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Acute inhibition of inducible nitric oxide synthase but not its absence suppresses asthma-like responses

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Abstract

In the present study we investigated the lymphocytes infiltration and other parameters of allergic lung inflammation comparing mice submitted to acute suppression of nitric oxide synthesis with mice deficient in inducible nitric oxide synthase ($NOS2^{-/-}$) gene. At weekly intervals C57Bl/6 mice, wild type and $NOS2^{-/-}$ were sensitized twice with ovalbumin-alumen and challenged twice with ovalbumin aerosol and lungs examined 24 h later. In wild type mice, treatment with nitric oxide synthase inhibitor, N^{ω}-nitro-L-arginine-methyl-ester (L-NAME) or aminoguanidine (i.p., 30 min before each ovalbumin challenge) caused a significant decrease in bronchoalveolar lavage cell number: eosinophils (90%), lymphocytes $NK1.1^{+}$ (70%), $T\gamma\delta^{+}$ (50%), $CD4^{+}$ (55%), $CD8^{+}$ (60%) and $B220^{+}$ (65%). Both inhibitors abolished airway hyperreactivity and significantly reduced mucus secretion (L-NAME 64%; aminoguanidine 58%). Surprisingly, in $NOS2^{-/-}$ mice these parameters of allergic lung inflammation were not significantly different when compared with wild type mice. In addition, treatment of $NOS2^{-/-}$ mice with L-NAME or aminoguanidine did not affect these parameters. Thus, acute inhibition of NOS2 activity inhibits asthma-like responses but absence of NOS2 has no affect.

Keywords: Nitric oxide; Eosinophil; Airway inflammation; Asthma; Hyperreactivity

1. Introduction

Asthma is a chronic inflammatory disease of the airways characterized by lymphocytes and eosinophil infiltration, where Th2-dependent mechanisms play a central role in eosinophil recruitment to the airways, mucus hyper secretion and airway hyperreactivity.

The involvement of $NK1.1^{+}$ and γδ T cells in allergic lung inflammation has been previously documented. We have shown a clear increase in the number of γδ and NK1.1 T cells as well as of eosinophils in the bronchoalveolar lavage of immunized mice after ovalbumin challenge (Gama Landgraf et al., 2003). It was also demonstrated that depletion or absence of $NK1.1^{+}$ cells before immunization inhibits lung eosinophilia in a mouse model of asthma (Korsgren et al., 1999; Lisbonne et al., 2003) and that γδ T cells appear to be required for development of a Th2 mediated lung inflammation in mice (Zuany-Amorim et al., 1998).

Nitric oxide seems to have an important role in amplifying and perpetuating Th2 mediated inflammatory responses. It has been speculated that the large amount of nitric oxide generated in the asthmatic airways may result in suppression of Th1 cells and a concomitant reduction of interferon-γ (IFN-γ) levels, leading to proliferation of Th2 cells (reviewed by Ricciardolo, 2003).

Exhaled nitric oxide was found in pediatric (Colon-Semidey et al., 2000) and adult asthmatics (Alving et al., 1993; Kharitonov et al., 1994). Nitric oxide can be generated enzymatically by three distinct isoforms of nitric oxide synthases: NOS1, NOS2 and NOS3 expressed by different cell types present in normal or inflamed lung tissue. NOS1 and NOS3 isoforms are expressed constitutively and produce low levels of nitric oxide whereas NOS2

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isoform is induced by bacterial products and/or pro-inflammatory cytokines (Meurs et al., 2003).

The exhaled nitric oxide detected in asthmatics appear to be derived from inducible nitric oxide synthase (or NOS2) expressed by bronchial epithelial cells or immuno-inflammatory cells following allergen exposure of sensitized mice (reviewed by Yates, 2001; Keller et al., 2005). It is also clear that pulmonary NOS2 expression is up-regulated in the lungs of asthmatics (Barnes and Liew, 1995; Kharitonov et al., 1994; Yates, 2001). However, the role of NOS2 in asthma as a pro- or anti-inflammatory mediator is still unresolved. Experimental models of asthma in mice have shown that acute inhibition of NOS2 activity either suppresses (Trifilieff et al., 2000) or exacerbates airway inflammation and chemokine expression (Blease et al., 2000). Controversial data are also found in studies with $NOS2^{-/-}$ mice. Xiong et al. (1999) showed that $NOS2^{-/-}$ animals presented a diminished airway inflammation while others clearly showed that airways inflammation is fully expressed in $NOS2^{-/-}$ mice (De Sanctis et al., 1999; Rodriguez et al., 2003).

Although it is well established that nitric oxide exerts a differential effect on T cell subpopulations, blocking the development of Th1 without affecting Th2 lymphocytes (Liew, 2002), the effect of nitric oxide on other lymphocyte populations has not been addressed.

In the present study we investigated the effect of inhibition of nitric oxide synthesis, by treatment of immunized mice with NOS inhibitors, before antigen challenge, on lymphocytes ($NK1.1^+$, $T\gamma\delta^+$, CD_4^+, CD_8^+, B^+) number in bronchoalveolar lavage fluid and on other parameters of lung inflammation as eosinophil infiltration, increased airway reactivity to methacholine and mucus secretion. In addition, we compared the allergic lung inflammation in mice submitted to acute suppression of nitric oxide synthesis, as above, with the inflammation that occurs in the absence of NOS2 by using mice that are deficient in NOS2 gene.

2. Materials and methods

2.1. Animals

Male wild type and NOS2 knockout ($NOS2^{-/-}$), C57Bl/6 mice weighing 20–25 g, 6–8 weeks old, from our own animal facilities were housed in a room with 12 h light–dark cycle with water and food ad libitum. Animal care and research protocols were in accordance with the principles and guidelines adopted by the Brazilian College of Animal Experimentation (COBEA) and approved by the Biomedical Sciences Institute/USP — Ethical Committee for Animal Research (CEEA).

2.2. Immunization protocol

Mice were sensitized on days 0 and 7 by intraperitoneal injection of a mixture containing 50 μg of ovalbumin and 1 mg of $Al(OH)_3$ in saline (a total volume of 0.2 ml). At 14 and 21 days after the first immunization the animal were challenged by exposure to an aerosol of ovalbumin (grade III, Sigma) generated by an ultrasonic nebulizer (ICEL US-800, SP, Brazil) delivering particles of 0.5–10 μm diameter at approximately 0.75 cc/min for 20 min. The concentration of ovalbumin in the nebulizer was 2.5% wt/vol.

The control group consisted of animals immunized as described and challenged with saline solution. Groups of animals received either the N^{ω}-nitro-L-arginine methyl ester (L-NAME, 30 mg/kg i.p.) or its inactive enantiomer, D-NAME, (30 mg/kg i.p.) or aminoguanidine (100 mg/kg i.p.), 30 min before each challenge.

2.3. Bronchoalveolar lavage

The animals were killed by injection of ketamine/xylazine (50 μl of a 100 mg/ml solution, i.p.) 24 h after exposure to the second aerosol challenge. A tracheal cannula was inserted via a midcervical incision and the airways were lavaged twice with 1 ml of phosphate-buffered saline (PBS, pH 7.4 at 4 °C).

2.4. Total and differential cell counts

The bronchoalveolar lavage fluid was centrifuged at 170 $\times g$ for 10 min at 4 °C, the supernatant was removed, and the cell pellet was resuspended in 0.5 ml of PBS. One volume of a solution containing 0.5% crystal violet dissolved in 30% acetic acid was added to nine volumes of the cell suspension. The total number of cells was determined by counting in a hematocytometer. Differential cell counts were performed after cytocentrifugation and staining with hematoxylin–eosin (Hema 3).

2.5. Flow cytometric analyses of lymphocytes

Phenotypic analysis of lymphocyte subpopulations were assessed by three color FACS, using a Facscalibur Cytometer equipped with Cell Quest software (Becton and Dickinson, San Jose, CA, USA), using gates defined by forward and side light scatter properties. Bronchoalveolar lavage cells were incubated with Fluorescein Isothiocyanate (FITC), R-Phycoerythrin (PE) or Cy-chrome-labeled monoclonal antibodies anti-CD_4 (clone H129.19), anti-CD_8 (clone 53-6.7), anti-CD45R/B220 (clone RA3-6B2), anti-Tγδ (clone GL3) or anti-NK-1.1 (clone PK136) and adjusted to 1×10^5 cells/ml in PBS supplemented with 5% fetal bovine serum and sodium azide (0.1%).

2.6. Evaluation of airway reactivity

The airway reactivity was evaluated as described by Gama Landgraf et al. (2003). Mice were immunized and submitted to two antigen aerosol exposures as described above. Twenty-four hours after the second antigen challenge, mice received an injection of ketamine/xylazine (50 μl of a 100 mg/ml solution, i.p.), the peritoneal cavity was cut open and animals were exsanguinated by section of the abdominal aorta. The thoracic cavity was then opened, the pulmonary artery was cannulated and perfused with 10 ml Krebs solution at 10 ml/min. A cannula was then inserted in the trachea; the lungs were removed carefully and perfused (5 ml/min) through the trachea with Krebs (37 °C, 95% O_2 and 5% CO_2) solution. A small incision was made in the lower end of each lobe to permit the outflow of the perfusate. The perfusion pressure was recorded in a Beckman R511A using Gould P23DB pressure

transducers. Increases over basal levels of perfusion pressure following bolus injection of methacholine were taken as a measure of constriction of the airways. Increase in perfusion pressure (cmH_2O) versus dose (μg methacholine) was measured for the entire recording period, areas under the curve calculated and results expressed as mean area under the curve (mm^2).

2.7. Histological analysis

Lungs were removed after the collection of bronchoalveolar lavage, perfused via the right ventricle with 10 ml PBS to remove residual blood, immersed in 10% phosphate-buffered formalin for 24 h and then kept in 70% ethanol until embedding in paraffin. Tissues were sliced (5 μm sections) and stained with hematoxylin/eosin for light microscopy examination or with periodic acid–Schiff (PAS)/hematoxylin for evaluation of mucus-producing cells. The intensity of mucus production was evaluated in each preparation and scores from 0 to 3 were attributed: 0 when none of the bronchi show any sign of mucus; 1, 2 or 3 when 25%, 50% or more than 50% of the bronchi epithelium was covered by mucus, respectively. Values shown for each lung represent the sum of 10 bronchi scored randomly at ×250 magnification.

2.8. Drugs and reagents

N^{ω}-nitro-L-arginine methyl ester (L-NAME), N^{ω}-nitro-D-arginine methyl ester (D-NAME), aminoguanidine bicarbonate, ovalbumin grade III, methacholine and fetal bovine serum were purchased from Sigma Chemical Co. (St. Louis, MO, USA); the monoclonal antibodies to lymphocyte markers from BD Pharmingen (San Diego, CA, USA); the Hema 3 from Biochemical Sciences Inc. (Swedesboro, NJ) and aluminum hydroxide gel (Rehydragel) from Reheis Inc. (Berkley Heights, NJ, USA).

2.9. Statistical analysis

Data are expressed as the means ± S.E.M. Statistical evaluation of the data was carried out by analysis of variance (ANOVA) and sequential analysis of differences among means was done by Turkey's contrast analysis. A P value lower than 0.05 was considered to be significant. $P<0.05$, $P<0.01$ and $P<0.001$ were marked with one, two or three asterisks, respectively.

3. Results

3.1. Cells in the bronchoalveolar lavage fluid

3.1.1. Lymphocytes

We first examined the lymphocyte population present in bronchoalveolar lavage fluid of animals treated or not with L-NAME or aminoguanidine. Mice immunized with ovalbumin were submitted to two ovalbumin aerosol challenges and bronchoalveolar lavage was performed 24 h after the second aerosol challenge. Bronchoalveolar lavage fluid cells were incubated with monoclonal antibodies to the cell markers NK 1.1^+, $T\gamma\delta^+$, CD_4^+, CD_8^+, $B220^+$as described in Materials and methods and submitted to flow cytometric analysis. Fig. 1 shows that whereas these cell populations are either undetectable or found in very low number in the control group (immunized given saline aerosol), a significant increase in all cell populations is observed in ovalbumin-immunized and challenged group. Treatment with L-NAME or aminoguanidine i.p., 30 min before each of the antigen challenges, induced a significant decrease in the NK 1.1^+, $T\gamma\delta^+$, CD_4^+, $CD_8^+$and $B220^+$cells in the bronchoalveolar lavage fluid.

Next we examined the lymphocytes population in control and allergic $NOS2^{-/-}$ mice. As shown in Fig. 2, the immunized and challenged $NOS2^{-/-}$ mice, similarly to the wild type, also presented a significant lymphocyte influx. The conflicting results obtained with pharmacological inhibition of nitric oxide and genetic deficiency of NOS2 prompted us to investigate whether L-NAME or aminoguanidine treatments are inhibiting the asthma phenotype through mechanisms that are independent of its effect on NOS2 activity. To test this hypothesis, we treated $NOS2^{-/-}$ mice with the nitric oxide inhibitors. As shown in Fig. 2, these treatments did not significantly change the number of the lymphocyte subtypes.

3.1.2. Eosinophils

In the control group (immunized mice submitted to saline aerosol), the number of eosinophils present in bronchoalveolar lavage fluid is very low (less than 1 cell/ml). In the immunized and antigen challenged lungs there is a marked increase in bronchoalveolar lavage fluid eosinophils in both wild type (106.6 ± 27 cells/ml) and $NOS2^{-/-}$ mice (115.2 ± 21.3 cells/ml). The numbers are not significantly different between the strains (Fig. 3). Eosinophils were the predominant cell type in the experimental groups accounting for 50% (wild type) and 55% ($NOS2^{-/-}$) of the total cells in the bronchoalveolar lavage fluid.

Groups of immunized mice received intraperitoneal injections of L-NAME, D-NAME or aminoguanidine, 30 min before each of the antigen aerosol challenges. Fig. 3 shows that in the wild type mice, treatment with L-NAME or aminoguanidine decreased significantly the number of eosinophils in the bronchoalveolar lavage fluid whereas treatment with D-NAME has no effect. In $NOS2^{-/-}$ mice, treatment with these drugs did not affect eosinophil number in bronchoalveolar lavage fluid.

3.2. Reactivity of the airways to methacholine

Groups of immunized mice were submitted to two ovalbumin aerosol challenges (experimental group) or saline (control group) and the lungs were removed and perfused through the trachea 24 h later as described in Materials and methods and methods. Bolus injection of increasing doses of methacholine (0.1 to 100 μg) caused bronchoconstriction measured as increase in perfusion pressure. Fig. 4 shows that in the wild type and $NOS2^{-/-}$ immunized groups the reactivity of the airways to methacholine was significantly higher than that of the control group (around 30% and 18% higher comparing areas under the curves of wild type and $NOS2^{-/-}$ mice, respectively). The maximal response of immunized and challenged lungs to methacholine was greater than that of control lungs but was not different when comparing wild type and $NOS2^{-/-}$ (E_{max} 32.4 ± 0.6 vs 25.29 ± 0.4 and 31.1 ± 0.8 vs 26.1 ± 1.5 cmH_2O, for wild type and $NOS2^{-/-}$, respectively). The EC_{50} values were also similar for both strains (1.5 μg approximately).

Airway hyperreactivity was significantly reduced by pretreatment of the wild type mice with the NOS inhibitors, L-NAME and aminoguanidine. However, in the $NOS2^{-/-}$ mice this

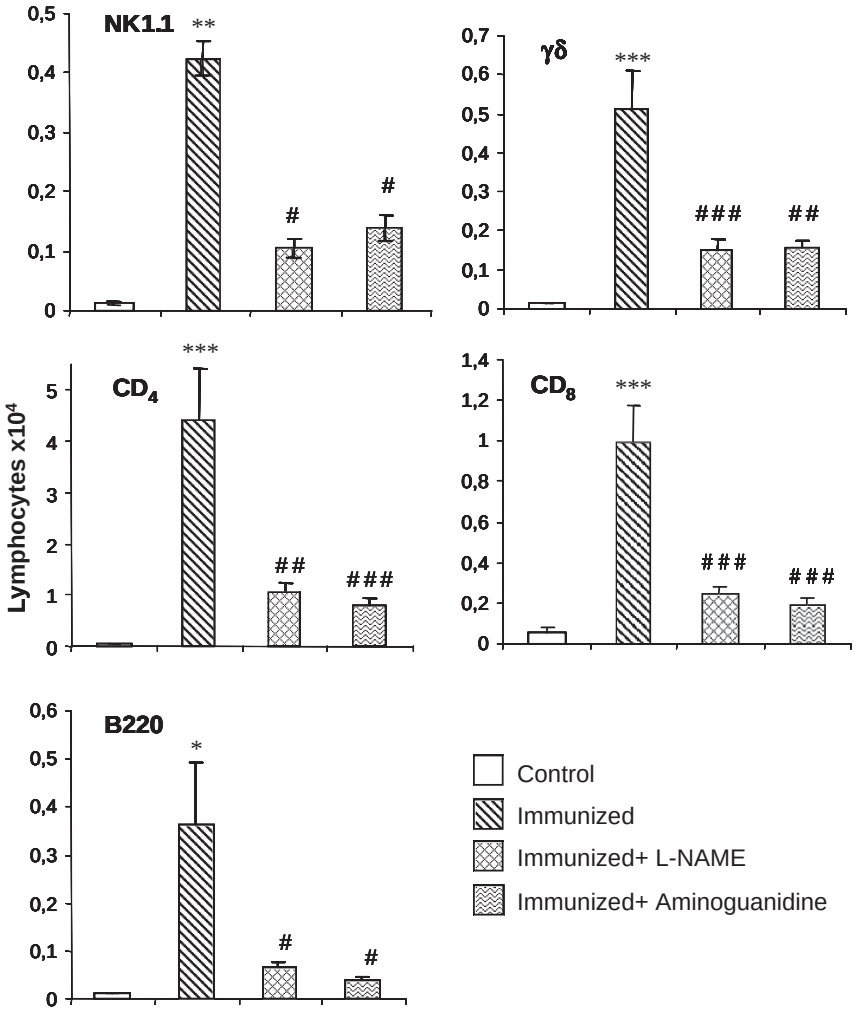

Fig. 1. The effect of treatment of mice with N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine on the bronchoalveolar lavage lymphocyte subpopulations. C57Bl/6 wild type were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization. Drugs were given i.p 30 min before each aerosol challenge. Bronchoalveolar lavage was performed 24 h after the second challenge. The cells were incubated with fluorochrome-labeled monoclonal antibodies to NK1.1, Tγδ, CD_4, CD_8, and B220, submitted to FACS analysis. Results are the mean ± S.E.M. of 6–8 animals group. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ in comparison with control group and $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ in comparison with immunized group.

treatment did not cause any effect (Fig. 4). Mice treated with D-NAME develop the same level of hyperreactivity as the non-treated groups (data not shown).

3.3. Histology of the lungs

The intensity of mucus production was evaluated in bronchi ($n=10$) and scored from 0 to 3 as described in Materials and methods. In the control group none of the bronchi contained mucus (score 0). In contrast, the immunized groups of wild type and $NOS2^{-/-}$ mice were positive for mucus, the mean of scores was similar in both groups (1.5 approximately). Mucus plugs were not observed in any group. It can be seen in Fig. 5 that L-NAME or aminoguanidine significantly inhibited mucus production in wild type mice, whereas they did not affect mucus production in $NOS2^{-/-}$ mice.

Examination of hematoxylin/eosin stained lung slides revealed a marked inflammatory infiltrate around small vessels and bronchi of immunized and challenged wild type (Fig. 6A) and $NOS2^{-/-}$ mice (Fig. 6C). Treatment with L-NAME strongly inhibited cellular infiltrate in wild type mice (Fig. 6B) whereas no effect was seen in $NOS2^{-/-}$ mice (Fig. 6D).

4. Discussion

Our results show that the inhibition of endogenous NOS2 decreased many aspects of inflammation but total absence of NOS2 isoform by gene deletion has no effect on these events.

The model used here for the study of allergic lung inflammation consisted of immunization with ovalbumin using aluminium hydroxide as adjuvant followed, 21 days later, by two ovalbumin aerosol challenges, one week apart. This model presented many of the characteristic features of allergic asthma such as: eosinophil and lymphocyte infiltration, mucus production and airway hyperreactivity.

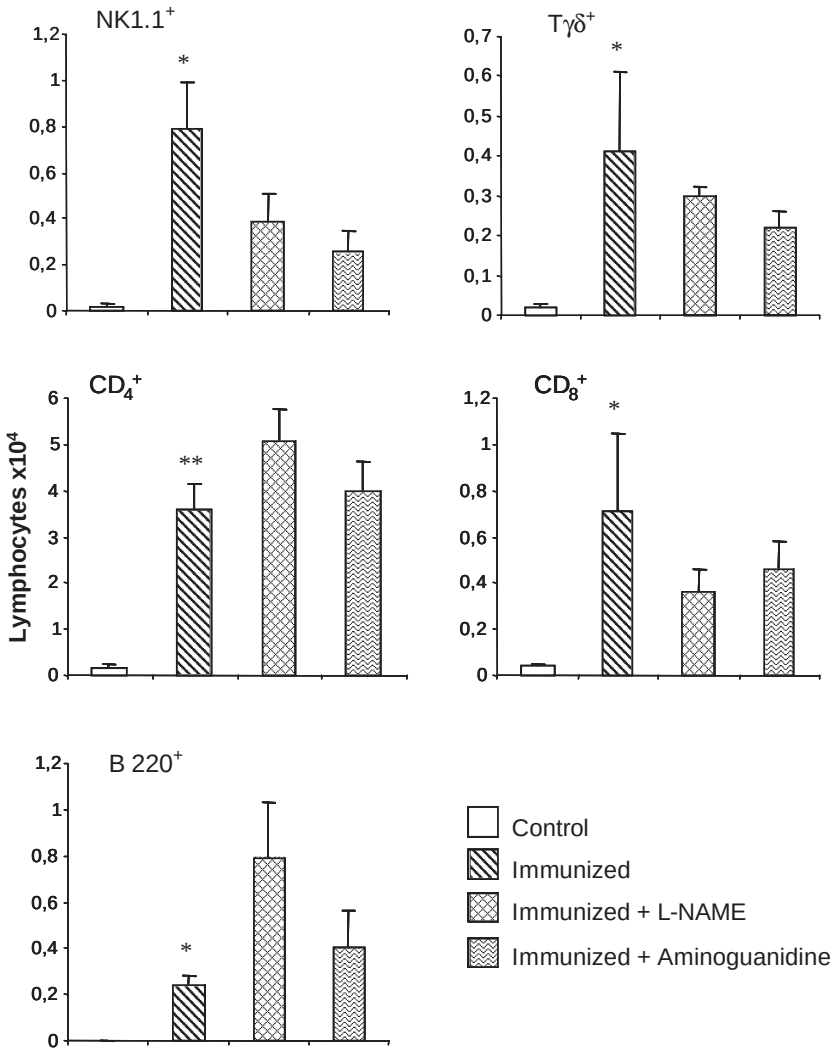

Fig. 2. The effect of treatment of mice with N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine on the bronchoalveolar lavage lymphocyte subpopulations. NOS2$^{-/-}$ mice were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization. Drugs were given i.p 30 min before each aerosol challenge. Bronchoalveolar lavage was performed 24 h after the second challenge. The cells were incubated with fluorochrome-labeled monoclonal antibodies to NK1.1, Tγδ, CD_4, CD_8, and B220, submitted to FACS analysis. Results are the mean±S.E.M. of 6–8 animals group. *$P<0.05$ and **$P<0.01$ in comparison with control group.

In this model of lung inflammation, we have observed a clear increase in the number of Tγδ$^+$ and NK1.1$^+$as well as of CD_4^+, CD_8^+, and B lymphocytes in the bronchoalveolar lavage fluid of immunized mice following antigen challenge, corroborating previous findings from our laboratory (Gama Landgraf et al., 2003).

Acute inhibition of endogenous nitric oxide synthesis reduced all different lymphocyte populations. In the NOS2$^{-/-}$ mice the number of the different subtypes appearing after antigen challenge was not significantly different from the wild type. This finding would imply that endogenous nitric oxide is important for lymphocyte accumulation in general, but that the long-term deficiency in nitric oxide production, caused by deletion of NOS2 can somehow be compensated for or overcome. Recently, it was shown that NOS2-mediated signals are important for homeostatic T cell maintenance by controlling apoptosis and memory expansion (Vig et al., 2004). Thus, nitric oxide derived from NOS2 appears to be involved in various T cell activities. It will be of interest to determine the mechanism by which NOS2 governs T cell migration in wild type animals. In sharp contrast, T cell recruitment in NOS2-deficient animals was not affected at all. Given the fact that T cell homeostasis is maintained in NOS2-deficient animals, it is likely that T cell physiology in these animals is independent of NOS2. It follows that allergen-induced T cell migration in NOS2-deficient animals does not rely on nitric oxide production.

The treatment with the NOS inhibitors, L-NAME and aminoguanidine, administered before each antigen challenge in wild type mice reduced the number of eosinophils in the bronchoalveolar lavage fluid. This result is compatible with that reported by Feder et al. (1997) which also reported inhibition of the influx of eosinophils into the

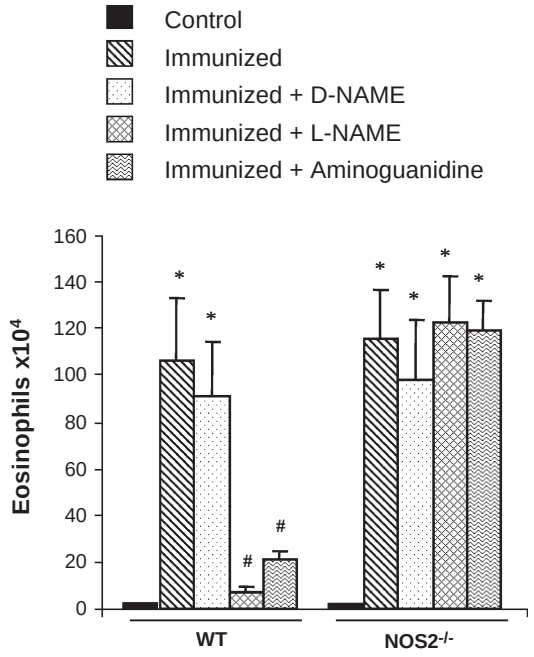

Fig. 3. The effect of treatment of mice with N^{ω}-nitro-L-arginine methyl ester (L-NAME), aminoguanidine and N^{ω}-nitro-D-arginine methyl ester (D-NAME) on the bronchoalveolar lavage eosinophils. C57Bl/6 wild type and NOS2$^{-/-}$ were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization. Drugs were given i.p 30 min before each aerosol challenge. Bronchoalveolar lavage was performed 24 h after the second challenge. Eosinophils were counted in cytocentrifuge preparation of bronchoalveolar lavage cells stained with hematoxylin/eosin. Results are the mean±S.E.M. of 8–10 animals group. *$P<0.01$ in comparison with the control group and $^{\#}P<0.05$ in comparison with the immunized group.

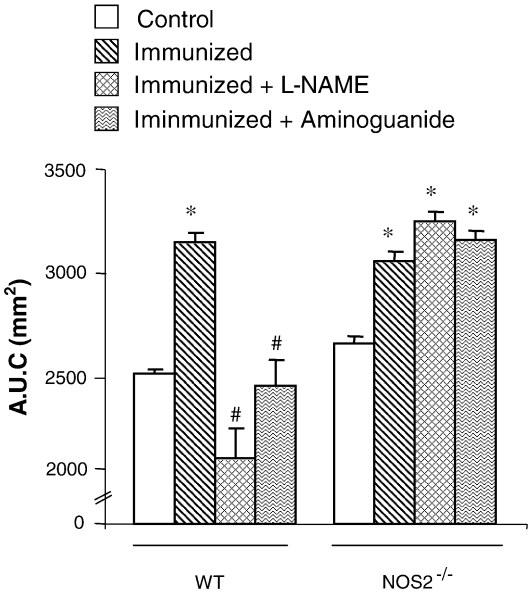

Fig. 4. The effect of treatment of mice with N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine on airway reactivity to methacholine. C57Bl/6 wild type and NOS2$^{-/-}$ were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization. Drugs were given i.p 30 min before each aerosol challenge. Twenty-four hours after the second challenge, the lungs were removed, perfused via the trachea and increases in perfusion pressure to bolus injection of methacholine were recorded. Results are expressed as mean area under the curve±S.E.M. of 6–7 animals group. *$P<0.001$ in comparison with the control group and $^{\#}P<0.001$ in comparison with the immunized group.

bronchoalveolar lavage fluid and lung tissue of allergic mice by L-NAME and aminoguanidine.

The decrease of the cells count was associated with a decrease in lung hyperreactivity to methacholine: L-NAME or aminoguanidine pre-treatment abolished the hyperreactivity of wild type mice.

It is known that nitric oxide generated by constitutive NOS has a bronchoprotective role in normal airways since NOS inhibitors potentiate basal lung airway reactivity (De Sanctis et al., 1997; Folkerts and Nijkamp, 1998). Moreover, it was shown that NOS1 isoform, controls the induction of airway hyperreactivity in murine allergic asthma (De Sanctis et al., 1999). Since the inhibitors used in this study could also inhibit constitutively NOS activity, we examined the effect of the treatment with L-NAME or aminoguanidine in NOS2-deficient mice. We found that treatment with NOS inhibitors did not affect airway hyperreactivity in NOS2$^{-/-}$ strain. Thus, it appears that the hyperreactivity seen in wild type mice is due to NOS2 activity. However, it is puzzling to understand why NOS2-deficient mice develop airway allergic inflammation and hyperreactivity. Taken together, our findings indicate that in wild type or NOS2$^{-/-}$ mice airway

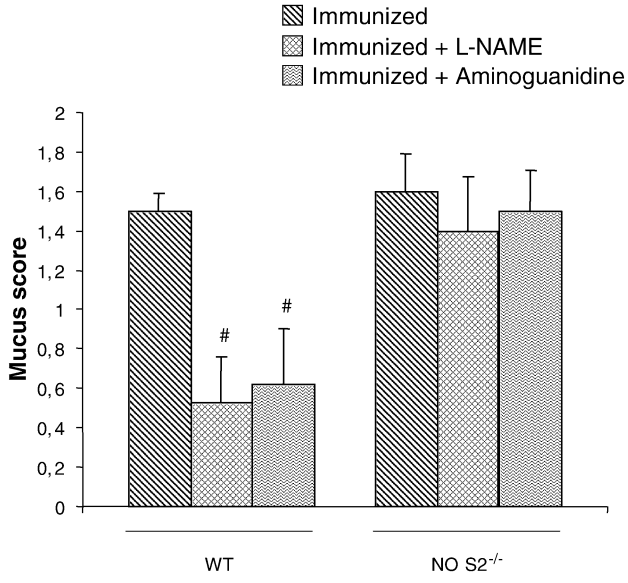

Fig. 5. The effect of treatment of mice with N^{ω}-nitro-L-arginine methyl ester (L-NAME) and aminoguanidine on mucus production. C57Bl/6 wild type and NOS2$^{-/-}$ were immunized with an i.p. injection of ovalbumin/alumen, one booster injection 7 days later, and two ovalbumin aerosol challenges (2.5%, 20 min) on days 14 and 21 post immunization. Drugs were given i.p. 30 min before each aerosol challenge. Lungs were removed 24 h after the second antigen challenge, embedded in paraffin, sliced and stained with PAS/hematoxylin for evaluation of mucus. Results are the mean±S.E.M. of 7–8 animals group. $^{\#}P<0.05$ in comparison with the immunized group.

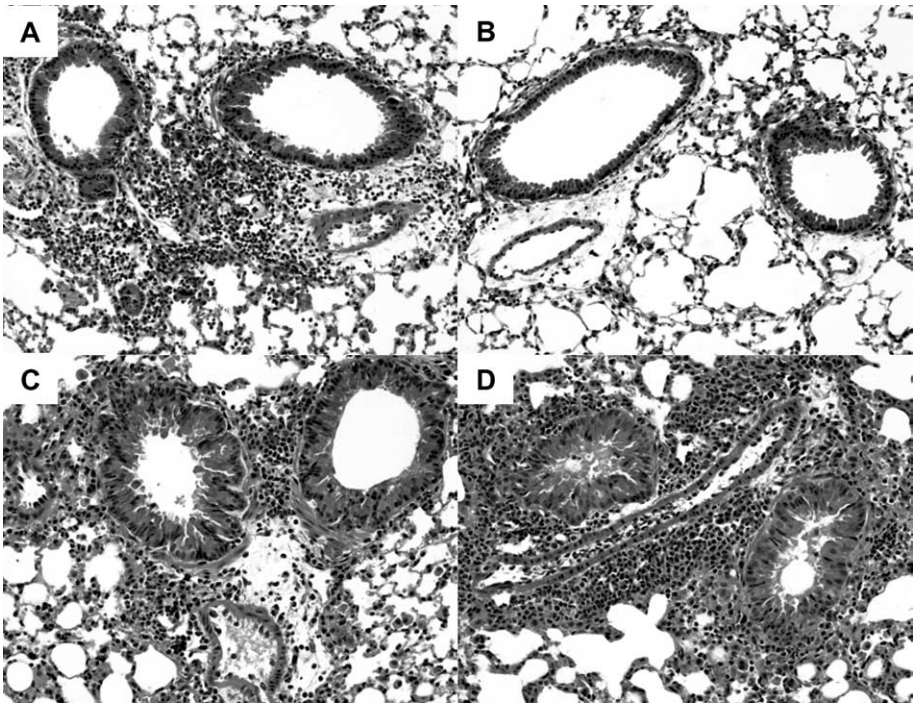

Fig. 6. Photomicrography of pulmonary parenchyma from C57Bl/6 wild type and NOS2$^{-/-}$ mice 24 h after induction of allergic lung inflammation. *Panel A*: lungs from immunized C57Bl/6 wild type mice that received two antigen aerosol challenges showing, peribronchiovascular cells infiltration, hematoxylin/eosin, 400X. *Panel B*: lungs of immunized C57Bl/6 wild type mice pre-treated with L-NAME, 30 mg/kg before each challenge — note the marked inhibition of the peribronchiovascular infiltrate; hematoxylin/eosin, 400X. *Panel C*: lungs from immunized C57Bl/6 NOS2$^{-/-}$ mice that received two antigen aerosol challenges showing peribronchiovascular infiltration, hematoxylin/eosin, 400X. *Panel D*: lungs of immunized NOS2$^{-/-}$ mice pre-treated with L-NAME, 30 mg/kg before each challenge — note that there is no difference in the peribronchiovascular infiltrate in comparison with the non-treated NOS2$^{-/-}$ group; hematoxylin/eosin, 400X.

hyperreactivity is associated with lung inflammation. However, in wild type animals, lung inflammation is orchestrated by nitric oxide, whereas in an organism devoid of NOS2 other factors are involved in the development of allergic inflammation.

Actually, the role of nitric oxide in the airway hyperreactivity is controversial (Ricciardolo et al., 1997; Ricciardolo, 2003; Keller et al., 2005). Some studies in guinea pigs have shown NOS inhibitors potentiate acute bronchoconstriction induced by allergen inhalation (Persson et al., 1993; Mehta et al., 1997), whereas other studies in vitro and in vivo showed that the hyperreactivity induced by allergen is not affected by pre-treatment with NOS inhibitors (de Boer et al., 1996; Mehta et al., 1997). In a recent clinical study, NOS2 inhibitors were able to decrease the exhaled nitric oxide levels in asthmatics patients but the benefit of this treatment on clinical symptoms was not reported (Hansel et al., 2003).

Mucus production was also not significantly different when comparing NOS2$^{-/-}$ and wild type mice. In the later the mucus score was strongly inhibited by pre-treatment with NOS inhibitors. Nitric oxide generated by NOS2 seems to stimulate mucus secretion and this is in accordance with Adler et al. (1995) who showed that the increased secretion of mucus induced by inflammatory stimuli is inhibited by LNMMA. Moreover, nitric oxide donors increased mucus secretion in human airways in vitro (Nagaki et al., 1995).

Our results suggest that in the model of lung allergic inflammation employed here, the NOS2 activity has pro-inflammatory effect in the wild type mice, since pre-treatment with aminoguanidine which is more selective against NOS2 (Barnes et al., 1998; Schuiling et al., 1998) markedly inhibited the airway inflammation measured by the decrease in the number of eosinophils, airway hyper-reactivity and mucus production.

We found that NOS2$^{-/-}$ mice develop airway inflammation of the same intensity that the wild type animals, corroborating previous findings (De Sanctis et al., 1999; Rodriguez et al., 2003). In contrast, Xiong et al. (1999) showed that eosinophilic infiltration of allergic airway disease is markedly suppressed in mice deficient for NOS2 production, apparently due to pronounced IFN-γ expression in these animals. These conflicting data may, in part, be explained by the fact that they were obtained in different animal species using different protocols of immunization and challenge. Also, different nitric oxide isoforms may be expressed in lung cells and tissues depending on the stage of the inflammation process. The mapping of the isoforms during the evolution of the disease would be important to better

understand the development of this complex disease. Moreover, it is of interest to determine the cell type responsible for nitric oxide production during allergic inflammation.

Although in animals with NOS2 deficiency the migration of ovalbumin-sensitized T cells to the lung was not impaired, in wild type animals nitric oxide was clearly important for the infiltration of these cells. Thus, we favor the notion that in wild type mice the nitric oxide generated as consequence of ovalbumin challenge is critically involved with the migration of T lymphocytes including NK1.1 and γδ T cells into the lung. In turn, these cells would initiate and orchestrate the Th2-driven intrapulmonary inflammation that is associated with eosinophil infiltration, hyperreactivity and mucus production. However, we have no explanation for the paradoxical effect of acute NOS2 suppression and lack of NOS2 in asthma responses.

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