

Original article

Neonatal exposure with LPS and/or allergen prevents experimental allergic airways disease: Development of tolerance using environmental antigens

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Background: Studies show that children in rural environments develop less asthma and allergic rhinitis than their urban counterparts. This may be a result, in part, of neonatal exposure to environmental antigens such as LPS and/or early exposure to allergens.

Objective: This study examined the effects of neonatal allergen and/or LPS exposure on subsequent immune responses to allergen.

Methods: Newborn mice were exposed to LPS and/or ovalbumin. At age 6 weeks, these animals were sensitized and challenged with ovalbumin, and airway inflammation, hyperresponsiveness, and cytokine expression were assessed. **Results:** Animals exposed to LPS in the neonatal period developed T cells expressing CD25 and IL-10 on sensitization and challenge. They demonstrated abrogation of airway hyperresponsiveness and significant decreases in IL-13 from bronchoalveolar lavage fluid and in specific IgE. IL-4-expressing spleen cells were also significantly decreased. Mice exposed in the neonatal period to ovalbumin demonstrated airway hyporesponsiveness after subsequent ovalbumin sensitization and challenge and did not produce specific IgE. In contrast, these animals showed increases in IFN- γ . Animals exposed to both LPS and ovalbumin developed a response characterized by IL-10 and IFN- γ -expressing T cells. **Conclusion:** This suggests that mucosal antigen exposure in the neonatal period results in inhibition of allergic responses to environmental allergens. Early LPS exposure directs mucosal responses toward tolerance, whereas ovalbumin exposure follows the T_H1-type response on subsequent sensitization.

Clinical implications: This study suggests that prevention of airways allergy may be best achieved by appropriate exposure of the airway mucosa early in life to environmental antigens. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: Asthma, allergic rhinitis, developmental immunology, allergy prevention, animal model, T-regulatory cells, lipopolysaccharide

Allergic sensitization of the airways with common aeroallergens is associated with the development of asthma and allergic rhinitis and affects as much as 30% of the general population. Asthma and allergy are the most frequent chronic diseases in children, with significant associated morbidity.^{1,2} Children in rural environments are somewhat protected from the development of atopic disease and asthma, and this protection may be a result of early exposure to immune stimuli including allergens and endotoxins, such as LPS.³⁻⁹ Essential to the development of allergy is the activation of T_H2-type T cells on contact with allergen. Nonallergic individuals respond to allergen by developing either a T_H1-type or T-regulatory (TR)-type immune response.¹⁰⁻¹³ Although individuals with allergy appear to be genetically predisposed to follow a T_H2-type response pattern,¹⁴⁻¹⁶ epidemiologic studies suggest that environmental influences can redirect responses toward T_H1 or TR.^{9,15-18} The hygiene hypothesis, as proposed by Strachan¹⁹ in 1989, theorized that early stimulation of the airways with microbes results primarily in the stimulation of T_H1 responses that, through a form of feedback inhibition, reduced the tendency for T_H2 expression in the airways. Failure to stimulate the airway appropriately therefore would result in increases in airway sensitization along a T_H2 pathway.²⁰ Recent evidence suggests that nonallergic airway responses are characterized by the activation of TR cells that actively inhibit both T_H1 and T_H2-type responses.^{11-13,21-24}

We hypothesized that asthma and allergy result from failure to tolerize actively the airway mucosal immune system early in life through antigen stimulation. Thus, if early responses are not appropriately directed, subsequent mucosal contact with innocuous aeroallergens would result in allergic sensitization. Although the prenatal immune system is predisposed to T_H2 cell activation,^{14,25} postnatal maturation of the immune response, driven by contact with antigen, may take several different pathways depending on the stimulus.^{9,18,26} Epidemiologic evidence suggests that the prenatal and neonatal immune system can be influenced by these environmental stimuli and that

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Abbreviations used

BAL: Bronchoalveolar lavage
 KLH: Keyhole limpet hemocyanin
 NALT: Nasal associated lymphoid tissue
 OVA: Ovalbumin
 TLR4: Toll-like receptor 4
 TR: T-regulatory

exposure to antigens in the neonatal period may result in organ-specific tolerance.^{6,9,16,17,27}

In the current study, we exposed neonatal mice within the first week of life to the allergen ovalbumin and/or the endotoxin LPS and examined subsequent immune responses in adult mice. Our findings demonstrate that early local exposure with LPS results in generation of CD25⁺, IL-10-expressing TR cells on subsequent sensitization and challenge with novel allergen, ovalbumin. Moreover, our data also indicate that neonatal exposure ovalbumin results in elaboration of ovalbumin-specific CD4⁺ T cells expressing IFN- γ on subsequent challenge, characteristic of T_H1-type responses. Taken together, these data suggest that neonatal immune stimulation influences mucosal immunoregulation directing subsequent responses toward functional tolerance, either through elaboration of TR or T_H1 cells, and ultimately inhibits the generation of allergies and asthma.

METHODS

Animals

Pregnant female BALB/C mice were obtained from Harlan-Sprague Dawley (Indianapolis, Ind) and were housed in a conventional animal facility at the Meakins-Christie Laboratories. All studies followed Canadian Council of Animal Care (CCAC) guidelines and were approved by the Animal Care Committee at McGill University. BALB/C mice were used in all experiments as a well standardized strain for models of allergic asthma. We have previously characterized the allergic responses using our sensitization and challenge protocol with this strain of mice.²⁸ For all experiments, *in vivo*, 8 to 10 animals were used per group. Animals remained in good condition until completion of the experimental protocol.

Neonatal antigen exposure

From the 3rd day of life, animals were divided into 6 groups and were exposed to antigen by intranasal application of 5 μ L in each nare daily for 10 days as detailed in Fig 1, A. Groups received one of the following solutions: normal saline (NS), 1% ovalbumin (grade V; Sigma-Aldrich, St Louis, Mo), 1.0 μ g LPS (*Escherichia coli* serotype 0111:B4; Sigma-Aldrich), 1% keyhole limpet hemocyanin (KLH; Sigma-Aldrich), and 1.0 μ g LPS in 1% ovalbumin.

Ovalbumin sensitization and challenge

At the age of 6 weeks, awake animals were sensitized daily for 10 days by local instillation of 5 μ L in each nare of 1% ovalbumin in PBS as previously described.^{28,29} Animals were then challenged on 5 consecutive days and assayed 24 hours after the final challenge (Fig 1, A).

Assessment of lung hyperresponsiveness by methacholine challenge

Bronchial responsiveness was measured 24 hours after ovalbumin challenge by using a small animal ventilator (FlexiVent; SCIREQ Montreal, Quebec, Canada) as described in detail elsewhere.²⁹ Maximal resistance measurements were obtained before and after graded doses of methacholine were given. Results are reported at low-dose methacholine (10 mg/kg).

Measurement of ovalbumin-specific IgG and IgE

Ovalbumin-specific IgG and IgE were assessed by ELISA as described previously.²⁸ For assessment of ovalbumin-specific IgG, standard mouse purified mouse IgG (Zymed Laboratories, San Francisco, Calif) was used, serially diluted. Ovalbumin-specific IgE was performed by using an ELISA kit (OPTeia IgE Kit; Pharmingen, San Diego, Calif) with the following modifications. Serum was incubated overnight with protein G sepharose beads. Plates were coated with ovalbumin before the addition of serum. Standards were plated and ELISA performed as per protocol (OPTeia mouse IgE set; Pharmingen).

Bronchoalveolar lavage

Immediately postmortem, lungs were lavaged with ice-cold 0.9% NaCl. The supernatant was stored at -20°C for IL-13 measurement. Cells were spun onto glass slides and stained by using the Diffquick method (Dade Behring Inc, Deerfield, Ill). Differential cell counts were obtained manually under light microscopy. Cells 2 to 3 $\times$ 100 were counted per slide and means obtained.

RNA extraction and RT-PCR

All materials for RNA extraction and RT-PCR were purchased from Invitrogen (Life Technologies, Carlsbad, Calif). Total RNA was extracted from nasal associated lymphoid tissue (NALT) by using Trizol reagent. RNA was treated with DNase according to manufacturer's instructions. cDNA synthesis was performed with SuperScript II RT. PCR conditions were 2 minutes at 94°C , 45 seconds at 94°C , 30 seconds at 54°C , 1 minute 30 seconds at 72°C for 35 cycles, then for 10 minutes at 72°C , performed in a DNA thermal cycler (MJ Research, Cambridge, Mass). The products were visualized by electrophoresis and analyzed by FluorChem Imaging Systems (Alpha Innotech, San Leandro, Calif). The following PCR primers were used: IL-10 5'-CTGAGGCGCTGTCATCGATT-3' and 5'-AGGTCCTGGAGTCCAGCAGA-3'; GAPDH 5'-GCCATGGACTGTGGTCATGA-3' and 5'-TTCACCACCATGGAGAAGGC-3'.

T-cell purification and culture

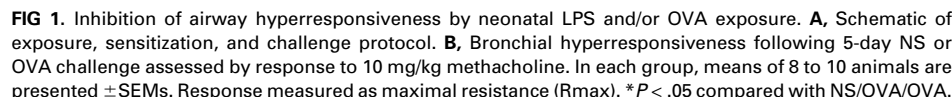
Single-cell suspensions of T cells were prepared from whole spleen as described previously. Splenocytes were then cultured for 4 days at 37°C in the presence of 100 $\mu\text{g/mL}$ ovalbumin. Supernatant was stored at -20°C for later cytokine assays, and T cells were stained for intracellular cytokines.

Cytokines detection by ELISA

Murine IL-10, IFN- γ ELISA kits were purchased from BD PharMingen (San Diego, Calif), and murine IL-13 ELISA kit was purchased from BioSource (Camarillo, Calif). IL-10, IL-13, and IFN- γ in the supernatant from ovalbumin-stimulated T-cell cultures and IL-13 in the bronchoalveolar lavage (BAL) were quantified following the manufacturer's instructions.

Flow cytometric analysis of intracellular cytokines

MACS-purified CD4⁺ T cells (Mouse CD4 Microbeads; Miltenyi Biotec, Auburn, Calif) were cultured in the presence of anti-CD3 (0.5



ovalbumin sensitization and challenge. To determine the specificity of this inhibitory effect, animals were also exposed to another allergen, KLH, in the neonatal period and subsequently sensitized and challenged with ovalbumin as adults. These animals showed no inhibition of airway hyperresponsiveness and behaved similarly to animals in the neonatal NS exposure group. These data show that animals exposed to individual allergens early in life develop antigen-specific hyporesponsiveness. Interestingly, animals treated with LPS as neonates failed to develop airway hyperresponsiveness to ovalbumin sensitization and challenge, suggesting that the immunomodulatory effects of LPS are antigen nonspecific.

The results are expressed as means $\pm$ SEMs. Statistical significance was measured by 1-way ANOVA followed by Tukey post hoc tests for individual group comparisons using SPSS software.

We then quantified the levels of the T_H2 cytokine IL-13 in BAL fluid from these groups after challenge. There were modest, statistically significant increases in IL-13 in BAL from NS/ovalbumin (OVA)/OVA and KLH/OVA/OVA mice. These results are consistent with those we have previously shown using the local sensitization and challenge model.²⁹ Mice that had been exposed to LPS and/or ovalbumin as neonates exhibited significantly decreased IL-13 levels in BAL fluid after ovalbumin challenge compared with mice sensitized and challenged with

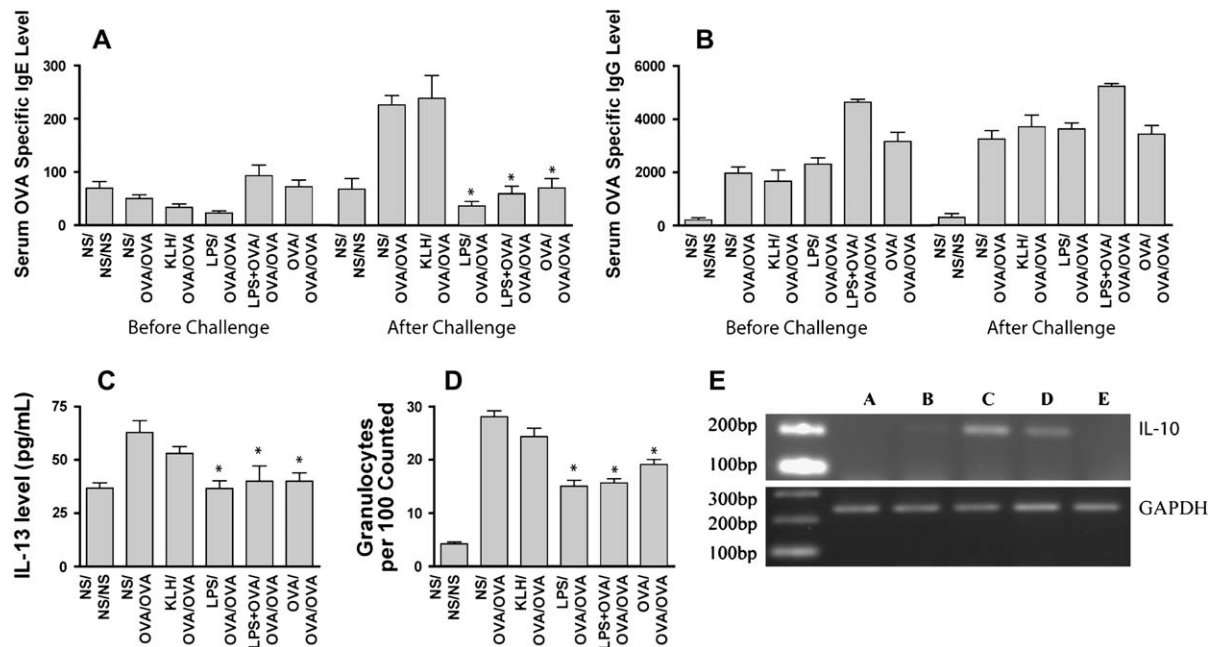


FIG 2. Neonatal OVA and/or LPS exposure inhibits development of specific allergic responses *in vivo*. Means are presented $\pm$ SEMs. **A** and **B**, OVA-specific IgE and IgG before and after OVA challenge. $*P < .01$. **C**, IL-13 production from BAL fluid after challenge. $*P < .05$. **D**, Granulocyte counts in BAL postchallenge. $*P < .001$. **E**, IL-10 mRNA expression in NALT by RT-PCR after challenge; lane A, NS/NS/NS; B, NS/OVA/OVA; C, LPS/OVA/OVA; D, LPS+OVA/OVA/OVA; E, OVA/OVA/OVA. GAPDH gene expression is also shown for comparison.

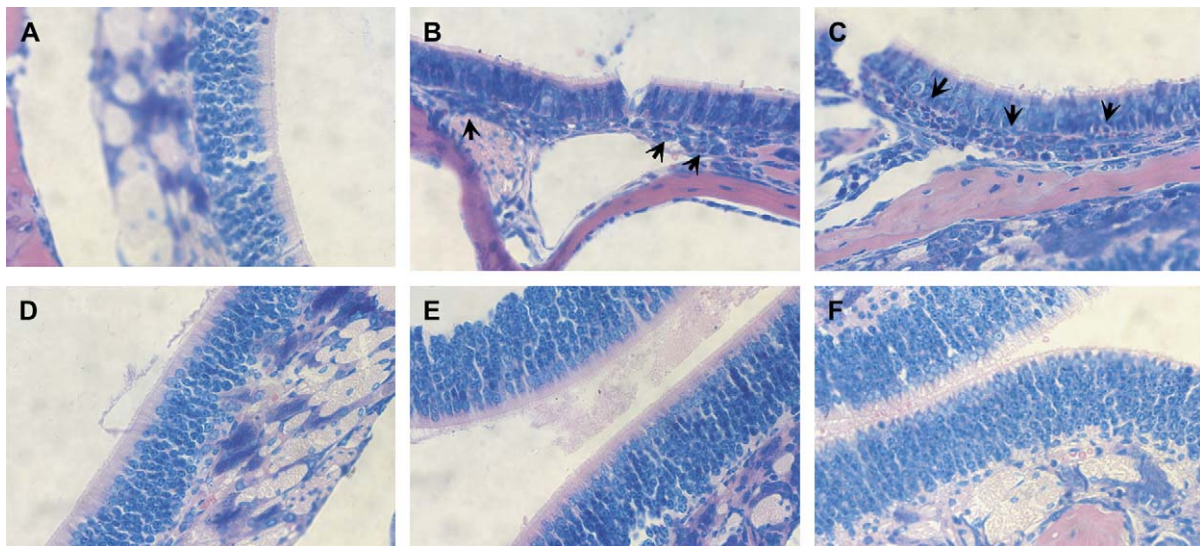


FIG 3. Neonatal exposure inhibits eosinophilic inflammation in the upper airway. Histology sections of BALB/C nares 24 hours after final challenge. Sections were stained with Giemsa stain. Original magnification 400 $\times$. Examples of eosinophils are marked by arrows. **A**, NS/NS/NS; **B**, NS/OVA/OVA; **C**, KLH/OVA/OVA; **D**, LPS/OVA/OVA; **E**, LPS+OVA/OVA/OVA; **F**, OVA/OVA/OVA.

ovalbumin (Fig 2, C). As well, neonatal exposure to ovalbumin or LPS inhibited the subsequent ovalbumin-induced increase in BAL fluid granulocytes (Fig 2, D). These data demonstrate that very early exposure to either specific allergen or LPS affects the characteristics of subsequent immune responses such that T_H2 -type responses are inhibited.

To determine whether this immunomodulation was secondary to the formation of a TR-type response, we assessed expression of the regulatory cytokine IL-10 in the NALT. The primary site of both allergen and LPS deposition in nonanesthetized mice is the upper airway.²⁸⁻³⁰ We therefore examined cytokine expression in NALT. As shown in Fig 2, E, IL-10 mRNA was upregulated in

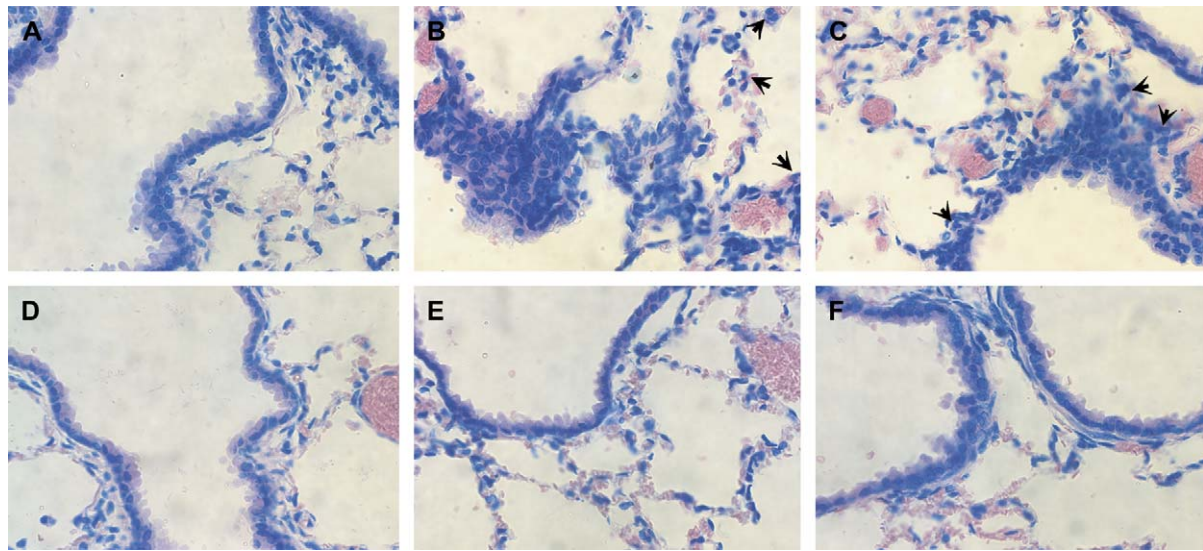


FIG 4. Neonatal exposure inhibits eosinophilic inflammation in the lower airway. Histology sections of BALB/C lung 24 hours following final challenge. Sections were stained with Giemsa stain. Original magnification 400 $\times$. Examples of eosinophils are marked by arrows. **A**, NS/NS/NS; **B**, NS/OVA/OVA; **C**, KLH/OVA/OVA; **D**, LPS/OVA/OVA; **E**, LPS+OVA/OVA/OVA; **F**, OVA/OVA/OVA.

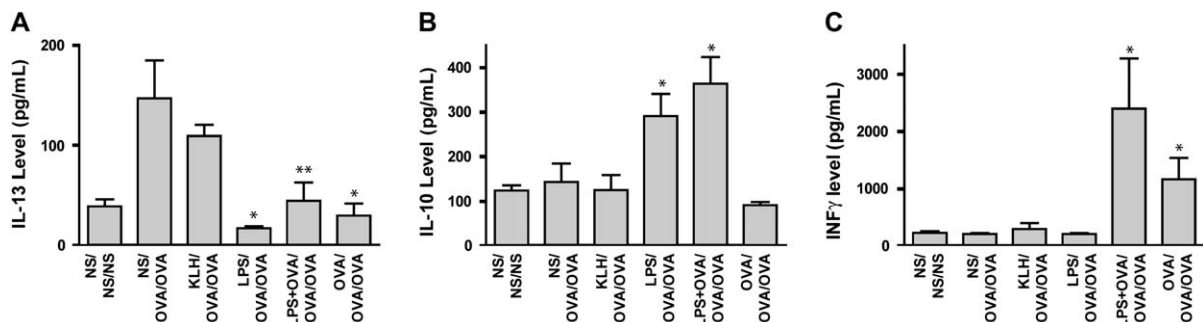


FIG 5. Neonatal exposure to LPS and/or OVA results in alterations in cytokine expression following OVA sensitization and challenge in OVA-cultured splenocytes. Cytokine levels in supernatants were assessed by ELISA. **A**, IL-13 levels $\pm$ SEMs. * $P < .01$, ** $P < .05$. **B**, IL-10 levels $\pm$ SEM. * $P < .05$. **C**, IFN- γ levels $\pm$ SEMs. * $P < .05$.

animals receiving LPS or LPS and ovalbumin during the neonatal period, suggesting that, at this local mucosal site, the cytokine milieu is consistent with the promotion of TR cells.

Exposure to LPS or allergen in neonates inhibits airways inflammation after allergen sensitization and challenge

We examined ovalbumin-induced inflammation in both the nares and lung sections from these mice. After ovalbumin sensitization and challenge, eosinophils were recruited to the upper airways in mice that received either NS or KLH as neonates (Fig 3, B and C) compared with saline-exposed nonsensitized mice (Fig 3, A). In contrast, after ovalbumin challenge, eosinophils were not detected in the upper airways in mice that received LPS and/or ovalbumin as neonates (Fig 3, D-F). Examination of the lower airway also demonstrated increased cellularity and

recruitment of eosinophils in lungs of mice receiving neonatal NS or KLH exposure and subsequently sensitized and challenged with ovalbumin (Fig 4, B and C). In contrast, the lower airways of mice with early LPS and/or ovalbumin exposure (Fig 4, D-F) did not differ from those of NS-exposed, NS-sensitized, and NS-challenged mice (Fig 4, A). Taken together, these data suggest that neonatal exposure to LPS results in changes in the mucosal immune response such that subsequent stimulation using novel allergens results in inhibition of allergen-induced airways inflammation and hyperresponsiveness.

Neonatal exposure to LPS or allergen results in alterations in cytokine expression after allergen sensitization and challenge in cultured splenocytes

After ovalbumin sensitization and challenge, splenocytes were harvested and cultured with ovalbumin, and

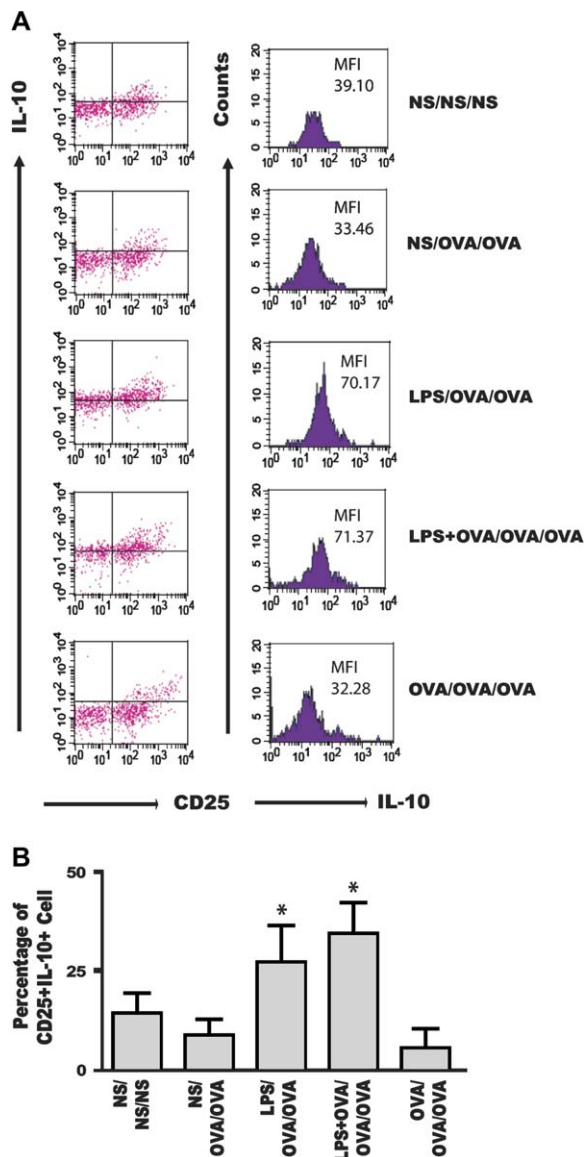


FIG 6. Neonatal exposure induces CD25/IL-10 TR-type cells in OVA-cultured splenocytes. **A**, IL-10/CD25⁺ double-stained cells are shown in the right upper quadrant, and mean fluorescence intensities (MFIs) of IL-10 staining in CD25⁺ cells are reported for 1 representative experiment. **B**, Percent of CD25⁺/IL-10-expressing cells in OVA-cultured splenocytes from each group. Means from 3 independent experiments are shown $\pm$ SEMs. * $P < .05$ compared with NS/OVA/OVA.

levels of IL-13 (T_H2), IL-10 (TR), and IFN- γ (T_H1) were quantified in the supernatants. Splenocytes from animals exposed as neonates to NS or KLH expressed abundant IL-13 (Fig 5, A). In contrast, splenocyte IL-13 levels from mice treated LPS and/or ovalbumin as neonates did not differ from those produced by mice exposed only to NS (Fig 5, A). Importantly, splenocytes from mice exposed to LPS as neonates produced significant amounts of IL-10 compared with all other groups, providing evidence that neonatal exposure of mice to LPS skews ovalbumin-dependent cytokine production to favor a

regulatory-type cytokine profile (Fig 5, B). Interestingly, splenocytes from mice exposed to ovalbumin in the neonatal period produced significantly elevated levels of IFN- γ , a T_H1-type cytokine (Fig 5, C). Finally, as shown in Fig 5, B and C, splenocytes from mice neonatally exposed to both LPS and ovalbumin produced IL-10 and IFN- γ . In all groups, cells cultured in the absence of ovalbumin did not reveal any significant changes in cytokine levels compared with the negative controls (data not shown), providing evidence that the alterations in cytokine expression were dependent on culture with antigen.

LPS exposure induces CD25⁺/IL10⁺ TR cells

To evaluate the phenotype of T cells derived from these animals, splenocytes were cultured with ovalbumin and the phenotype of the CD4⁺ cells evaluated by flow cytometry. As shown in Fig 6, LPS and LPS plus ovalbumin exposure resulted in a significant increase in IL-10-expressing CD25⁺ T cells, a regulatory cell phenotype, compared with all other groups. Interestingly, early exposure to ovalbumin alone did not stimulate the expression of this T-cell phenotype. These data are consistent with those shown in Fig 2, E, in which IL-10 mRNA levels in the NALT were upregulated only in mice exposed as neonates to LPS, and not ovalbumin or KLH.

To define cytokine expression patterns in these mice further, CD4⁺ cells derived from splenocytes cultured with ovalbumin were analyzed for intracellular IFN- γ and IL-4 expression. As shown in Fig 7, ovalbumin sensitization and challenge after neonatal NS exposure (NS/OVA/OVA) results in an increase in IL-4 expression compared with the negative control group (NS/NS/NS). Early ovalbumin exposure (OVA/OVA/OVA) induced a population of IFN- γ -expressing T cells. LPS exposure alone (LPS/OVA/OVA) inhibits the stimulation of IL-4-expressing T cells on allergen stimulation. Taken altogether, these data suggest that early exposure to the immunomodulator LPS results in the formation of CD25⁺IL-10-producing T cells and inhibits the elaboration of T_H2-type IL-4-expressing cells. Early allergen exposure stimulates, in this model, a T_H1-type IFN- γ -expressing T-cell population. Coexposure of LPS plus ovalbumin results in the development of both TR and T_H1-type cellular responses to subsequent allergen sensitization.

DISCUSSION

We examined the effects of neonatal mucosal antigenic stimulation on subsequent mucosal immune responses in adult mice. Allergic airways result from inappropriate immune responses to otherwise innocuous antigens.⁷ Our data support the hypothesis that airways allergy, both asthma and allergic rhinitis, results from failure to tolerize actively the airway mucosal immune system early in life through antigen stimulation.^{26,31,32} If the early response is not appropriately directed, subsequent mucosal contact

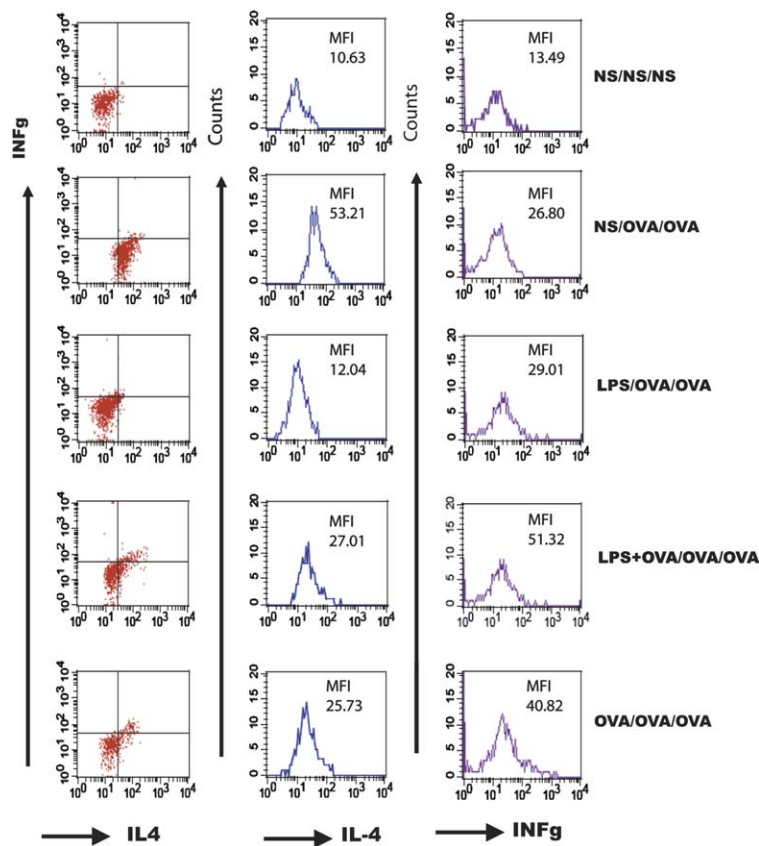


FIG 7. Neonatal exposure to OVA induces CD4⁺ cells expressing IFN- γ , whereas LPS exposure inhibits formation of IL-4 expressing CD4⁺ T cells compared with NS-exposed mice after OVA sensitization and challenge. Intracellular cytokine staining from OVA-splenocyte cultures derived from mice treated as described in Fig 1 assessed by flow cytometry. Results are representative of 3 independent experiments.

with innocuous aeroallergens results in allergic sensitization.

Accumulating evidence suggests that the immune mechanism responsible for protection from aberrant pathological T_H2 responses is active CD4⁺ peripheral immune tolerance mediated by the TR subset.^{12,13,23,24,33} Active suppression involves production of antigen-specific T_H cells that may inhibit T_H2 cell development through elaboration of the cytokine IL-10.³³⁻³⁵ IL-10 has been shown to downregulate both T_H1 and T_H2 cell responses.³⁴⁻³⁶ Significantly, regulatory T cells producing IL-10 protect against the development of allergic asthma in animal models.^{35,36} In this study, we have demonstrated that intranasal neonatal exposure to LPS results in upregulation of IL-10-producing T cells and precludes the development of the allergic phenotype in these mice.

LPS acts on immune cells by binding to receptors, CD14, and Toll-like receptor 4 (TLR4), found on antigen-presenting cells.³⁷ The effects of LPS exposure on the development of asthma have been explored in rodent models.^{9,14,27,38-47} Tulic et al⁴⁶ have shown in a rat model that LPS given immediately before or as long as 4 days after intraperitoneal sensitization with ovalbumin results in decreased ovalbumin-specific IgE and prevention of

inflammatory changes and lung eosinophilia as well as reduced airways hyperresponsiveness after challenge. In contrast, LPS given after sensitization results in increased inflammation and increased vascular leakage.⁴⁷ In another study, mice were exposed to intranasal LPS at 3 weeks of age immediately before allergic sensitization.³⁹ As in the rat model, airway hyperresponsiveness was decreased after treatment, although the duration of this effect was not explored. The effect of LPS exposure during gestation has also been studied.^{27,38} Pregnant mice were exposed to LPS before and during pregnancy. Immune responses in the pups after sensitization and challenge showed a reduction in the expression of T_H2-type cytokines, but there was no effect on airway hyperresponsiveness. Taken together, these studies and others illustrate the immunomodulatory effects of LPS exposure and suggest that timing of exposure affects outcome of the subsequent immune responses.^{9,14,27,38-47} We have shown not only that LPS exposure results in expression of TR-type cells and inhibition of the allergic response but also that this effect, if initiated in the neonatal period, lasts at least into adolescence/early adulthood, in our model. Interestingly, neonatal LPS exposure does not significantly increase IFN- γ production, suggesting that timing of exposure affects the characteristics of subsequent responses.

Recently LPS was shown to decrease the allergen-induced expression of T_H2 cytokines in nasal explant tissue from atopic children but not atopic adults.⁴⁸ Expression of TLR4 was decreased in adult tissue compared with that of children, suggesting that LPS may have the greatest immunomodulatory effects early in life. A recent study examined peripheral dendritic cell cytokine expression after LPS stimulation in culture.¹¹ These investigators showed diminished IL-10 expression in dendritic cells derived from atopic versus nonatopic children and suggested that an intrinsic ability to upregulate IL-10 production may also be an important factor in preventing allergy. Finally, polymorphisms in TLR4 were found to be independently associated with asthma development in Swedish school-aged children.⁴⁹ Taken together, these data suggest that LPS exposure may influence the development of atopic diseases in children.

We have shown that early exposure to LPS or allergen has a significant effect on the development of allergic responses later in life. In this study, LPS exposure resulted in inhibition of allergen-specific IgE, T_H2 cytokine expression, and airways inflammation and hyperresponsiveness in animals sensitized several weeks after the exposure period. We have also demonstrated upregulation of $CD4^+/CD25^+$ T cells expressing IL-10 in the LPS-exposed mice after challenge and an increase in IFN- γ -expressing T cells in ovalbumin-exposed mice. This has led us to speculate that inhibition of allergic sensitization may occur via both antigen independent (as in the LPS group) and antigen-dependent (as in the ovalbumin-exposed group) pathways and that appropriate mucosal stimulation very early in life may prevent the development of allergic airways diseases.

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