

Exacerbated Th2-mediated airway inflammation and hyperresponsiveness in autoimmune diabetes-prone NOD mice: a critical role for CD1d-dependent NKT cells

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The NOD mouse has proved to be a relevant model of insulin-dependent diabetes mellitus, closely resembling the human disease. However, it is unknown whether this strain presents a general bias toward Th1-mediated autoimmunity or remains capable of mounting complete Th2-mediated responses. Here, we show that NOD mice have the capacity to develop a typical Th2-mediated disease, namely experimental allergic asthma. In contrast to what might have been expected, they even developed a stronger Th2-mediated pulmonary inflammatory response than BALB/c mice, a strain that shows a typical Th2 bias in this model. Thus, after allergen sensitization and intra-nasal challenge, the typical features of experimental asthma were exacerbated in NOD mice, including enhanced bronchopulmonary responsiveness, mucus production and eosinophilic inflammation in the lungs as well as specific IgE titers in serum. These hallmarks of allergic asthma were associated with increased IL-4, IL-5, IL-13 and eotaxin production in the lungs, as compared with BALB/c mice. Notwithstanding their quantitative and functional defect in NOD mice, CD1d-dependent NKT cells contribute to aggravate the disease, since in OVA-immunized CD1d^{-/-} NOD mice, which are deficient in this particular T cell subset, airway eosinophilia was clearly diminished relative to NOD littermates. This is the first evidence that autoimmune diabetes-prone NOD mice can also give rise to enhanced Th2-mediated responses and might thus provide a useful model for the study of common genetic and cellular components, including NKT cells that contribute to both asthma and type 1 diabetes.

Key words: Th1/Th2 cells / Autoimmunity / Diabetes / Allergy / Cytokines

Received	5/5/03
Revised	10/11/03
Accepted	8/12/03

1 Introduction

Type 1 insulin-dependent diabetes mellitus (IDDM) is an autoimmune disorder characterized by lymphocyte-mediated destruction of insulin-producing β -cells in the pancreatic Langerhans' islets [1]. The nonobese diabetic (NOD) mouse has proved a relevant model for IDDM, closely resembling the human disease [2]. This strain develops spontaneous diabetes resulting from an autoimmune response directed against the pancreatic

islets of Langerhans, with subsequent destruction of the insulin-producing β -cells, insulin deficiency and hyperglycemia [2]. This process is preceded by macrophage, B and T lymphocyte infiltration of pancreatic islets [2].

Autoreactive T cells play a major pathogenic role, as demonstrated by the capacity of purified cells or T cell clones to transfer the disease to immuno-incompetent NOD mice [2, 3]. Among these, the contribution of Th1 cells seems to be determinant [4–8]. Indeed, destructive insulinitis is paralleled by predominant secretion of IFN- γ , IL-2, IL-12, TNF- α and IL-18, while the Th2 type cytokine IL-4 is associated with non-destructive insulinitis [4, 9–11]. The role of Th1 cells as inducers of diabetes is also supported by findings obtained with cytokine/anti-cytokine treatments. Thus, systemic treatment with IL-12, which

[DOI 10.1002/eji.200324151]

Abbreviations: **BALF**: Bronchoalveolar lavage fluid **BHR**: Bronchopulmonary hyperreactivity **IDDM**: Insulin-dependent diabetes mellitus **PAS**: Periodic acid-Schiff

promotes the development of Th1 cells, accelerates disease onset in NOD mice [9]. Conversely, diabetes is delayed by systemic treatment with anti-IFN- γ mAb or IL-12 antagonists [12, 13].

Converging data indicate that cytokine imbalance plays a key role in disease emergence, with overexpression of Th1 diabetogenic T cells together with a defective differentiation of Th2 autoreactive T lymphocytes, probably due to inappropriate regulatory cells [3, 4]. Consistent with this view, efficient induction of regulatory Th2 cells in NOD mice has been achieved by systemic treatment with IL-4 or its local expression in pancreatic β -cells in insulin-promoter-IL-4 transgenic mice [14, 15]. Based on the Th1/Th2 paradigm, it has been postulated that IL-4 induces a shift in the balance of anti-islet T cells from the pathogenic Th1 toward a less harmful Th2 phenotype.

Whether the defective differentiation of autoreactive Th2 lymphocytes in NOD mice is due to a more general defect in the efficient induction of Th2 cells is still unclear. NOD mice can be manipulated to mount Th2 responses against conventional antigens including myelin basic protein, hen egg lysozyme or β -galactosidase [16, 17]. However, induction of such unipolar Th2 responses has been obtained only when NOD mice were neonatally immunized, whereas the ability of adult NOD mice to develop Th2 responses against exogenous antigens has not been investigated.

Here, we addressed this issue by evaluating whether adult NOD mice are capable of developing a typical Th2-mediated response outside the pancreas. To do so, we studied a Th2-mediated pulmonary inflammatory disease, *i.e.* experimental allergic asthma, which is induced after allergen sensitization and intra-nasal (i.n.) challenge with ovalbumin (OVA). We evaluated the principal symp-

toms of allergic asthma in NOD mice, namely elevated serum IgE, mucus production, eosinophilia, and enhanced bronchial reactivity. These are all associated with a Th2 cytokine profile, which is obligatory for disease development [18, 19].

In the present report, we show that NOD mice display a complete asthmatic phenotype with increased Th2 responses and more severe bronchial inflammation and hyperresponsiveness than BALB/c mice, known for their typical Th2 bias in this model. From these findings, we conclude that adult NOD mice are not generally Th1 biased and can develop exacerbated Th2-mediated responses.

2 Results

2.1 OVA-challenged NOD mice exhibit airway eosinophilia and lung inflammation

Experimental OVA-dependent allergic asthma is characterized by airway eosinophilia and mucus hyperproduction [18]. These parameters were compared in NOD and BALB/c mice, a strain which develops a typical Th2 bias in this model [19]. Fig. 1A shows that total cell counts in bronchoalveolar lavage fluid (BALF) were augmented after i.n. challenge with OVA both in NOD and in BALB/c mice immunized with 1 or 10 μ g of OVA. The number of eosinophils was even higher in BALF of NOD than of BALB/c mice for either dose (Fig. 1B), reaching 28% of total cells in NOD versus 7% in BALB/c mice when 10 μ g of OVA were injected. The difference between NOD and BALB/c mice was even more striking at the low dose, which induced a ten-fold increase of eosinophils, as compared with a four-fold increase in mice having received 10 μ g of OVA.

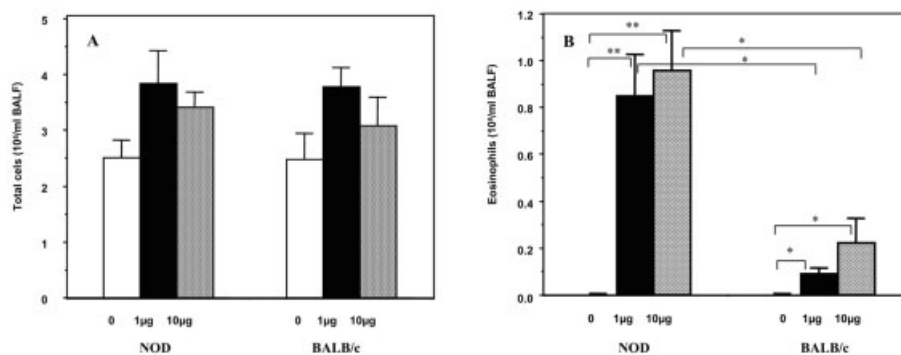


Fig. 1 Eosinophil recruitment into the BALF of NOD and BALB/c mice after OVA-immunization and challenge. (A) Total and (B) differential cell counts were determined in the BALF of NOD and BALB/c mice immunized with 1 μ g or 10 μ g OVA and challenged i.n. with 10 μ g OVA. Each value represents the mean $\pm$ SEM of 6–18 mice. Significant differences were observed between saline and OVA-sensitized and challenged mice, and between NOD or BALB/c animals, as indicated. * $p < 0.005$ and ** $p < 0.0001$.

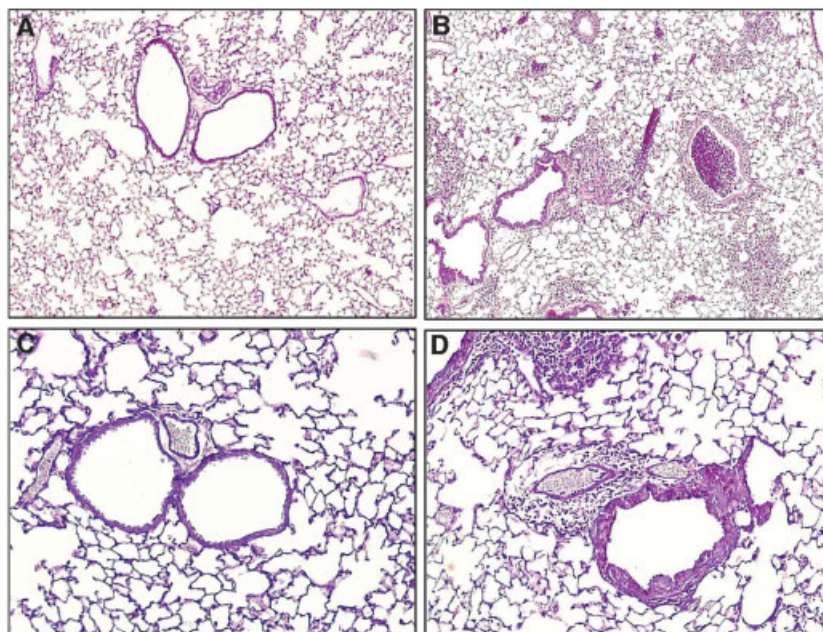


Fig. 2. OVA-induced inflammation and mucus production in the lungs of NOD mice. Lung sections from NOD mice immunized with 1 μ g OVA and challenged with saline (A and C) or 10 μ g OVA (B and D) were stained with HE and PAS. (A, B) Representative lung sections stained with HE (magnification $\times 100$) from saline- or OVA-challenged NOD mice. Bronchioles without inflammation or dense perivascular and peribronchiolar infiltrates mainly composed of eosinophils are present in lung of saline- or OVA-challenged NOD mice, respectively. (C, D) PAS-stained slides (magnification $\times 100$) show that mucin is absent from the epithelium of NOD mice challenged with saline, while it is increased in the bronchiolar lining cells in OVA-challenged NOD mice.

Since OVA challenge induces eosinophil migration into BALF after lung eosinophil sequestration [20], we examined this feature in NOD mice. We observed intense airway inflammation, characterized by peribronchiolar infiltrates composed mainly of eosinophils (Fig. 2B) and intense mucus production (Fig. 2D). Conversely, eosinophil recruitment and mucosal cells in the bronchiolar lining cells were virtually absent in saline-challenged animals (Fig. 2A and Fig. 2C) or in OVA-challenged non-immunized animals (data not shown).

These findings clearly show that OVA immunization and challenge induce both eosinophilia and airway inflammation in NOD mice.

2.2 NOD mice produce high levels of Th2 cytokines and eotaxin after OVA challenge

Allergic airway inflammation is accompanied by the generation of IL-4, IL-5 and IL-13 as well as CCR3 chemokines, such as eotaxin [18–21]. Fig. 3 shows elevated levels of IL-4, IL-5 and eotaxin in BALF and lungs of NOD mice after OVA challenge, but no difference was noted for IL-13 (Fig. 3). In both compartments, NOD mice challenged with OVA produced higher cytokine levels than

BALB/c mice and IFN- γ was detected in neither strain (data not shown).

2.3 NOD mice secrete OVA-specific serum IgE following immunization and challenge

To investigate the peripheral immune response in immunized and challenged mice, we measured serum levels of OVA-specific IgE, the production of which depends on both IL-4 and IL-13 in this model [18, 22, 23]. Fig. 4 shows that OVA immunization induced specific IgE in both NOD and BALB/c mice, with increased titers after OVA challenge. As expected from the absence of IFN- γ in these conditions, IgG2a was detected in neither strain (data not shown). These findings support the concept that OVA-induced changes in NOD mice result from a strong Th2 immune response.

2.4 Anti-CD4 mAb treatment inhibits Th2 cytokine secretion, OVA-specific IgE production and airway eosinophilic inflammation in NOD mice

It is well established that CD4⁺ T cells are the major population responsible for eosinophilic airway inflammation,

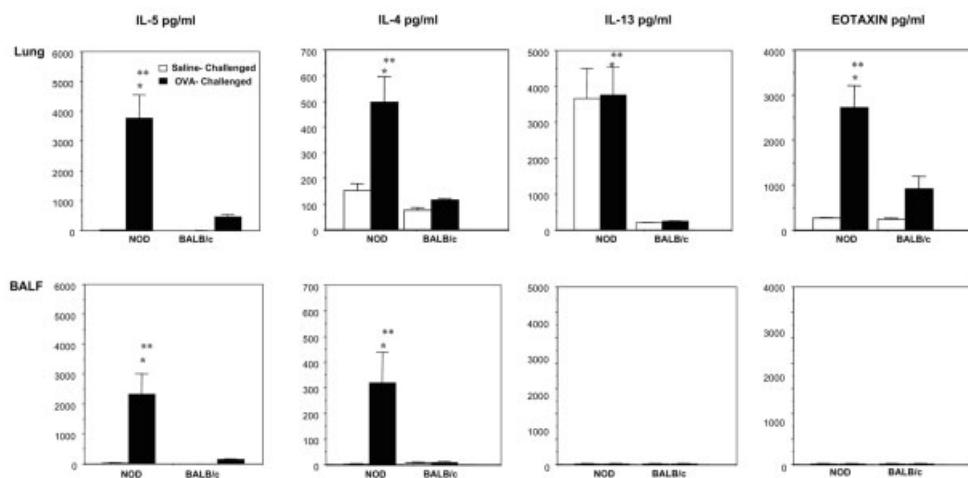


Fig. 3. Th2 cytokines and eotaxin in lung homogenates and BALF from OVA-sensitized and challenged mice. IL-5, IL-4, IL-13 and eotaxin levels in lung homogenates (upper panels) and BALF (lower panels) of NOD and BALB/c mice immunized with 1 μ g OVA and challenged i.n. with 10 μ g OVA or saline were collected immediately after methacholine provocation and measured by ELISA. Data are expressed as means $\pm$ SEM. Significant differences were observed between saline and OVA-sensitized and challenged mice, and between NOD and BALB/c mice, as indicated. * p < 0.005 compared to control and ** p < 0.005 compared to BALB/c animals.

Th2 cytokine and IgE production in murine allergic asthma [18]. For this reason, we examined the consequences of CD4 depletion on the development of experimental asthma in NOD mice. As depicted in Fig. 5, anti-CD4 mAb treatment blocked airway eosinophilia as well as IL-4 and IL-5 production in the lung. Under the same conditions, OVA-specific IgE production was also suppressed (Fig. 5), indicating that CD4 cells are also determinant for the development of allergic asthma in diabetes-prone mice.

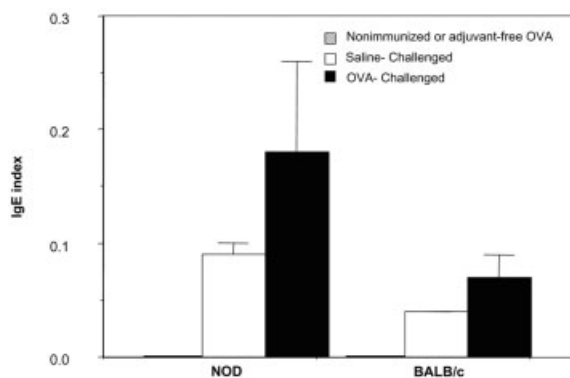


Fig. 4. OVA-specific IgE production in NOD and BALB/c mice. NOD and BALB/c mice were immunized with 1 μ g OVA and challenged i.n. with 10 μ g OVA or saline. Three days later, animals were bled and OVA-specific IgE levels were determined in the serum by ELISA. Results are expressed as mean $\pm$ SEM of at least six mice.

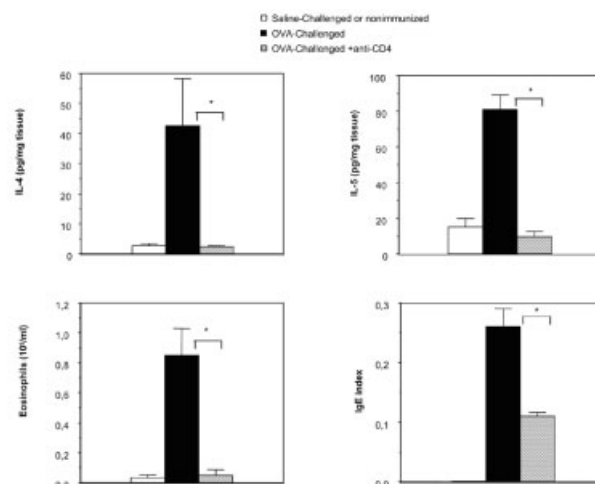


Fig. 5. Effects of the anti-CD4 mAb treatment on Th2 cytokine levels, OVA-specific IgE production and airway eosinophilic inflammation in NOD mice. Groups of NOD mice were immunized (day 0) with 10 μ g OVA and boosted (day 7) with 10 μ g OVA. Three days later, these mice were treated i.p. with anti-CD4 mAb (0.5 mg/mouse) for 3 consecutive days. After the last treatment, mice were challenged i.n. with 10 μ g OVA (day 14). In one set of experiments BALF and lungs were collected 24 h after the OVA-challenge. IL-4 and IL-5 levels were measured in the supernatants of lung biopsies (see Sect. 4) and eosinophil numbers into the BALF. In another group, mice were sacrificed after 72 h and the serum levels of OVA-specific IgE were determined by ELISA. Results represent the mean $\pm$ SEM of five mice. * p < 0.02.

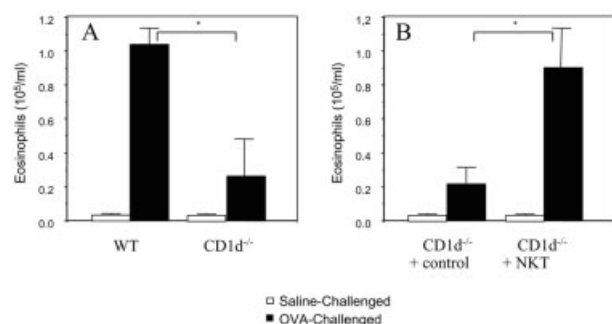


Fig. 6. Marked decrease in specific airway eosinophilia and IL-5 production in OVA-challenged CD1d^{-/-} NOD mice: restoration by adoptive transfer of NKT cells before challenge. Groups of WT and CD1d^{-/-} NOD mice were immunized with 1 μg OVA and challenged i.n. with 10 μg OVA or saline. Eosinophil numbers were measured 1 day after the challenge into the BALF. In one set of experiments (B), groups of OVA-immunized CD1d^{-/-} mice were adoptively transferred 24 h before challenge with enriched CD1d/ α -galactosylceramide-positive splenocytes from WT mice. As controls, CD1d^{-/-} mice were adoptively transferred 24 h before challenge with splenocytes from CD1d^{-/-} animals. Results are expressed as mean $\pm$ SEM of five to seven mice. * $p < 0.05$ and ** $p < 0.01$.

2.5 Airway eosinophilic inflammation and IL-5 production are markedly reduced in NKT cell-deficient NOD mice

Others and we have recently documented that CD1d-restricted NKT cells are required for experimental allergic asthma in mice which are not susceptible to autoimmune diseases [24, 25]. Although NKT cells from NOD mice have been shown to be quantitatively and functionally deficient [26–28], we evaluated whether they are nevertheless critical for developing an exacerbated disease in NOD mice. As shown in Fig. 6A, OVA-immunized CD1d^{-/-} NOD mice, which are deficient in the CD1d-restricted NKT cell subset, exhibited impaired airway eosinophilia and decreased IL-5 in the lung (data not shown) as compared to their control littermates. However, IL-4 and IL-13 production in the lungs as well as OVA-specific IgE titers in the serum remained unaffected (data not shown). To confirm the implication of NKT cells in the severity of eosinophilic inflammation in NOD mice, we reconstituted OVA-sensitized CD1d^{-/-} NOD mice with splenic NKT cells 24 h before airway challenge with OVA. Adoptive transfer of syngenic CD1d-restricted NKT cells fully restored the capacity of CD1d^{-/-} NOD mice to develop airway eosinophilia, while spleen cells from CD1d^{-/-} mice did not (Fig. 6B).

2.6 OVA-sensitized and -challenged NOD mice develop bronchopulmonary hyperreactivity (BHR)

Several components of airway inflammation, such as eosinophil-derived mediators, Th2 cytokines like IL-13, IL-5, IL-4 and elevated serum IgE titers are associated with the development of BHR, although their specific mechanism of action remains controversial [18]. Since these elements are effectively enhanced in NOD mice, we addressed their capacity to express BHR. As shown in Fig. 7, immunization with 1 μg OVA, promoted a significant BHR after methacholine inhalation, which was even stronger than in BALB/c mice receiving 10 μg of OVA, *i.e.* the optimal dose in this strain. BHR required both immunization and challenge with OVA, since it did not occur in NOD mice immunized with alum alone and challenged with OVA (data not shown).

3 Discussion

Diabetes in NOD mice occurs through a Th1-mediated autoimmune process [4–8]. For this reason, this strain is generally considered “Th1-prone”. However, the ability of adult NOD mice to mount Th2-mediated diseases remains unexplored. This issue is important with regard to the Th1/Th2 paradigm, which predicts that Th1- and Th2-mediated diseases are mutually exclusive.

In the present study, we demonstrate that NOD mice can develop an exacerbated Th2-mediated pulmonary inflammatory response, which implies that adult NOD mice are not generally Th1-biased but conserve the capacity to mount strong Th2 responses to exogenous Ag. Indeed, after immunization and i.n. challenge with OVA, adult NOD mice expressed a typical Th2 phenotype both locally and systemically, including the induction of elevated IL-4, IL-5, IL-13 and eotaxin secretion by lungs, along with increased OVA-specific IgE levels in serum. Moreover, this clear-cut Th2 profile was concomitant with the appearance of several symptoms of airway inflammation in the lungs.

Overall, the Th2-mediated inflammatory response to OVA developed by NOD mice was more pronounced than in the BALB/c strain, which is known for its propensity to mount intense Th2 responses [29]. This finding led us to explore the role of CD4⁺ T cells which play a key role in OVA-induced asthma in BALB/c mice [30]. *In vivo* depletion of CD4⁺ T cells in NOD mice abolished tissue eosinophilia, lung-derived Th2 cytokine production and increased OVA-specific serum IgE. Thus the general notion that CD4⁺ T cells are obligatory for the development and expression of the disease applies to NOD

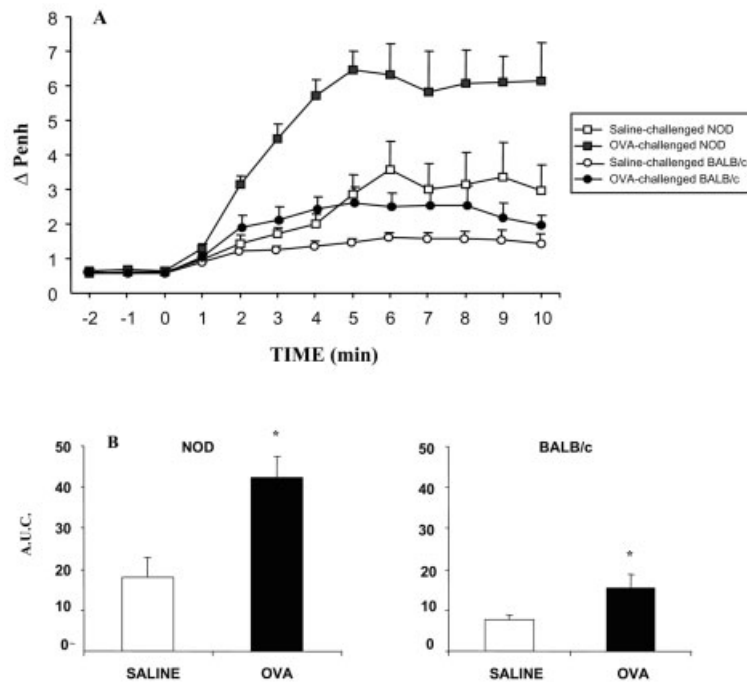


Fig. 7. Antigen-induced bronchopulmonary hyperreactivity in NOD and BALB/c mice. NOD and BALB/c mice were immunized with 1 μ g and 10 μ g OVA, respectively. One day after saline or OVA challenge, BHR was evaluated using the barometric plethysmographic method. The graphics correspond to the Penh (A) and the area under the curve (A.U.C.) (B), calculated for 10 min, and express the intensity of BHR (see Sect. 4). Each value represents the mean $\pm$ SEM of 6–18 mice. Significant differences were observed between saline and OVA-sensitized and challenged mice, and between NOD and BALB/c mice, as indicated. * $p < 0.005$.

mice. This is also true for BHR, another hallmark of allergic asthma engendered by augmented Th2 responses. Here again, the response was exacerbated and more pronounced than in BALB/c mice, which are currently used in studies on OVA-induced asthma [19]. Thus, NOD mice display a complete asthmatic phenotype with increased Th2 responses and more severe bronchial inflammation and hyperresponsiveness than BALB/c mice.

What are the immunological mechanisms accounting for the different intensities of the Th2 response in NOD and BALB/c mice? Several reports point to a role of IFN- γ -secreting Th1 cells in the modulation of Th2-mediated allergic inflammatory responses [18, 31, 32]. Since IFN- γ was not detected in lungs and BALF of either strain, it cannot account for the differences observed. In agreement with this finding, other authors have suggested that the immune regulation of asthma and allergic diseases is much more complex than a mere imbalance between Th1 and Th2 cells. In particular, Th2 cell-induced inflammation could not be diminished in an adoptive transfer model with antigen-specific Th1 cells, which did effectively reduce airway mucus production and eosinophilia, when they were given in excess [32, 33].

Among the other cellular components that may provide an explanation of our findings, T regulatory (Tr) cells deserve particular attention. Thus, recent studies demonstrate that allergen-specific Tr cells are critically involved in down-regulating Th2 effectors and inflammation in asthma [34–37]. Yet, it remains to be examined whether in a model of immune dysregulation like that of NOD mice, allergen-specific Tr cells are defective. NKT cells, another subset of Tr lymphocytes regulate immune responses in several models of autoimmunity in the NOD strain, including EAE and type 1 diabetes [28, 38, 39]. They have recently been shown to be required in allergen-induced Th2 airway inflammation in mice, which are not susceptible to autoimmune diseases [24, 25]. We report here that this notion applies to NOD mice, as far as airway eosinophilia and IL-5 production in lungs are concerned. These hallmarks of allergic asthma are significantly reduced in NKT-deficient NOD mice, dropping approximately to the same levels as in BALB/c mice. Yet, contrasting with non-autoimmune mouse strains [25, 26], production of the Th2-type cytokine IL-4 and specific Th2-mediated IgE levels were not affected in NKT cell-deficient NOD mice, as compared with their control littermates. In this regard, it should be underlined that the

IL-4 production by NKT from NOD mice is partially deficient [26–28] and needs to be activated by the specific NKT cell ligand α -galactosylceramide to efficiently counteract the development of the Th1-mediated autoimmune disease type 1 diabetes [28, 39]. Therefore, it would be important to further elucidate the origin of the particular efficiency of NKT cells to promote eosinophilia and IL-5 production in the NOD genetic background. Altogether, these findings lead us to conclude that the difference between the intensities of Th2 responses in NOD and BALB/c mice could in part be explained by NKT cells.

More generally, the NOD strain can be proposed as a new experimental model to address the question of the common immunological factors that influence susceptibility to type 1 diabetes and asthma. This approach might provide new insights for the clinical situation since emerging evidence suggests that the current increase of Th2-mediated allergies in developed countries correlates with that of Th1-mediated autoimmune diseases [40]. To this end, the large panel of congenic NOD strains that have been developed to map the genetic regions controlling this affection [41] might also be useful for the identification of new genes controlling asthma. Since NOD mice were also suitable for allergen (OVA)-dependent allergic asthma in terms of BHR they also allow the dissection of the complex interactions between immunological and non-immunological mechanisms controlling asthma.

4 Materials and methods

4.1 Mice

NOD wild-type (WT) and CD1d^{-/-} mice (backcrossed for at least six generations into the NOD background) [39], and BALB/c mice were bred and maintained in our animal facility under specific pathogen-free conditions. For experiments, 7–8-week-old males were used.

4.2 Immunization, booster and challenge protocols

Mice were immunized (day 0) and boosted (day 7) by subcutaneous injections of 1 or 10 μ g OVA (ICN Biomedicals, Inc, OH) adsorbed to 1.6 mg alum (Merck, Darmstadt, Germany) adjuvant in 0.4 ml saline. Seven days after the second immunization, mice received an intra-nasal (i.n.) challenge with 10 μ g OVA or saline under ketamine anesthesia (Imalgene 1000, 35 mg/kg body mass, Merial, Lyon, France).

4.3 Measurement of bronchopulmonary hyperreactivity

Bronchopulmonary hyperreactivity (BHR) was assessed 24 h after the i.n. challenge by delivering an aerosol of methacholine for 20 s at 100 mM (Sigma Aldrich, Stenheim,

Germany) to unrestrained, conscious mice placed in a plethysmographic chamber (EMKA technologies, Paris, France). The index of airflow obstruction was expressed as enhanced pause (Penh), calculated as: $\text{Penh} = [\text{Te (expiratory time)} - \text{Tr (relaxation time)}] / \text{Tr} \times [\text{Pef (peak expiratory flow)} / \text{Pif (peak inspiratory flow)}]$. The values of Penh were expressed per minute and are the average of three determinations recorded every 20 s. In order to simplify the interpretations, the area under the curve (A.U.C.) was calculated for 10 min after methacholine inhalation. The resulting A.U.C. values are indicated in graphs and represent the quantitative expression of BHR.

4.4 Serum and bronchoalveolar lavage fluids

Immediately after evaluating BHR, mice were anesthetized by the intraperitoneal injection of urethane (ethylcarbamate, 2 g/kg body mass, Sigma Aldrich, Stenheim, Germany) and bled for serum antibody and cytokine determinations by sandwich enzyme-linked immunosorbent assay (ELISA). The abdominal cavity was opened, the trachea was cannulated and lungs were lavaged with 2 ml of sterile PBS solution. Total and differential cell counts were determined in the bronchoalveolar-lavage fluid (BALF). BALF were centrifuged and supernatants were removed and stored at -20°C until cytokine measurement.

4.5 Measurement of OVA-specific IgE in the serum

OVA-specific IgE were determined by sandwich ELISA. Microplates were coated with appropriate anti-isotype capture mAb, purified from cell culture supernatants and incubated overnight at 4°C . After washing, the plates were blocked with PBS containing 1% of BSA (fraction V, Euro-medex, Mundolsheim, France) for 2 h at room temperature (RT). Samples were then added and incubated overnight at 4°C . The plates were washed and 10 μ g/ml of biotinylated OVA was added for 2 h at RT. After washing, the ExtrAvidin peroxidase conjugate was added for 45 min at RT. The OVA-biotin and ExtrAvidin conjugate was revealed by tetramethylbenzidine substrate (Kirkegaard Perry Laboratories, Maryland). Data were expressed as IgE index calculated as follows: $\text{IgE index} = [\text{OD sample} - \text{OD buffer only}] / [\text{OD positive control} - \text{OD buffer only}]$.

4.6 Determination of lung histology

Immediately after BALF collection, the lungs were perfused to eliminate residual blood, removed and fixed in paraformaldehyde. Paraffin-embedded tissues were sliced and 3- μ m sections were stained with hematoxylin and eosin (HE) for optic microscopy examination or with periodic acid-Schiff (PAS) for determination of mucus-producing cells.

4.7 Lung homogenates

Lungs were removed and homogenized in PBS. Lung homogenates were centrifuged for 15 min at 1600 $\times$ g at 4°C .

Supernatants were removed and stored at -20°C until cytokine measurement.

4.8 Lung explants

On the day following i.n. challenge, lungs were perfused through the pulmonary artery with saline to remove residual blood. They were chopped into 16 small pieces, four of which were used per well. Lung biopsies were incubated for 24 h at 37°C in 5% CO_2 atmosphere in AIM V medium (Life Technologies, Grant Island, NY) supplemented with 1% L-glutamine (Life Technologies) and gentamicin ($10\text{ }\mu\text{g/ml}$, Sigma). The supernatants were harvested and stored at -20°C until cytokine assay. Results are expressed in pg/ml of cytokine produced per mg of lung tissue (dry weight), which was determined after removal of medium.

4.9 Cytokine and eotaxin levels in the BALF, lung homogenates and lung biopsies

IL-4, IL-5, IL-13, IFN- γ and IL-12 levels in the BALF, lung homogenates and lung biopsies were measured by ELISA with appropriate capture mAb and with biotinylated detection mAb according to the manufacture's protocol (PharMingen, San Diego, CA). Conjugation of streptavidin-peroxidase (Amersham-Pharmacia, Les Ulis, France) was revealed using peroxidase substrate (Sigma). The cytokine concentrations were expressed as pg/ml based on a regression curve established for each assay with recombinant murine cytokines (PharMingen). The sensitivity of assays was $\sim 10\text{ pg/ml}$.

4.10 Anti-CD4 mAb treatment

Three days after OVA-immunization (day 7), NOD mice were treated i.p. with 0.5 mg/mouse anti-CD4 mAb (clone GK 1.5) for three consecutive days. After the last treatment, the mice were challenged i.n. with $10\text{ }\mu\text{g}$ OVA (day 14).

4.11 Adoptive transfer

Spleen cells from WT or CD1d $^{-/-}$ NOD mice were depleted of CD8 $^{+}$ T cells, CD62L $^{+}$, and CD19 $^{+}$ B cells labeled with the corresponding mAb with anti-rat-coated magnetic beads (Dynal, Compiègne, France). Among the mAb used for cell depletion CD8 (clone 53.67) and CD62L (clone Mel14) were purified in our laboratory while the CD19 (1D3) was purchased from PharMingen. The enriched negative selected NKT spleen cell population (containing 1×10^5 invariant V α 14 NKT cells as assessed by CD1d/ α -galactosylceramide tetramer staining) [25], as well as negative controls (equivalent numbers of similarly depleted spleen cells from CD1d $^{-/-}$ mice) were injected i.v. into CD1d $^{-/-}$ mice that had been immunized twice with OVA.

4.12 Statistical analysis

Results are expressed as means $\pm$ standard error of the mean (SEM). Mann-Whitney non-parametric or Student's *t*-test were used for analysis of significance among the groups.

Acknowledgements: We thank Dr. L. Van Kaer and Dr. M. Kronenberg for providing the CD1d $^{-/-}$ NOD mice [39] and the CD1d-tetramer, respectively. We acknowledge Dr. Elke Schneider for critically reviewing the manuscript. We thank Diane Damotte for pathological analysis of lung sections. We thank also Olivier Babin for histology technical support and Isabelle Renault for managing the specific pathogen-free mouse colonies. This work was supported by Institute funds from the Association pour la Recherche sur le cancer (no. 5889). L.M.A. and R.Z. were supported by personal grants from the INSERM and Fundação de Amparo à Pesquisa do Estado de São Paulo (Brazil), and from the Association pour la Recherche sur le Cancer, respectively.

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