

Supporting Information

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SI Materials and Methods

Induction of Tolerance or Inflammation and Assessment of Airway Inflammation. Mice were tolerized by daily exposure to aerosolized OVA (1% wt/vol in PBS) for 20 min/day for 10 days consecutively. Ten days after completion of these treatments, mice were challenged by immunization with two i.p. injections of 10 μ g per ml OVA and 1 mg of alum (Intergen) on days 21 and 27 of the protocol. Seven days later, mice were again exposed to aerosolized 1% OVA daily for an additional 7 days.

Acute and chronic asthma models were used for induction of airway inflammation (1, 2). The acute model essentially consisted of the challenge phase of the tolerance protocol: two injections of OVA/alum followed by treatment with aerosolized OVA. The chronic model consisted of i.p. injections of OVA/alum on days 1, 14, and 21 followed by aerosol treatments with 1% OVA on days 28–29, 35–36, 42–43, and 47–48. In each of these models, assessment of airway inflammation was carried out on the day after the completion of the protocol as described previously (1).

Bronchoalveolar lavage (BAL) was performed, and total as well as differential cell counts in the BAL fluid were assessed as previously described (3). Histologic examination was carried out after fixing lung tissues in Streck tissue fixative, paraffin embedding, and H&E staining. Inflammation in lung tissues was blindly scored on a scale from 1 to 5 (4), with 1 being indicative of no inflammation and 5 representing the highest degree of inflammation. At least 6 fields were scored for each section. The data are presented as the mean \pm SD for each section.

Assessment of Airway Mucus. The presence of mucus in cells and in the airways was detected by using a periodic acid-Schiff staining kit (Richard Allan Scientific) with minor modifications. Deparaffinized, rehydrated histologic sections were stained sequentially with periodic acid solution, Schiff reagent, hematoxylin, and bluing reagent. Stained sections were then dehydrated with anhydrous alcohol and cleared with xylene substitute before mounting. Random images were then taken in a blinded fashion of 10 airways from both proximal and distal locations. These images were scored as “blocked” or “open” depending on the amount of mucus plugging the airways. A percentage of blocked airways per animal was then estimated and compiled into the average percentage of blocked airways per group. The average percentage of blocked airways in each group is shown \pm SD.

Measurement of Airway Hyperresponsiveness. Airway responsiveness (AHR) to methacholine (MCh) was assessed at 24 h after the final challenge by OVA inhalation by using the forced oscillation technique to measure respiratory mechanics as previously described by using the FlexiVent system (SCIREQ) (5, 6). From the analysis, the following indices of lung function were derived: Newtonian resistance (R_n , a measure of central airways caliber), tissue resistance (Gti-abbreviated G, a measure of either ventilatory inhomogeneity or tissue viscoelastic properties), and elastance (Hti-abbreviated H, a measure of lung elasticity or airway closure).

Mice were anesthetized with pentobarbital sodium (90 mg/kg i.p.+ 5 mg/kg after intubation of animals) diluted in saline. Tracheostomy was then preformed, and a metal 18-gauge canula was inserted into the trachea. The measurements of airway resistance (R_n) were taken first at baseline by exposing to nebulized PBS and then after exposure to ascending doses of MCh (3.125 mg/ml, 6.25 mg/ml, 12.5 mg/ml, and 25 mg/ml). The

peak response for each variable was then determined, and the percentage of change from baseline, measured at the beginning of the protocol, was calculated.

OVA-Specific IgE and IgG2a ELISAs. OVA-specific IgE was measured by ELISA as described previously by us (1, 7). OVA-specific IgG2a was measured essentially as described previously (8). 96-well microtiter plates were coated with 50 μ g per ml of OVA in 0.1 M NaHCO₃ by overnight incubation at 4°C. The plates were washed with 0.05% PBS-Tween 20 and blocked with 3% BSA-PBS for 2 h at room temperature. One hundred microliters of diluted serum samples in 1% BSA-PBS were added to wells, and the plates were incubated overnight at 4°C. After washing with PBS-Tween 20, 100 μ l of biotinylated anti-mouse IgG2a (BD Biosciences, 1 μ g/ml) was added to the wells for 2 h. After washing, streptavidin-horseradish peroxidase (Amersham Biosciences, 1:1,000) was added, and incubation was continued for 30 min at room temperature. The plates were then washed, and 100 μ l of substrate solution (R&D Systems) were added. The reaction was terminated by adding 100 μ l of 1 M H₂SO₄, and absorption at 450 nm was read.

As standards for anti-OVA IgE and anti-OVA IgG2a, pooled sera from animals immunized with OVA/alum and boosted or from animals immunized with OVA/CpG ODN and repeatedly challenged with aerosolized OVA were used, respectively. The standard curves were generated by linear regression analyses of the absorbances versus serial dilutions of the reference sera. The standards were assigned arbitrary units of 10⁴ units per ml for IgE and 25 \times 10⁵ units per ml for IgG2a.

SI Results

IDO^{-/-} Mice Are Able to Mount Tolerance but Attenuated Allergic Airway Inflammation in the Absence of IDO. In mice subjected to the acute inflammation model by i.p. sensitization with OVA/alum followed by challenge with aerosolized OVA, surprisingly, the level of Th2 cytokines detected in the bronchoalveolar lavage fluid (BALF) derived from IDO^{-/-} mice was lower than that detected in the BALF obtained from WT mice (Fig. S1).

OVA-specific IgE in the serum of immunized IDO-deficient mice was also lower compared with that in the serum of WT mice (Fig. S1). Although these differences in cytokine and serum IgE levels between the IDO^{-/-} mice and the WT controls did not reach statistical significance, the trend was observed in multiple experiments, suggesting a possible involvement of IDO in the establishment of Th2 responses in the airways. In addition, the BALF IFN- γ level, although relatively low as would be expected when using a Th2-inducing model, was higher in IDO^{-/-} animals, further suggesting a shift away from Th2 under IDO-deficient conditions (Fig. S1C).

Interestingly, although OVA-sensitized IDO^{-/-} mice displayed a relatively lower Th2-type airway inflammation, the spleen T cells from these mice showed enhanced immune response upon antigen stimulation *in vitro*, including T cell proliferation and both Th1 and Th2 cytokine production (Fig. S1E and F), which is in agreement with reports in the literature (9–11). These results indicated that the animals used in these studies were not altered with regard to what has been described for IDO-mediated inhibitory function in the spleen but suggested a unique role for this molecule in the lung. In subsequent experiments, given that we saw decreased rather than increased Th2 responses in the lung in the absence of IDO, we focused on

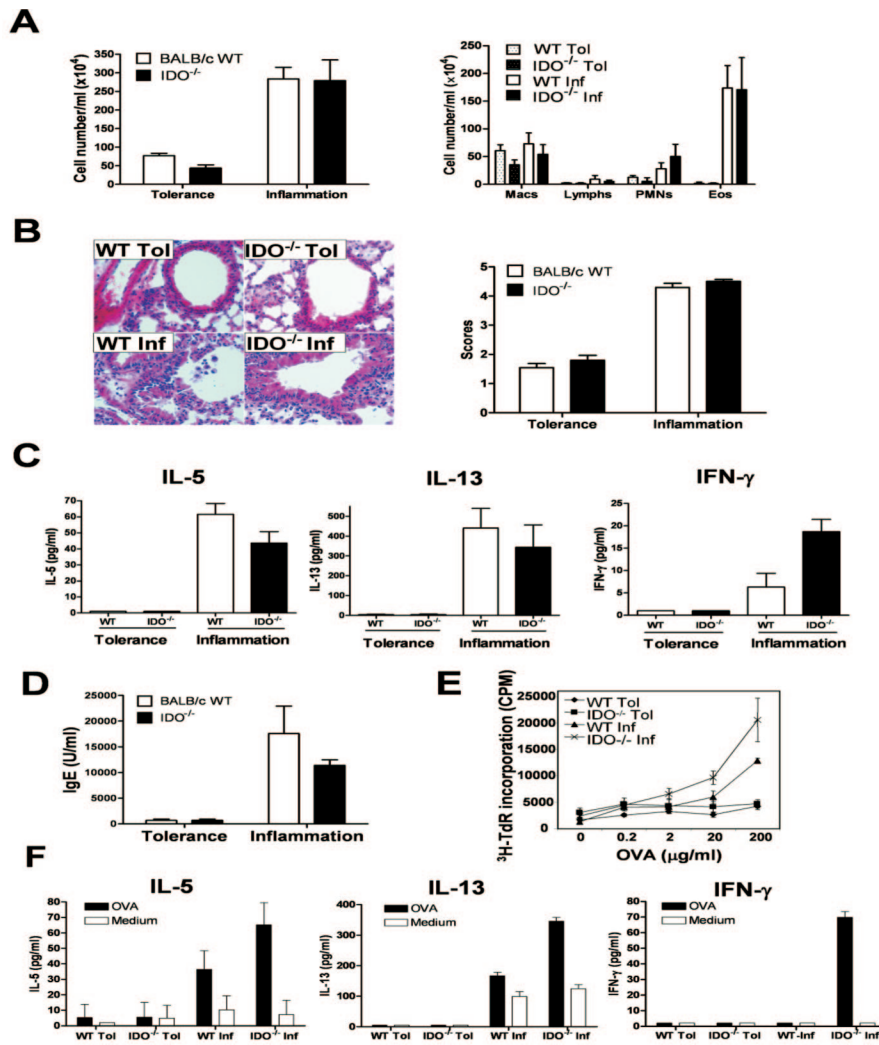
effects of IDO deficiency on inflammation rather than tolerance in the airways.

Lower Cell Numbers in Lung-Draining LNs of Mice Subjected to Th1 or Th2 Model of Lung Inflammation and Lower IL-4/IL-5 but Not IL-13 Recall Response Induced by mDCs from OVA/cholera toxin (CT) model.

The numbers of total, CD4⁺ T cells, and mDCs were lower in the LNs of IDO-deficient mice compared with those in WT mice regardless of the model (Fig. S3A). In the lung, although the difference in total and CD4⁺ T cells numbers between WT and IDO^{-/-} mice was still observed when cells were isolated from

OVA/CT-immunized mice, it was less remarkable in the case of OVA/CpG-immunized mice (Fig. S3A). We next examined the ability of equal numbers of mDCs isolated from the lungs of the immunized WT and IDO^{-/-} mice to stimulate a recall response in the primed T cells from the LNs of the mice by using equal numbers of LN cells. A lower response was detected when mDCs from IDO-deficient mice were used except in the case of IL-13 and IFN- γ (Fig. S3B). It is interesting to note the differential effects on IL-4/IL-5 versus IL-13 given that B7(CD80/86)-CD28 costimulation has been shown to be important for IL-4/IL-5 but not IL-13 production in previous studies (12, 13).

1. Ostroukhova M, et al. (2004) Tolerance induced by inhaled antigen involves CD4(+) T cells expressing membrane-bound TGF-beta and FOXP3. *J Clin Invest* 114:28–38.
2. McMillan SJ, Xanthou G, Lloyd CM (2005) Manipulation of allergen-induced airway remodeling by treatment with anti-TGF-beta antibody: effect on the Smad signaling pathway. *J Immunol* 174:5774–5780.
3. Zhang DH, et al. (1999) Inhibition of allergic inflammation in a murine model of asthma by expression of a dominant-negative mutant of GATA-3. *Immunity* 11:473–482.
4. McMillan SJ, Bishop B, Townsend MJ, McKenzie AN, Lloyd CM (2002) The absence of interleukin 9 does not affect the development of allergen-induced pulmonary inflammation nor airway hyperreactivity. *J Exp Med* 195:51–57.
5. Irvin CG, Bates JH (2003) Measuring the lung function in the mouse: the challenge of size. *Respir Res* 4:4.
6. Bates JH, Irvin CG (2003) Measuring lung function in mice: the phenotyping uncertainty principle. *J Appl Physiol* 94:1297–1306.
7. Oriss TB, et al. (2005) Dynamics of dendritic cell phenotype and interactions with CD4+ T cells in airway inflammation and tolerance. *J Immunol* 174:854–863.
8. Spergel JM, et al. (1998) Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J Clin Invest* 101:1614–1622.
9. Swanson KA, Zheng Y, Heidler KM, Mizobuchi T, Wilkes DS (2004) CD11c+ cells modulate pulmonary immune responses by production of indoleamine 2,3-dioxygenase. *Am J Respir Cell Mol Biol* 30:311–318.
10. Hwu P, et al. (2000) Indoleamine 2,3-dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. *J Immunol* 164:3596–3599.
11. Mellor AL, et al. (2005) Cutting edge: CpG oligonucleotides induce splenic CD19+ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN Type 1 signaling. *J Immunol* 175:5601–5605.
12. Scales HE, et al. (2004) Effect of inducible costimulator blockade on the pathological and protective immune responses induced by the gastrointestinal helminth *Trichinella spiralis*. *Eur J Immunol* 34:2854–2862.
13. Urban J, et al. (2000) IL-13-mediated worm expulsion is B7 independent and IFN-gamma sensitive. *J Immunol* 164:4250–4256.



SI Fig. 5

Fig. S1. IDO^{-/-} mice are susceptible to induction of immune tolerance (Tol). Immune tolerance to OVA was induced in IDO^{-/-} or BALB/c wild-type mice by inhalation of aerosolized OVA. Induction of tolerance was assessed by challenge with i.p. immunization with OVA and alum adjuvant followed by additional aerosol treatments. Airway inflammation (Inf) was induced by i.p. OVA/alum followed by aerosol treatments. (A) Total (Left) and differential (Right) cell counts in BALF recovered from mice undergoing tolerance or inflammation. The results are shown as mean cell number per ml BALF per animal ± SD. MACs, macrophages; Lymphs, lymphocytes; PMNs, neutrophils; and Eos, eosinophils. (B) H&E staining (Left; ×400 magnification) and severity scores (Right) of lung tissue (5 highest, 1 lowest; n = 4 mice per group). (C) BALF cytokines as determined by ELISA. (D) OVA-specific serum IgE was determined by ELISA. Shown are mean values ± SD. (E) Proliferation of total spleen cell cultures and cytokine production (F) in response to OVA *in vitro* as assessed by [³H]thymidine incorporation and ELISA, respectively. Data shown are mean ± SD. *, P < 0.05. The experiment was repeated three times with similar results.

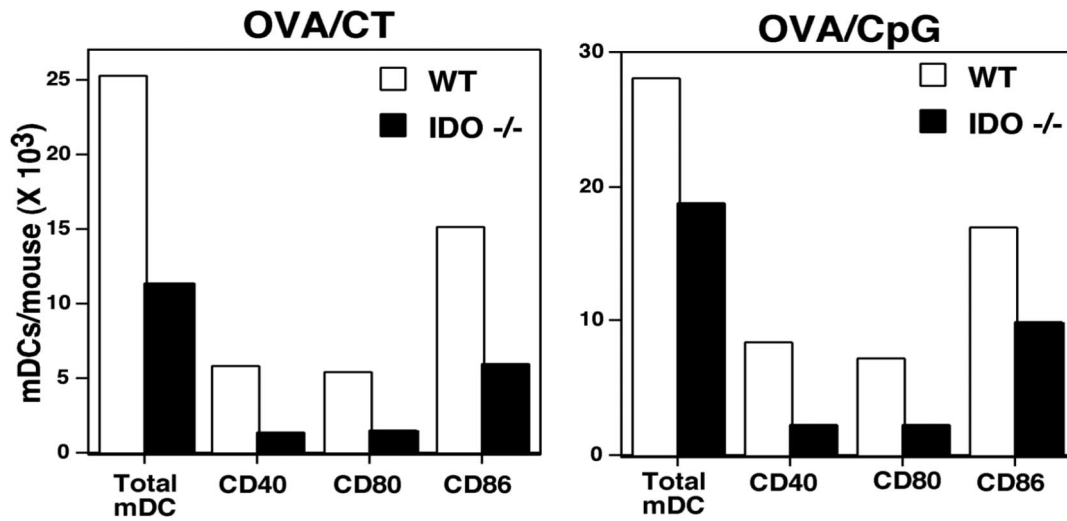


Fig. S2. The total number of lung-draining lymph node mDCs expressing costimulatory molecules is reduced in $IDO^{-/-}$ mice. Mice were treated daily for 3 consecutive days with intranasal administration of OVA/CT or OVA/CpG. Five days after the last intranasal treatment, lung-draining lymph node mDCs were partially purified before analysis by flow cytometry. The mDCs were identified by expression of CD11c and high-level MHC Class II as described previously (7). The percentage of such cells was used to calculate the total number of mDCs per mouse. The mDCs were also assessed for expression of the costimulatory molecules CD40, CD80, and CD86. These data are expressed as the number of cells per mouse expressing each of the molecules. The percentage of cells staining positively with appropriate isotype control antibodies was subtracted before performing the calculations.

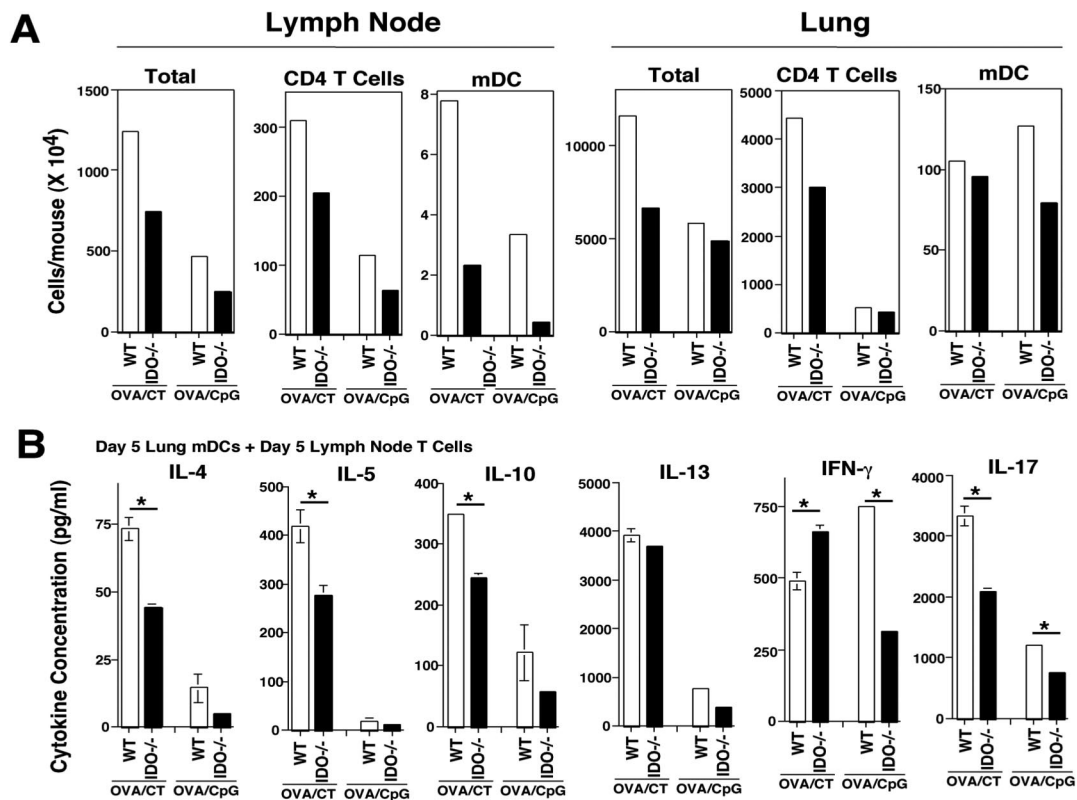


Fig. 53. Lower cell numbers in lung-draining lymph nodes of mice subjected to the Th1 (OVA/CpG) or Th2 (OVA/CT) model and lower cytokine production elicited by lung mDCs in a recall response. Mice were subjected to the OVA/CT or OVA/CpG model (Fig. 4), and 5 days after the last antigen instillation, lung-draining LNs and lung tissue were harvested. (A) From both LNs and lungs, the number of total cells, CD4⁺ T cells, and mDCs was calculated based on cell counts and flow cytometry (*Left*). (B) mDCs were purified from lung tissue by cell sorting and were used to stimulate LN T cells. The recall cytokine response was assessed after 48 h of culture by multiplex assay.

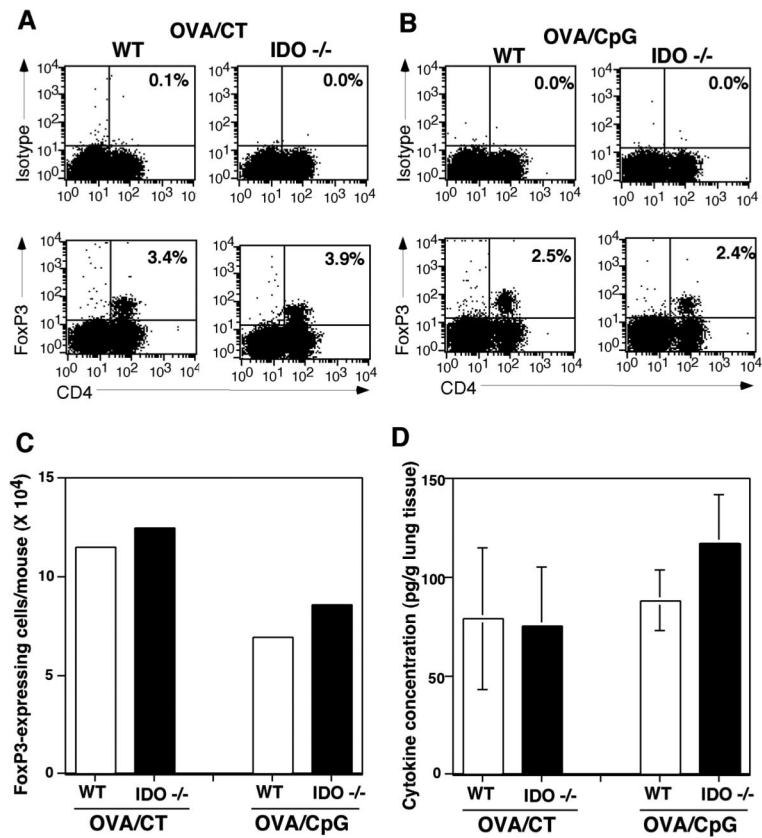


Fig. S4. IDO^{-/-} mice do not exhibit any detectable defect in regulatory T cells. (A–C) Wild-type and IDO^{-/-} mice were treated daily for 3 consecutive days with intranasal administration of OVA/CT or OVA/CpG. Five days after the last intranasal treatment, lung tissue T cells were assessed for expression of FoxP3 by intracellular staining and flow cytometry. (A) Isotype and FoxP3 staining on CD4⁺ T cells after OVA/CT treatment. (B) Isotype and FoxP3 staining on CD4⁺ T cells after OVA/CpG treatment. (C) The percentages of FoxP3 expression were used to calculate the number of FoxP3 expression cells under each of the conditions. (D) Wild-type and IDO^{-/-} mice were treated intranasally with OVA/CT or OVA/CpG as described above and then were rested for a week followed by challenge with aerosolized OVA for 10 days. One day after completion of treatments, whole lung extracts were prepared and assessed for expression of IL-10 by multiplex assay. The extracts were also examined for expression of TGF- β , but none was detected (data not shown).

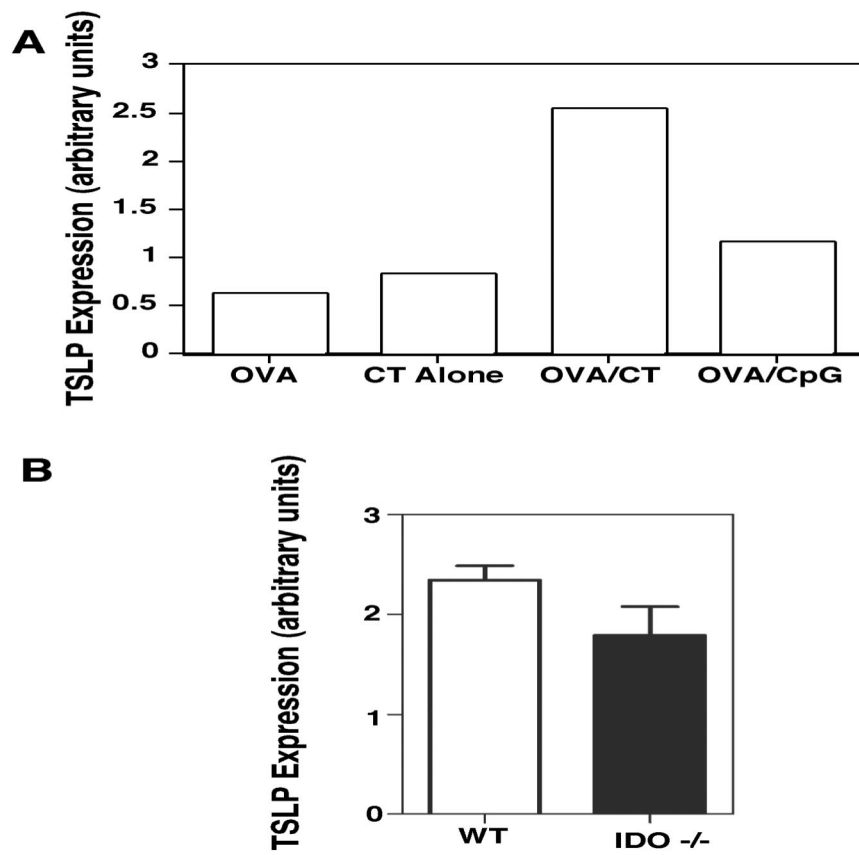


Fig. 55. Selective expression of TSLP in the lung of OVA/CT-treated mice is not defective in $IDO^{-/-}$ mice. (A) Wild-type mice were treated daily for 3 consecutive days with intranasal administration of OVA, CT alone, OVA/CT, or OVA/CpG ODN. Five days after the last intranasal treatment, mRNA was extracted from whole lung tissue and subjected to quantitative RT-PCR to assess TSLP expression. (B) Wild-type and $IDO^{-/-}$ mice were similarly treated and assessed for lung tissue expression of thymic stromal-derived lymphopoietin (TSLP).