The Journal of Immunology

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J. Immunol. 2006;177;8484-8492
http://www.jimmunol.org/cgi/content/full/177/12/8484

The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Prenatal Stress Enhances Susceptibility of Murine Adult Offspring toward Airway Inflammation[1]

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Allergic asthma is one of the most prevalent and continuously increasing diseases in developed countries. Its clinical features include airway hyperresponsiveness and inflammation upon allergen contact. Furthermore, an emerging area of research subsumed as fetal programming evaluates the impact of environmental insults in utero on the incidence of diseases in later life. The aim of this study was to identify whether prenatal exposure to stress, which constitutes a severe environmental insult, perpetuates airway inflammation in later life. Our experiments were performed in mice and revealed that prenatally stressed adult offspring indeed show an increased vulnerability toward airway hyperresponsiveness and inflammation. Furthermore, we provide persuasive insights on dysregulated pathways of the cellular and humoral immune response upon Ag challenge in prenatally stressed adult offspring, reflected by a Th2 greater Th1 adaptive immune response and increased CCR3 and IgE levels in vivo. Additionally, APCs derived from prenatally stressed offspring trigger clonal expansion of Th2 cells in vitro. We also deliver experimental evidence for a reduced corticotrophin-releasing hormone expression in the paraventricular nucleus of adult offspring in response to prenatal stress. Furthermore, behavioral analyses indicate an increase in anxiety in these mice. In conclusion, our data will facilitate future research aiming to identify the individual impact, hierarchy, and redundancy of multiple key protagonists in airway inflammation in an interdisciplinary context. This will foster the substantiation of disease-prevention strategies, such as asthma, during the prenatal period. *The Journal of Immunology,* **2006, 177: 8484–8492.**

Asthma is one of the most prevalent and continuously increasing diseases of the 21st century in developed countries (1, 2). Two pathological entities, allergic and nonallergic, are generally and collectively referred to as asthma, and clinical features of both entities comprise recurrent attacks of dyspnea caused by airway hyperresponsiveness and inflammation (3, 4). The most common form of asthma, allergic asthma, is defined as bronchial constriction and Th2-dominated airway inflammation upon allergen contact, whereby the Th2 predominance is a result of high levels of IL-4, IL-5, IL-9, and IL-13 (4). Among these cytokines, IL-4 clearly plays a decisive role because it perpetuates the Th2 bias in $CD4^+$ T cells and triggers a humoral immune response toward up-regulation of IgE, a prototypic feature of allergies (5, 6). Furthermore, eosinophil accumulation in the airways is a hallmark of allergic airway inflammation. Eosinophil migration depends on chemokine receptor (CCR) expression, predominantly involving CCR3 (7, 8).

It has become increasingly apparent that the primary risk factor for the development of an allergic disease such as asthma is a genetic predisposition, because children of allergic parents are more prone to allergic diseases than children of nonallergic parents. Such genetically determined predisposition to allergic diseases, which include allergic rhinitis, allergic asthma, and atopic eczema, is termed atopy. Strikingly, the presence of atopic diseases in the mother propagates the onset of allergic diseases in the offspring considerably stronger than atopic diseases of the father (9). Such observations from epidemiological studies obviously challenge genetic predispositions as the sole cause for allergic diseases. In line with this, it has recently been introduced that allergic diseases may be aggravated by environmental factors during the perinatal period (10). Aggravating agents include maternal smoking, nutrition supply, and pollution (11, 12).

An emerging area of research addresses the identification of environmental insults in utero causing permanent functional changes of the offspring. Characteristically, such insults are not overtly teratogenic, yet may result in an increased susceptibility to disease and/or dysfunction later in the life span. This scientific hypothesis of the fetal origin of adult diseases or fetal programming has been developed by epidemiology studies, initially accentuated by Barker (13). Most of the subsequent studies in this area have concentrated on repulsively altered nutrition in utero and its prominent influence on multiple aspects of adult health and disease risk, such as cardiovascular diseases, obesity, and diabetes mellitus type 2 (14, 15). More recently, the list of programming agents during pregnancy has been amended and comprises air/water pollution, endocrine agonists and antagonist, corticosteroid exposure, maternal smoking, bacterial stimuli (endotoxin), and stress perception (16–18).

As yet, pathways of an increased vulnerability to develop allergic diseases in response to environmental insults in utero remain to be elucidated. Persuasive and instructive models must be made available to provide convincing experimental evidence for a causal

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Received for publication July 26, 2006. Accepted for publication September 26, 2006.

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[1] M.K.P.K. was supported by the new investigator program of the Charité and S.M.B. is a Fellow of the Schering Research Foundation. This work was made possible by research grants from the Charité to P.C.A. and M.K.P.-K.

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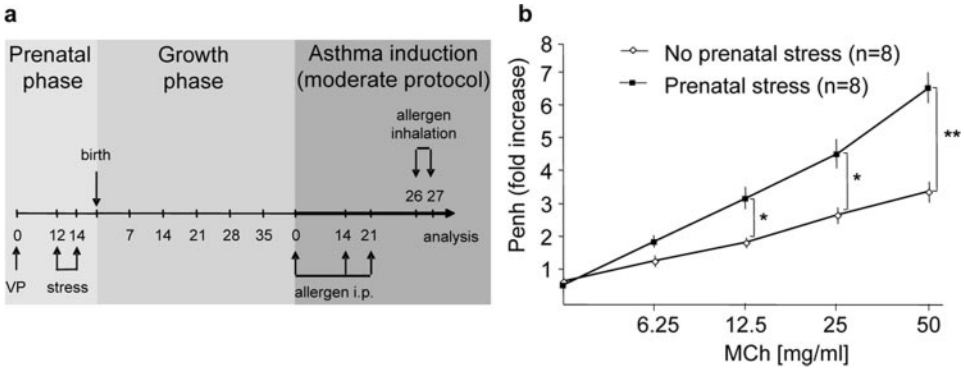

FIGURE 1. Prenatal stress exposure increases airway hyperresponsiveness. *a*, Pregnant female BALB/c mice were stressed on gestation days 12 and 14. At the age of 42 days, the offspring were immunized three times i.p. The allergen provocation (inhalation) was performed two times: moderate protocol on days 26 and 27 after initial i.p. sensitization. *b*, In vivo airway response was analyzed by whole-body plethysmography using increasing dosages of aerosolized MCh. Data are expressed as fold increase of Penh values for each concentration of MCh relative to PBS Penh values (±SD). The raw Penh data are depicted in Table II.

relationship between fetal programming and the onset of allergic diseases in the offspring to understand the individual impact, hierarchy, and redundancy of multiple key protagonists in allergic diseases. In this study, we provide such a model by using maternal stress exposure during pregnancy as an environmental insult aggravating allergic diseases in the offspring. We show that prenatal stress exposure increases susceptibility to allergic airway hyperresponsiveness and inflammation, clinical features that are accompanied by a Th2-dominated immune response systemically as well as locally in the lung of adult mice upon prenatal stress exposure. Clearly, stress during pregnancy leads to elevated levels of maternal stress hormones (19), whereby the majority of the stress-elevated cortisol levels within the maternal circulation will be inactivated at the placenta. However, 10–20% of the maternal cortisol will still pass into the fetal system, which likely influences not only fetal cortisol concentrations, but also the maturation of the hypothalamic pituitary adrenal axis (20). Thus, we further aimed to characterize corticotrophin-releasing hormone (CRH)[4] expression in the brain of adult offspring and observed a reduced CRH expression in the paraventricular nucleus (PVN) of prenatally stressed offspring. Additionally, behavioral analyses indicate an increase in anxiety in prenatally stressed offspring. Using this model, it may now be further substantiated in interdisciplinary research approaches how programming agents can exert profound alterations of fetal development, which may subsequently lead to effective disease prevention strategies (e.g., with respect to atopic diseases).

Materials and Methods

Mice

BALB/c mice were purchased from Charles River Laboratories and maintained in an animal facility with a 12-h light/dark cycle. Animal care and experimental procedures were followed according to institutional guidelines and conformed to the requirements of the state authority for animal research conduct (Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit Berlin).

Maternal stress exposure

BALB/c females at 6 wk of age were mated with 8-wk-old BALB/c male mice. Successful mating was assumed upon the appearance of a vaginal plug. Pregnant females were segregated and divided into two different subgroups. One group served as a control and was left undisturbed during pregnancy. The other group was exposed to stress on days 12 and 14 during gestation.

Exposure to sound stress was applied for the duration of 24 h. The sound was emitted by a rodent repellent device (Conrad Electronic) with 70 dB at a frequency of 300 Hz in intervals of 15 s. The stress device was placed into the mouse cage so that the mice could not escape the sound perception.

Delivery of litters was documented, and females with their respective litter were kept in individual cages and left undisturbed for a period of 21 days, except for documentation of weight. Then, offspring were weaned and gender distribution was documented. Only female offspring were used for subsequent allergen challenges. The rationale for using exclusively female mice was to avoid any stress exposures during adolescence and early adulthood in the offspring. Because male mice are known to fight when kept in one cage, which constitutes a strong stressor, we refrained from using male offspring for subsequent experiments.

OVA protocols applied on offspring

At 42 days of age, female offspring were sensitized to OVA by three i.p. injections of 10 μg OVA (grade VI; Sigma-Aldrich), emulsified in 1.5 mg $Al(OH)_3$ (Pierce) in a total volume of 200 μl (days 1, 14, and 21). Mice were placed in a Plexiglas chamber and exposed to aerosolized OVA (0.1 g reconstituted in 5 ml of PBS, grade V; Sigma-Aldrich) for 20 min on days 26 and 27 upon the first i.p. injection (Fig. 1*a*).

In vivo airway reactivity

Airway hyperresponsiveness was assessed 24 h after the last aerosol challenge (day 28) as in vivo lung function to inhaled methacholine (MCh; Sigma-Aldrich), analyzed by whole-body plethysmography (EMKA Technologies). Briefly, each mouse was placed in a main chamber of the body plethysmograph. Aerosolized PBS and MCh in increasing concentrations (6–50 mg/ml) were nebulized ultrasonically through an inlet of the main chamber for 3 min, respectively. Pressure differences between the main and a reference chamber caused by volume and resultant pressure changes during inspiration and expiration of the mouse were measured and averaged for 3 min after each nebulization. As an index of airway obstruction, enhanced pause (Penh) values were calculated, and airway reactivity was expressed as an increase on Penh values for each concentration of MCh (raw Penh data). The fold increase of Penh was calculated by dividing the Penh value of each MCh concentration through the Penh value achieved at the PBS aerosolization.

Serum levels of total and OVA-specific Ig

On day 29, blood samples were taken out of tail veins, and levels of total IgE and OVA-specific IgE, IgG1, and IgG2a were measured by means of ELISA, as previously described (21). Levels of OVA-specific Abs were related to pooled standards generated in our laboratories and expressed as arbitrary units per milliliter.

Bronchoalveolar lavage (BAL)

On day 28, lungs underwent lavage through a tracheal drain tube twice with 1 ml of cold PBS. Cytospin slides were stained with Diff-Quick (Dade

[4] Abbreviations used in this paper: CRH, corticotrophin-releasing hormone; PVN, paraventricular nucleus; MCh, methacholine; BAL, bronchoalveolar lavage; PAS, periodic acid-Schiff; CBA, cytometric bead array; DC, dendritic cell; EPM, elevated plus maze; Penh, enhanced pause.

Behring), and BAL cells were differentiated on the basis of morphological criteria by counting 100 cells under a light microscope.

Histology

The right ventricles were cannulated and the lungs were rinsed with 10 ml of PBS to remove intravascular blood. Brains from OVA-sensitized animals were taken, frozen in dry ice, and stored at −70°C. Samples were snap frozen and sectioned. Sections were either stained with H&E for evaluation of the lung morphology or stained with periodic acid-Schiff (PAS) as a measure of mucus production.

In vitro production of cytokines

On day 28, upon initiation of sensitization protocols, mice of all groups were sacrificed. For in vitro cytokine production, blood mononuclear cells were isolated by lysis of the erythrocytes and resuspended in culture medium AimV. BAL cells were washed with RPMI 1640 and resuspended in AimV. Cells were cultured in 96-well round-bottom plates at 37°C in a 5% CO_2 atmosphere with PMA/ionomycin and OVA at a concentration of 250 μg/ml. After 24 h, supernatants were harvested, and levels of cytokines were assessed by means of cytometric bead arrays (CBA).

Flow cytometry

Flow cytometry was performed using our standard protocol (22). Briefly, lung cells were washed twice with buffer (PBS supplemented with 1% BSA; Sigma-Aldrich and 0.1% sodium acid; Sigma-Aldrich). Normal mouse serum (2%) was added to avoid nonspecific binding by Fc receptors. Cells were then incubated for 30 min at 4°C with the respective mAb against surface Ags. The cells were then washed and fixed using Fix solution (BD Biosciences) for 30 min at 4°C in the dark. Afterward, the cells were washed, resuspended in PBS, and acquired. As controls, cells were stained with the corresponding isotype-matched mAb. The acquisition was performed using a FACSCalibur (BD Biosciences). Flow cytometer instrument compensation was set in each experiment using single-color stained samples. Data were analyzed by using CellQuest software (BD Biosciences). Flow cytometry results were expressed as percentage of cells positive for the surface marker evaluated.

Isolation and purification of lung dendritic cells (DCs)

DC were isolated by digestion of fragments of the lung at 37°C for 30 min with a mixture of DNase I (fraction IX; Sigma-Aldrich) and mg/ml Collagenase Typ IA-S (Sigma-Aldrich) followed by a dissociation for 5 min with 10 mM EDTA. The $CD11c^+$ cell fraction was isolated using magnetic cell sorting according to the manufacturer's instruction (Miltenyi Biotec). Briefly, lung suspensions were incubated for 30 min at 4°C with biotinylated hamster anti-mouse CD11c diluted 1/100 in labeling buffer (PBS supplemented with 2 mM EDTA). After washing, cells were incubated (15 min, 4°C) with Streptavidin MicroBeads (Miltenyi Biotec) and processed using magnetic cell sorting to collect $CD11c^+$ cells. Flow cytometry analysis revealed that ~90% of selected cells expressed CD11c.

Coculture experiments

$CD4^+$ T cells were isolated from the spleen of BALB/c with a $CD4^+$ Isolation Kit (Miltenyi Biotec) according to the manufacturer's instruction and seeded (2×10^5 cells/well) in 96-well plates as responders with lung-derived DC (1×10^4 cells/well) from nonprenatally stressed, sham-treated offspring and prenatally stressed, sham-treated offspring (stimulator:responder ratio was 1:5). Cell cultures were performed at 37°C in 5% CO_2 for 72 h in RPMI 1640 supplemented with antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin), 2 mM L-glutamine, 1 mM pyruvate, and 10% FCS. For analysis of cumulative cytokines, cell culture supernatants were collected and analyzed.

Cytokine determination by CBA

Cytokines were analyzed in cell culture supernatants using CBA. Briefly, 24-h culture supernatants were harvested and stored at −70°C until cytokine determination. IL-4, IL-5, TNF-α, IFN-γ, and IL-2 were detected simultaneously using the mouse Th1/Th2 cytokine CBA kit (BD Pharmingen). Briefly, 50 μl of each sample was mixed with 50 μl of mixed capture beads and 50 μl of the mouse inflammation PE detection reagent consisting of PE-conjugated anti-mouse cytokines. The samples were incubated at room temperature for 2 h in the dark. After incubation with the PE detection reagent, samples were washed once and resuspended in 300 μl of washing buffer before acquisition on a FACSCalibur cytometer (BD Biosciences). Data were analyzed using CBA software (BD Biosciences). Standard curves were generated for each cytokine using the mixed cytokine standard provided by the kit. The concentration of each cytokine in cell supernatants was determined by interpolation to the corresponding standard curve.

CRH mRNA hybridization and quantification of brain sections

Fresh-frozen brains were sectioned on a cryostat at 15 μm, thaw mounted onto slides, and stored at −70°C. Sections containing PVN (bregma, −0.82 mm) and amygdala (bregma, −1.46 mm) were processed by in situ hybridization according to previously published protocols (23). Briefly, sections were fixed in 4% paraformaldehyde and pretreated with triethanolamine/acetic anhydride before incubation in standard hybridization buffer (37°C for 20 h). Hybridization buffer contained the [^{35}S]dATP-labeled oligonucleotide probe (185,000 cpm/45 μl/slide with a parafilm coverslip) for CRH (complementary to bases 496–537) (24). The following day, sections were stringently washed in SSC (at 58°C), dried, and dipped in photographic gel emulsion (K5; Ilford Imaging). Slides were exposed for 8 wk, and then developed, fixed, counterstained with H&E, and coverslipped. Analysis of CRH mRNA hybridization was made in the PVN and the central nucleus of the amygdala and the medial amygdala. The number of positive cells and the grain area per cell were analyzed in four brain sections per mouse. Positive cells were defined as having greater than three times the background number of silver grains per cell and were counted within eight PVN or amygdala profiles per mouse as defined in the study by Franklin and Paxinos (25). Hybridization was evaluated by measuring the silver grain area over individual neurons within the region of interest (×40 objective) using a computer-aided image analysis system (Open Lab; Improvision). Twenty positive cells were analyzed per mouse and background measurements were taken from tissue adjacent to the area that exhibited no evident hybridization; average background from four cells was subtracted from the silver grain area of positive cells. The slides were coded so that during the quantitative analysis the experimenter was unaware of the treatment group to which each slide belonged.

Behavioral analysis

The elevated plus maze (EPM) (26) was used to assess anxiety-like behavioral responses in adult offspring after prenatal stress exposure, which has been designed to present the animal with a conflict between the exploration of a novel environment and its reluctance to move away from sheltering walls. The EPM consisted of two open (30 cm × 5 cm) and two closed (30 cm × 5 cm × 15 cm) arms, extending from a common central platform (5 cm × 5 cm). The apparatus was constructed from black Plexiglas and elevated to a height of 50 cm above the floor. All testing was conducted between 8:00 a.m. and 11:00 a.m. Mice were placed on the central platform facing an open arm. Open- and closed-arm entries were documented over a period of 5 min. An arm entry was defined when a mouse entered an arm with all four paws. EPM was analyzed by an investigator blind to the experimental groups.

Statistical analysis

Data of two independent experiments were analyzed. Pairs of groups were compared with a Student's *t* test. Significance was set at a p of 0.05.

Results

Establishment of a mouse model on fetal programming of allergic asthma

Our main task within the present study was to establish a mouse model on fetal programming of allergic diseases. In this study, two well-characterized experimental models were combined: first, the exposure to sound stress during pregnancy, which has been shown to constitute a challenge to fetal tolerance if exposure to stress is performed during the peri-implantation period (22). Because teratogenesis had to be avoided and a vulnerable window for fetal lung development and immune system programming is likely present later in gestation (27), we changed the stress intervention time point during gestation from the peri-implantation period to the postimplantation period. Thus, pregnant female mice were exposed to stress on gestation days 12 and 14 (Fig. 1*a*) for a duration of 24 h, respectively. We observed that the offspring of prenatally stressed mice had a significantly lower birth weight compared with the offspring of nonstressed mice on day 2 after birth (Table I), which was no longer detectable on day 4 and beyond. The overall

Table I. *Weight development during the first 10 days after birth and survival rate of prenatally stressed and nonstressed offspring (mean ± SD)*[a]

Prenatal Stress	Weight on Day 2 after Birth (g)	Weight on Day 4 after Birth (g)	Weight on Day 6 after Birth (g)	Weight on Day 10 after Birth (g)	No. of Living Offspring
Nil	2.3 ± 0.2	3.2 ± 0.3	4.4 ± 0.6	6.9 ± 0.7	5.4 ± 2.1
Gestation days 12 and 14	1.9 ± 0.2**	3.1 ± 0.3	4.1 ± 0.3	6.6 ± 0.5	5.4 ± 2.1

[a] Because gender differentiation was not possible, all offspring were subjected to weighting, whereby only female mice were used for subsequent OVA sensitation.

**, $p < 0.01$ of prenatally stressed offspring compared to nonstressed offspring.

survival rate of offspring from stressed and nonstressed mice did not significantly differ (Table I).

Second, we exploited a widely used mouse model of allergic airway disease by OVA sensitization and aerosol challenge of offspring from stressed female mice and respective controls once they reached adulthood. In this study, an allergen aerosol challenge for 2 days upon systemic sensitization (moderate protocol, Fig. 1*a*) has been applied (28, 29). Airway hyperresponsiveness was evaluated by whole-body plethysmography and is shown as fold increase of enhanced pause ($Penh_{max}$), which mimics the exponential increase in airway constriction. The raw Penh data describing the enhanced pause independent of the response to control are depicted in Table II.

Strikingly, stress challenge during pregnancy clearly revealed a significant increase in airway hyperresponsiveness upon allergen aerosol challenge in the offspring (Fig. 1*a* and Table II). Thus, these results provide clear-cut experimental evidence that prenatal stress renders murine offspring more susceptible toward allergic airway hyperresponsiveness. Because it is a crucial step to link the right time window during pregnancy to an increased susceptibility to airway hyperresponsiveness, we tested variations of the time window and observed that a single exposure of stress given on gestation day 12 did not suffice to induce an increased vulnerability to develop airway hyperreagibility (data not shown).

Besides airway hyperresponsiveness as a frequent clinical feature of asthma, we aimed to investigate the effect of prenatal stress challenge on an additional clinical read-out parameter of asthma, the airway inflammation. Airway inflammation is triggered by an increased leukocyte influx, predominantly eosinophils, into the lung. We observed that prenatal stress exposure resulted in the relative increase of eosinophil recruitment into the lung, as detected in the BAL (Fig. 2*a*). Fig. 2*b* shows representative examples of cytospins prepared to evaluate the relative leukocyte distribution in BAL. Absolute numbers of leukocyte subsets in BAL supported these observations. Data are given in Table III.

Next, we independently confirmed airway inflammation and increased mucus production in the lung by analysis of lung tissues from OVA- and sham (PBS)-treated offspring upon H&E (Fig. 2*c*) or predigested PAS (Fig. 2*d*) staining.

Prenatal stress challenges humoral immune response in OVA-sensitized adult offspring

In addition to the cellular immune response, our goal was to investigate humoral immune-response pathways in our experimental setting. We primarily focused on levels of total IgE in serum, because IgE is a key mediator of allergic asthma. As depicted in Fig. 3*a*, the total amount of IgE significantly increased in OVA-sensitized adult offspring in response to prenatal stress compared with nonstressed, OVA-sensitized offspring. Markedly, OVA-specific IgE did not differ between prenatally stressed and nonstressed animals (Fig. 3*b*), providing clear confirmation that the differences of clinical asthma features among the various groups of mice are not due to a variation in the effectiveness of sensitization. The detection of allergen-specific immunoglobulins IgG1 (Fig. 3*c*) and IgG2a (Fig. 3*d*) confirms an induction of a humoral immune response instead of tolerance upon sensitization. Because we know from previous experiments in adult, nonpregnant animals that were sensitized and challenged with OVA that stress exposure does not alter IgE, IgG1, or IgG2a levels (28), we refrained from taking blood from the mothers during pregnancy to avoid additional stress. However, hypothetically, changes of maternal IgE may interfere with the offspring's IgE production.

Prenatal stress challenges the Th1/Th2 balance in OVA-sensitized adult offspring

Published evidence discloses the pivotal role of a balanced cytokine equilibrium in preventing allergic diseases, whereby a Th2 > T_H1 immune disequilibrium contributes to the pathogenesis of allergic diseases (30). Hence, we investigated the cytokine profile of blood- and BAL-derived leukocytes in our experimental groups of adult offspring upon in vitro restimulation with the OVA allergen.

Table II. *Raw data (mean ± SD) on analysis of airway hyperresponsiveness*[a]

No. of Offspring/ Group	Day of Prenatal Stress	Sensitation Protocol	Base Line[b]	PBS[c]	MCh[d] (6 mg)	MCh[d] (12 mg)	MCh[d] (25 mg)	MCh[d] (50 mg)
8	Nil	Moderate	0.66 ± 0.13	0.68 ± 0.21	0.84 ± 0.19	1.22 ± 0.28	1.76 ± 0.58	2.16 ± 0.53
8	2	Moderate	0.59 ± 0.11	0.53 ± 0.10	0.99 ± 0.35	1.70 ± 0.90	2.42 ± 1.37	3.51 ± 1.68*
9	Nil	None (PBS)	0.49 ± 0.08	0.47 ± 0.10	0.65 ± 0.22	1.22 ± 0.67	1.82 ± 0.75	2.15 ± 0.79
11	2	None (PBS)	0.56 ± 0.14	0.59 ± 0.13	0.96 ± 0.40	1.51 ± 0.76	1.86 ± 1.08	2.26 ± 1.41

[a] Penh; data depict the enhanced pause independent of the response to PBS.

[b] Penh (mean), as measured over 3 min at the beginning of the body plethysmography, when no aerosol challenge with PBS or MCh had been induced (basal Penh value).

[c] Penh (mean), as measured over 3 min after aerosol challenge with PBS in the body plethysmograph (PBS Penh value).

[d] Penh (mean), as measured over 3 min after aerosol challenge with increasing dosage of MCh in the body plethysmograph (MCh Penh value).

*, $p < 0.05$ of prenatally stressed, OVA-treated offspring vs nonstressed, OVA-treated offspring.

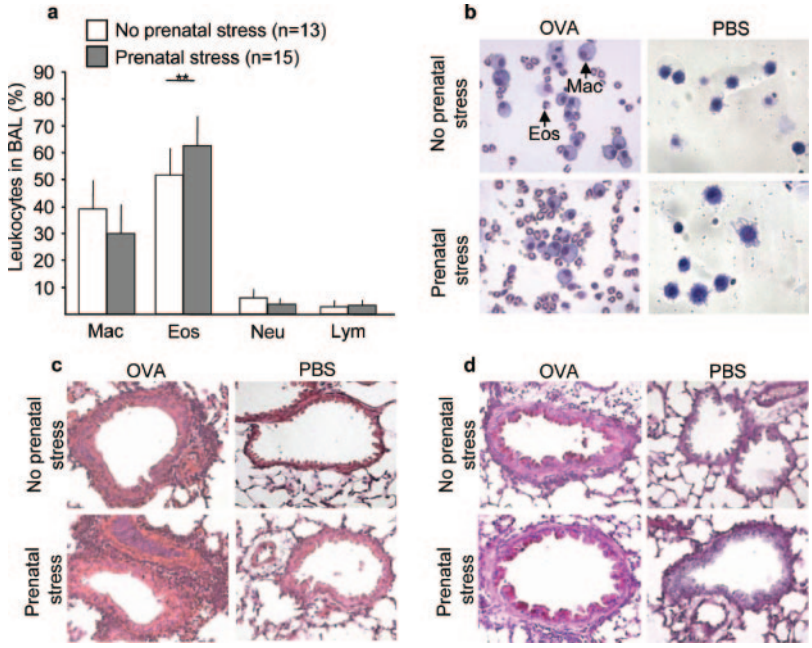

FIGURE 2. Prenatal stress increases airway inflammation. *a*, Cells of BAL were collected from OVA-sensitized offspring, cytospins were prepared, and leukocyte differentiation was performed to obtain percentages of macrophages (Mac), eosinophils (Eos), neutrophils (Neu), and lymphocytes (Lym) among BAL leukocytes from the respective groups. Values are given as mean per group ± SD. Absolute numbers of leukocyte distribution are shown in Table III. *b*, Representative examples of BAL leukocyte differentiation (cytospins) of nonprenatally or prenatally stressed, OVA-immunized offspring (*left panel*) and PBS-immunized offspring (*right panel*). Arrows, The most abundant leukocyte subsets, macrophages, and eosinophils. *c*, Lung tissue of nonprenatally or prenatally stressed, OVA-immunized offspring (*left panel*) was stained with H&E to confirm airway inflammation compared with PBS-treated offspring (*right panel*). *d*, Predigested PAS staining of lung tissue from nonprenatally or prenatally stressed, OVA-immunized offspring (*left panel*) and PBS-treated offspring (*right panel*) was performed to detect increased mucus production.

Using CBA, we observed a significant increase of IL-4 secretion in blood of OVA-sensitized offspring upon intrauterine stress challenge (Fig. 4*a*). Secretion of TNF-α, a prototypic Th1 cytokine, was reduced in blood lymphocytes after prenatal stress (Fig. 4*b*). The ratio of all Th2/Th1 cytokines analyzed by CBA (IL-2, IL-4, IL-5, TNF-α, and IFN-γ) compliments the results and reveals a net dominance of Th2 cytokines (Fig. 4*c*). In line with the Th2 > Th1 cytokine profile in blood, we observed a significantly reduced secretion of TNF-α in BAL-derived leukocytes of prenatally stressed offspring (Fig. 4*d*). In BAL cell suspensions, levels of secreted IL-4 were below the sensitivity of the method and could not be detected. Representative examples of CBA analysis in supernatants from blood and BAL-derived leukocyte cultures are depicted in Fig. 4, *e* and *f*.

Prenatal stress increases CCR3$^+$ cells in OVA-sensitized adult offspring

Because the chemokine receptor CCR3 has been proposed to orchestrate eosinophil migration into the lung, we investigated the percentage of CCR3-expressing leukocytes in lung tissue after enzymatic digestion (31). In prenatally stressed mice, an increased percentage of chemokine receptor CCR3$^+$ cells among lung-derived leukocytes could be detected (Fig. 4*g*, histograms are shown in Fig. 4*h*).

Prenatal stress alters APCs toward clonal expansion of T_H2 cells in vitro

Next, we tested which Th1/Th2 cytokine pattern is induced in naive T cells by lung-derived APCs (e.g., DC after prenatal stress exposure). In this study, we analyzed the cytokine levels in supernatants of CD11c$^+$ DC in coculture with naive T cells derived from the spleen of untreated BALB/c mice. We deliberately chose to use CD11c$^+$ APCs from non-OVA-sensitized offspring to mimic Ag presentation upon primary Ag contact. Nonetheless, the animals underwent the entire procedure involved in sensitization, such as i.p. injections and aerosol provocation, using PBS instead of OVA to exclude the stress associated with the sensitization procedure that affects Ag presentation. Strikingly, dramatic alterations of cytokine profiles resulting in a Th2 > Th1 cytokine ratio in culture supernatants (Fig. 5*a*) as a result of altered key cytokines, including TNF-α, IFN-γ, IL-5, IL-4, and IL-2 (Fig. 5, *b–f*), occurred when CD11c$^+$ lung cells derived from prenatally stressed adult offspring were compared with nonstressed offspring.

Airway hyperresponsiveness and inflammation in non-OVA-sensitized offspring

As controls, some of the offspring were injected and aerosol challenged with PBS instead of OVA sensitization. As shown in Fig. 6*a* and Table II, airway hyperresponsiveness was lower compared with OVA-sensitized mice and no significant differences could be observed between prenatally stressed or nonstressed offspring. Furthermore, upon PBS injection and aerosol challenge, a weak influx of leukocytes could be detected in the BAL, which comprised predominantly macrophages (Fig. 6*b* and Table III). Similar to the OVA-sensitized mice, a significant decrease of TNF-α secretion in blood could be detected upon stress challenge (Fig. 6*c*); however, the concentration of IL-4 secreted by blood leukocytes

Table III. *Absolute number (mean ± SD) of BAL leukocyte subsets*

No. of Offspring/ Group	Day of Prenatal Stress	Sensitization Protocol	Macrophages ($\times 10^3/\mu$l)	Eosinophils ($\times 10^3/\mu$l)	Neutrophils ($\times 10^3/\mu$l)	Lymphocytes ($\times 10^3/\mu$l)
13	Nil	Moderate	108 ± 44	144 ± 55	16 ± 7	7 ± 7
15	2	Moderate	94 ± 46	195 ± 64	12 ± 6	9 ± 3
7	Nil	None (PBS)	69 ± 31	0.1 ± 2	1.4 ± 1	0.4 ± 1
5	2	None (PBS)	69 ± 11	0.0 ± 0	0.9 ± 1	0 ± 0

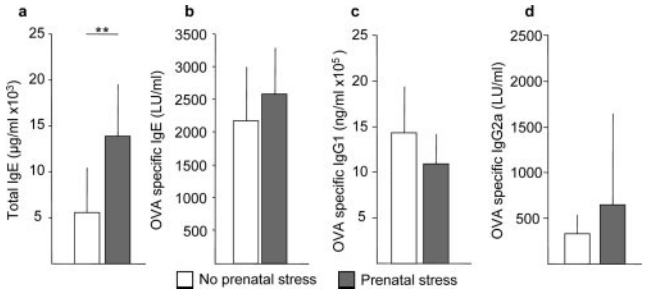

FIGURE 3. Prenatal stress increases total IgE production. *a*, Serum levels of total IgE (±SD) in prenatally stressed and nonstressed OVA-treated offspring (**, $p < 0.01$). *b*, OVA-specific Ab response (±SD) comparing prenatal stress and nonprenatal stress in OVA-immunized and challenged offspring. *c*, OVA-specific IgG1 (±SD) and OVA-specific IgG2a (±SD) in OVA-immunized and challenged offspring in dependence of prenatal stress (*d*).

was not altered by exposure to prenatal stress (Fig. 6*d*). No differences could be detected with respect to the Th2:Th1 cytokine ratio (Fig. 6*e*) and TNF-α secretion by BAL leukocytes (Fig. 6*f*) in control or prenatally stressed, PBS-treated offspring (Fig. 6*e*).

Effects of prenatal stress on CRH gene expression in the brain and behavior

CRH expression could mainly be detected in the parvocellular neurons in the PVN, which mediate hypothalamic pituitary adrenal axis responses. The *CRH* mRNA hybridization signal was stronger in the PVN of control offspring (Fig. 7*a*), compared with prenatally stressed mice (Fig. 7*b*). Quantification of *CRH* mRNA expression revealed that the silver grain area per cell in the PVN was significantly reduced by prenatal stress (Fig. 7*c*). There was no significant difference in *CRH* mRNA expression in either region of the amygdala between the two groups (data not shown). Furthermore, exposure to prenatal stress significantly reduced the percentage of open-arm entries (Fig. 7*d*) and time spent in open arms (Fig. 7*e*),

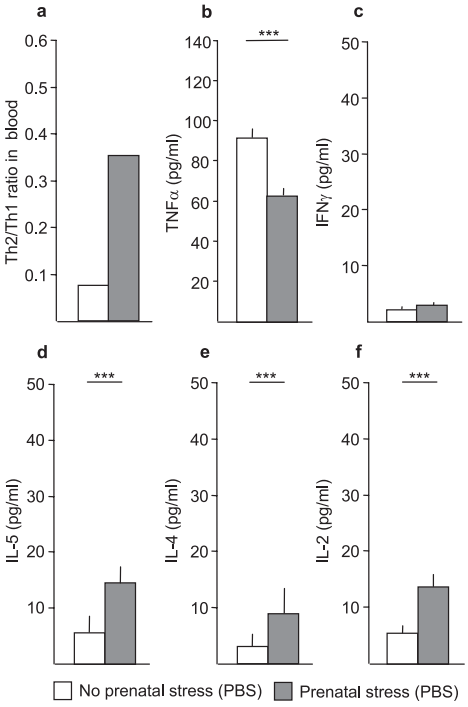

FIGURE 5. Prenatal stress exposure primes T cells toward the secretion of a Th2 cytokine profile in vitro. *a*, Th2:Th1 ratio in supernatants of naive T cells cocultured with lung $CD11c^+$ cells from different experimental groups, calculated by the sum of IL-4 and IL-5 levels divided by the sum of TNF-α and IFN-γ levels. An increase of Th2 over Th1 cytokines was detectable in supernatants from prenatally stressed mice (*, $p < 0.001$). *b–f*, Analysis of individual cytokine levels (TNF-α, IFN-γ, IL-5, IL-4, and IL-2) in respective supernatants.

which indicates an increase in anxiety-like behavior compared with the control group. There were no significant differences with respect to the number of closed-arm entries between the groups

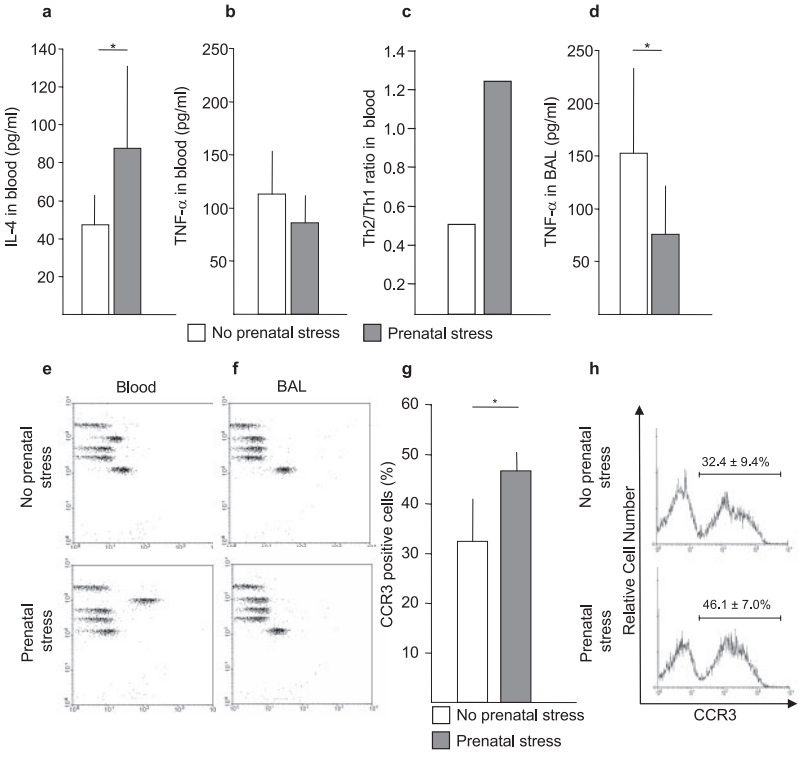

FIGURE 4. Prenatal stress strongly induces a Th2 immune response in prenatally stressed compared with nonprenatally stressed mice in experimental allergic airway disease. *a* and *b*, Cytokine determination in supernatants of leukocytes isolated from blood of OVA-immunized and challenged animals upon allergen stimulation over 24 h. Cytokines were measured by CBA (*, $p < 0.05$). *c*, The ratio of Th2:Th1 cytokine was calculated by adding the individual concentrations (picograms per milliliter) of IL-4 and IL-5, and dividing this sum by the sum of TNF-α and IFN-γ. An increase in Th2 cytokines over Th1 was observed in supernatants from prenatally stressed mice. *d*, TNF-α concentration (picograms per milliliter ± SD) secreted from BAL cells 24 h after allergen stimulation in OVA-immunized and challenged animals (*, $p < 0.05$). *e* and *f*, Representative examples of CBA analysis for cytokines in supernatants of leukocytes isolated from the blood (*left panel*) or the BAL (*right panel*) of OVA-immunized and challenged stressed and nonstressed animals. From *top* to *bottom*, the bead clouds show IL-2, IL-4, IL-5, IFN-γ, and TNF-α. We did not consider IL-2 when calculating the Th2:Th1 coefficient given in *c*, because this cytokine cannot easily be assigned to either classification. *g*, Mean percentage ± SD of $CCR3^+$ lung leukocytes in response to prenatal stress in OVA-immunized and challenged animals, as analyzed by flow cytometry. *h*, Representative histograms on flow cytometry results to detect CCR3 positivity among lung leukocytes in the respective groups.

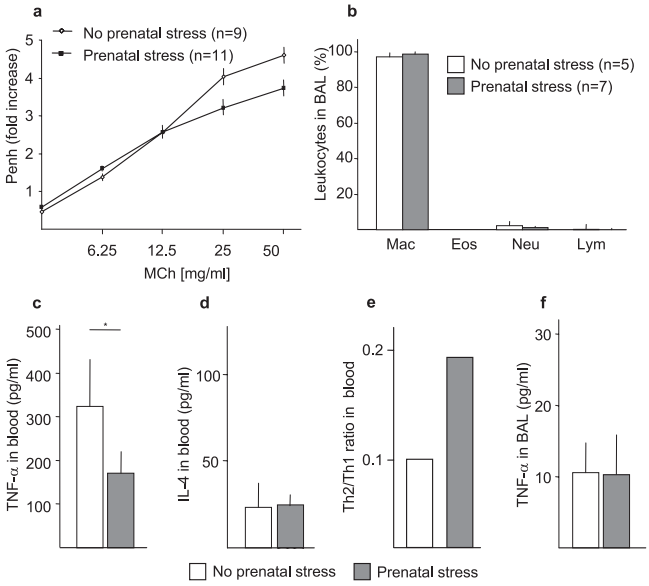

FIGURE 6. Prenatal stress does not affect airway responsiveness or inflammation in BAL of sham-immunized animals. *a*, Pregnant BALB/c mice were stressed on days 12 and 14 of gestation. At the age of 42 days, the offspring were sham immunized with PBS three times i.p. The PBS provocation (inhalation) was performed either on day 26 or 27 after injection. In vivo airway response to MCh was performed with an increasing dosage of aerosolized MCh, identical to the protocol used to generate data presented in Fig. 1. Data are expressed as increases of Penh values for each concentration of MCh relative to PBS Penh values (±SD). The raw Penh data are depicted in Table II. *b*, Cells of BAL fluid were collected from offspring followed by sham sensitization and aerosol provocation with PBS and were differentiated. Depicted are the mean values (±SD) of the relative numbers of cells in the BAL. The absolute cell numbers are listed in Table III. *c* and *d*, Cytokine determination (TNF-α, IL-4) in supernatants of leukocytes from the blood of PBS-immunized offspring. Cytokines were measured by CBA; ∗, $p < 0.05$. *e*, The ratio of the Th2:Th1 cytokine was calculated by dividing the sum of IL-4 and IL-5 by the sum of TNF-α and IFN-γ. An increase in Th2 cytokines over Th1 was observed in supernatants from prenatally stressed mice. *f*, TNF-α concentration (picograms per milliliter) secreted from BAL cells in sham-sensitized animals.

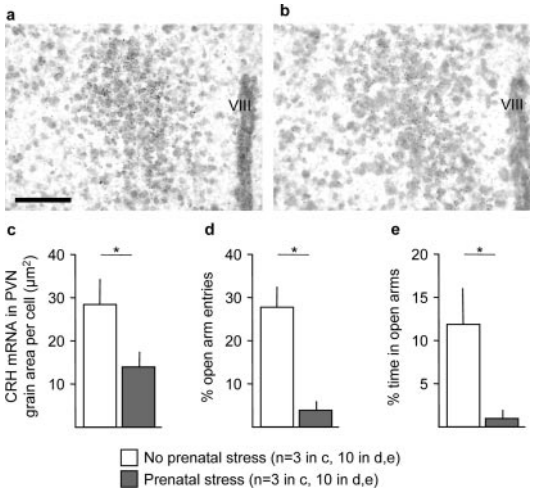

FIGURE 7. Expression of *CRH* mRNA expression in the PVN. Photomicrographs showing hybridization in the parvocellular PVN in control (*a*) and prenatally stressed mice (*b*). VIII, third ventricle; scale bar, 100 μm. *c*, Effect of prenatal stress on *CRH* mRNA expression in the brains of adult offspring. Data are mean ± SEM silver grain area per cell for *CRH* mRNA in the PVN; ∗, $p < 0.05$ one-tailed Student's *t* test. *d*, Percentage of open-arm entries of mice in EPM behavioral testing. *e*, Time spent in open arms in EPM analysis.

(data not shown), which verifies that the anxiogenic-like behavior seen in the prenatally stressed mice is not due to a general reduction in locomotor activity.

Discussion

Fetal programming of allergic asthma: introducing a mouse model

In the present study, we introduced an experimental approach to investigate pathways of fetal programming of allergic diseases. Besides the delineation of an increased vulnerability toward asthma-specific clinical features, such as airway hyperresponsiveness and inflammation in prenatally stressed adult offspring, we provide persuasive insights on dysregulated pathways of the cellular and humoral immune response upon Ag challenge in adulthood. Furthermore, we delivered experimental evidence for a reduced CRH mRNA expression in the PVN of adult offspring in response to prenatal stress, as well as behavioral changes, such as a reduction in locomotor activity and an increase in anxiety.

Vulnerable time window during fetal development programming allergic asthma

A crucial step toward the validation of an experimental model on fetal programming of asthma was clearly to define the most vulnerable time window for environmental insults during gestation. In this study, lung organogenesis provided an essential sentinel period for programming agents. In rodents, lung organogenesis is divided into four stages: 1) pseudoglandular stage, 2) canalicular stage, 3) terminal sac stage, and 4) alveolar stage (27). The rationale behind our experimental design with a prenatal stress exposure on gestation days 12 and 14, respectively, was based on the assumption that a stress insult during the most vulnerable period of organogenesis, the pivotal pseudoglandular stage, most likely programs the susceptibility to develop asthma in later life.

In humans, lung development begins at the third week of pregnancy with the formation of an entodermal diverticulum, from which the two lung buds are formed and elongate into the primary bronchi, followed by extensive branching and alveolization to ensure the development of a vast mucosal surface necessary for an efficient gas exchange after birth (32). The bronchial tree differentiates into multiple divisions during lung development during the first 6 mo of pregnancy and final maturation of the bronchial tree occurs after birth (32), whereby the time window of the stress exposure we chose in the present mouse model falls into this gestational period.

Strikingly, it has been hypothesized that the lung of adults suffering from asthma shares morphological features with the fetal lung, such as the appearance of epithelial-mesenchymal trophic units (33). Whether this is due to regression of lung tissue upon initial maturation or due to impairment of organogenesis and fetal lung maturation ab initio remains yet to be enlightened.

Besides lung organogenesis, another auspicious aspect for research on vulnerable targets involved the prevalence of asthma comprising alterations of immune system programming. The development of the immune system, including the development of the repertoire of reactive lymphocytes that will exist in postnatal life, begins prenatally and alterations of the fetal immune environment lead to impaired immune responses in later life (34). Presently, we observed that prenatally stressed offspring display profound alterations of cellular and humoral immune-response pathways to allergen challenge, including a modified leukocyte distribution in BAL, an influx of CCR3 cells into the lung, and a

Th2 > Th1 cytokine profile. Furthermore, cell populations of innate immunity, operating at the interface between the immune system and the pathogen world, have been altered by prenatal environmental insults, as shown by our results on the DC-mediated increase of Th2 cytokines by T cells in prenatally stressed offspring. Our observations on the humoral immune response revealing increased levels of IgE in blood of prenatally stressed offspring amend published evidence arising from human pregnancies demonstrating that maternal stress perception is associated with an increase in cord blood IgE levels (35). In this study, published evidence indicated that stress perceived by young mothers due to chronic caregiving results in an increased IgE level in the offspring in the first 2 years of life, associated with classical clinical signs of allergies in early childhood (36).

An explanation why TNF-α secretion is systemically decreased in the adult offspring following prenatal stress exposure remains speculative; because these data are in agreement with published evidence demonstrating a decrease of TNF-α secretion upon stimulation with endotoxin in rhesus monkeys 2 years after prenatal stress exposure (37), it may be that maternal up-regulation leads to fetal down-regulation, so contributing to the programming of decreased TNF-α response on stress.

In the present study, an established approach of OVA sensitization to analyze clinical and pathophysiological characteristics of asthma-like symptoms in murine models has been used. Evidently, a limitation of such an OVA-induced allergic airway inflammation model is the lack of spontaneously occurring asthma-like clinical symptoms when mice are not sensitized. Clearly, such a spontaneous murine asthma model would be highly desirable in the context of fetal programming. Furthermore, the exposure to stress during gestation may have resulted in an overall heightened sensitivity state in these offspring because it affected birth weight and behavior in adult offspring. Also, the reduced expression of *CRH* in the PVN in prenatally stressed adult offspring might result from stress-induced increased exposure to maternal corticosterone during pregnancy, negatively feeding back to the fetal/neonatal PVN and down-regulating *CRH* gene expression. This would mirror the effect of chronic stress on *CRH* expression in adults (38). Thus, future use of this model in interdisciplinary research endeavors may reveal whether the offspring express increased susceptibilities toward other chronic and/or atopic diseases.

More than a decade ago, Barker (13) emphasized the scientific hypothesis of a fetal basis of adult diseases. Since then, most of the subsequent trials in this area have concentrated on malnutrition during pregnancy as an environmental insult, affecting multiple aspects of adult health and disease risk, such as the occurrence of cardiovascular or metabolic diseases. More recently, maternal stress perception during pregnancy has been recognized as another pivotal environmental insult (39). In this study, we presented substantial experimental evidence that stress during pregnancy aggravates asthma in offspring in later life by severe alteration of the immune response to allergens and likely by impairment of lung organogenesis. This model may now be used to further demonstrate the individual impact, hierarchy, and redundancy of multiple key protagonists in fetal programming of asthma.

Acknowledgments

We thank E. Hagen, P. Moschansky, P. Busse, C. Seip, and B. Handjiski for their assistance and continuous support in generating this work. Furthermore, we are grateful for the contributions by V. Parker, funded by a summer vacation studentship from the British Society for Neuroendocrinology. M. K. Pincus-Knackstedt, S. M. Blios, and P. C. Arck are part of the Embryo Implantation Control Network of Excellence, cofinanced by the European Commission throughout the FP6 framework program Life Science, Genomics, and Biotechnology for Health.

Disclosures

The authors have no financial conflict of interest.

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