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# ICOS-Expressing CD4 T Cells Induced via TLR4 in the Nasal Mucosa Are Capable of Inhibiting Experimental Allergic Asthma

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**Modulation of adaptive immune responses via the innate immune pattern recognition receptors, such as the TLRs, is an emerging strategy for vaccine development. We investigated whether nasal rather than intrapulmonary application of Protollin, a mucosal adjuvant composed of TLR2 and TLR4 ligands, is sufficient to elicit protection against murine allergic lower airway disease. Wild-type, Tlr2<sup>-/-</sup>, or Tlr4<sup>-/-</sup> BALB/c mice were sensitized to a birch pollen allergen extract (BPEX), then received either intranasal or intrapulmonary administrations of Protollin or Protollin admixed with BPEX, followed by consecutive daily BPEX challenges. Nasal application of Protollin or Protollin admixed with BPEX was sufficient to inhibit allergic lower airway disease with minimal collateral lung inflammation. Inhibition was dependent on TLR4 and was associated with the induction of ICOS in cells of the nasal mucosa and on both CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>-</sup> T cells of the draining lymph nodes (LNs), as well as their recruitment to the lungs. Adoptive transfer of cervical LN CD4<sup>+</sup>ICOS<sup>+</sup>, but not CD4<sup>+</sup>ICOS<sup>-</sup>, cells inhibited BPEX-induced airway hyperresponsiveness and bronchoalveolar lavage eosinophilia. Thus, our data indicate that expansion of resident ICOS-expressing CD4<sup>+</sup> T cells of the cervical LNs by nasal mucosal TLR4 stimulation may inhibit the development of allergic lower airway disease in mice. *The Journal of Immunology*, 2012, 189: 2793–2804.**

Allergic asthma is a chronic disease characterized by airway hyperresponsiveness (AHR), airway inflammation, and intermittent obstruction, as well as airway remodeling. Immunologically, allergic asthma is Th2 cell dominated, marked by expression of cytokines such as IL-4, IL-5, and IL-13, as well as peribronchial inflammatory cell infiltrates including eosinophils and neutrophils (1). Inhibition of allergic asthma by immunomodulation or regulation of the adaptive immune system via manipulation of innate immune processes is an attractive therapeutic strategy. Ligands of the TLR family, such as the synthetic lipoprotein PAM3CSK4 (2, 3), LPS (4–6), resiquimod (7, 8), and CpG oligodeoxynucleotides (9, 10), which activate TLR2, TLR4, TLR7/8, and TLR9, respectively, are capable

of inhibiting experimental allergic asthma in animal models. In this context, we examined the immunomodulatory potential of Protollin, a mucosal vaccine adjuvant composed of TLR2 and TLR4 ligands, in a murine model of experimental allergic asthma (11). Protollin was determined to be safe and well-tolerated up to a dose of 1.5 mg LPS when administered via the intranasal route in phase I (12) and II (13) human clinical trials, and has been tested as an intranasal vaccine adjuvant in animal models of influenza (14), respiratory syncytial virus (15), severe acute respiratory syndrome (16), plague (*Yersinia pestis*) (17), and measles (18) infection, as well as in models of Alzheimer's disease (19). Protollin consists of LPS molecules (TLR4 ligand) from *Shigella flexneri*, a Gram-negative bacterium, noncovalently incorporated in nanomolecular vesicles formed by proteosomes, consisting of purified hydrophobic outer-membrane proteins from *Neisseria meningitidis* (shown to signal via a TLR2/1 heterodimer complex) (20, 21).

Harnessing the therapeutic potential of adjuvants while minimizing toxicity is a central aspect of vaccine design. Administration of TLR2 or TLR4 ligands into the lower airways carries the potential danger of exacerbating lower airway inflammation by promoting monocytic/neutrophilic inflammation (22). Alternatively, the nasal mucosa is populated by lymphoid tissues (23), as well as the recently described M cells (24), which can serve as effective inductive sites for immune responses, while minimizing exposure of the lower airways to TLR ligands. Thus, we assessed the immunomodulatory potential of nasal application of Protollin as an adjuvant in intranasal immunotherapy of lower airway disease, in a murine model of birch pollen allergic asthma, and focused on the changes in CD4<sup>+</sup> T cell responses induced by Protollin in vivo via the nasal mucosa.

TLR adjuvants can provide signals that suppress or divert the T cell response to an allergen away from the Th2 inflammatory

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BPEX, birch pollen allergen extract; Ers, respiratory system elastance; GATA-3, GATA-binding protein-3; ICOS-L, ICOS ligand; LN, lymph node; MCh, acetyl- $\beta$ -methylcholine; NALT, nasal-associated lymphoid tissue; PNU, protein nitrogen unit; Pro/BPEX, Protollin admixed with BPEX; Rrs, respiratory system resistance; Treg, T regulatory cell; wt, wild-type.

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response. The specific anatomical microenvironment targeted by the adjuvant, the activation state of dendritic cells, and the cytokine milieu provide the signals that direct lineage-specific differentiation of naive T cells into Th1, Th2, Th17, T follicular helper, or T regulatory cell (Treg) subsets (25). LPS is a TLR ligand that can support the development of diverse Th responses and, in fact, has been shown to promote the differentiation of each of these T cell lineages depending on the particular tissue compartment (26–29). The dose of LPS is also an important determinant of the ensuing T cell response (28, 30), and the timing of LPS exposure in relation to disease onset or development, as well as the age of animals at the time of exposure influences whether LPS amplifies or attenuates allergic disease (31). We were interested in characterizing the T cell response associated with the inhibition of experimental allergic asthma by Protollin. The induction of IL-12 (32), IFN- $\gamma$  (4) and/or IL-10 (33, 34) has been associated with the inhibition of allergic airway disease by LPS; TLR2 ligands have also been shown to inhibit experimental asthma in mice by promoting a Th1 response (3). Thus, we hypothesized that nasal application of Protollin would inhibit experimental asthma by promoting Th1 or Treg responses. ICOS is expressed by all of the aforementioned T cell subsets and is important in the differentiation (35), effector (36, 37), proliferative (38), migratory (39) and memory (40) functions of T cells. A number of recent reports have shown that ICOS expression on Tregs enhances the function, proliferation and survival of these cells and has critical immunoregulatory implications (41). Our data demonstrate that nasal application of Protollin is sufficient to inhibit the development of allergic lower airway disease, mediated by TLR4-dependent stimulation of the nasal mucosa and potential amplification of ICOS-expressing CD4<sup>+</sup> T cell populations.

## Materials and Methods

### Animal treatments

Six- to 8-wk-old female wild-type (wt) BALB/c mice were purchased from Charles River, Canada. Tlr2 knockout mice on a BALB/c background were generated in the Animal Care Facilities of the McGill University Health Centre, and Tlr4 knockout mice were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in a conventional or specific pathogen-free animal facility under a 12-h light/dark cycle with free access to food and water. All animals were sensitized on day 0 with a single 0.15-ml i.p. injection of 20 protein nitrogen units (PNU) birch pollen allergen extract (BPEX; Greer Laboratories, Lenoir, NC) and 3 mg alum hydrogel (Alum hydrogel 2%; Brenntag Biosector, Frederiksund, Denmark). This BPEX extract is used for clinical purposes in intradermal desensitization, and thus is low in endotoxin (only 114 endotoxin units/mg protein, equivalent to a dose of  $\sim$ 1.5 endotoxin units/kg mouse body weight). Experimental procedures were approved by the McGill University Animal Care Committee.

### Experimental asthma and nasal immunotherapy protocol

On each of days 7, 10, and 13 after sensitization (Fig. 1), awake mice received nasal applications, that is, without anesthesia, of  $15 \pm 5 \mu\text{l}$  of either PBS, BPEX (25 PNU), Protollin alone (15  $\mu\text{g}$ ), or Protollin admixed with BPEX (Pro/BPEX; 15  $\mu\text{g}$ /25 PNU). Previously, nasal application of similar volumes of Evans blue dye indicated  $>90\%$  deposits in the nose/upper airways, with the remaining portion depositing largely in the gastrointestinal tract, with very little in the lungs (42). Protollin consisted of a 1:1.1 ratio of Neisserial proteins to *Shigella* LPS at a concentration of 1  $\mu\text{g}/\mu\text{l}$  LPS, resulting in an intranasal dose of  $\approx 15 \pm 5 \mu\text{g}$  LPS and Neisserial proteosomes on each of the indicated days. Some mice were administered an equivalent dose of 15  $\mu\text{g}$ , or a lower dose of 2  $\mu\text{g}$ , Protollin alone or Pro/BPEX, in a volume of 20  $\mu\text{l}$  sterile PBS, intranasally under light isoflurane (4%) anesthesia to test the effects of Protollin deposited in the lower airways. On days 15, 16, and 17, mice were also allergen challenged intranasally under light isoflurane anesthesia with a dose of 25 PNU BPEX in a volume of 36  $\mu\text{l}$  sterile PBS. Control mice were sham challenged with sterile PBS only.

### Assessment of allergen-induced AHR

On day 19, 48 h after the final allergen challenge, mice were anesthetized with an injection of xylazine hydrochloride (10 mg/kg, i.p.) followed by i.p. administration of sodium pentobarbital (32 mg/kg). Once anesthesia was achieved, as assessed by loss of response to external stimuli, in some experiments a catheter was inserted in the jugular vein of the mouse (for i.v. methacholine delivery only). In all experiments, the mice were tracheostomized using a 19G metal cannula and connected via the endotracheal cannula to a commercial small-animal ventilator (FlexiVent; SCIREQ, Montreal, QC, Canada). The animal was ventilated at a respiratory rate of 150 breaths/min and tidal volume of 10 ml/kg against a positive end expiratory pressure of 3 cm H<sub>2</sub>O. The mouse was then paralyzed with a 1-mg/kg pancuronium bromide i.v. or i.p. injection before the measurement of baseline respiratory mechanics. A 1.2 s, 2.5-Hz single-frequency forced oscillation maneuver was performed at 10-s intervals, and respiratory system resistance (Rrs) and elastance (Ers) were calculated with commercial software. In some experiments, doubling doses of methacholine (acetyl- $\beta$ -methylcholine [MCh]; Sigma-Aldrich Canada, Oakville, ON, Canada) from 20 to 640  $\mu\text{g}/\text{kg}$  were delivered i.v. In other experiments, doubling concentrations of MCh from 15.6 to 125 mg/kg were delivered to the mouse as an aerosol using a 4-s nebulization period synchronized with inspiration at a nebulization duty cycle of 50%. Allergen-induced AHR was assessed by MCh-induced bronchoprovocation by recording the peak Rrs and Ers for each dose of MCh administered.

### Assessment of airway inflammation

On day 19, bronchoalveolar lavage (BAL) was performed using saline and a protease-inhibitor mixture. The recovered cell pellet was used to measure the total number of cells in the BAL, and cytopins were prepared and stained with Diff-Quick stain (Diff-Quik method; Medical Diagnostics, Dürdingen, Germany) for differential cell counts. A panel of cytokine proteins was assayed in the BAL supernatant using a Bioplex system (Bio-Rad Laboratories, Mississauga, ON, Canada).

### Measurement of BPEX-specific serum IgE

On day 19, blood was collected by exsanguination into serum separator tubes and left at room temperature to clot. Samples were centrifuged at  $4000 \times g$  for 5 min, and the serum was collected and stored at  $-20^\circ\text{C}$ . Plates were coated overnight with 20  $\mu\text{g}/\text{ml}$  BPEX for measurement of BPEX-specific serum IgE by ELISA (BioLegend, San Diego, CA), according to the manufacturer's instructions.

### mRNA analysis of nasal-associated lymphoid tissues

On day 14 (Fig. 1), 24 h after the final nasal application of PBS, BPEX, Pro, or Pro/BPEX, nasal-associated lymphoid tissues (NALT) were harvested, immersed in RNA later, and then stored at  $-80^\circ\text{C}$ . After thawing, tissues were homogenized and RNA was extracted using the RNeasy Minikit (Qiagen, Toronto, ON, Canada). Reverse transcription was performed using Superscript II enzyme (Invitrogen, Life Technologies, Burlington, ON, Canada). Real-time PCR was used to measure the expression (relative to the housekeeping gene, S9) of cytokines relevant to Th cells and Tregs, IL-4, IFN- $\gamma$ , IL-17A, IL-10, IL-27, and TGF- $\beta$ , transcription factors GATA-binding protein-3 (GATA-3), T-Box expressed in T cells (T-bet), RAR-related orphan receptor  $\gamma$ , Foxp3, and B cell lymphoma 6, as well as costimulatory molecules, ICOS, CTLA-4, and programmed cell death-1 mRNA.

### Flow cytometric analysis of cervical lymph node and lung Th cells and Tregs

To further characterize the CD4<sup>+</sup> Th cell and Treg response induced by Protollin, we harvested cervical lymph nodes (LNs) and lungs from BPEX-sensitized mice on day 16 or 17 (Fig. 1), 24 h after one or two BPEX allergen challenges, respectively, after three administrations (days 7, 10, and 13) of either PBS or Protollin alone. Superficial cervical LNs were isolated and placed in RPMI 1640 medium, containing 8% heat-inactivated FBS, 2 mM L-glutamine, 50  $\mu\text{g}/\text{ml}$  gentamicin, and 10 mM HEPES. The mouse was then tracheostomized, cannulated, and the lungs were inflated with 1 ml sterile PBS (Invitrogen), supplemented with 0.2 Wünsch units/ml collagenase from *Clostridium histolyticum* (type XI-S), 1000 Dornase units/ml DNase I (type II-S) (Sigma-Aldrich Canada, Oakville, ON, Canada), and 0.5 mM Ca<sup>2+</sup>. Lungs were then excised, minced with forceps and a scalpel blade, and incubated on an orbital shaker at  $37^\circ\text{C}$  for 1 h. The digestion was inhibited by the addition of cold complete RPMI 1640 medium with 2 mM EDTA (Invitrogen) and 50  $\mu\text{M}$  2-ME. The lung digest was then repeatedly passed through a 16G needle and then through a 70-

$\mu\text{m}$  BD Falcon cell strainer and centrifuged. RBCs were lysed with ammonium chloride solution. Cells were counted using a Beckman Coulter A<sup>c</sup>T Counter, plated in a 96-well culture plate, and incubated for 15 min on ice with mouse BD Fc Block (BD Biosciences, Mississauga, ON, Canada). The cells were then stained for 20 min with FITC-conjugated rat anti-mouse CD4 mAb followed by PE anti-CD25 (BD Biosciences) or PE anti-ICOS (BioLegend, San Diego, CA), or the appropriate isotype control Ab. ICOS were then fixed with BD Cytotfix/Cytoperm solution, incubated with 1% BSA in BD Perm/Wash solution, and finally stained with allophycocyanin anti-Foxp3 mAb or isotype control Ab (eBioscience, San Diego, CA). Cell acquisition was performed using the BD FACSCalibur and analyzed with Cell Quest Pro software (BD Biosciences). For flow cytometric analysis of CD4<sup>+</sup> T cell cytokines, cells isolated from cervical LNs and lungs were stimulated for 5 h with PMA (10 ng/ml), ionomycin (250 ng/ml), and BD GolgiStop in RPMI 1640 medium containing 8% heat-inactivated FBS, 2 mM L-glutamine, 50  $\mu\text{g}/\text{ml}$  gentamicin, and 10 mM HEPES at 37°C. The cells were then stained with anti-mouse CD4 FITC, ICOS PE, and either IFN- $\gamma$  PE-Cy7, IL-4 allophycocyanin, or IL-10 allophycocyanin (BD Biosciences).

### *In vivo neutralization of IL-10*

Coinciding with their first administration on day 7, mice receiving Protollin alone were injected i.p. with 50  $\mu\text{g}$  of either isotype control or anti-IL-10 mAb (BD Biosciences). In addition, the mice received intranasal administrations, under isoflurane anesthesia, of 25  $\mu\text{g}$  of either Ab on days 7 and 10. After Protollin and Ab administrations, mice were challenged with BPEX on days 15–17 and underwent lung function testing, as well as blood and BAL collection on day 19.

### *Adoptive transfer of FACS-sorted CD4<sup>+</sup>ICOS<sup>+</sup> or CD4<sup>+</sup>ICOS<sup>-</sup> cells*

On day 14, cervical LNs were harvested and pooled from BPEX-sensitized mice that received nasal applications on days 7, 10, and 13 of either PBS or Protollin alone, and isolated cells were stained with anti-CD4 FITC and anti-ICOS PE mAb. CD4<sup>+</sup>ICOS<sup>+</sup> or CD4<sup>+</sup>ICOS<sup>-</sup> cells were then sorted by flow cytometry using a Beckman Coulter MoFlo cell sorter. Because of the rarity of CD4<sup>+</sup>ICOS<sup>+</sup> cells, a maximum of 0.3 to 0.4 million cells of either of the sorted populations were adoptively transferred i.p. in 0.3 ml sterile PBS (on day 14) to mice that had been sensitized with BPEX in parallel with the sacrificed animals but that had not received any intranasal treatments. Control animals received an injection of only sterile PBS as a sham adoptive transfer ("mock AT"). Mice were then challenged on days 15–17 and underwent lung function testing in response to MCh, as well as BAL collection on day 19.

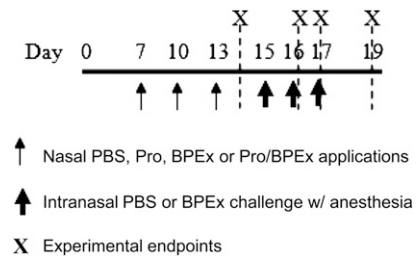
### *Statistical analysis*

Airway responses to MCh bronchoprovocation were analyzed in GraphPad Prism version 5 (GraphPad software, San Diego, CA) by two-way ANOVA followed by Bonferroni posttests comparing all experimental groups with each other. One-way ANOVA and post hoc Newman-Keuls tests were used for all other analyses involving three or more groups, or unpaired Student *t* test was used in the case where only two experimental groups were compared. Data were log-transformed before statistical analysis when not normally distributed.

## Results

### *Nasal application of Protollin prevents allergen-induced lower airway inflammation*

The effects of intranasal instillation of Protollin, with or without anesthesia, on allergen-induced airway inflammation were compared. Protollin (15  $\mu\text{g}$ ) administered to awake animals without anesthesia, before the BPEX challenge (Fig. 1), significantly reduced BAL eosinophil and neutrophil numbers on day 19 compared with mice that received PBS before the challenge (Fig. 2Aii, 2Aiii). BAL lymphocyte numbers, however, were not significantly reduced (Fig. 2Aiv). In contrast, Protollin (15  $\mu\text{g}$ ) administered with anesthesia before the BPEX allergen challenge caused significant airway inflammation, as indicated by substantially higher total BAL cell counts compared with mice given PBS or an equivalent dose of Protollin without anesthesia, followed by BPEX challenge (Fig. 2Ai). Differential cell counts of BAL fluid from mice that received



**FIGURE 1.** Acute experimental asthma protocol. All animals were sensitized with BPEX/alum (i.p.) on day 0. Animals received nasal applications, while conscious (i.e., without anesthesia), of either PBS, Protollin alone (Pro alone), BPEX, or Pro/BPEX, indicated by thin arrows, followed by intranasal challenge with light anesthesia of PBS or BPEX, indicated by thick arrows. Dashed lines and X indicate days on which samples were harvested and/or airway responses to MCh were assessed; day 14: NALT harvested for RNA extraction; day 16: cervical lymph nodes and lungs harvested for flow cytometric analysis of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> (Treg) cells or CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> cells, as well as intracellular cytokine staining for IL-4, IL-10, and IFN- $\gamma$ ; day 17: lungs harvested for flow cytometric analysis of Tregs or CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> cells; day 19: assessment of airway responses to MCh using the flexiVent, followed by blood and BAL collection.

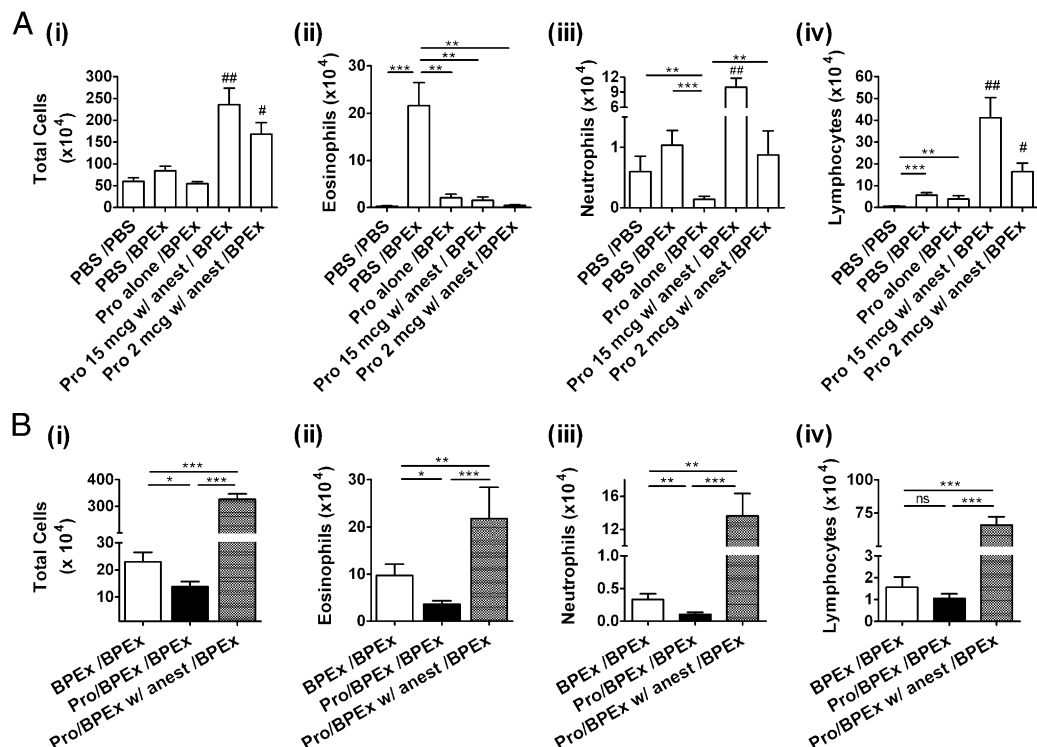
Protollin with anesthesia demonstrated marked neutrophilia (Fig. 2Aiii) and elevated macrophage (Supplemental Fig. 1A) and lymphocyte (Fig. 2Aiv) numbers, whereas eosinophils (Fig. 2Aii) were virtually absent. Administration of a lower dose of Protollin (2  $\mu\text{g}$ ) to anesthetized animals before the BPEX challenge also resulted in airway inflammation that was characterized by increased macrophage numbers (Supplemental Fig. 1A), as well as lymphocytes (Fig. 2Aiv); in this case, neutrophil numbers were not increased beyond those of PBS-treated, BPEX-challenged mice, and eosinophilia was still completely inhibited (Fig. 2Aii, 2Aiii). Overall, delivery of Protollin into the mouse lungs, even at the lower dose, was sufficient to cause considerable airway inflammation.

When Protollin was delivered admixed with BPEX (Pro/BPEX/BPEX), intranasally without anesthesia, mice had significantly lower BAL eosinophil and neutrophil numbers compared with mice given nasal applications of BPEX alone before the BPEX allergen challenge (BPEX/BPEX; Fig. 2Bii, 2Biii). Pro/BPEX delivered with anesthesia caused severe airway inflammation similar to that observed when Protollin was administered alone with anesthesia (Fig. 2Bi), except that eosinophils were also increased in the BAL fluid (Fig. 2Bii). Thus, we determined that nasal application of Protollin was sufficient to inhibit allergen-induced lower airway eosinophilia and neutrophilia, while evoking less inflammation compared with the intrapulmonary administration.

### *Nasal application of Protollin is sufficient to inhibit AHR*

To determine whether nasal application of Protollin as an adjuvant with BPEX inhibited the development of AHR to subsequent allergen challenge, we assessed airway responses to increasing doses of MCh. In animals that received only nasal applications of PBS, those that were BPEX allergen challenged exhibited significantly greater increases in Ers (Fig. 3A), as well as Rrs (Supplemental Fig. 1B), in response to increasing doses of i.v. MCh compared with control animals sham-challenged with PBS. Nasal application of BPEX alone before the BPEX challenge significantly reduced airway responsiveness at the highest dose of MCh compared with animals that received PBS before the challenge, indicating tolerogenic effects of the allergen extract itself mediated via the nasal mucosa. Mice given Protollin mixed with BPEX (Pro/BPEX) had significantly reduced MCh-induced changes in Ers and Rrs compared with mice that received nasal applications





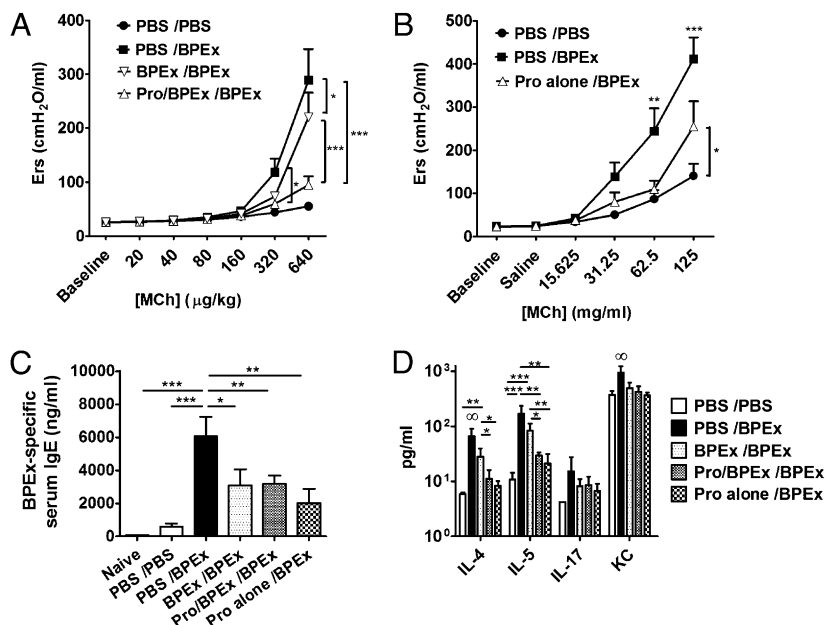
**FIGURE 2.** Intranasal application of Pro/BPEX or Protollin alone (Pro alone) to conscious animals is sufficient to inhibit allergen-induced airway inflammation while evoking less collateral inflammation in comparison with intrapulmonary administration. Total inflammatory cells (**Ai, Bi**), eosinophils (**Aii, Bii**), neutrophils (**Aiii, Biii**), and lymphocytes (**Aiv, Biv**) in BALs collected on day 19 after nasal application without anesthesia of PBS, Pro alone, BPEX, or Protollin combined with BPEX (Pro/BPEX), or intranasal application with anesthesia of Protollin, 15 or 2  $\mu$ g, or Pro/BPEX, followed by sham (PBS) or allergen (BPEX) challenge.  $n = 5-19$  animals/group from  $>3$  independent experiments (number signs indicate significant difference from other groups: # $p < 0.01$ , ## $p < 0.001$ ). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

of PBS or BPEX alone. Thus, the combined nasal administration of Protollin and BPEX to conscious animals before BPEX challenge inhibited the development of allergen-induced AHR. In a separate experiment, mice received nasal instillations of PBS or Protollin alone before the BPEX challenge, and airway responses to aerosolized MCh were determined. Even on its own, nasal Protollin administration prevented the development of AHR, as reflected in changes in Ers (Fig. 3B) and Rrs (Supplemental Fig. 1C).

#### Nasal application of Protollin inhibits allergen-induced airway Th2 cytokines and serum birch pollen-specific IgE

Cytokine measurements in BAL fluid collected 48 h after the final challenge (day 19) indicated that nasal administration of the allergen extract, BPEX, on its own, without anesthesia, partially, but significantly, reduced the allergen-induced Th2 cytokines IL-4 and IL-5 (Fig. 3D). Mice that received Protollin mixed with BPEX or Protollin alone before BPEX challenge had even lower IL-4 and

**FIGURE 3.** Intranasal application of Pro/BPEX or Protollin alone (Pro alone) to conscious animals before allergen challenge is sufficient to prevent allergic airway disease. Airway responses (Ers) to i.v. (**A**) ( $n = 8-19$  animals/group from  $>3$  independent experiments) or aerosolized (**B**) ( $n = 6-8$  animals/group from  $>3$  independent experiments) MCh 48 h after intranasal sham (PBS) or allergen (BPEX) challenges following nasal application to conscious mice of either PBS, BPEX, Pro alone, or Pro/BPEX. (B) Double and triple asterisks indicate significant difference of PBS/BPEX from Pro alone/BPEX. BPEX-specific serum IgE quantified by ELISA; all groups were BPEX sensitized, except "Naive" (**C**);  $n = 4-9$  animals/group from  $>3$  independent experiments, and cytokines in the BAL fluid quantified by multiplex assay (**D**) 48 h after PBS or BPEX challenges;  $n = 6$  animals/group from 4 independent experiments ( $\infty$  indicates significant difference from all other groups). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .



IL-5 levels in the BAL fluid, comparable with levels in sham (PBS)-challenged mice. The neutrophil chemoattractant, KC, was also significantly lower in all experimental groups compared with its level in PBS-treated, BPEX-challenged mice. Despite trends indicating that nasal application of Protollin resulted in lower allergen-induced IL-17 (Fig. 3D) and IFN- $\gamma$  levels (data not shown), there was no statistically significant change in these cytokines. IL-2, IL-10, and TNF- $\alpha$  were below the level of detection (not shown). Thus, congruent with the paucity of inflammatory cells in the BAL fluid of Pro/BPEX or Protollin alone-treated mice, these mice also had reduced airway Th2 cytokines and KC, without indication of induction of alternative proinflammatory or anti-inflammatory cytokines. Furthermore, nasal application of BPEX alone, Protollin mixed with BPEX, or Protollin alone significantly reduced serum BPEX-specific IgE production associated with allergen challenge (Fig. 3C). The combination of Protollin with BPEX did not further reduce BPEX-specific IgE compared with BPEX or Protollin administration alone.

*Nasal application of Protollin inhibits allergic airway disease via TLR4 and not TLR2*

Protollin was administered intranasally to conscious allergen-sensitized Tlr2 or Tlr4 knockout mice, before BPEX allergen challenge, as indicated for wt mice (Fig. 1). Protollin significantly inhibited AHR (Fig. 4A), BAL eosinophilia (Fig. 4C), and serum IgE (Fig. 4D) in Tlr2<sup>-/-</sup> mice to a comparable degree as in wt mice. However, Protollin failed to inhibit AHR and BAL eosinophilia in Tlr4<sup>-/-</sup> mice (Fig. 4B, 4C). Serum IgE was not significantly induced in BPEX-challenged Tlr4<sup>-/-</sup> mice, indicating that TLR4 additionally contributes to aspects of experimental allergic asthma in this model.

*Nasal application of Protollin affects T cell transcription factor and costimulatory molecule expression in NALT*

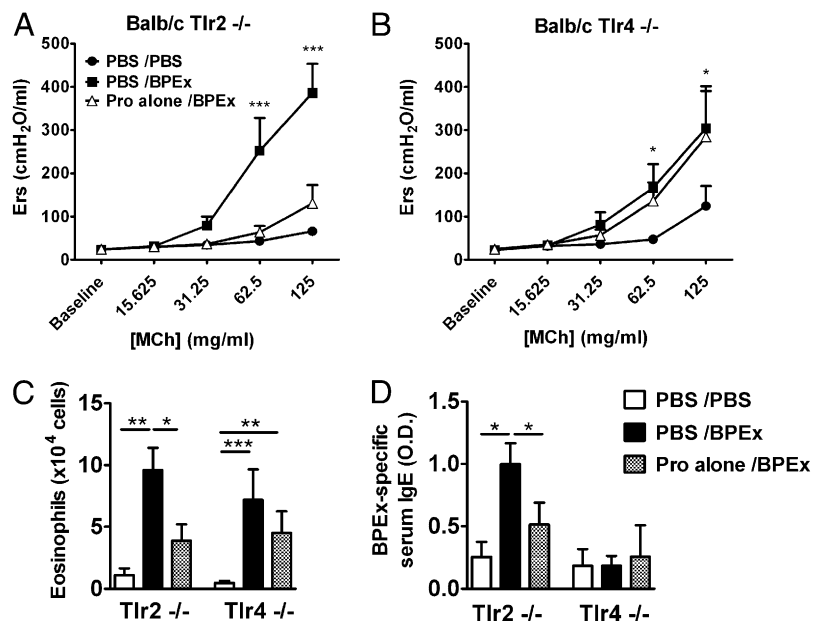
Given that nasal application of Protollin inhibited the allergen challenge-associated Th2 response, we wished to assess the early effects of Protollin administration on the local T cell response in the nasal mucosa, by quantifying T cell transcription factor and costimulatory molecule expression. To this end, we harvested NALT from mice on day 14 (Fig. 1), 24 h after the final nasal application

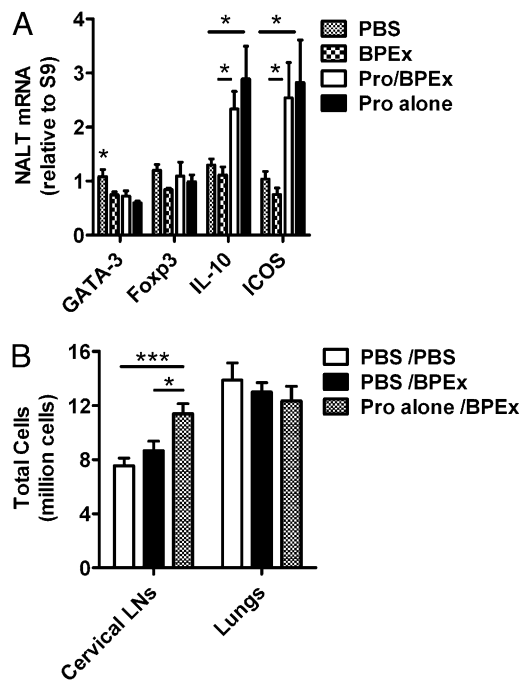
of either PBS, Protollin alone, BPEX alone, or Protollin mixed with BPEX, in the absence of allergen challenge. Nasal application of BPEX alone, Protollin alone, or Protollin mixed with BPEX significantly inhibited the expression of the Th2 master transcription factor GATA-3 (Fig. 5A). The expression of IL-4, Th1-associated IFN- $\gamma$  and T-bet, as well as TNF- $\alpha$  and Treg-associated IL-27, TGF- $\beta$  (data not shown), and Foxp3 (Fig. 5A) was unaffected by the application of Protollin to the nares. We also found no significant induction of the coinhibitory molecules CTLA-4 or programmed cell death-1 that have been associated with negative regulation of T cell responses and allergic airway disease (data not shown). Protollin also did not induce the inhibitor of T cell proliferation, indoleamine-2,3-dioxygenase, or the transcription factor, Egr-2, associated with Tr1 regulatory cells and T cell anergy (data not shown). However, Protollin administration alone, or in combination with BPEX, significantly induced the expression of ICOS, as well as the anti-inflammatory cytokine IL-10 (Fig. 5A). The induction of these molecules and inhibition of GATA-3 expression indicated early immunomodulatory effects of Protollin on the local T cell response in the nasal mucosa, preceding airway exposure to allergen.

*Nasal application of Protollin induces ICOS on CD4<sup>+</sup> T cells in the cervical LNs and recruitment to the lungs on allergen challenge*

To assess the effects of intranasal Protollin on the induction of ICOS and Tregs in the superficial cervical LNs that drain the nasal mucosa, as well as in the lungs, we harvested these tissues on day 16, 24 h after a single BPEX challenge following intranasal applications of either PBS or Protollin on days 7, 10, and 13 to examine by flow cytometry the frequency of CD4<sup>+</sup> T cells expressing either ICOS or CD25, as well as the transcription factor Foxp3. Protollin caused an increase in the total number of cells in the LNs, but not in the lungs (Fig. 5B), and significantly increased ICOS expression among CD4<sup>+</sup> cells in the LNs compared with animals that received only PBS before sham or allergen challenges (Fig. 6A, 6Ci). Although both CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>-</sup> cells were increased in the LNs (Fig. 6B, 6Ci, 6Cii), Protollin primarily induced ICOS<sup>+</sup>Foxp3<sup>-</sup> cells. However, the ratio of LN CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> cells to CD4<sup>+</sup>ICOS<sup>-</sup>Foxp3<sup>+</sup> cells was significantly increased as compared with that in PBS-

**FIGURE 4.** The inhibition of allergic airway disease by intranasal Protollin application is TLR4 and not TLR2 dependent. Airway responses (Ers) to aerosolized MCh in Tlr2<sup>-/-</sup> (A) (n = 10–13 animals/group from >3 independent experiments) or Tlr4<sup>-/-</sup> (B) (n = 6–8 animals/group from 4 independent experiments) mice 48 h after intranasal sham (PBS) or allergen (BPEX) challenges following nasal application to conscious mice of either PBS or Pro alone. BAL eosinophil numbers (C) (n = 8–12 animals/group) and BPEX-specific serum IgE (D) (n = 7–11 animals/group) in Tlr2 or Tlr4<sup>-/-</sup> mice after nasal applications without anesthesia followed by sham or allergen challenge. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.





**FIGURE 5.** Intranasal Protollin application has an immunomodulatory effect preceding allergen exposure on T cells in the nasal mucosa and causes draining cervical LN cell counts to increase. Expression of T cell transcription factors, costimulatory molecules, and cytokines quantified by real-time RT-PCR in NALT harvested from BPEx-sensitized mice (day 14) 24 h after final nasal application without anesthesia of PBS, BPEx, Protollin combined with BPEx (Pro/BPEx), or Protollin alone (Pro alone) (A) ( $n = 4-5$  samples/group from  $>3$  independent experiments, each sample consisting of NALT pooled from 3 animals). Total cell numbers from cervical LNs ( $n = 16-18$  samples from  $>3$  independent experiments) or lungs ( $n = 15$  samples/group from  $>3$  independent experiments) harvested (day 16) 24 h after a single PBS or BPEx challenge after nasal applications without anesthesia of either PBS or Protollin (B). \* $p < 0.05$ , \*\*\* $p < 0.001$ .

treated sham or BPEx-challenged mice, further affirming the induction of ICOS on Tregs of Protollin-exposed mice (Fig. 7A). In comparison, absolute numbers of LN  $CD4^+CD25^+Foxp3^+$  and  $Foxp3^-$  cells were similarly augmented in BPEx-challenged mice pretreated with either PBS or Protollin, as compared with sham-challenged mice (Fig. 7B), and the relative ratios of these populations were not significantly affected by Protollin exposure (Fig. 7A). Overall, Protollin did not increase  $Foxp3$ -expressing Tregs at this time point beyond those found in PBS-exposed, allergen-challenged mice, whether in the LNs (Fig. 7B) or the lungs (data not shown). Importantly, in contrast with wt BALB/c mice, nasal application of Protollin to Tlr4 knockout mice failed to increase either the percentage (Fig. 6Ciii) or absolute number (Fig. 6Civ) of draining cervical LN  $CD4^+ICOS^+$  cells, indicating a TLR4-dependent induction of ICOS that correlated with the inhibition of allergic airway disease.

In contrast with wt LNs, there was no concomitant increase in ICOS expression on wt lung  $CD4^+$  cells of Protollin-treated mice after a single allergen challenge (data not shown). However, a significant increase was observed 24 h after two BPEx challenges (Fig. 8), supporting a recruitment of ICOS-expressing  $CD4^+$  cells to the lungs. ICOS expression among lung  $CD4^+Foxp3^+$  cells appeared to have been equivalently induced in BPEx-challenged mice regardless of whether they had received prior Protollin (Fig. 8A). However, Protollin significantly increased the absolute number of  $CD4^+ICOS^+Foxp3^+$  cells (Fig. 8C). Protollin also significantly increased ICOS expression among  $CD4^+Foxp3^-$

lung cells (Fig. 8B, 8D), and both  $CD4^+ICOS^+Foxp3^+$  and  $CD4^+ICOS^+Foxp3^-$  populations were increased by Protollin relative to  $CD4^+ICOS^-Foxp3^-$  cells (Fig. 8E and 8F, respectively). Thus, nasal applications of Protollin before allergen challenge induced ICOS-expressing  $CD4^+Foxp3^+$  and  $CD4^+Foxp3^-$  cells, initially in the cervical LNs draining the nasal mucosa and later in the lungs.

#### Nasal application of Protollin inhibits allergic airway disease independently of IL-10

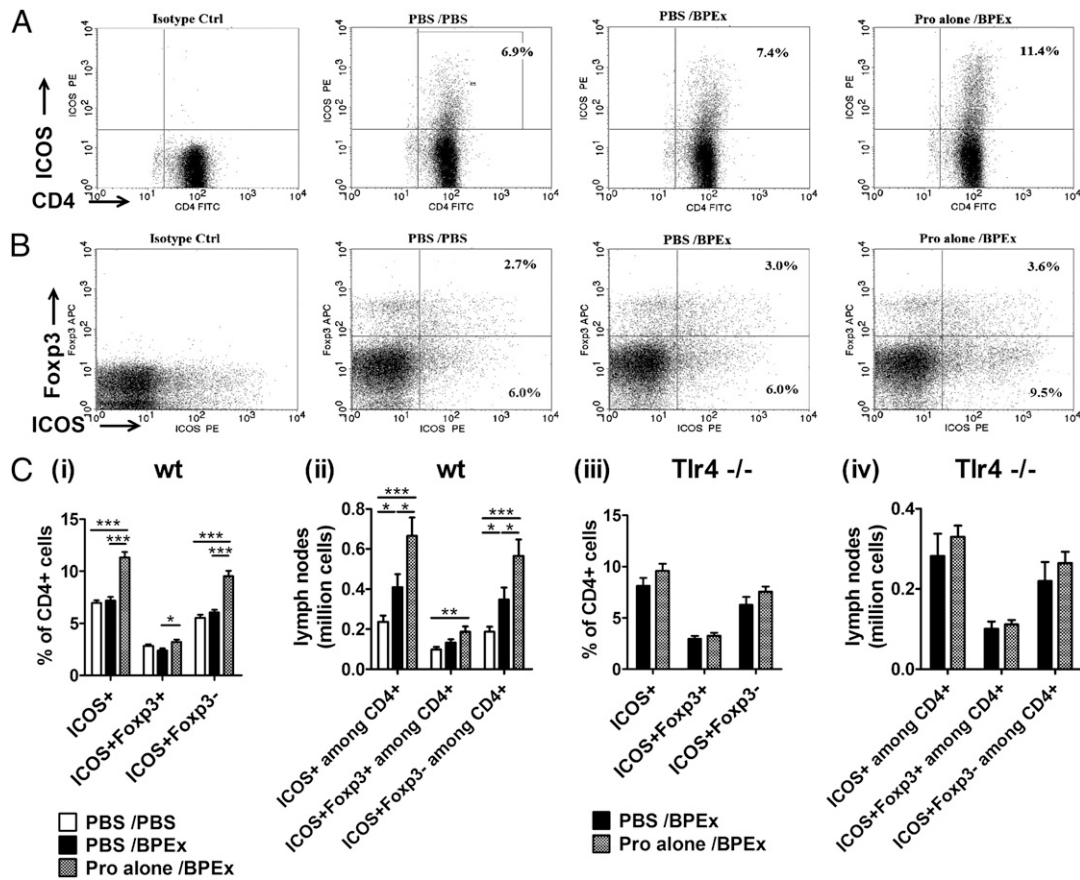
Our finding that intranasal Protollin augmented both ICOS and IL-10 mRNA levels in the NALT, together with reports of high ICOS expression being associated with IL-10-producing T cells, led us to investigate whether the  $CD4^+ICOS^+$  cells induced by Protollin preferentially expressed IL-10. Flow cytometric analysis of cervical LN intracellular cytokines was performed 24 h after a single BPEx challenge (day 16). No changes were found in IL-4 or IFN- $\gamma$  expression in  $CD4^+$  cells or the  $CD4^+ICOS^+$  subset (data not shown); however, Protollin exposure increased the number of IL-10-expressing  $CD4^+ICOS^+$  cells and the ratio of IL-10- to IFN- $\gamma$ -expressing  $CD4^+ICOS^+$  cells in the cervical LNs (Fig. 9A). No concomitant changes in IL-4, IFN- $\gamma$ , or IL-10 expression were found in the lungs, in either the  $CD4^+$  or  $CD4^-$  populations (data not shown), further indicating the absence of a direct effect of Protollin on the lungs. Twenty-four hours after two allergen challenges, Protollin increased lung  $CD4^+ICOS^+IL-10^+$  cells relative to PBS-treated sham-challenged, but not allergen-challenged, mice (Fig. 9B). Furthermore, although administration of anti-IL-10 mAb during the period of Protollin administrations increased BAL lymphocyte numbers (Fig. 9Ciii) compared with injection of isotype control Ab, airway responses to MCh (Fig. 9Ci) and BAL eosinophilia (Fig. 9Cii) were unaltered after BPEx challenges. Thus, in vivo neutralization of the IL-10 cytokine did not abolish Protollin's inhibition of experimental asthma.

#### ICOS induced via the nasal mucosa plays a role in inhibiting allergic airway disease

On day 14 after BPEx sensitization, mice received a sham adoptive transfer, an adoptive transfer of FACS-sorted  $CD4^+ICOS^+$  or  $CD4^+ICOS^-$  cells isolated from the pooled superficial cervical LNs of BPEx-sensitized, intranasal PBS-treated mice, or  $CD4^+ICOS^+$  cells from Protollin-treated mice to assess whether the TLR4-dependent induction of ICOS by Protollin was involved in the inhibition of allergic airway disease. After BPEx challenges, mice that had received prior adoptive transfer of LN  $CD4^+ICOS^+$  cells from either Protollin- or PBS-treated mice had significantly lower airway responses to MCh compared with those that received  $CD4^+ICOS^-$  cells from PBS-treated mice or that underwent sham adoptive transfer (Fig. 10A, 10B). Thus, specifically, ICOS-expressing  $CD4^+$  cells from the LNs draining the nasal mucosa had the capacity to inhibit allergen-induced AHR. Furthermore, adoptive transfer of LN  $CD4^+ICOS^+$  cells from mice that received nasal administrations of Protollin significantly reduced total BAL inflammatory cell numbers (Fig. 10C) and, specifically, eosinophils (Fig. 10D).

## Discussion

Modulation of adaptive immune responses via the innate immune pattern recognition receptors, such as the TLRs, is an emerging strategy for vaccine development. Defining the conditions and mechanisms by which these receptors can regulate inflammation in a safe manner is complicated by the diversity of immunological effects propagated by their cognate ligands. In this article, we show that nasal application of Protollin, a mucosal adjuvant that has been



**FIGURE 6.** Intranasal Protollin application elicits TLR4-dependent induction of ICOS expression and amplification of ICOS-expressing CD4<sup>+</sup> T cell populations in the draining cervical LNs. Representative dot plots of CD4-gated LN cells from wt BALB/c mice stained for CD4 and ICOS (A), as well as Fcp33 (B) harvested on day 16, 24 h after a single PBS or BPEX challenge following nasal applications of either PBS or Protollin alone (Pro alone). Percentage of LN CD4-gated cells that are ICOS<sup>+</sup>, ICOS<sup>+</sup>Fcp33<sup>+</sup>, ICOS<sup>+</sup>Fcp33<sup>-</sup>, and absolute cell numbers from wt BALB/c mice (Ci and Cii, respectively) or Tlr4<sup>-/-</sup> mice (Ciii, Civ); n = 6–8 samples/group from at least 3 independent experiments. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

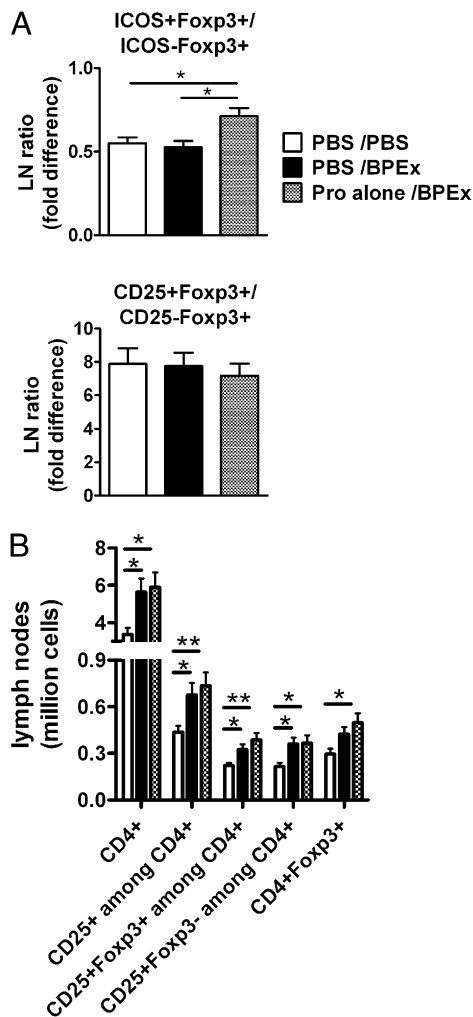
determined safe in human clinical trials and composed of TLR2 and TLR4 ligands, is sufficient to inhibit the development of allergic lower airway disease. Compared with intrapulmonary delivery, nasal application of Protollin is associated with less collateral lung inflammation and may confer reduced toxicity and an increased safety profile. Interestingly, the inhibition of experimental asthma by nasal application of Protollin is dependent on TLR4 and not TLR2, and is associated with elevated numbers of ICOS-expressing CD4<sup>+</sup> T cells in the draining cervical lymphoid tissues and lungs, with no increase in CD4<sup>+</sup>CD25<sup>+</sup>Fcp33<sup>+</sup> lymphocytes. Adoptive transfer of CD4<sup>+</sup>ICOS<sup>+</sup> cells from the cervical LNs of either PBS- or Protollin-treated mice before allergen challenge inhibits subsequent allergic airway disease, suggesting that these cells have the capacity to inhibit the development of allergic airway disease and can be amplified by TLR4 stimulation of the nasal mucosa.

Epidemiological evidence supports a role for microbial exposure in ensuring the maturation of the immune system in a balanced manner that prevents predisposition to Th2-associated responses (43–45). LPS and its receptor TLR4 have been associated with specific gene–environment interactions that may either protect or exacerbate allergic disease (46, 47), demonstrating the complexity of the relationship between microbial exposure and immune responses. The biological activity of LPS and its potential toxicity for the airway epithelium is due to its diphosphorylated lipid A portion (48); thus, derivatives with lower inflammatory capacity such as the monophosphoryl lipid A (49) are required for use in vaccines. Although the hydrophobic lipid A portions of LPS are

less toxic because they are shielded within vesicles formed by the neisserial proteosomes in Protollin (11), we found that intrapulmonary administration of 15 μg Protollin before allergen challenge caused marked monocytic, neutrophilic, and lymphocytic lung inflammation, while inhibiting eosinophilia. In addition, combined Protollin/BPEX administration with anesthesia before challenge amplified all types of inflammatory cells in the BAL, including eosinophils. Thus, intranasal, rather than intrapulmonary, administration appears to be a safer modality for mucosal vaccines, such as Protollin, given that nasal Protollin application to conscious animals was sufficient to inhibit experimental lower airway allergic disease while evoking less collateral pulmonary inflammation compared with an equivalent dose administered with anesthesia.

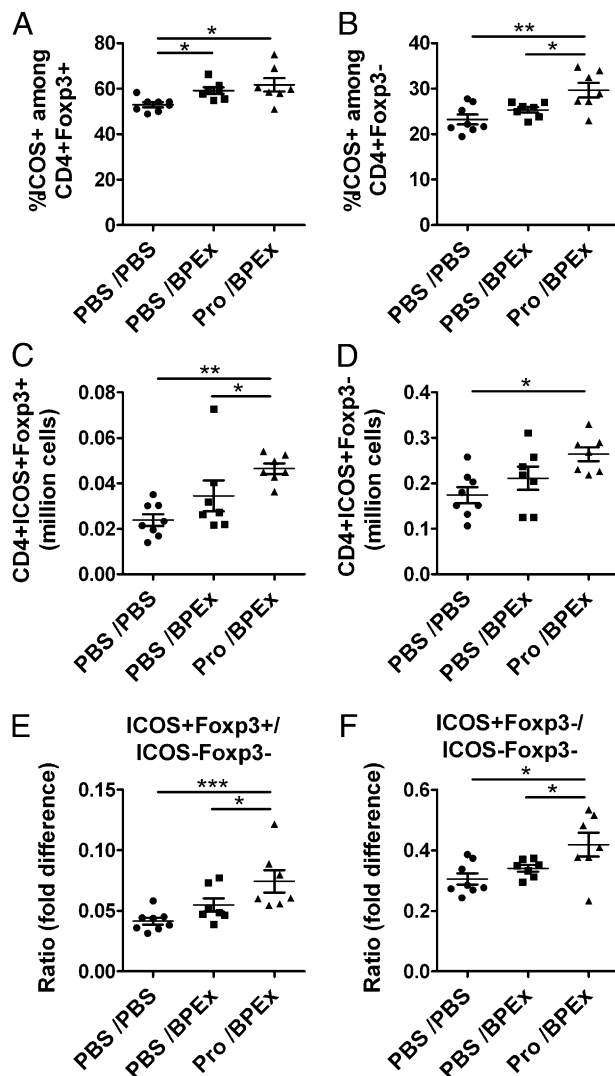
In rodents, the nasal mucosa contains nasopharynx-associated lymphoid tissues that are found dorsal to the cartilaginous soft palate; analogous structures exist in the human upper airways (50). The NALT also mediates Ag-specific tolerance to nasally deposited Ag (51) and, thus, supports both positive and negative regulatory signals for the immune system that are propagated by the draining cervical LNs (52). We found that nasal application of the allergen extract BPEX, on its own, before the allergen challenge resulted in partially reduced pulmonary responsiveness to methacholine, BAL Th2 cytokines, and BPEX-specific serum IgE, indicating that intranasal BPEX administration mediates tolerogenic lower airway and systemic effects. Nasal application of Protollin on its own also inhibited allergic airway disease in a TLR4-





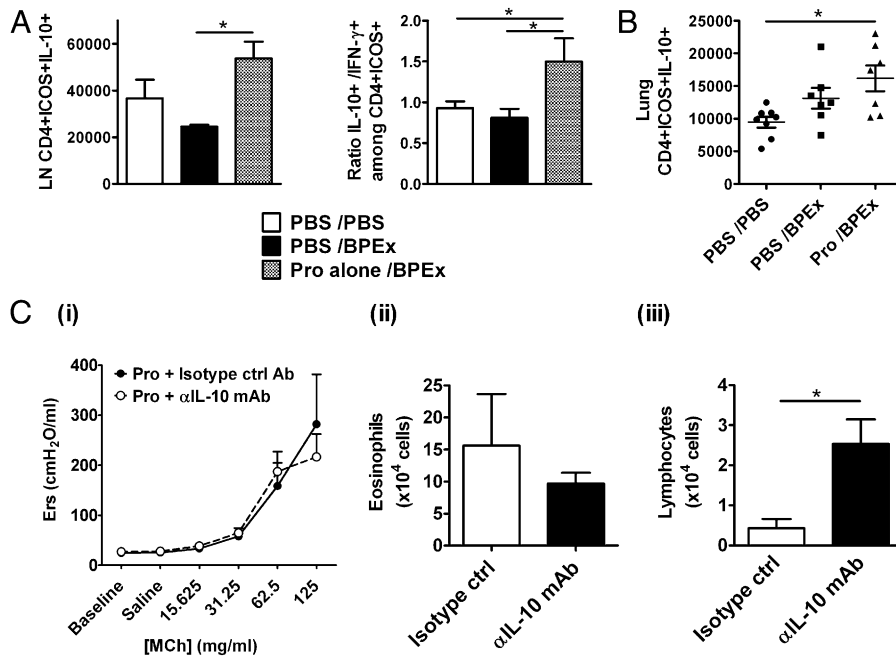
**FIGURE 7.** Intranasal Protollin application significantly affects the relative proportion of cervical LN CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> and ICOS<sup>+</sup>Foxp3<sup>-</sup> cells but does not affect CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells. Ratio of LN CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> to CD4<sup>+</sup>ICOS<sup>-</sup>Foxp3<sup>+</sup> cells, as well as ratio of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> to CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> cells (**A**). Absolute numbers of LN CD4<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>, CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup>, or total CD4<sup>+</sup>Foxp3<sup>+</sup> cells (**B**);  $n = 6$  samples/group from >3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ .

dependent manner, suggesting that LPS is the active component of the adjuvant in this model. Protollin's protective effects in another murine model, as a component of an intranasal respiratory syncytial virus vaccine, were also mediated by TLR4-MyD88 signaling and not TLR2 (53). Interestingly, the *N. meningitidis* outer membrane vesicle vaccine, which is related in composition to Protollin, has also been shown to depend on TLR4 and not TLR2 activation (54). The combined nasal application of BPEX with Protollin produced a greater inhibition of AHR compared with BPEX on its own and reduced BAL granulocyte numbers. Intrapulmonary LPS administration to mice has been shown to prevent experimental allergic asthma, associated with an absence of allergen-specific Th2 or Th1 cytokines, suggested to be anergy (55). Other studies have implicated Th1 responses, because of elevated expression of the transcription factor T-bet (56) or a requirement for IL-12 (32), and/or IL-10-producing CD4<sup>+</sup> cell responses (34, 57), in the inhibition of Th2-mediated allergic airway disease, depending on the timing of intrapulmonary LPS exposure in relation to disease onset or development. We aimed to characterize the CD4 T cell response associated with the TLR4-



**FIGURE 8.** Intranasal Protollin application may promote the recruitment of ICOS-expressing CD4<sup>+</sup> T cell populations to the lungs. Percentage of lung CD4<sup>+</sup>Foxp3<sup>+</sup> and CD4<sup>+</sup>Foxp3<sup>-</sup> cells expressing ICOS [(**A**) and (**B**), respectively], harvested from wt BALB/c mice on day 17, 24 h after two PBS or BPEX challenges following nasal applications of either PBS or Protollin alone (Pro alone), as well as absolute cell numbers [(**C**) and (**D**), respectively]. Proportion of CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> or ICOS<sup>+</sup>Foxp3<sup>-</sup> cells relative to CD4<sup>+</sup>ICOS<sup>-</sup>Foxp3<sup>+</sup> cells [(**E**, **F**);  $n = 7-8$  samples/group from 3 independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

dependent inhibition of experimental allergic airway disease via the nasal mucosa by nasal Protollin administration, but found no induction of IFN- $\gamma$  mRNA in the NALT, or protein in CD4<sup>+</sup> cells of the superficial cervical LNs or lungs, as well as in BAL fluids. Expression of T-bet in the NALT was also unaffected by Protollin (data not shown). We explored an alternative immunoregulatory mechanism, namely, the induction of Tregs by Protollin in the NALT, cervical LNs, and lungs. Neither Foxp3 expression nor CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg numbers were significantly altered; however, we found the exclusively T cell-expressed ICOS to be significantly induced in the NALT after nasal application of Protollin, revealing an immunomodulatory effect on T cells in the nasal mucosa. We confirmed by flow cytometry that ICOS expression was substantially increased in CD4<sup>+</sup> cells of the superficial cervical LNs, but not of the lungs after Protollin administrations and a single allergen challenge. At a later time point, after two allergen challenges, we also found elevated ICOS



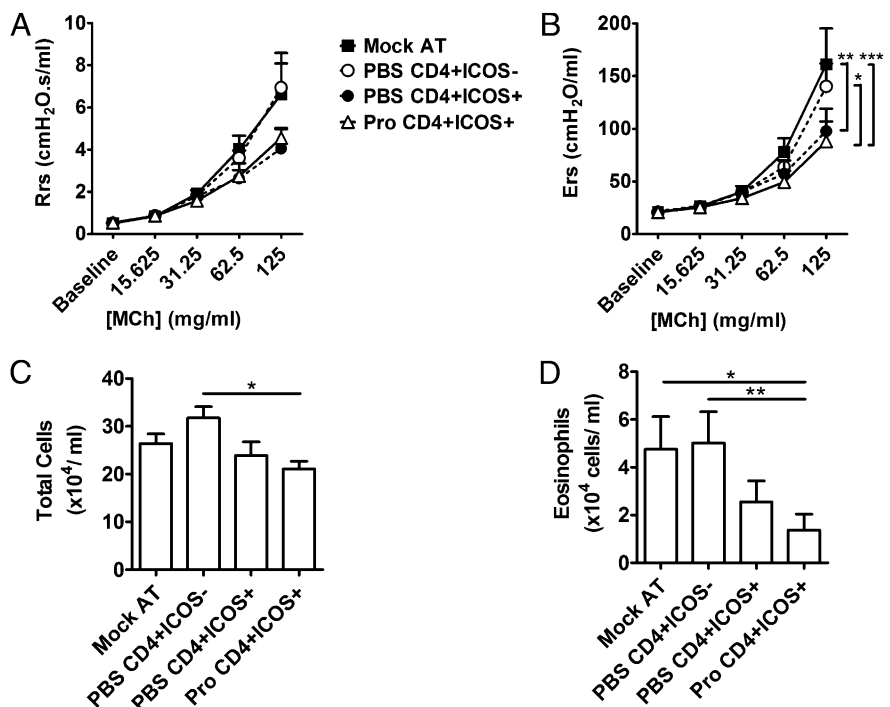
**FIGURE 9.** Intranasal Protollin application induces IL-10-expressing CD4<sup>+</sup>ICOS<sup>+</sup> T cells, but IL-10 is dispensable for the inhibition of allergic airway disease. Intracellular cytokine staining and flow cytometry were used to assess IL-10 expression among anti-CD4, anti-ICOS Ab-stained cervical LN cells harvested on day 16, 24 h after a single sham (PBS) or allergen (BPEX) challenge of mice after nasal applications of PBS or Protollin. The ratio of IL-10<sup>+</sup> to IFN- $\gamma$ <sup>+</sup> cells among LN CD4<sup>+</sup>ICOS<sup>+</sup> cells is also shown (A); *n* = 6 animals/group from >3 independent experiments. Absolute number of CD4<sup>+</sup>ICOS<sup>+</sup>IL-10<sup>+</sup> cells harvested from the lungs on day 17, 24 h after two PBS or BPEX challenges following nasal applications of PBS or Protollin (B); *n* = 7–8 animals/group from 3 independent experiments. Airway responses to MCh (Ers) (Ci), BAL eosinophil (Cii), and lymphocyte (Ciii) numbers evaluated after BPEX challenge of mice that received in vivo administrations of either neutralizing anti-IL-10 mAb or isotype control Ab coinciding with intranasal Protollin administrations; *n* = 4 animals/group from 2 independent experiments. \**p* < 0.05.

expression among lung CD4<sup>+</sup> cells, as well as an induction in both CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> and ICOS<sup>+</sup>Foxp3<sup>-</sup> populations, indicating possible migration of these cells from the draining LNs. This latter observation is supported by a recent report that ICOS expression enhances CD4 T cell migration to the lungs (39).

ICOS expression has been identified as a distinguishing marker conferring enhanced function and survival among human (58) and

murine (41) Tregs. Interestingly, Protollin increased the proportion of LN CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> cells to ICOS<sup>-</sup>Foxp3<sup>+</sup> cells, without a simultaneous impact on the ratio of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> to CD25<sup>-</sup>Foxp3<sup>+</sup> cells, indicating a selective induction of ICOS among Tregs. We also observed a specific induction of CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> and not CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in the lungs. Thus, our data establish the induction of ICOS rather than CD4<sup>+</sup>

**FIGURE 10.** ICOS-expressing CD4<sup>+</sup> cells of the cervical LNs have the capacity to inhibit allergic airway disease. Airway responses (Rrs and Ers) to aerosolized MCh of allergen-challenged wt BALB/c mice receiving prior injection of sterile PBS (mock AT), or adoptive transfer of CD4<sup>+</sup>ICOS<sup>+</sup> or CD4<sup>+</sup>ICOS<sup>-</sup> cells pooled from the cervical LNs of either intranasal PBS- or Protollin-treated mice [(A) and (B), respectively]. Total inflammatory cells and eosinophils [(C) and (D), respectively] in BAL fluid collected immediately after assessment of airway responses; *n* = 5–7 animals/group from 3 independent experiments. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.



CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs as a correlate of the inhibition of AHR. The increased relative ICOS but not CD25 expression among CD4<sup>+</sup>Foxp3<sup>+</sup> cells from Protollin-treated mice suggests that these CD4<sup>+</sup> cells may consist of distinct subpopulations that are differentially modulated by Protollin, or that the induced ICOS-expressing cells constitute only a minute subset of the CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> population. An alternative explanation may be that Protollin induces ICOS equivalently among CD25<sup>+</sup>Foxp3<sup>+</sup> and CD25<sup>-</sup>Foxp3<sup>+</sup> Tregs.

Protollin-induced, ICOS-expressing CD4<sup>+</sup> cells in the cervical LNs were, however, predominantly Foxp3<sup>-</sup>. High ICOS expression has been reported in IL-10–producing cells such as the peripheral regulatory Tr1 cells (59). Interestingly, IL-10 expression in the NALT was increased by Protollin administration compared with PBS and by combined Protollin/BPEX administration compared with BPEX alone. In addition, nasal application of Protollin before allergen challenge increased the numbers of CD4<sup>+</sup>ICOS<sup>+</sup>IL-10<sup>+</sup> cells in the cervical LNs, as well as their proportion relative to IFN- $\gamma$ <sup>+</sup> cells, without concomitant increases in lung CD4<sup>+</sup>ICOS<sup>+</sup>IL-10<sup>+</sup> cells. Intrapulmonary administration of LPS at a dose that is comparable with our intranasal application has been reported to inhibit experimental OVA-induced asthma by inducing lung resident myeloid-derived suppressor cells to produce IL-10 (33). We also found no increase in IL-10 expression by CD4<sup>-</sup> cells in the lungs at any time point (data not shown), further supporting the absence of a direct effect of Protollin on the lungs. After two allergen challenges, Protollin-treated mice had higher numbers of CD4<sup>+</sup>ICOS<sup>+</sup>IL-10<sup>+</sup> cells in the lungs; however, this was not significantly different compared with PBS-treated, allergen-challenged mice, and no increase in IL-10 protein levels could be detected in the BAL fluid at a later time point. Finally, the administration of anti-IL-10 mAb had no effect on the inhibition of AHR or BAL eosinophilia by Protollin, despite significant effects on other inflammatory cell populations in the BALs. Thus, despite preferential expression of IL-10 by Protollin-induced CD4<sup>+</sup>ICOS<sup>+</sup> cells of the LNs draining the nasal mucosa, this cytokine did not appear to mediate the inhibitory effects of Protollin in this model.

ICOS deficiency in humans precipitates either immunodeficiency or autoimmunity, indicating its importance in immunoregulation (37). Through adoptive transfer studies, we confirmed that ICOS-expressing CD4<sup>+</sup> cells from the cervical LNs of Protollin-treated mice were capable of inhibiting the development of allergic airway disease, indicating that the TLR4-dependent induction of ICOS via the nasal mucosa can inhibit lower airway allergic disease. However, we did not confirm whether the CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>+</sup> or CD4<sup>+</sup>ICOS<sup>+</sup>Foxp3<sup>-</sup> populations mediated this inhibition and whether their recruitment to the lungs was necessary for protection. There is evidence to suggest that either population could be inhibitory, given that ICOS–ICOS ligand (ICOS-L) interaction alone has been shown to induce immunoregulatory negative feedback (60). Consistent with our findings, ICOS<sup>+</sup> cells inducing T cell anergy without a requirement for IL-10 have been identified in both humans and mice (61, 62). Interestingly, the ICOS–ICOS-L interaction was reported to be necessary in only one of these reports, indicating that ICOS ligation is not always required for the inhibitory function of ICOS<sup>+</sup> cells. T cells that rely on IL-10 and ICOS–ICOS-L interactions have also been shown to inhibit AHR and allergic airway inflammation (63); however, Foxp3 expression was not reported in any of these studies. Significantly, a recent report by Whitehead et al. (64) demonstrated that induction of ICOS<sup>+</sup>Foxp3<sup>+</sup> T cells mediated the natural suppression of IL-17–dependent allergic airway disease that occurs with repeated allergen challenge. This was IL-10 independent,

despite its expression by these cells, and was rather mediated by the cytokine IL-35 (64). The authors proposed that targeting this pathway might be of therapeutic value for the treatment of allergic asthma in humans. Interestingly, both aforementioned studies that demonstrated ICOS-dependent spontaneous resolution of murine allergic airway disease after allergen challenge used the experimental allergen OVA, known to be tainted with significant levels of LPS. Whether the contaminating LPS has any bearing on the induction of ICOS in these studies was not described. In this article, we show that the mucosal adjuvant Protollin induces ICOS among CD4<sup>+</sup>Foxp3<sup>+</sup> and Foxp3<sup>-</sup> cells via the nasal mucosa in a TLR4-dependent manner. TLR4 stimulation is known to enhance ICOS-L expression in innate immune cells (65, 66); our finding that TLR4 induces ICOS provides additional insight into the regulation of CD4 T cell responses by innate immune stimuli. Finally, that the adoptive transfer of equivalent numbers of CD4<sup>+</sup>ICOS<sup>+</sup> cells pooled from intranasal PBS or Protollin-treated mice appeared to yield the same inhibitory effects suggests that the cervical LNs inherently contain resident CD4<sup>+</sup>ICOS<sup>+</sup> cells that possess an intrinsic regulatory capacity, and that stimulation of the nasal mucosa via TLR4 serves to amplify this population to a threshold that permits the inhibition of allergic airway disease in this model. Our work is supported by a report that specifically proliferating CD4<sup>+</sup>CD25<sup>+</sup> and CD25<sup>-</sup> T cell subsets isolated from mouse cervical LNs possessed the capacity to transfer tolerance to delayed-type hypersensitivity (51).

In conclusion, we find that nasal application of Protollin is sufficient to prevent the development of lower airway allergic disease in response to subsequent allergen exposure, with minimal inflammation of the lungs, and that this inhibition is dependent on TLR4, but not TLR2. It is associated with an induction of ICOS expression in the nasal mucosa and on CD4<sup>+</sup> T cells of the draining LNs and lungs. Finally, we show that resident CD4<sup>+</sup>ICOS<sup>+</sup> cells of the cervical LNs have the capacity to inhibit allergic airway disease in mice and can be amplified via TLR4 stimulation of the nasal mucosa.

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## Disclosures

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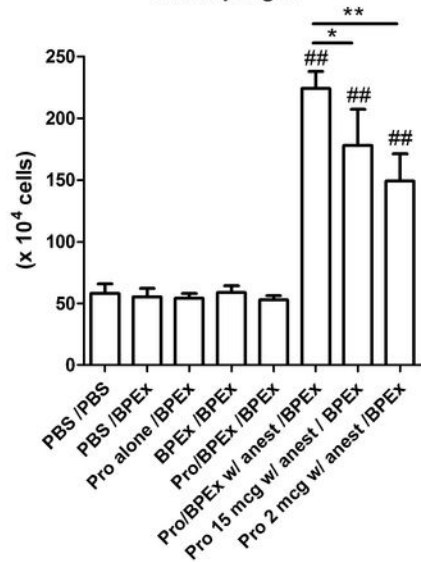
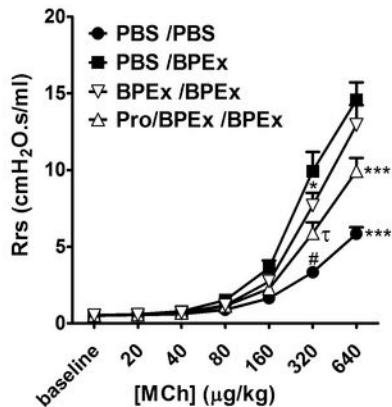
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## Supplementary Figure Legends

**Supplementary Figure 1. Intranasal application of Protollin does not induce significant inflammation in the lungs, as compared to intrapulmonary administration of an equivalent or lower dose, and is sufficient to inhibit AHR (respiratory system resistance) to increasing doses of MCh.** Macrophage numbers (A) in BALs collected on day 19 after nasal application without anesthesia of PBS, Protollin alone, BPEX, or Protollin combined with BPEX (Pro/BPEX), or intranasal application with anesthesia of Protollin , 15 or 2  $\mu$ g, or Pro/BPEX, followed by sham (PBS) or allergen (BPEX) challenge; n=5-19 animals/group from > 3 independent experiments. All groups receiving intranasal applications of Protollin alone or Pro/BPEX with anesthesia were significantly different from groups receiving nasal applications without anesthesia (<sup>##</sup> p < 0.001). Airway responses (respiratory system resistance) to i.v. (B; n=8-19 animals/group from > 3 independent experiments) or aerosolized (C; n=6-8 animals/group from > 3 independent experiments) MCh 48 hrs after intranasal sham (PBS) or allergen (BPEX) challenges following nasal application to conscious mice of either PBS, BPEX, Pro alone, or Pro/BPEX (\* indicates significant difference of BPEX /BPEX from PBS /BPEX; <sup>†</sup> indicates difference of Pro/BPEX /BPEX from PBS /BPEX; <sup>#</sup> indicates difference of PBS /PBS from all other groups in Supp. Fig. 1B; \*\* and \*\*\* in Supp. Fig. 1C indicate significant difference of PBS /BPEX from Pro alone /BPEX).

**A****Macrophages****B**

\*\*\* p < 0.001 significantly different from all other groups

# p < 0.001 significantly different from all other groups, except p < 0.05 compared to Pro/BPEx/BPEx

τ p < 0.001 compared to PBS/BPEx

\* p < 0.05 compared to PBS/BPEx

**C**