c⁺ CD45 RA⁻ CD24^{hi} CD172α^{lo}) subset, whereas lower panels represent the CD8⁻ splenic DC equivalent (CD11c⁺ CD45 RA⁻ CD24^{lo} CD172α^{hi}) subset. (B) Flt-3-derived BMDCs from B6-deficient and TLR2-deficient mice were stimulated *in vitro* with BPP-OVA (1 μ g/ml), LPS (1 μ g/ml), or CpG (5 nmol/ml) for 18 hours, and cell-free supernatants were analyzed for cytokine production using a cytokine bead array. Untreated Flt-3-derived BMDCs (Unp) served as negative controls. The results obtained are representative of two independent experiments. (C) B6-deficient (solid lines) or TLR2-deficient (dotted lines) mice ($n = 3$) were treated with a single intranasal dose of BPP-OVA (1 μ g/mouse). Six hours later, lung DCs (upper panel) and mediastinal lymph node DCs (lower panel) were analyzed by flow cytometry for the expression of CD86 and MHC Class II. Gray histograms represent untreated mice. FSC, forward scatter; WT, wild-type.

that BPP-OVA exerted little or no impact on the endogenous population, and any response so generated did not inhibit the function of transferred Th2-biased cells.

Alternatively, we used RAG2-deficient recipient mice for adoptive transfer experiments (Figure 1B). As these mice lack an endogenous CD4⁺ T-cell repertoire, any reduction in Th2

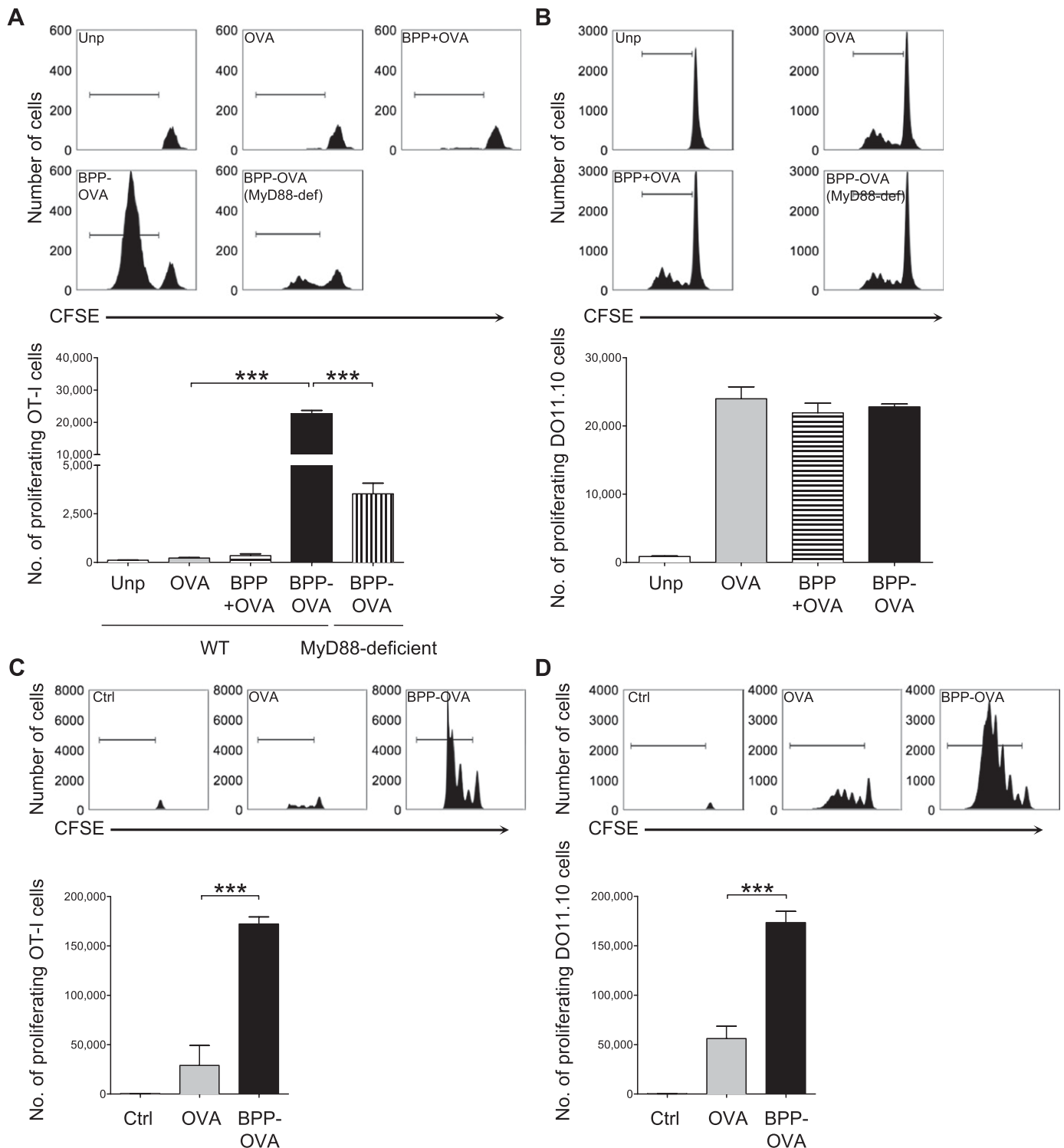


Figure 3. BPP-OVA treatment enhances antigen presentation by DCs. (A and B) *In vitro* antigen presentation. (A) Sorted lung DCs (1×10^4) from B6-deficient and MyD88-deficient mice were pulsed *in vitro* with BPP-OVA (1 μ g/ml), BPP (1 μ g/ml) plus OVA (100 μ g/ml), or OVA alone (100 μ g/ml), and were used to stimulate 5×10^4 carboxyfluorescein succinimidyl ester (CFSE)-labeled transgenic mice with ovalbumin-specific T cell receptors on CD4⁺ T cells (OT-I mice) cells. (B) A similar experiment, except that antigen-pulsed sorted lung DCs from BALB/c mice were used to stimulate DO11.10 cells *in vitro*. Unpulsed DCs (Unp) served as negative controls. The number of proliferating OT-I or DO11.10 cells (CFSE^{low}) were determined using flow cytometry after 65 hours of coculture. Data represent the means $\pm$ SEMs of triplicate measurements for each condition ($n = 3$; *** $P < 0.001$). (C and D) *In vivo* antigen presentation. We adoptively transferred 3×10^6 CFSE-labeled OT-I (C) or DO11.10 (D) cells into naive B6 or BALB/c mice, respectively, which were then treated with a single i.n. dose of BPP-OVA (1 μ g/mouse) or OVA alone (25 μ g/mouse). Untreated mice (Ctrl) were used as negative controls. The number of proliferating OT-I or DO11.10 cells (CFSE^{low}) from mediastinal lymph nodes of treated mice were evaluated using flow cytometry after 60 hours. Upper panels show representative CFSE proliferation profiles, and lower panels show the quantitative data of proliferated (CFSE^{low}) cells. Data are shown as means $\pm$ SEMs, and are representative of two independent experiments ($n = 3$ mice per group, pooled analysis; *** $P < 0.001$).

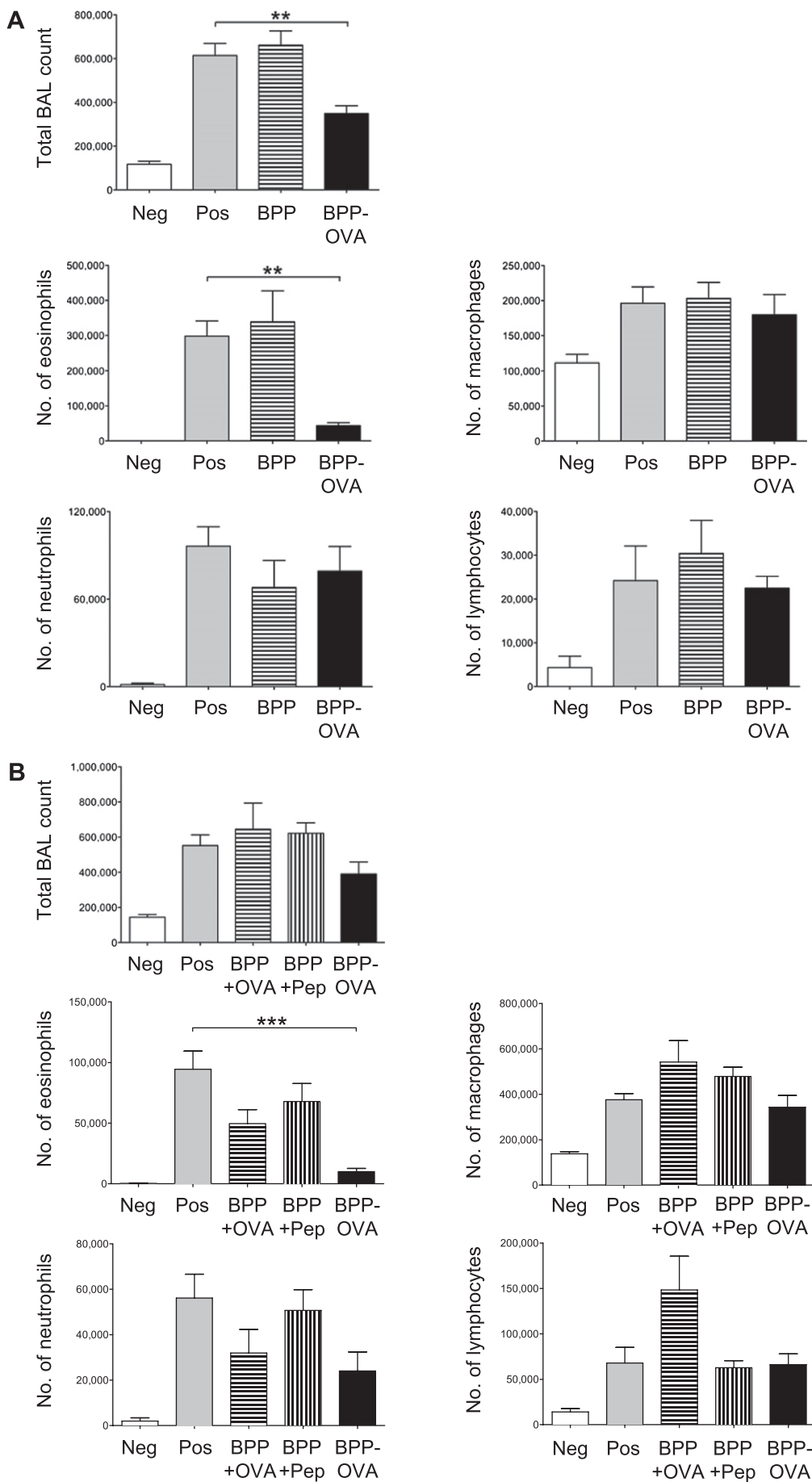


Figure 4. BPP-OVA treatment impairs allergic airway inflammation in mice. (A). BALB/c mice were sensitized to OVA and alum on Days 0, 14, and 21, and challenged by inhalation with OVA on Days 35 and 36. A week before the first challenge, mice were treated with a single i.n. dose of BPP-OVA or BPP (1 μ g/mouse). Control mice were not treated, and were challenged with either OVA (Pos, positive control) or PBS (Neg, negative control). Panels represent total leukocytes and numbers of infiltrating leukocyte subpopulations from the bronchoalveolar lavage (BAL) fluid. (B) We adoptively transferred $3-5 \times 10^6$ Th2-biased cells into naive BALB/c mice, which were treated a day later with BPP-OVA (1 μ g/mouse, i.n.), BPP (1 μ g/mouse, i.n.) plus OVA (25 μ g/mouse, i.n.), or BPP plus OVA₂₅₇₋₂₆₄ and OVA₃₂₃₋₃₃₉ peptides (1 μ g/mouse each, i.n.). Three days after treatment, mice were challenged twice on consecutive days with OVA (25 μ g/mouse, i.n.). Untreated mice were either challenged with PBS (Neg, negative control) or with OVA (Pos, positive control). Panels represent total leukocytes and numbers of infiltrating leukocyte subpopulations from the BAL fluid. Data represent means $\pm$ SEMs, and constitute the pooled analysis of two independent experiments ($n = 9-10$ mice per group; $**p < 0.01$ and $***p < 0.001$).

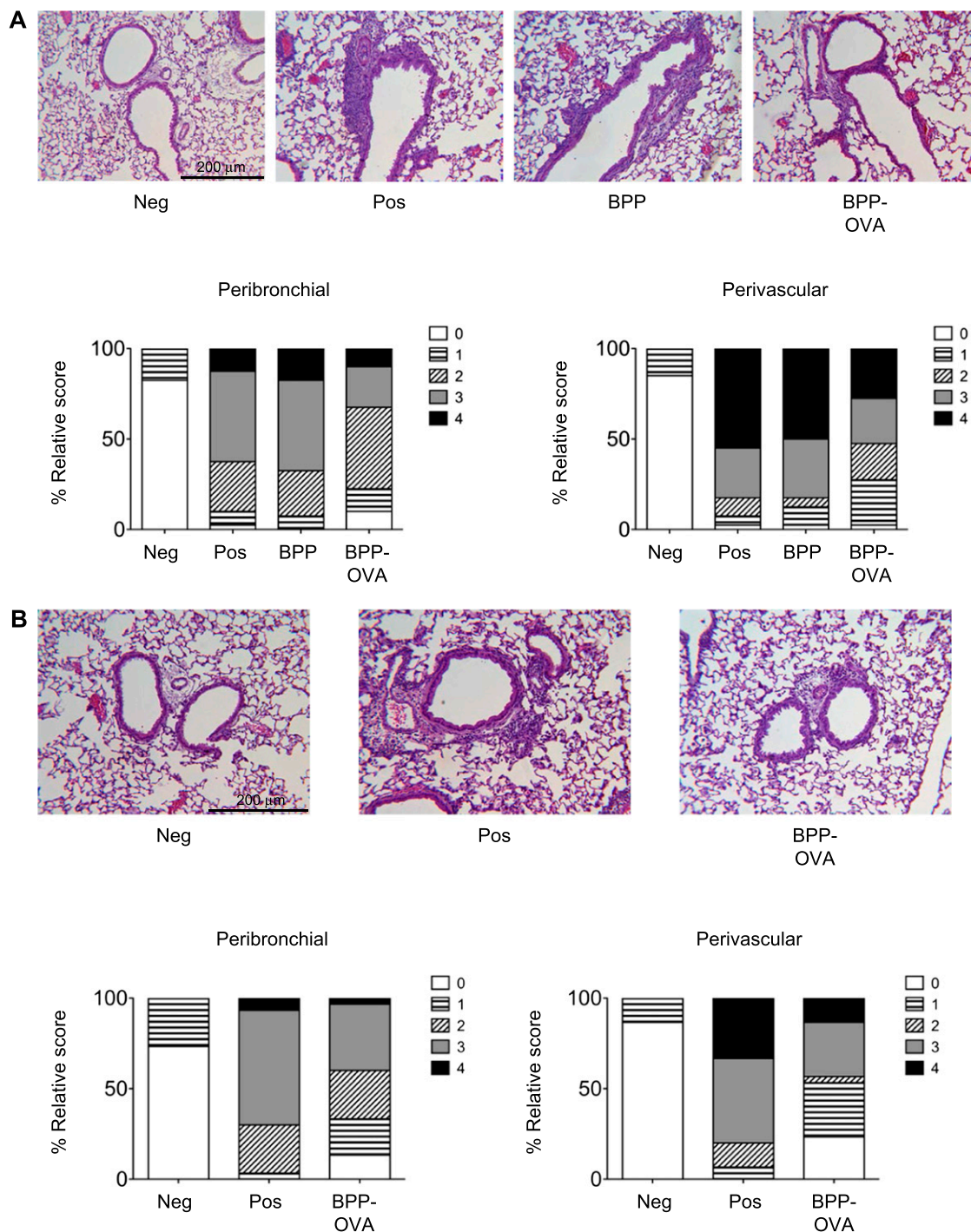


Figure 5. Total cellular infiltration in lung tissue. (A) BALB/c mice were sensitized using OVA and alum (10 μ g per mouse and 1.5 mg per mouse, respectively), were treated with either BPP (1 μ g/mouse) or BPP-OVA (1 μ g/mouse), and were challenged with OVA (1%, via inhalation), as described in Figure 1A. Control mice remained untreated and were challenged with either OVA (positive control) or PBS (negative control). (B) We adoptively transferred $3-5 \times 10^6$ Th2-biased cells into naive BALB/c mice, which were treated a day later with BPP-OVA (1 μ g/mouse, i.n.) and then challenged with OVA (25 μ g/mouse, i.n.), as described in Figure 1B. Control mice were left untreated, and were challenged with either OVA (positive control) or PBS (negative control). *Upper panels* represent lung histology analyses (magnification, $\times 100$; hematoxylin and eosin staining), whereas *lower panels* show relative scores for respective groups (0, normal; 1, a few cells; 2, a ring of inflammatory cells one layer deep; 3, a ring of inflammatory cells two to four layers deep; 4, a ring of inflammatory cells more than four cells deep). Data are representative of two independent experiments ($n = 3-4$ mice per group).

responses by BPP-OVA would be attributed to its direct impact on the transferred Th2-biased cells. After the adoptive transfer of Th2-biased cells, RAG2-deficient mice were treated with BPP-OVA, followed by allergen challenge. As with the BALB/c mice, we observed a strong reduction in airway eosinophilia in RAG2-deficient mice that had been treated with BPP-OVA (Figure 6B).

Furthermore, the direct *in vitro* stimulation of Th2-biased cells with BPP-OVA did not lead to T-cell activation, implying that the observed effects on Th2-biased cells required antigen presentation (data not shown).

The BPP-OVA construct also included a MHC Class I-restricted OVA epitope. To exclude bystander effects mediated by CD8⁺ T

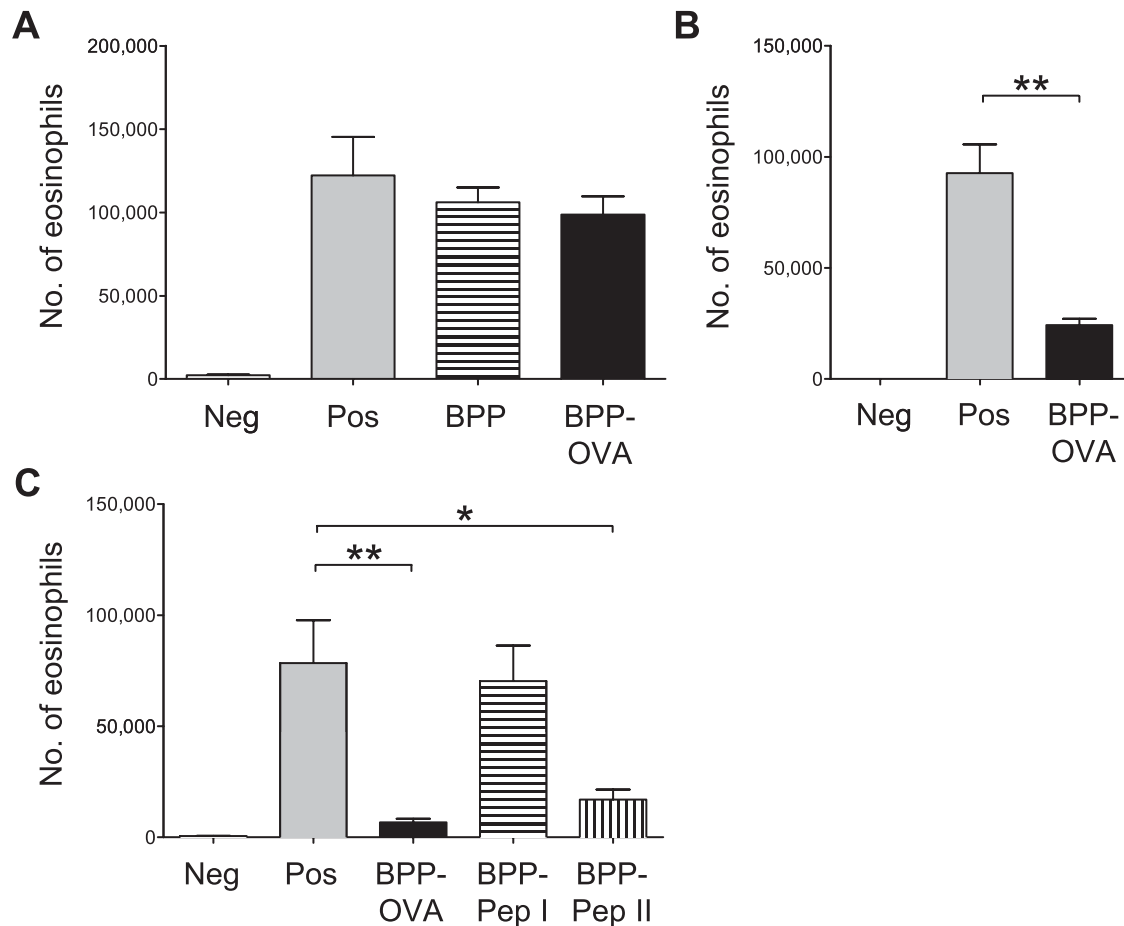


Figure 6. BPP-OVA treatment primarily affects antigen-specific Th2-biased cells. (A) BALB/c mice were treated with BPP-OVA (1 μ g/mouse, i.n.) or BPP (1 μ g/mouse, i.n.), or were left untreated. Three days after treatment, $3-5 \times 10^6$ Th2-biased cells were adoptively transferred into mice, and were challenged consecutively every day for 2 days with OVA (25 μ g/mouse, i.n.). Untreated mice were challenged with either PBS (Neg, negative control) or OVA (Pos, positive control). (B) We adoptively transferred $3-5 \times 10^6$ Th2-biased cells into recombination-activating gene 2 (RAG2)-deficient mice, which were treated a day later with BPP-OVA (1 μ g/mouse, i.n.) and challenged twice on Days 5 and 6 with OVA (25 μ g/mouse, i.n.). Untreated mice were challenged with either PBS (Neg, negative control) or OVA (Pos, positive control). (C) We adoptively transferred $3-5 \times 10^6$ Th2-biased cells into BALB/c mice, which were then treated with different BPP constructs the following day. Treatment groups included BPP-OVA (1 μ g/mouse, i.n.), MHC Class I-restricted epitope (BPP-Pep I; 1 μ g/mouse, i.n.), and BPP-Pep II (1 μ g/mouse, i.n.). Mice were challenged twice on Days 5 and 6 with OVA (25 μ g/mouse, i.n.). Untreated mice were challenged with either PBS (Neg) or OVA (Pos). All panels represent numbers of infiltrating eosinophils in the BAL. Data are shown as means $\pm$ SEMs, and are representative of two independent experiments ($n = 3-5$ mice per group, * $P < 0.05$ and ** $P < 0.01$).

cells, we used two other BPP constructs in which BPP was either conjugated only to an MHC Class I-restricted epitope (BPP-Pep I) or Class II-restricted epitope (BPP-Pep II) of OVA. If a strong CD8⁺ T cell response was sufficient to reduce airway eosinophilia, then treatment with BPP-Pep I would be able to inhibit the Th2-biased response. Th2-biased cells were adoptively transferred intravenously into BALB/c mice, which were then treated intranasally with BPP-OVA, BPP-Pep I, or BPP-Pep II and subsequently challenged (Figure 1B). BAL analysis revealed that, unlike BPP-OVA, BPP-Pep I did not ameliorate airway eosinophilia. Moreover, BPP-Pep II alleviated airway eosinophilia (Figure 6C), implying that the effect on Th2-biased cells was primarily a CD4⁺ T cell-dependent response.

BPP-OVA Treatment Leads to IFN- γ Production from Th2-Biased Cells

To determine the effects of BPP-OVA treatment on Th2-biased cells, we adoptively transferred these cells into BALB/c mice, followed by an intranasal administration of BPP-OVA or BPP alone. Three days after treatment, Th2-biased cells (CD4⁺

KJ1.26⁺) were purified from the lungs (Figure 7A) and were restimulated *in vitro*, as already described. BPP-OVA treatment did not affect the numbers of Th2-biased cells in lung tissue (data not shown). Cytokine analysis of cell-free supernatants (Figure 7B) revealed that the Th2-biased cells isolated from BPP-OVA-treated mice produced significantly higher amounts of IFN- γ , compared with cells from untreated mice. IL-5 production was highly decreased, which was in agreement with the decreased levels of airway eosinophilia. Th2-biased cells were not found to be hyporesponsive as a result of excessive antigen stimulation, because the concentrations of IL-4 and IL-13 remained relatively unchanged. Treatment with BPP alone did not lead to either a decrease of IL-5 or an increase in IFN- γ secretion by Th2-biased cells. This result is in line with observations that BPP alone did not alleviate airway eosinophilia. To validate whether the BPP-OVA-induced IFN- γ production by Th2-biased cells was crucial in impairing airway eosinophilia, we treated mice with anti-IFN- γ antibodies before airway challenges. Th2-biased cells were adoptively transferred into BALB/c mice, followed by BPP-OVA treatment as before (Figure 1B). Anti-IFN- γ antibodies were administered on Days 3 and 4 before antigen challenge.

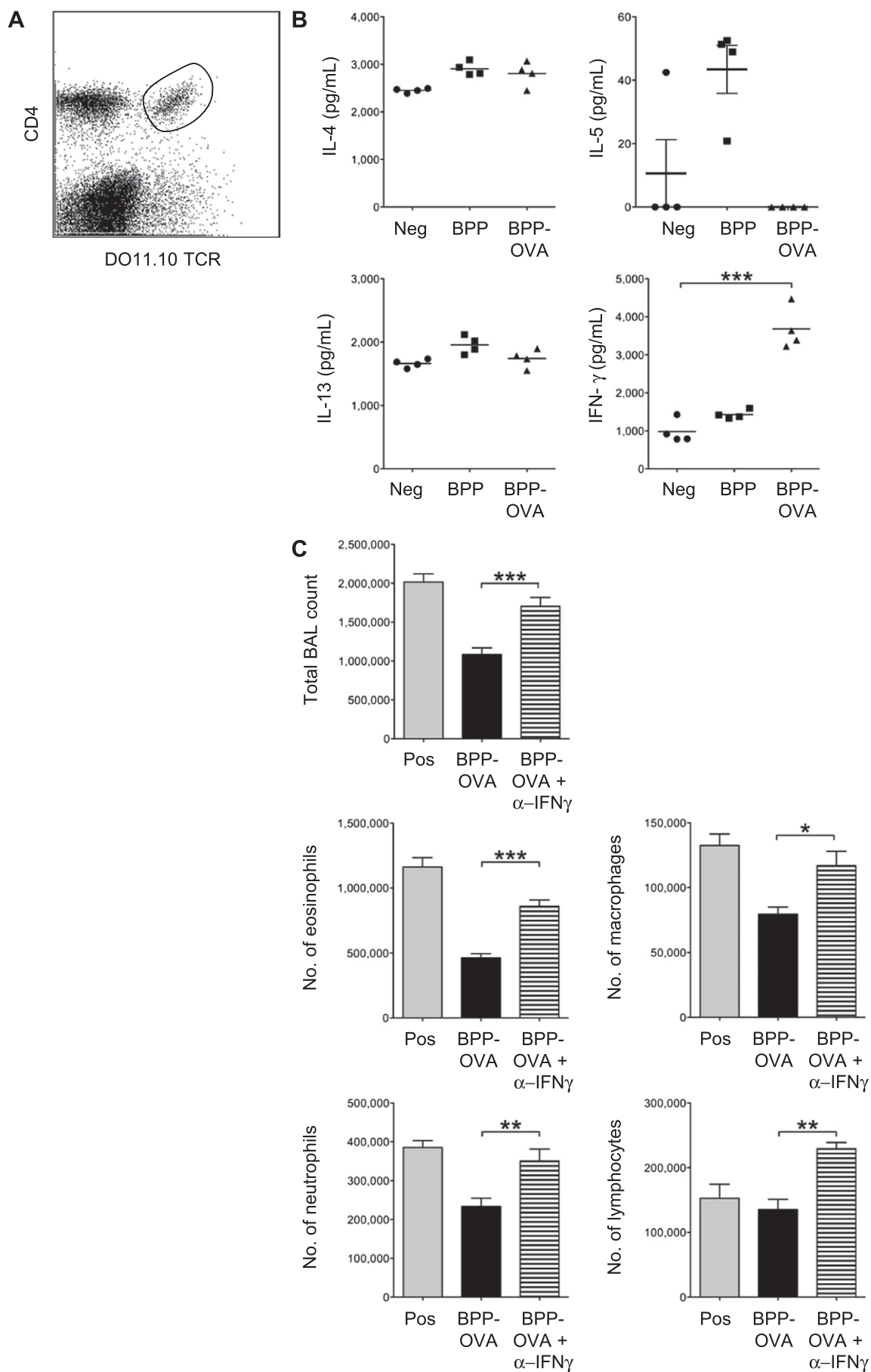


Figure 7. IFN- γ production and reduced IL-5 production by Th2-biased cells after BPP-OVA treatment play a role in reducing airway eosinophilia. (A) We adoptively transferred $3\text{--}5 \times 10^6$ Th2-biased cells into BALB/c mice, which were treated the following day with BPP-OVA (1 $\mu\text{g}/\text{mouse}$, i.n.) or BPP (1 $\mu\text{g}/\text{mouse}$, i.n.), or were left untreated (Neg, negative control). Three days after treatment, lungs of each group were pooled and digested. Th2-biased cells could be sorted from the lung tissue, as shown in a representative flow cytometric plot. (B) Sorted Th2-biased cells were restimulated *in vitro* with OVA_{323–339} peptide pulsed splenic DCs for 72 hours. Cell-free supernatants were analyzed for IL-4, IL-5, IL-13, and IFN- γ , using cytokine bead array. Data are shown as means $\pm$ SEMs, and are representative of two independent experiments ($n = 4$; *** $P < 0.001$). (C) We adoptively transferred $3\text{--}5 \times 10^6$ Th2-biased cells into naive BALB/c mice, which were treated a day later with BPP-OVA (1 $\mu\text{g}/\text{mouse}$, i.n.). On Days 3 and 4, mice either received 3 μg of anti-IFN- γ antibodies ($\alpha\text{-IFN-}\gamma$) i.p., or were left untreated. Positive control mice (Pos) were not treated with BPP-OVA and anti-IFN- γ antibodies. Mice were challenged twice on Days 5 and 6 with OVA (25 $\mu\text{g}/\text{mouse}$, i.n.). Panels represent total leukocytes and numbers of infiltrating leukocyte subpopulations from the BAL fluid. Data are shown as means $\pm$ SEMs, and represent a pooled analysis of two independent experiments ($n = 10$ mice per group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Anti-IFN- γ antibody treatment completely reversed the ability of BPP-OVA to reduce airway eosinophilia (Figure 7C). These results strongly suggest that induced IFN- γ production and a concomitant decrease in IL-5 production from previously Th2-biased cells could contribute to the alleviation of allergic airway disease.

DISCUSSION

By using BPP-OVA, we demonstrated that TLR2 activation, with concomitant antigen delivery in the form of a conjugate to its ligand, resulted in redirecting an existing Th2 response to a Th1 phenotype. These data provide insights into the functional diversity and plasticity of primed T-cell responses for therapeutic interventions. A previous study by Knothe and colleagues explored the impact of BPP administration in a murine model of allergic airway disease (14). Seven repetitive doses of BPP along with allergen in sensitized mice did not decrease either airway eosinophilia or concentrations of Th2 cytokines in BAL, compared with mock-treated mice. Only in comparison to mice with amplified airway inflammation were some effects observed. Although no IFN- γ was detected in the BAL, the authors reported that the administration of BPP and allergen diminished the expression levels of both IL-4 and IFN- γ in total mRNA isolated from mediastinal LN cells. In contrast, our study clearly demonstrated that a single dose of BPP-OVA as a construct could significantly diminish airway eosinophilia as well as Th2 cytokines, whereas BPP was ineffective. We speculate that the discrepancies observed in these studies are attributable to major differences in the models used, doses administered, and most importantly the use of BPP-OVA in the form of a conjugate. Our model system was designed to better define the immunoregulatory effects of BPP and BPP-OVA at a cellular level.

Several reasons led us to favor BPP instead of MALP-2. First, BPP exhibits improved solubility and pharmacokinetic properties, which made it a more attractive candidate as a mucosal adjuvant. Second, because it is chemically synthesized, it is endotoxin-free and additionally offers the possibility of directly linking antigenic epitopes to it during synthesis.

Lung DCs play a crucial role not only for sensitization to inhaled antigens, but also in the maintenance of allergic inflammation (25, 26). Upon TLR ligation, lung DCs up-regulate several costimulatory molecules on their surface, and also secrete various cytokines. Both properties have been used to characterize DC maturation status (27, 28). The intranasal application of BPP-OVA led to the activation of lung DCs and mediastinal lymph node DCs. In addition to direct DC activation, indirect effects mediated by factors such as granulocyte/macrophage colony-stimulating factor and thymic stromal lymphopoietin, as produced by airway epithelial cells, may have facilitated these changes (27, 29). Our results using BMDCs and TLR2-deficient mice strongly suggest that BPP exerts a direct effect on lung and mediastinal lymph node DC activation.

BPP-OVA significantly enhanced the antigen presentation capacity of lung DCs and splenic DCs *in vitro*. We also showed that, for the cross-presentation of soluble antigen, DCs pulsed with a mixture of antigen and BPP were not as efficient as DCs pulsed with BPP-OVA, and these findings are similar to those observed for CpG-OVA conjugates (30). We hypothesize that the chemical coupling ensures that the TLR2 signal and antigen are delivered in the same endocytic compartment, which would result in optimized and enhanced antigen presentation and T-cell stimulation (31). Similarly, Blander and Medhizitov have also shown that the efficiency of antigen presentation is dependent on TLR signaling, accompanying the antigen (32).

Using TLR4 agonists such as LPS, the direct activation of lung DCs was demonstrated to be essential for efficient antigen presentation and the subsequent priming of T cells in mediastinal lymph nodes (29). In line with these investigations, we showed that the local application of BPP-OVA led to efficient antigen presentation via MHC Class I and Class II to antigen-specific T cells in a TLR2-dependent manner. The application of the TLR 9 agonist CpG to sensitized mice before allergen challenge can reduce airway eosinophilia, an effect partly attributable to reduced DC migration (5). However, this does not hold true in the case of BPP application, as we and others (17) did not observe reduced airway inflammation when MALP-2 or BPP was administered alone before challenge. Furthermore, we could not find evidence for impaired lung DC migration to draining lymph nodes (data not shown).

We used different murine models of allergic airway disease, in which antigen-specific Th2-biased T-cell responses were generated either by sensitization or by the adoptive transfer of Th2-biased T cells (12). In the case of allergic airway disease, lung DCs play an important role in stimulating an efficient effector function in antigen-specific Th2 cells. They also aid in the recruitment of effector Th2 cells to the lung via the secretion of chemokines such as CCL17 and CCL22 (27, 33). TLR agonists could potentially modulate the immunostimulatory capacity of lung DCs, which in turn might affect the function of Th2-biased cells (34). Low doses of LPS, a TLR4 agonist, were found to be essential in inducing Th2 responses to inhaled antigen, whereas higher doses of LPS were found to exert a suppressive effect (35, 36). CpG suppressed airway allergic responses not only by affecting the migration of lung DCs to draining LN, but also by impairing their T-cell stimulation activity (5).

Using a sensitization model of allergic airway disease, we observed that a single dose of BPP-OVA was effective in alleviating airway eosinophilia. However, BPP-OVA treatment did not seem to affect other parameters of allergic airway inflammation such as the production of mucus (data not shown). In later experiments, we also showed that BPP-OVA administration did not impair IL-13 production by Th2-biased cells, which may explain the observed results. Because the sensitization model could involve various other cell types, we used the adoptive transfer model, which would potentially register only the

influence on Th2-biased cells. Treatment with BPP-OVA seems to exert a rate-limiting effect on the function of Th2-biased cells, because we observed a similar reduction in airway eosinophilia. Similar results were obtained using lipoprotein I, a TLR2/4 agonist, which alleviated allergic airway disease when coadministered with the antigen (16). Revets and colleagues concluded that lipoprotein I induced adaptive Th1 responses by modulating antigen-presenting cell (APC) function (16). The role of TLR2 with respect to Th2 responses has been widely debated, because paradoxical effects have been observed with TLR2 agonists such as Pam3Cys and peptidoglycan (34). Using a murine model of allergic airway disease, it was observed that either PAM3Cys (37) or peptidoglycan (38), administered at the time of sensitization, exacerbated Th2 responses. On the other hand, when these agonists were administered before sensitization, they alleviated airway inflammation (39). Furthermore, the administration of BPP and IFN- γ in combination, but not alone, was demonstrated to alleviate airway eosinophilia in both a classic OVA model (17) and a chronic model of airway hypersensitivity using ragweed pollen antigen (21).

We observed that the administration of BPP-OVA to naive mice led to a strong CTL response, which was T helper cell-dependent. Based on these results, we would expect that the local administration of BPP-OVA to mice could also affect the endogenous CD4⁺ T-cell population. Contrary to this hypothesis, by using T cell-deficient mice or administering BPP-OVA before T-cell transfer, we observed that the responses from the endogenous population exerted little or no impact on Th2-biased cells. However, some impact cannot be entirely ruled out, because the mice were challenged 3 days after treatment, and this time period may not have been sufficient for the generation of a robust Th1 response from a naive CD4⁺ T-cell population. However, our data using RAG-deficient mice strongly favor the hypothesis that the effect is directly exerted on antigen-specific Th2-biased cells. In addition, the BPP-Pep II construct was sufficient to mitigate the Th2 response, hence suggesting that a CTL response played little or no role in the observed decrease in airway eosinophilia in our model. We could also infer from these results that T cell receptor engagement was crucial, because treatment with the BPP-Pep I construct could not alleviate airway eosinophilia, thereby providing evidence that the inhibitory effect of BPP-OVA treatment was antigen-specific. Th2-biased cells were also found to express very low concentrations of TLR2, and BPP-OVA-treated Th2-biased cells, unlike DCs, could not stimulate naive CD4⁺ T cells (*data not shown*). Thus, we may infer that the effects of BPP-OVA treatment on Th2-biased cells were dependent on professional APCs such as lung DCs.

Tregs were shown to inhibit the effector function of Th2-biased cells. In a murine model of allergic airway disease, sublingual immunotherapy with OVA allergen and PAM3CSK4, a TLR2 agonist, led to an enhanced Th1/Treg phenotype. The investigators demonstrated that PAM3CSK4-treated DCs induced IFN- γ and IL-10 production from CD4⁺ T cells (19). Contrary to these results, other investigators demonstrated that PAM3CSK4 treatment suppressed airway inflammation by promoting a Th1 response independent of regulatory T cells (40). In the present investigation, the percentage of Forkhead box P3 cells among Th2-biased cells ranged between 10% and 15%, and remained unchanged even upon BPP-OVA treatment, compared with control samples (Figure E4). Furthermore, because we did not detect IL-10 or IL-17 in cultures of restimulated Th2-biased cells, we believe that neither Tregs nor Th17 responses are induced upon BPP-OVA treatment.

The reduced airway eosinophilia after BPP-OVA treatment could be explained by an altered phenotype of Th2-biased cells. The inhibition of IL-5 production by Th2-biased cells could

explain the reduced airway eosinophilia and hence reduced allergic airway disease. Among Th2 cytokines, IL-5 plays a major role in the proliferation, differentiation, and activation of eosinophils (41). Hogan and colleagues clearly demonstrated that IL-5 production by CD4⁺ T cells was sufficient for the development of airway eosinophilia (42). The same group, in another investigation, also demonstrated that IL-5 not only regulated eosinophilia in the airways and bloodstream, but also regulated the eotaxin-mediated chemotaxis of eosinophils in the lungs (43). In line with these findings, we demonstrated that Th2-biased cells isolated from the lungs of BPP-OVA-treated mice showed significantly diminished concentrations of IL-5, providing a plausible explanation for the decreased airway eosinophilia.

We also demonstrated that antigen-specific Th2-biased cells from lungs of BPP-OVA-treated mice produced significantly higher concentrations of IFN- γ , compared with control mice. Inhibiting IFN- γ with specific antibodies reversed the ameliorated eosinophilic inflammation upon BPP-OVA treatment. These results indicate that the induction of IFN- γ plays a pivotal role in the mode of action of BPP-OVA. Counteracting a Th2-biased response with Th1 cytokines was previously shown to be effective in alleviating airway inflammation. Treatment with nebulized IFN- γ before airway sensitization was shown to impair airway eosinophilia and airway hyperreactivity (44). A recent study also demonstrated that IFN- γ produced by T cells could inhibit the differentiation of myeloid progenitor cells to eosinophils in the bone marrow (45). Treatment with TLR2 agonists such as lipoprotein I led to an enhanced production of IFN- γ from restimulated LN cells, along with increased IFN- γ transcript levels in CD4⁺ T cells (16). Similarly, Weigt and colleagues reported that the autologous culture of lymphocytes from *Dermatophagoides pteronissinus* allergen 1 allergic individuals with DCs pulsed with allergen, MALP-2, and IFN- γ led to an enhanced production of IFN- γ (20). These investigations indicate that the induced IFN- γ impaired airway eosinophilic inflammation.

In conclusion, our results indicate that local treatment using BPP-OVA in sensitized mice leads to the alleviation of a Th2-biased response. The induction of IFN- γ and a concomitant decrease in IL-5 production in Th2-biased cells alleviates allergic airway disease. This study expands our understanding of the role of TLR signaling and antigen presentation by DCs in modulating a fine-tuned, primed T-cell response and in therapeutic interventions against allergies.

Author disclosures are available with the text of this article at www.atsjournals.org.

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Online Data Supplement

TLR2 agonist-allergen coupling efficiently redirects Th2 cell responses and inhibits allergic airway eosinophilia

Jayendra Kumar Krishnaswamy, Adan Chari Jirmo, Abdul Mannan Baru, Thomas Ebensen, Carlos A. Guzmán, Tim Sparwasser, Georg M.N. Behrens

Materials and Methods

Generation of bone marrow-derived dendritic cells (BMDC): Flt-3 derived BMDC were generated from B6 mice and TLR2-deficient mice as described previously [E1].

Dendritic cell enrichment: For isolation of dendritic cells (DC) from lungs [E2, E3], mediastinal lymph nodes [E4] and spleen [E5], organs were harvested from mice (B6, BALB/c or MyD88-deficient), digested using DNase I and collagenase (Worthington, NJ, USA). Lung and splenic DC were enriched by density gradient using Nycodenz (Axis Shield PoC, Oslo, Norway). Splenic DC were further enriched with a cocktail of rat anti-mouse monoclonal antibodies against Gr1, Ery, B220, CD19 and CD3 antigens and goat anti-rat IgG coupled magnetic beads (Biomag, Qiagen, Germany). Enriched lung DC were FACS sorted as CD11c^{int} MHCII^{hi} cells on FACS Aria (BD Biosciences, Heidelberg, Germany). Purity of DC obtained was usually 95-98%.

In vitro and in vivo T cell proliferation assays: OVA-specific TCR transgenic CD8⁺ and CD4⁺ T cells were purified from the lymph nodes (LN) of OT-I and DO11.10 mice respectively, according to the manufacturer's protocol (CD8⁺ and CD4⁺ T-cell enrichment set, BDTM Imag, BD Biosciences, Heidelberg, Germany) [E3]. Purified cells were labelled with 5µM carboxyfluorescein diacetate succinimydyl ester (CFSE), as described previously [E5]. For the *in vitro* proliferation assays, lung or splenic DC were pulsed with OVA alone (100 µg/mL), or OVA co-administered with BPP (1µg/mL) or BPP-OVA (1µg/mL) for 1 hour at 37°C. Cells were washed and cultured in a ratio of 1x10⁴ DC to 5x10⁴ T cells at 37°C. After 60 hours, cells were stained with antibodies against CD8 (for OT-I cells) or CD4 and KJ1.26 (for DO11.10 cells). The total number of proliferating cells (CFSE^{low}) was determined using flow

cytometry. For *in vivo* proliferation assays, 3×10^6 CFSE labelled T cells were adoptively transferred (i.v.) into B6 or BALB/c mice. Mice received a single intranasal dose of OVA (25 μ g per mouse) or BPP-OVA (1 μ g per mouse). Proliferation of OT-I or DO11.10 cells in mediastinal LN was quantified after 60 hours by determining the number of CFSE^{low} cells [E3].

Histology: Twenty four hours after the last challenge, lungs were isolated from mice fixed in 4% formalin and embedded in paraffin as described previously [5]. Using a microtome, sections of thickness 3 μ were made and stained with H&E (Merck). From each mouse lung, atleast two sections (containing hilar structures and periphery) of all lobes of right and left lung were evaluated. Microphotographs were taken using a Laborlux K Microscope (LEITZ, Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany) with an Olympus DP71 camera. Peribronchial and perivascular inflammation of three to four mice per group were analyzed and scored as follows: 0: Normal; 1: Few cells; 2: A ring of inflammatory cells one layer deep; 3: A ring of inflammatory cells two to four layers deep; 4: A ring of inflammatory cells more than four cells deep [E3].

Supplementary Figure Legend

Figure E1: **Maturation of Flt-3 derived BMDC from BALB/c mice after TLR**

stimulation (A) Surface expression of CD86 and MHC class II on Flt-3 derived BMDC from BALB/c mice after overnight treatment with BPP-OVA (dotted lines, 1 $\mu\text{g/mL}$) or LPS (dashed lines, 1 $\mu\text{g/mL}$). Untreated Flt-3 derived BMDC served as negative controls (solid lines). Isotype controls are shown in gray histograms. The upper panels represent the CD8⁺ splenic DC equivalent (CD11c⁺ CD45 RA⁻ CD24^{hi} CD172 α ^{lo}) subset, whereas the lower panels represent the CD8⁻ splenic DC equivalent (CD11c⁺ CD45 RA⁻ CD24^{lo} CD172 α ^{hi}) subset. (B) Flt-3 derived BMDC from BALB/c mice were stimulated *in vitro* with BPP-OVA (1 $\mu\text{g/mL}$), LPS (1 $\mu\text{g/mL}$) or CpG (5 nmoL/mL) for 18 hours and cell free supernatants were analysed for cytokine production using cytokine bead array. Untreated Flt-3 derived BMDC (Unp) served as negative controls. The results obtained are representative of two independent experiments.

Figure E2: **Enhanced *in vivo* antigen presentation by BPP-OVA is TLR2-**

dependent. 3 x 10⁶ CFSE-labelled OT-I cells were adoptively transferred into naïve B6 or TLR2-deficient mice, which were then treated with a single i.n. dose of BPP-OVA (1 $\mu\text{g}/\text{mouse}$) or OVA alone (25 $\mu\text{g}/\text{mouse}$). Untreated mice (Ctrl) were used as negative controls. The number of proliferating OT-I cells (CFSE^{lo}) from mediastinal lymph nodes of treated mice (n=3 mice per group, pooled analysis) were evaluated using flow cytometry after 60 hours. Upper panels show representative CFSE proliferation profiles and lower panel show quantitative data of proliferated (CFSE^{low}) cells. Data shown are mean + SEM and are representative of two independent experiments (n=3 mice per group, pooled analysis; ***p<0.001).

Figure E3: **Profile of *in vitro* cultured Th2-biased cells.** (A) For *in vitro* generation of Th2-biased cells, DO11.10 cells were stimulated *in vitro* with OVA₃₂₃₋₃₃₉ peptide-pulsed splenic DC for 5 days in the presence of IL-2, IL-4 and anti-IFN γ . Culture comprised primarily of CD4⁺ DO11.10 TCR⁺ T cells indicating that Th2-biased cells were antigen specific. (B) Cells from the culture were restimulated *in vitro* with Leukocyte Activation Cocktail with BD GolgiPlugTM for 4-6 hours, and stained for intracellular expression of IL-4 and IFN γ and analyzed by flow cytometry.

Figure E4: **FOXP3⁺ cells in adoptively transferred Th2-biased cells.** 3-5 x 10⁶ Th2-biased cells were adoptively transferred into naïve BALB/c mice which were treated a day later with BPP-OVA (1 μ g/mouse, i.n.), OVA (25 μ g/mouse, i.n.) or left untreated (Negative). Three days later, mediastinal LN were isolated from mice, pooled and lysed. (A) Gating strategy employed for adoptively transferred Th2-biased cells (CD4⁺ DO11.10 TCR⁺). (B) Intracellular staining for FOXP3 in Th2-biased cells. Data shown are a representative of two independent experiments (n=3 mice per group).

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Figure E1

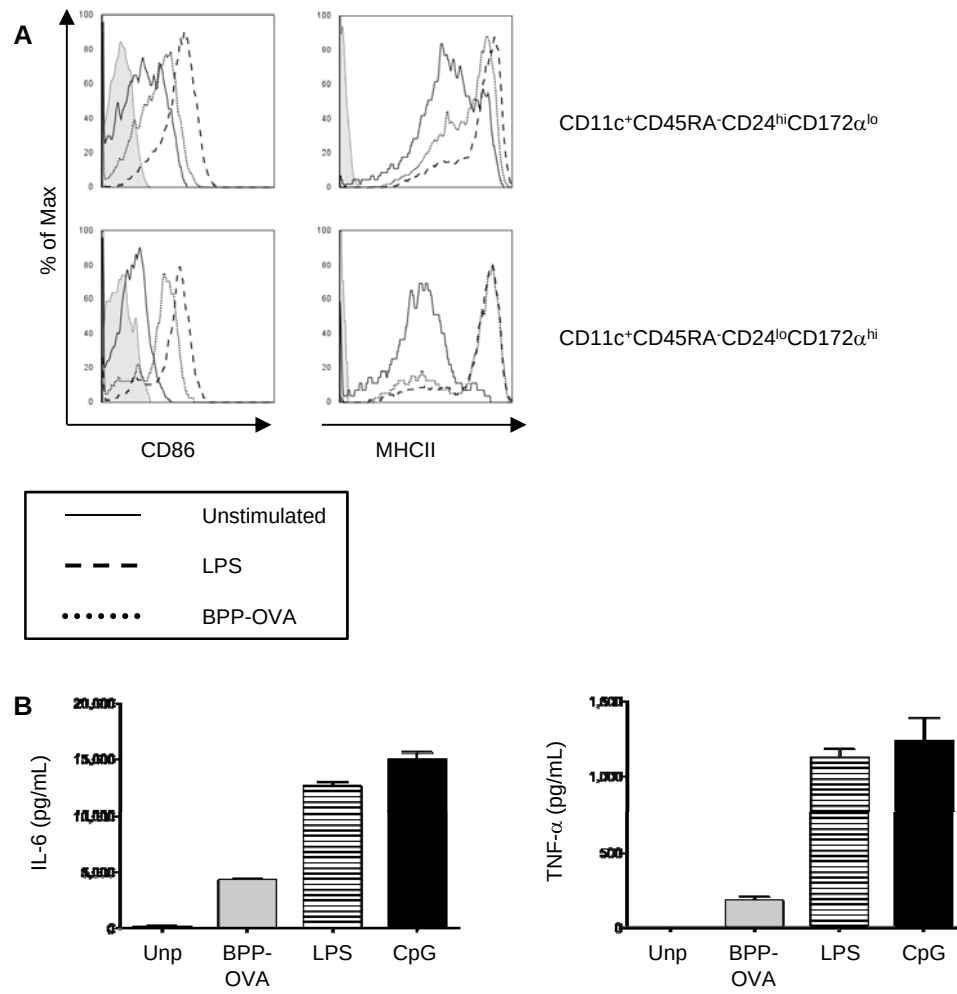


Figure E2

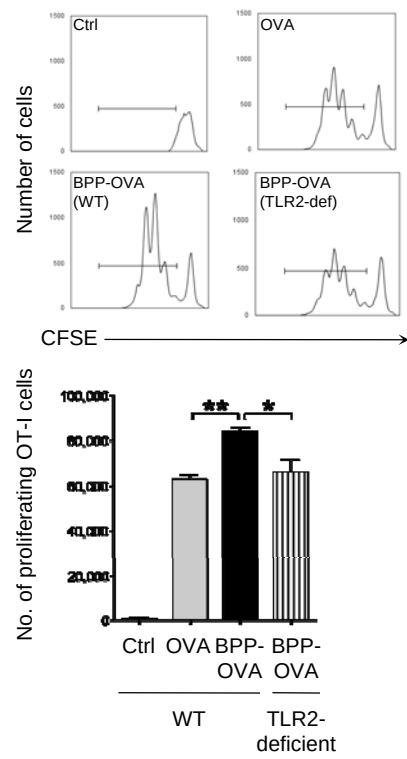


Figure E3

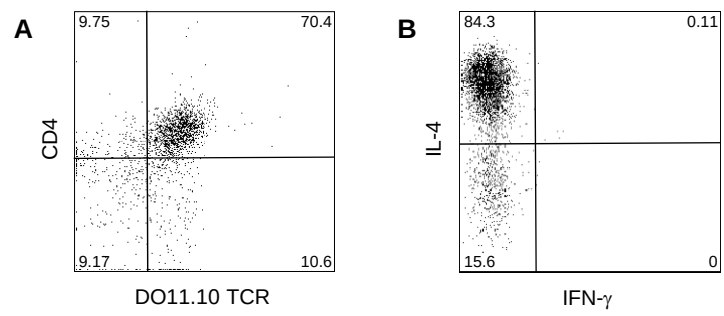


Figure E4

