

Epigenetic regulation in murine offspring as a novel mechanism for transmaternal asthma protection induced by microbes

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Background: Bronchial asthma is a chronic inflammatory disease resulting from complex gene-environment interactions. Natural microbial exposure has been identified as an important environmental condition that provides asthma protection in a prenatal window of opportunity. Epigenetic regulation is an important mechanism by which environmental factors might interact with genes involved in allergy and asthma development. **Objective:** This study was designed to test whether epigenetic mechanisms might contribute to asthma protection conferred by early microbial exposure.

Methods: Pregnant maternal mice were exposed to the farm-derived gram-negative bacterium *Acinetobacter lwoffii* F78. Epigenetic modifications in the offspring were analyzed in T_H1- and T_H2-relevant genes of CD4⁺ T cells.

Results: Prenatal administration of *A lwoffii* F78 prevented the development of an asthmatic phenotype in the progeny, and this effect was IFN- γ dependent. Furthermore, the *IFNG* promoter of CD4⁺ T cells in the offspring revealed a significant protection against loss of histone 4 (H4) acetylation, which was closely associated with IFN- γ expression. Pharmacologic inhibition of H4 acetylation in the offspring abolished the asthma-protective

phenotype. Regarding T_H2-relevant genes only at the *IL4* promoter, a decrease could be detected for H4 acetylation but not at the *IL5* promoter or the intergenic T_H2 regulatory region conserved noncoding sequence 1 (*CNS1*).

Conclusion: These data support the hygiene concept and indicate that microbes operate by means of epigenetic mechanisms. This provides a new mechanism in the understanding of gene-environment interactions in the context of allergy protection. (J Allergy Clin Immunol 2011;■■■:■■■-■■■.)

Key words: Asthma, allergy, epigenetics, histone acetylation, hygiene hypothesis, T cells

Allergic bronchial asthma is a multifactorial disorder of the lung initiated by both genetic and environmental factors.¹ Epigenetic regulation represents one important mechanism by which environmental factors might interact with genes involved in allergy and asthma development.² Although it has been shown that DNA methylation and histone modification might have a crucial role in the underlying processes of development and differentiation of T-cell subsets, including T_H1 and T_H2 cells,³ the detailed molecular and cellular nature of this relationship in asthma initiation or prevention remains to be uncovered.⁴

Recently, the concept of epigenetic regulation in asthma development has gained increasing attention.⁵ In particular, transplacental effects are within the focus of current research because microbial exposure in early life, being prevalent in farm settings, is linked to asthma protection at a later stage.^{6,7} Various microbial isolates and compounds were recently identified to exhibit an allergoprotective capacity.⁸⁻¹³ In essence, we find substantial differences between several microbes in terms of influence phenotype development in asthma models. Of these bacterial strains and components, the gram-negative farm-derived isolate *Acinetobacter lwoffii* F78 was shown to provide the strongest allergoprotective effect in a prenatal model of transmaternal experimental asthma protection (see Table E1 in this article's Online Repository at www.jacionline.org) and was therefore chosen for further mechanistic studies. First mechanistic studies have shown that transfer of allergoprotective effects is dependent on maternal Toll-like receptor signaling.¹³ In this study we hypothesized that maternal exposure to *A lwoffii* F78 during gestation might result in epigenetic regulation of immune response genes in CD4⁺ T cells in the offspring. To test this hypothesis, we used a well-established animal model of developmental asthma protection and examined possible changes in DNA methylation and histone

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Abbreviations used

BAL:	Bronchoalveolar lavage
CFSE:	Carboxyfluorescein succinimidyl ester
ChIP:	Chromatin immunoprecipitation
CNS1:	Conserved noncoding sequence 1
H3K27me3:	Trimethylated lysine 27 of histone 3
H4:	Histone 4
H4ac:	Acetylated histone 4
MNC:	Mononuclear cell
OVA:	Ovalbumin

modifications within the genomic regions of T_H1 and T_H2 cytokine genes after maternal exposure to *A. lwoffii* F78.

METHODS**Animals**

Six- to 8-week-old female BALB/c mice and BALB/cscid mice were obtained from Harlan Winkelmann (Borchen, Germany). Mice were kept under specific pathogen-free housing conditions. Water and an ovalbumin (OVA)-free diet were supplied *ad libitum*. All animal experiments were performed in accordance with German and international guidelines and were approved by local authorities (Regierungspraesidium Giessen).

Preparation of *A. lwoffii* F78

A. lwoffii F78 was grown and cultured as described previously.⁸ Living bacteria were washed in PBS and lyophilized. Lyophilized bacteria were shown to generate living colonies after reconstitution in PBS.

Prenatal *A. lwoffii* F78 exposure

According to protocols previously optimized for microbial concentration and timing of treatment,^{10,11,13} female BALB/c mice received intranasal applications of 10^8 colony-forming units of freeze-dried *A. lwoffii* F78 reconstituted in a final volume of 50 μ L of PBS 11, 9, 6, 4, and 2 days before mating, as well as every other day during the 3 weeks of pregnancy. Offspring did not receive any *A. lwoffii* F78 supplementation, and *A. lwoffii* DNA could not be detected in the lungs of offspring at the day of analysis. Age-matched control animals were sham treated with PBS.

Sensitization and challenge of offspring

At the age of 4 weeks, offspring of PBS (control)- or *A. lwoffii* F78-exposed mothers were sensitized to OVA by means of 2 intraperitoneal injections of 10 μ g of OVA (grade VI; Sigma, St Louis, Mo) emulsified in 1.5 mg of Al(OH)₃ (Pierce, Cheshire, United Kingdom) in a total volume of 200 μ L at days 25 and 39 of the experimental protocol. Sham-treated groups received PBS with Al(OH)₃. At days 44, 45, and 46, animals were exposed to aerosolized OVA (1% wt/vol diluted in PBS) for 20 minutes. Lung function was analyzed 24 hours after the last aerosol challenge, and another 24 hours later, mice were terminally anesthetized with ketamine plus xylazine for further analyses.

Treatment with anti-IFN- γ mAb

Offspring mice received intravenous injections of a neutralizing monoclonal rat IgG anti-mouse IFN- γ antibody (100 μ g in 100 μ L of PBS per mouse and treatment; MAB 485; R&D Systems, Minneapolis, Minn) on the day before and 30 minutes before each OVA aerosol challenge. Control animals received the same amount of IgG from rat serum antibody (control, Sigma).¹⁴

Garcinol treatment

Mice received intraperitoneal injections of the histone acetyltransferase inhibitor garcinol (Enzo Life Sciences, Plymouth Meeting, Pa) at a

concentration of 0.2 mg/kg body weight 1 day before and 30 minutes before each OVA aerosol challenge. For additional details, see the **Methods** section in this article's Online Repository at www.jacionline.org.

Assessment of airway reactivity

Lung function analysis was performed 24 hours after the last aerosol challenge by using noninvasive head-out body plethysmography, as previously described.¹⁵ Additional details on the **methods** used are provided in this article's Online Repository.

Measurement of antibody titers

Blood samples were taken from the maxillary vessels, and serum levels of anti-OVA IgE, anti-OVA IgG₁, and anti-OVA IgG_{2a} were measured by means of ELISA, as previously described.¹⁰

Bronchoalveolar lavage and differential cell counts

Bronchoalveolar lavage (BAL) was performed, as described previously,¹⁶ with the exception that lavage was performed only once with a volume of 1 mL. Additional details on the **methods** are provided in this article's Online Repository.

Lung histology and quantitative morphologic analysis of mucin production

Fixed lung tissues were embedded into paraffin, and 3- μ m sections were stained with periodic acid-Schiff for determination of goblet cell metaplasia. Additional details on the **methods** are provided in this article's Online Repository.

Preparation and stimulation of mononuclear cells

Spleens were meshed through a nylon sieve, followed by density gradient centrifugation (Pancoll; PAN-Biotech, Bayern, Germany) to obtain mononuclear cells (MNCs). MNCs (1×10^6 cells/well) were stimulated with anti-CD3 (0.5 μ g/mL) and anti-CD28 (2 μ g/mL) mAbs (BD, Franklin Lakes, NJ). After 24 hours, cell-free supernatants were harvested by means of centrifugation (300g for 10 minutes), and cytokine levels were assessed by means of ELISA.

Measurements of cytokines

Cell-culture supernatants were analyzed for IL-4, IL-5, IL-10, IFN- γ (Opteia Kit; BD Pharmingen, San Jose, Calif), and IL-13 production (CytoSet; Invitrogen, Carlsbad, Calif) by using the ELISA technique. Assays were performed as recommended by the manufacturers.

Measurement of proliferation

Proliferation measurement of MNCs and CD4⁺ T cells was performed with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, Ore), according to the manufacturer's instructions. Additional details on the **methods** are provided in this article's Online Repository.

Quantitative PCR

Total RNA of isolated CD4⁺CD25⁻ T cells was extracted (TriFast; Peqlab, Erlangen, Germany) and reverse transcribed (Omniscript RT Kit; Qiagen, Hilden, Germany) by using oligo (dT) primer. A RotorGene (Corbett Life Sciences Qiagen) and the QuantiTect SYBR Green PCR Kit (Qiagen) were used for quantitative real-time PCR of *IL4*, *IL5*, *IL10*, *IL13*, *IFNG*, and *CD3d* (housekeeping control gene). Quantitative PCR data were analyzed according to the previously described $2^{-(\Delta\Delta C_T)}$ method¹⁷ and normalized to *CD3* gene transcript levels. Primer sequences are detailed in **Table E2** in this article's Online Repository at www.jacionline.org.

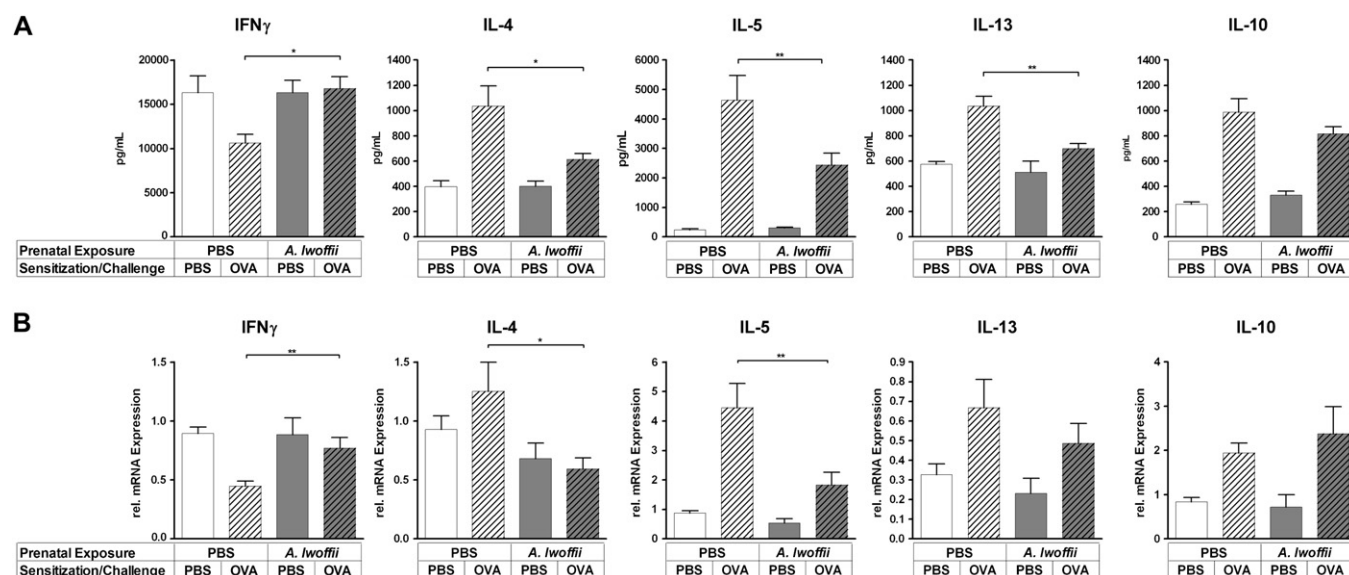


FIG 1. T_H1/T_H2 cytokine balance after prenatal *A. Iwoffii* F78 exposure. **A**, Concentrations of IFN- γ , IL-4, IL-5, IL-13, and IL-10 in the supernatant of anti-CD3/anti-CD28-stimulated mononuclear spleen cells from offspring from *A. Iwoffii* F78- or sham-exposed (PBS) mothers. **B**, Relative mRNA expression of IFN- γ , IL-4, IL-5, IL-13, and IL-10 in CD4⁺CD25⁺ splenic T cells. Data represent the results of 2 independent experiments, each with 4 to 8 individually analyzed animals per group.

Preparation and bisulfite conversion of genomic DNA, PCR, and pyrosequencing

CD4⁺CD25⁺ T cells were isolated from splenic MNCs by using MACS, as recommended by the manufacturer (Miltenyi Biotec, Bergisch Gladbach, Germany). Bisulfite conversion of genomic DNA was performed as described elsewhere.¹⁸ Template preparation was performed as described previously by Tost and Gut.¹⁹ Pyrosequencing was performed with the PyroMark ID System (Biotage AB, Uppsala, Sweden) and sequencing primers. Primer sequences are shown in Table E2. Additional details on the methods are provided in this article's Online Repository.

Chromatin immunoprecipitation

CD4⁺CD25⁺ T cells were isolated from splenic MNCs by using MACS, as recommended by the manufacturer (Miltenyi Biotec). DNA-protein interactions in isolated cells were cross-linked in the presence of 1% formaldehyde for 10 minutes at room temperature. Subsequently, chromatin immunoprecipitation (ChIP) was performed, as described previously,²⁰ with antibodies against acetylated H4 (H4ac; Upstate Biotechnology, Charlottesville, Va) and trimethylated lysine 27 of histone 3 (H3K27me3; Abcam, Cambridge, United Kingdom). Immunoprecipitated and eluted DNA was purified with QIAquick columns (Qiagen) and amplified by means of quantitative PCR. Primer sequences are shown in Table E2. All amplifications were performed in duplicate by using 2 μ L of DNA per reaction. The duplicate mean values were displayed as the percentage of immunoprecipitated DNA compared with total DNA (100% input), and the SE was calculated. Control ChIP was performed with a control antibody (rabbit IgG, Sigma) to ensure specificity (data not shown).

Statistical analysis

The number of independently performed experiments is mentioned in the legend of each figure. All numeric data are expressed as means $\pm$ SEs and analyzed for significance by using 3-factor ANOVA with the factors maternal *A. Iwoffii* F78 exposure, sensitization of the offspring, and garcinol treatment and by using Tukey analysis or the Student unpaired *t* test with *P* values of less than .05, less than .01, and less than .001. Calculations were performed

by use of GraphPad Prism software, version 4.01 (GraphPad Software Inc, La Jolla, Calif).

RESULTS

Asthma-protective effects by prenatal *A. Iwoffii* F78 exposure are associated with altered IFN- γ production

Protection from the development of an asthmatic phenotype by prenatal *A. Iwoffii* F78 exposure was accompanied by a marked shift in the T_H1/T_H2 cytokine profile (Fig 1). In offspring mice from *A. Iwoffii* F78-exposed mothers, T-cell *in vitro* stimulation of splenic MNCs revealed a significantly reduced capability to produce the T_H2 cytokines IL-4, IL-5, and IL-13. In contrast, the IFN- γ responsiveness was completely restored in these animals, whereas IL-10 production remained unaffected. Analysis of mRNA cytokine expression in anti-CD3/anti-CD28-stimulated CD4⁺CD25⁺ T cells confirmed these results. Furthermore, no differences were detected in CD4⁺CD25⁺ forkhead box protein 3-positive regulatory T-cell numbers in the progeny by means of FACS analysis (data not shown).

We examined whether the reduced T_H2 response might be the result of the restored IFN- γ production in the progeny because IFN- γ counterregulates many T_H2 cell activities^{21,22} (Fig 1). Therefore IFN- γ function was blocked by treating offspring of *A. Iwoffii* F78-exposed mothers with a neutralizing anti-IFN- γ mAb. This treatment completely abolished the *A. Iwoffii* F78-mediated effect, as evidenced by restored airway inflammation, goblet cell metaplasia, and development of airway hyperreactivity (Fig 2, A-C). Concurrently, functional blockade of IFN- γ resulted in significantly increased production of the T_H2 cytokines IL-4, IL-5, and IL-13 (Fig 2, D), indicating a regulatory effect of IFN- γ on T_H2 cytokine production in this model. Accordingly, the trend toward decreased OVA-specific IgE antibody levels in prenatally *A. Iwoffii* F78-exposed mice was also eliminated by anti-IFN- γ treatment (Fig 2, E).

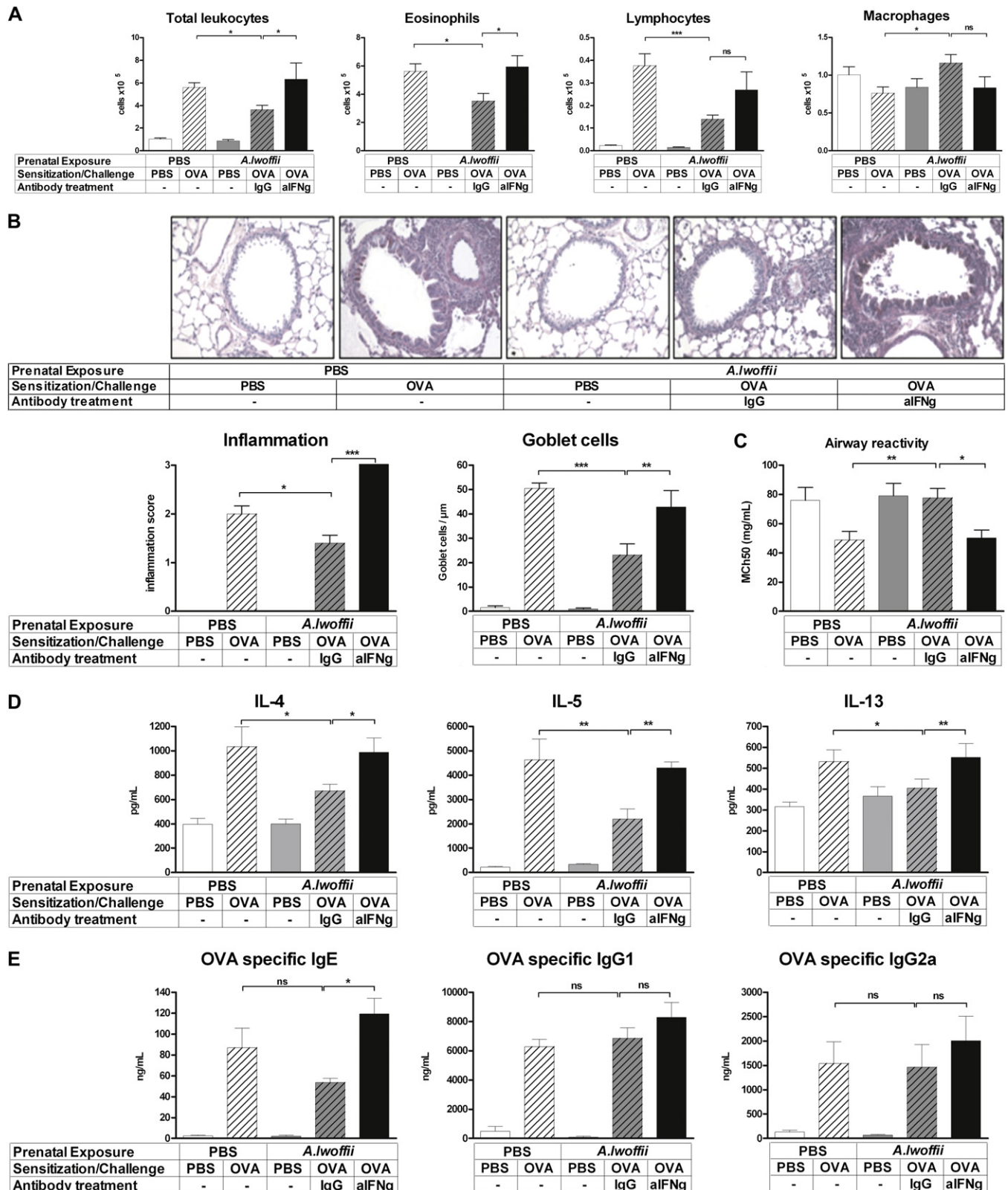


FIG 2. Effects of anti-IFN- γ treatment during OVA challenge in offspring of *A. Iwoffii* F78-exposed mothers. **A**, Differential leukocyte numbers in BAL fluids. **B**, Quantification of inflammation and mucus-producing goblet cells. **C**, Airway responsiveness to methacholine (MCH) in offspring. **D**, *In vitro* T_H2 cytokine production by restimulated mononuclear splenocytes. **E**, OVA-specific serum antibody levels (n = 8 mice per group).

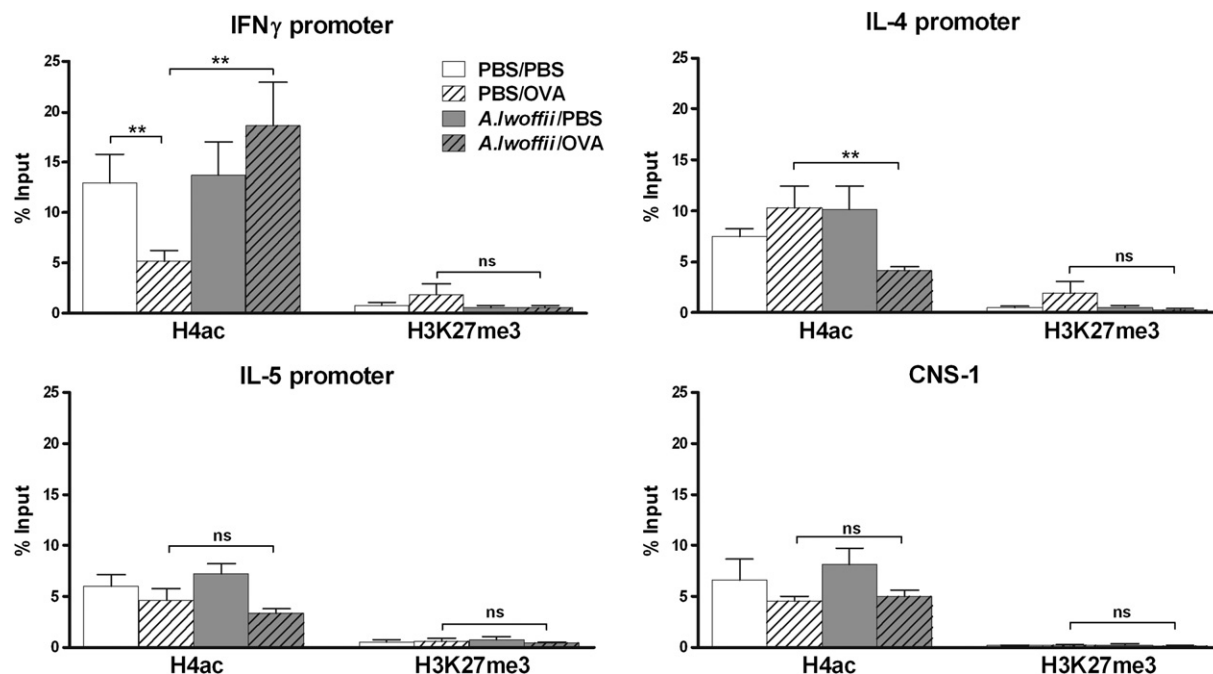


FIG 3. H4 acetylation within the *IFNG* and *IL4* promoters after prenatal *A lwoffii* F78 exposure. H4ac and H3K27me3 levels within the *IFNG*, *IL4*, and *IL5* promoters and the *CNS1* region were determined by means of ChIP in CD4⁺CD25⁺ T cells purified from spleens of offspring from *A lwoffii* F78- or sham-exposed (PBS) mothers. Data represent the results of 2 independent experiments, each with 4 to 8 individually analyzed animals per group.

Prenatal *A lwoffii* F78 exposure affects epigenetic modifications within genes of the T_H1/T_H2 loci

Epigenetic regulation by histone modification and DNA methylation has been shown to have a crucial role in the development of T_H1 and T_H2 cells (reviewed by Wilson et al³). ChIP was used to investigate the role of histone modifications at the *IFNG* promoter, the promoters for *IL4* and *IL5*, and the important T_H2 regulatory region conserved noncoding sequence 1 (*CNS1*) as a possible mechanism underlying the persistent alterations in cytokine production. H4ac (associated with transcriptionally active chromatin) and histone 3 lysine 27 trimethylation (H3K27me3; a marker of inactive chromatin), both of which are known to be modulated during T-cell differentiation,²³⁻²⁵ were analyzed in CD4⁺CD25⁺ T cells from prenatally *A lwoffii* F78- or sham-exposed offspring mice. OVA-sensitized and challenged progeny of sham-exposed mothers (PBS/OVA) showed reduced levels of H4ac at the *IFNG* promoter compared with PBS-sensitized progeny of sham-exposed mothers (PBS/PBS, Fig 3). This suggests that OVA sensitization/challenge affects the acetylation status of H4 at the *IFNG* promoter (Fig 3). *A lwoffii* F78 exposure alone (*A lwoffii*/PBS) had no effect on H4ac, as shown by a similar H4ac level compared with that of the sham-exposed control group (PBS/PBS). However, in offspring of mothers exposed to *A lwoffii* F78, the effect of OVA sensitization/challenge on H4ac was prevented. In contrast, no significant changes in H4ac levels were observed between all study groups at the promoter region of the T_H1 transcription factor signal transducer and activator of transcription 4, nor were respective mRNA levels significantly changed (see Fig E1 in this article's Online Repository at www.jacionline.org). Analysis of H4ac within the T_H2 locus revealed no differences at the *IL5* promoter and the *CNS1* region. Only at

the *IL4* promoter were reduced H4ac levels observed in sensitized/challenged offspring from *A lwoffii* F78-exposed mothers (Fig 3). In contrast, H3K27me3 was nearly undetectable at all analyzed loci in all study groups, suggesting that this repressive mark was not involved in the regulation of T_H1 and T_H2 cytokine production in this model.

Additionally, we investigated DNA methylation at the promoters of the *IFNG*, *IL4*, and *IL5* genes, as well as in the *CNS1* region. Six CpG positions known to play a role in IFN- γ expression²⁶ were analyzed, which showed only low site-specific absolute methylation levels (ranging from 11% to 17% in naive mice). Baseline methylation levels were markedly higher within T_H2 cytokine promoters, ranging from 29% to 60% for *IL4* and from 47% to 100% for *IL5*, respectively. Prenatal *A lwoffii* F78 exposure did not, however, induce any changes in the CpG methylation levels at all these sites (see Fig E2 in this article's Online Repository at www.jacionline.org). In contrast, methylation within the *CNS1* region was only discretely affected by prenatal *A lwoffii* exposure at 3 CpG positions (−8435 [+5% compared with the PBS/OVA group], −8384 [+8%], and −8374 [+11%]; see Fig E2).

Inhibition of histone acetylation by garcinol abolishes *A lwoffii* F78-mediated asthma protection in the progeny

In our model the strongest effect of prenatal *A lwoffii* F78 exposure on epigenetic marks was observed on H4ac at the *IFNG* promoter. Therefore we explored whether pharmacological inhibition of *de novo* histone acetylation with garcinol (a polyisoprenylated benzophenone known to inhibit histone acetyltransferases p300 and p300/CBP-associated factor,²⁷ thereby affecting *de novo*

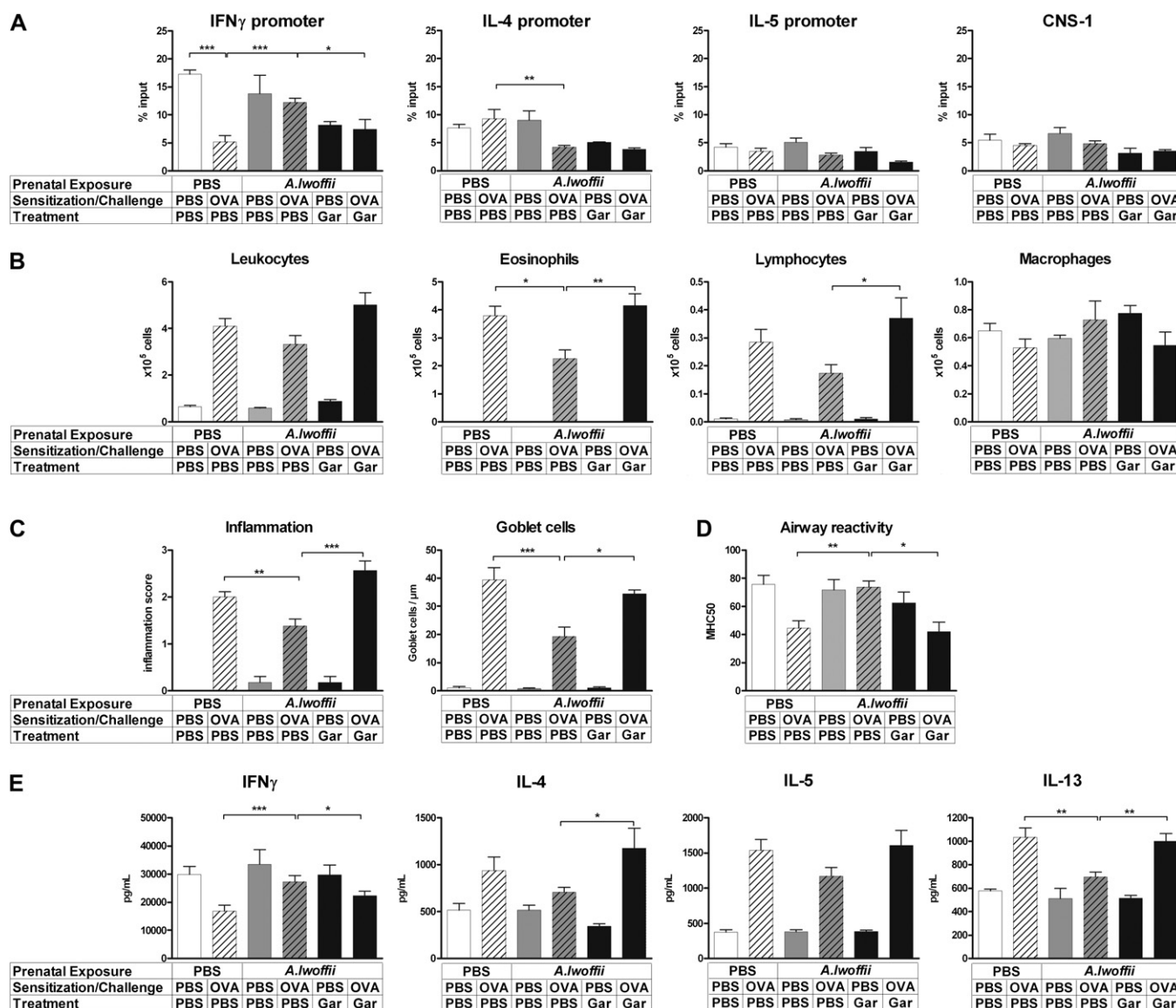


FIG 4. Effects of garcinol treatment during OVA challenge on H4 acetylation at the *IFNG* promoter of CD4⁺CD25⁺ splenic T cells and asthma protection by prenatal *A. Iwoffii* F78 exposure. **A**, H4ac within the *IFNG* and *IL4* promoters and *CNS1* (for control groups, see Fig E4). **B**, Differential leukocyte numbers in BAL fluid. **C**, Quantification of airway inflammation and mucus-producing goblet cells. **D**, Airway responsiveness to aerosolized methacholine (MCH). **E**, *In vitro* cytokine production by restimulated splenocytes. Gar, Garcinol. Data represent the results of 2 independent experiments, each with 4 to 8 individually analyzed animals per group.

acetylation) could restore the asthmatic phenotype. Initial experiments were performed to exclude possible nonspecific or cytotoxic effects of garcinol treatment on T cells. *Ex vivo* proliferation of anti-CD3/anti-CD28-stimulated, CFSE-labeled CD4⁺ T cells isolated from garcinol-treated mice remained completely unaffected. In addition, no effects were observed on *in vivo* proliferation of CFSE-labeled CD4⁺ T cells transferred into mice with severe combined immunodeficiency, which were subsequently treated with garcinol (see Fig E3 in this article's Online Repository at www.jacionline.org). Furthermore, intraperitoneal injection of garcinol was well tolerated by the mice because no changes in behavior, body weight, or coat appearance were observed.

Application of the compound was carried out in a time period that has been identified by the IFN- γ -blocking experiment. This

is further supported by the observation that prenatal exposure with *A. Iwoffii* F78 did not affect the sensitization to OVA but counteracts the development of airway inflammation and airway hyperresponsiveness triggered by OVA challenge.¹³ Garcinol treatment of sham-sensitized progeny from PBS-exposed mothers reduced H4ac levels at the *IFNG* promoter (see Fig E4 in this article's Online Repository at www.jacionline.org). Already low levels of H4ac in sensitized/challenged progeny from sham-exposed mothers were not further affected by garcinol treatment. However, garcinol treatment of progeny from *A. Iwoffii* F78-exposed mothers significantly prevented the *A. Iwoffii* F78-induced protection against the loss of H4ac at this locus. In contrast, garcinol treatment did not affect H4ac levels at the *IL4* promoter, the *IL5* promoter, and *CNS1* (Fig 4, A).

Consequently, garcinol treatment resulted in significantly reduced IFN- γ levels in anti-CD3/anti-CD28-stimulated splenic MNCs accompanied by increased IL-4, IL-5, and IL-13 production (Fig 4, E). Moreover, the asthmatic phenotype was fully restored by garcinol treatment of the progeny of *A. lwoffii* F78-exposed mothers, as indicated by a significant influx of eosinophils into the airway lumen (Fig 4, B), marked inflammatory cell accumulation in the lung tissue and goblet cell hyperplasia (Fig 4, C), and development of airway hyperreactivity to aerosolized methacholine (Fig 4, D). Garcinol treatment has no influence on the asthmatic phenotype of prenatally PBS-exposed animals (data not shown).

DISCUSSION

In this study we provide evidence that epigenetic regulation plays an important role in transmaternal asthma protection. This protection is dependent on the production of IFN- γ , as indicated by the abolishment of asthma protection after functional blockade of this cytokine by mAb treatment. Focusing on cytokine genes of the adaptive immune response, we discovered significant changes in H4 acetylation at the *IFNG* promoter and the *IL4* promoter, which were positively associated with IFN- γ and IL-4 protein levels. In contrast, H4 acetylation was not changed at the *IL5* promoter and at the genomic enhancer region *CNS1* of the T_H2 cytokine cluster, nor was CpG methylation significantly affected at *IFNG*, as well as at the T_H2 locus.

IFN- γ is a potent suppressor of T_H2 -driven allergic immune responses counteracting airway hyperresponsiveness, airway eosinophilia, and mucus production.^{21,22,28} In mice and human subjects, a number of studies have provided evidence for the pivotal role of IFN- γ in allergy protection (reviewed by Vuillemin et al²⁹). T cells from subjects at risk of atopic disease generally produce lower IFN- γ levels.³⁰ Accordingly, recent epidemiologic studies demonstrate that maternal farm exposure is significantly associated with a higher capacity of cord blood-derived MNCs to produce IFN- γ ,³¹ supporting a direct influence of environmental factors on prenatal T-cell maturation. This is in agreement with our findings in a murine model and illustrates the importance of the adaptive immune response in the regulation of transmaternal asthma protection.

Epigenetic processes are a consequence of complex gene-environment interactions that are known to modulate the phenotype without affecting the genotype. In this regard epigenetic processes contribute to the plasticity of gene expression and consequently are crucial for developmental processes and cell differentiation, including maturation of T_H cells.^{26,32-34} Both human and murine studies provide strong evidence for a regulatory role of epigenetic processes within the genomic locus on the production of IFN- γ .^{5,23,26,35} In particular, *in vitro* differentiation of murine unmethylated naive T_H0 cells did not influence CpG methylation under T_H1 -polarizing conditions, whereas T_H2 polarization was associated with hypermethylation of the respective CpG sites.²⁶ In our experiments no differences between treatment groups were observed with regard to CpG methylation levels within the proximal *IFNG* promoter.

In contrast, our data point toward a role of H4 acetylation in transcriptional control of the *IFNG* locus. H4 acetylation is an important mark for an open chromatin structure, and indeed, the changes observed in terms of H4 acetylation at this promoter were positively associated with changes in IFN- γ protein

production. Animals were treated with garcinol, a known inhibitor of histone acetyltransferases p300 and p300/CBP-associated factor, to further investigate the role of histone acetylation.^{27,36} Analysis of the H4 acetylation levels at the *IFNG* promoter indicated that garcinol treatment prevented the *A. lwoffii* F78-induced increases in *de novo* acetylation in this promoter. Furthermore, the *A. lwoffii* F78-mediated prevention of the asthmatic phenotype was abolished in these animals. These data suggest that histone acetylation at the *IFNG* gene promoter might represent an important mechanism in the process of asthma protection by microbial exposures in this model. However, because of the limitations of the model, we cannot rule out that epigenetic modifications of further immune-regulatory molecules might also be involved in the multifactorial process of asthma development and protection.

The reduced production of the T_H2 cytokines IL-4, IL-5, and IL-13 observed in the OVA-sensitized and challenged animals derived from *A. lwoffii* F78-treated mother mice is most likely due to a regulatory effect of the high levels of IFN- γ in these animals and might be linked to the observation that acetylation at the H4 histone of the IL-4 promoter is decreased. It is well known that IFN- γ has a strong inhibitory effect on the development and activation of T_H2 cells. Analyses of CpG methylation levels of the promoters of IL-4 and IL-5 provided no evidence for a role of CpG methylation in this context. Only in the genomic T_H2 enhancer region *CNS1* was an effect on CpG methylation observed in some of the 13 investigated CpG sites. These changes in methylation levels might further support the suppression of T_H2 cytokine protein production. Therefore protection against loss of H4 acetylation in the *IFNG* promoter together with increases in *CNS1* CpG methylation could work together in this context.

It is well known that epigenetic processes are critical in the regulation of IFN- γ production in human subjects and mice.^{23,25,26,35} Here we demonstrate an important role for H4ac in transcriptional control of the *IFNG* locus. H4ac is an important marker for an open chromatin structure, and indeed, the changes observed in terms of H4ac at the *IFNG* promoter positively correlate with changes in IFN- γ protein production.

It still remains unclear which signals are directly responsible for activation of histone-modifying enzymes, DNA methyltransferases, or both.³⁷⁻³⁹ It has been suggested that inflammatory conditions on several levels, including the production of IL-6, reactive oxygen species, and I κ B kinase α activation, might be involved in the activation of these enzymes. In particular, IL-6 could be a relevant mediator of the observed protection in our model because we have recently shown that the *A. lwoffii* F78-mediated effects are dependent on maternal Toll-like receptor recognition of this microbe, subsequently leading to a sustained release of IL-6.¹³

This is the first study indicating that asthma protection achieved by maternal exposure to microbes is associated with epigenetic regulation in the offspring. There are, of course, additional environmental factors that might exert their effects on the epigenetic level as well. Prenatal methyl-donor-rich diet,⁴⁰ exposure to airborne polycyclic aromatic hydrocarbons,⁴¹ or diesel exhaust particles⁴² were shown to be associated with changes in DNA methylation levels, the latter at the *IFNG* promoter. Here we observe for the first time that the gram-negative bacterium *A. lwoffii* F78 triggers characteristic alterations in histone modification at specific genomic loci. This is associated with altered cytokine production levels and subsequent prevention of the asthmatic phenotype. Those data obtained in animal models are now the

foundation to translate these observations into the human situation. Nevertheless, our data support the concept that maternal exposure plays an important role in allergy and asthma prevention of the next generation and that epigenetic regulation in the progeny is an important mechanism. Such epigenetic effects might be amenable to interventions to prevent asthma development.

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Clinical implications: Epigenetic modulation induced by prenatal exposure to microbes represents an important mechanism for the prevention of allergy and asthma in the offspring.

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METHODS

Preparation of garcinol

Garcinol was reconstituted in dimethyl sulfoxide at a concentration of 24 mg/mL and stored at -20°C . Before intraperitoneal treatment of the animals, the stock solution was further diluted 1:600 in PBS, resulting in a final concentration of 40 $\mu\text{g/mL}$. Control animals received PBS/0.16% (vol/vol) dimethyl sulfoxide.

Assessment of airway reactivity

Lung function analysis was performed 24 hours after the last aerosol challenge by using noninvasive head-out body plethysmography, as previously described,^{E1} a method that was shown to directly correlate with invasive lung function measurements of lung resistance.^{E2} Briefly, bronchial responsiveness was determined as changes in midexpiratory airflow in response to consecutive exposures to increasing concentrations (3, 6, 12.5, 25, 50, 75, 100, and 125 mg/mL) of aerosolized β -methacholine (Sigma). The methacholine concentration that induced a 50% reduction of baseline midexpiratory airflow was defined as MCH_{50} .

Principal comparison of noninvasive head-out body plethysmography with invasive Flexivent lung function analysis in our laboratory revealed concordance (data not shown).

BAL and differential cell counts

BAL was performed as described previously,^{E1} with the exception that lavage was performed only once with a volume of 1 mL. An automated Casy TT cell counter (Schaefer Systems, Reutlingen, Germany) was used to determine total leukocyte cell counts. For differential cell count analysis, cytopsin preparations were prepared, fixed, and stained with Diff-Quick (Merz & Dade AG, Dudingon, Switzerland).

Lung histology and quantitative morphologic analysis of mucin production

Directly after BAL, lungs were fixed with 6% paraformaldehyde. Lung tissues were embedded into paraffin, and 3- μm sections were stained with periodic acid-Schiff. Goblet cell metaplasia and the volume of epithelial mucin per surface area of the airway epithelial basal membrane was determined as described previously.^{E3} Inflammatory scores were quantified as follows: 0, normal; 1, few inflammatory cells; 2, 1 to 2 cell layers of inflammatory cells; and 3, 3 or more cell layers of inflammatory cells.

Measurement of proliferation

MNCs from garcinol-treated animals were labeled with 1 $\mu\text{mol/L}$ CFSE (Molecular Probes, Invitrogen) for 15 minutes at 37°C (according to the manufacturer's instructions) before stimulation with anti-CD3 and anti-CD28 to assess *ex vivo* proliferative capacity of cells. After 72 hours, cells were harvested and stained with anti-CD4-allophycocyanin (BD), and proliferation was determined by means of flow cytometry.

CD4^{+} T cells were isolated from spleens of BALB/c mice by means of MACS, as recommended by the manufacturer (Miltenyi Biotec), and labeled

with 1 $\mu\text{mol/L}$ CFSE to assess the *in vivo* proliferative capacity of cells. Cells (1×10^7) were transferred by means of intravenous injection into BALB/c^{scid} mice. Recipients were treated every other day with 0.2 mg/kg garcinol intraperitoneally. At day 8, proliferation of CFSE-labeled CD4^{+} T cells from the spleen was determined by means of flow cytometry.

Preparation and bisulfite conversion of genomic DNA, PCR, and pyrosequencing

$\text{CD4}^{+}\text{CD25}^{-}$ T cells were isolated from splenic MNCs by means of MACS, as recommended by the manufacturer (Miltenyi Biotec); resuspended in ATL buffer (Qiagen); and incubated with proteinase K (Sigma) at 37°C for 1 hour. Proteinase K was deactivated by means of incubation at 95°C for 15 minutes, and genomic DNA was stored at -80°C .

Bisulfite conversion of genomic DNA was performed as described elsewhere.^{E4} PCR products were generated in a 50- μL reaction volume with 10 pmol of forward and reverse primer and HotstarTaq PCR Master Mix (Qiagen). Template preparation was performed as described previously by Tost and Gut.^{E5} Pyrosequencing was performed with the PyroMark ID System (Biotage AB) and sequencing primers (see below). The position of CpG sites within the genomic sequence was determined in comparison with the transcription start of published full-length mRNA sequences NM_008337 (*IFNG*), NM_010558 (*IL5*), and NM_021283 (*IL4*). The *IL4* transcription start site was used for numbering of CpGs within *CNS1*. Primers sequence are shown in Table E2.

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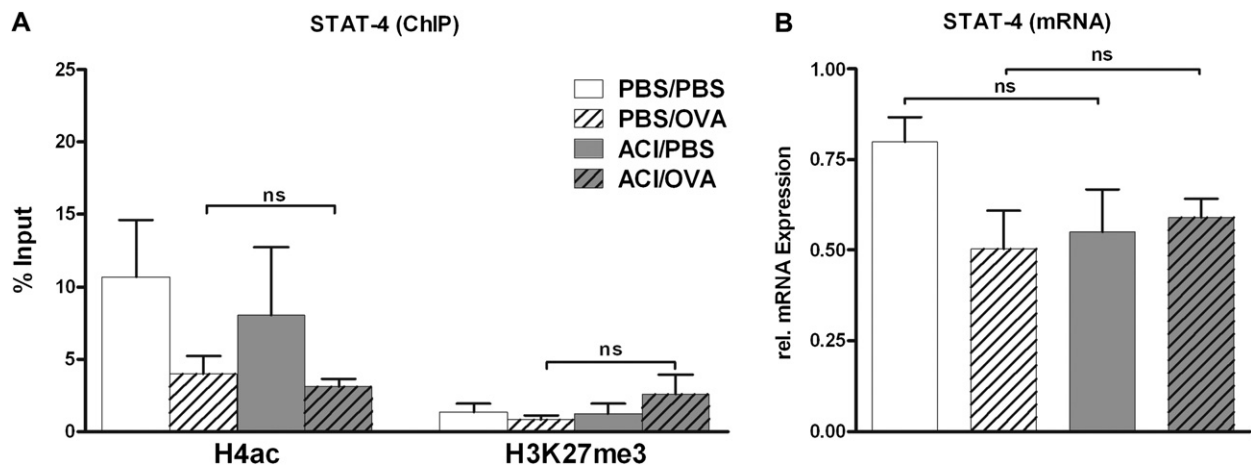


FIG E1. H4 acetylation within the STAT-4 promoter after prenatal A. Iloffii F78 exposure. H4ac and H3K27me3 levels within the STAT-4 promoter were determined by means of ChIP in CD4⁺ CD25⁺ T cells purified from spleens of offspring from A. Iloffii F78 - or sham-exposed (PBS) mothers. Data represent the results of 2 independent experiments, each with 4 to 8 individually analyzed animals per group.

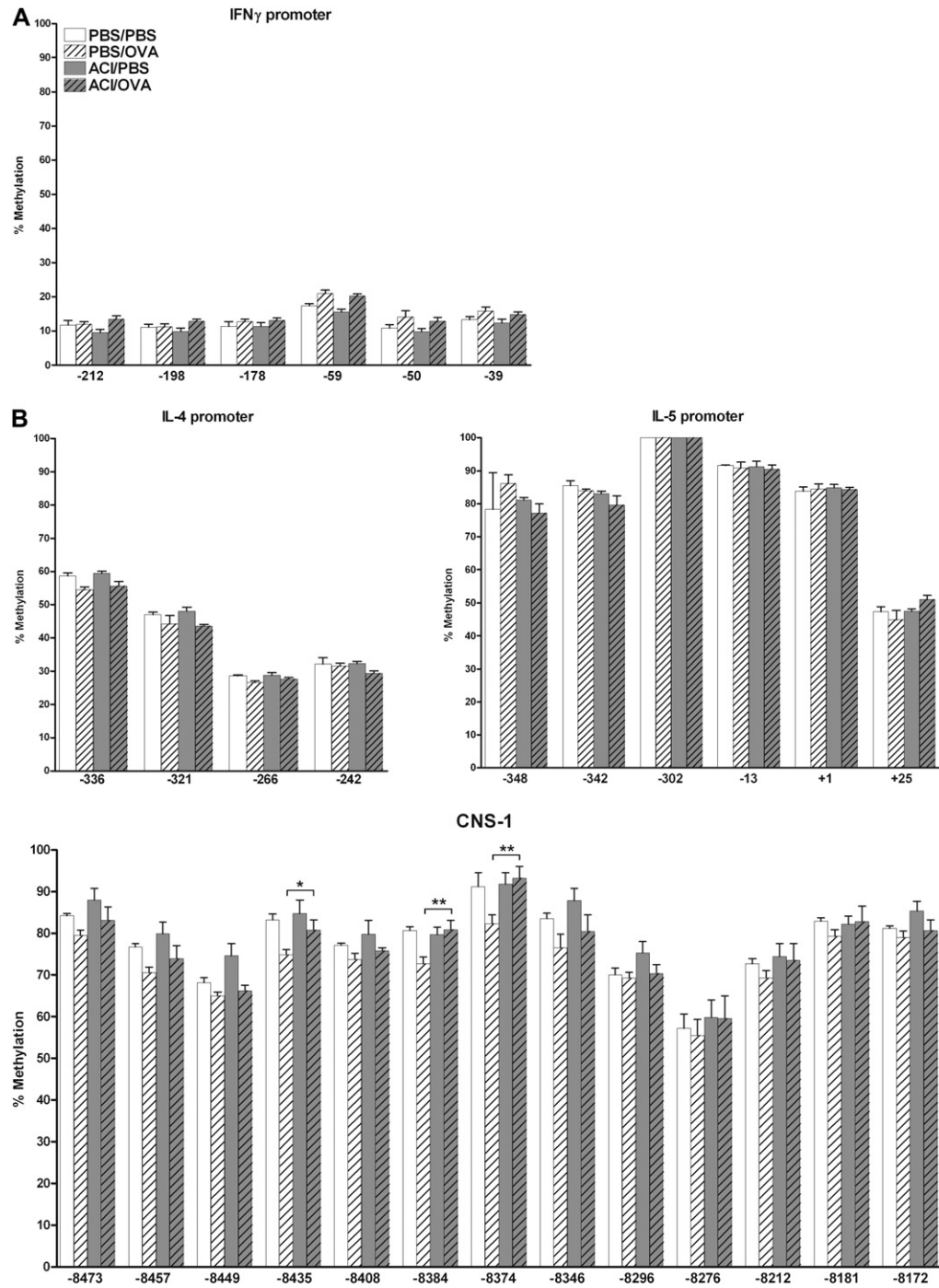


FIG E2. Modulation of DNA methylation within *CNS1* in splenic CD4⁺CD25⁺ T cells from offspring after pre-natal *A. Iwoffii* F78 exposure. Positions of CpG sites correspond to their positions upstream of the transcription start site of annotated sequence NM_008337 for *IFNG* (A), NM_010558 (*IL5*), and NM_021283 (*IL4*) for *IL4* and *CNS1* (B). Data represent results of 2 independent experiments, each with 4 to 8 individually analyzed animals per group.

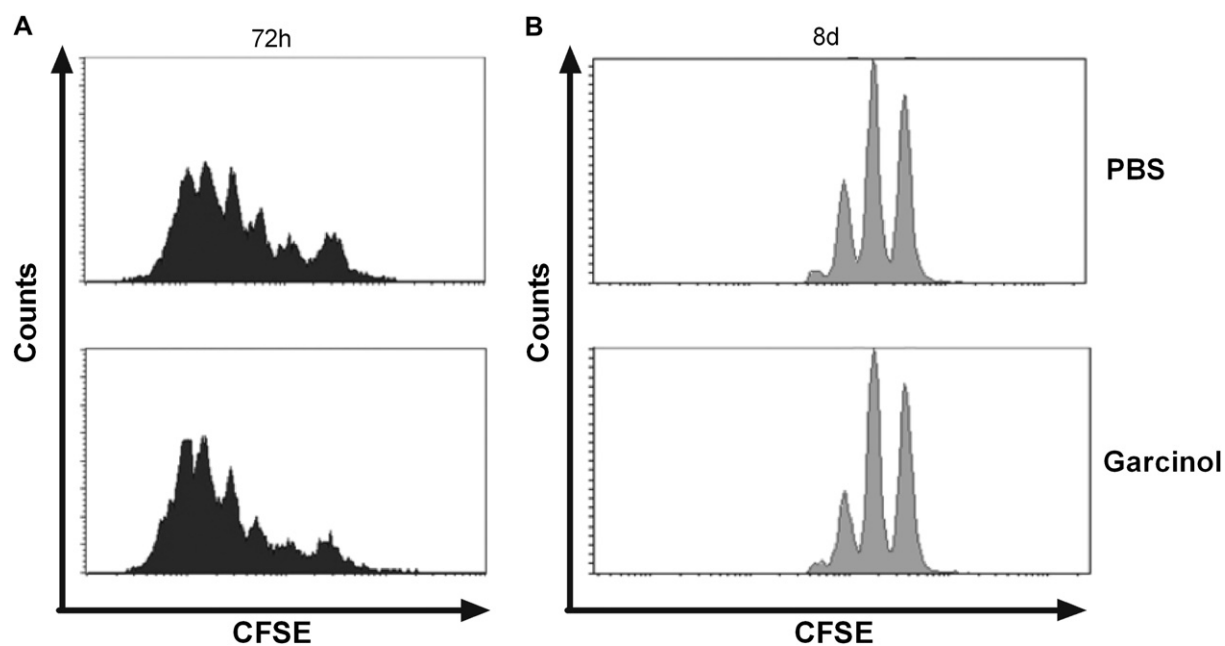


FIG E3. T-cell activation and proliferation after garcinol treatment. **A**, *Ex vivo* proliferation of splenic T cells of garcinol- and PBS-treated mice determined by means of CD4⁺ and CFSE labeling and subsequent FACS analysis. **B**, *In vivo* proliferation of splenic CFSE-labeled CD4⁺ T cells transferred from garcinol- or PBS-treated mice intravenously into BALB/c^{scid} mice determined at day 8 by means of FACS analysis. Data are from 1 representative mouse per group. Two independent experiments were performed, each with 2 to 4 individually analyzed animals per group.

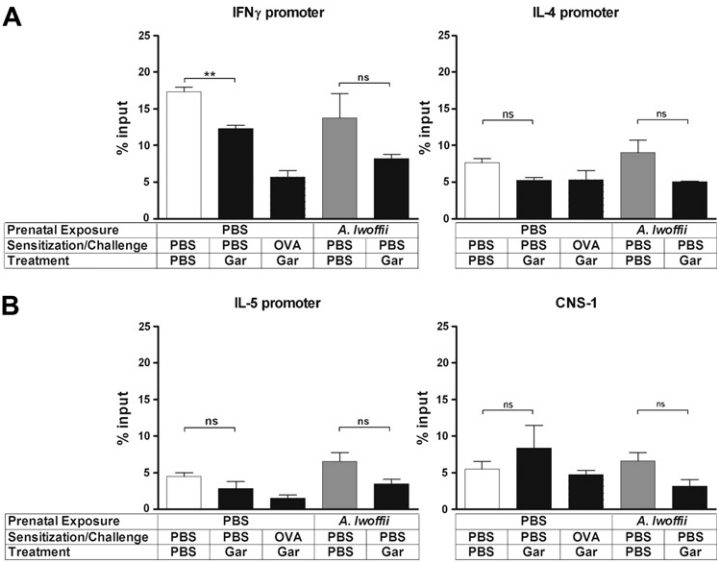


FIG E4. Modulation of H4 acetylation at the *IFNG* promoter by using the histone acetyltransferase inhibitor garcinol (control groups). H4ac levels within the *IFNG* and *IL4* promoter and *CNS1* in CD4⁺CD25⁺ splenic T cells from offspring treated with garcinol before each OVA challenge were analyzed by means of ChIP. Data represent the results of 2 independent experiments, each with 4 to 8 individually analyzed animals per group. *ns*, Not significant. Respective data of experimental groups are provided in Fig 4.

TABLE E1. Asthma-protective effect of different bacterial strains

Change in % compared with the sham-treated OVA group			
Exposure to:	<i>Acinetobacter lwoffii</i> F78 ^{E5}	<i>Lactococcus lactis</i> G121 ^{E6}	<i>Bacillus licheniformis</i> 467 ^{E7}
BAL§			
Leukocytes	−34 ± 5†	−36 ± 9†	−31 ± 16
Eosinophils	−92 ± 2‡	−88 ± 4‡	−89 ± 6‡
Lymphocytes	+120 ± 31*	+40 ± 26	+7 ± 27
Macrophages	+36 ± 9	+36 ± 21	+69 ± 15
Airway reactivity#	+144 ± 11*	+116 ± 13*	+53 ± 23
Change in % compared with the sham-treated OVA group			
Maternal exposure to:	<i>A lwoffii</i> F78 ^{E4}	<i>Lactococcus lactis</i> G121 (unpublished data)	LPS ^{E8}
BAL§			
Leukocytes	−56 ± 12*	−11 ± 13	−72 ± 5*
Eosinophils	−55 ± 11*	−8 ± 16	−63 ± 7†
Lymphocytes	−59 ± 9*	+3 ± 17	−55 ± 15
Macrophages	+31 ± 23	−31 ± 30	−55 ± 5*
Immunoglobulins			
Anti-OVA IgE	−31 ± 7	+1 ± 1	−66 ± 12*
Anti-OVA IgG ₁	−25 ± 16	−7 ± 6	−94 ± 3‡
Anti-OVA IgG _{2a}	−18 ± 28	+39 ± 30	+43 ± 41
Cytokines in spleen¶			
IFN-γ	+26 ± 7*	+17 ± 12	−27 ± 24
IL-5	−54 ± 8†	−40 ± 23	−75 ± 5†
Airway reactivity#	+64 ± 14†	+27 ± 12	−20 ± 27
Inflammation score**	−33 ± 5*	−15 ± 11	Not done

The table reviews previously published data as indicated by the mentioned references. The top panels show that female BALB/c mice were intranasally exposed to PBS (control), *A lwoffii* F78, *L lactis* G121, or *B licheniformis* starting 10 days before sensitization until aerosol challenge. After challenge, the asthmatic phenotype was analyzed. The bottom panels show female BALB/c mice were intranasally exposed to PBS (control), *A lwoffii* F78, or *L lactis* G121 before and during pregnancy. LPS application was performed intraperitoneally before mating and intranasally during pregnancy. Offspring were sensitized to OVA and OVA aerosol challenged, and the asthmatic phenotype was analyzed. Means ± SEs are shown with significances for PBS/OVA versus bacterium/OVA: * $P < .05$, † $P < .01$, and ‡ $P < .001$.

§Numbers of total leukocytes, eosinophils, macrophages, and lymphocytes in BAL fluid.

||Concentration of anti-OVA IgE, anti-OVA IgG₁, and anti-OVA-IgG_{2a} in serum of offspring.

¶Cytokine production of anti-CD3/anti-CD28–stimulated MNCs from spleens of offspring.

#Airway responsiveness to methacholine measured by means of head-out body plethysmography, as described in the [Methods](#) section. Shown is the methacholine concentration that causes a 50% reduction in baseline midexpiratory airflow.

**Quantification of inflammation in airways as described in the [Methods](#) section.

TABLE E2. Primer sequences

Bisulfite PCR		5'-3'
Py-IFN- γ		
s	GGT TTG AGA AGT TTA AGA GTT TTT TTA	
as	<i>Biotin</i> - CCT ACA CCT CTC TAA CTT CCA ATT TTA TAC CTA ATC	
Py-CNS-1		
s	TAT AAG GGT TGT AGG AAG AGT AAT GTA GTT TTT ATA T	
as	<i>Biotin</i> - CAC TTT AAA CTA ATC AAA CCT AAA ACT ATA TCC ATA	
Py-IL-4		
s	GTT AAA GTT TTA TTT TAT GGT TTT GTT TGT TTT AT	
as	<i>Biotin</i> - ACA AAT AAA TTT TCC TAT AAA ATC AAA CCA ATT AA	
Py-IL-5		
s	GGG GAT AAA AAA GAA GGT GTT TAA GAA AAG T	
as	<i>Biotin</i> - AAA CAC TCA AAT ACA AAA ACA TCC TTC TCA TAA CT	
Pyrosequencing		5'- 3'
IFN- γ seq 1	ATT TTA TAA GAA TGG TAT AGG TG	
IFN- γ seq 2	AAA ATT AAA AAA AAA TTT GTG	
CNS-1 seq 1	AAT GTA GTT TTT ATA TAT TGG TTT AT	
CNS-1 seq 2.1	TTG AGA GGG GAT TAT TTT TTA T	
CNS-1 seq 3.1	TAT GGG GTT TTT TTT GGT TG	
IL-4 seq 2	TTT TTT TGA TAT TAT TTT GTT	
IL-5 seq 1	AAG AAG GTG TTT AAG AAA AG	
IL-5 seq 2	TTT TTT AGA GAG AGA ATA AAT TG	
ChIP		5'- 3'
Ch-IFN- γ		
s	CAT ACC CTT TCC TTG CTT TTC	
as	TTG TGG GAT TCT CTG AAA GCA	
Ch-CNS-1		
s	GGG AGT TTC TTA GGC CCT CT	
as	CCC CCT CTC ACT GTG AAA AC	
Ch-IL-4		
s	TCT GCC TCC ATC ATC CTT CT	
as	ACA CCA TAA TCG GCC TTT CA	
Ch-IL-5		
s	ACC CTG AGT TTC AGG ACT CG	
as	TCC CCA AGC AAT TTA TTC TCT C	
Ch-STAT4		
s	AGA GGA ATG GCG TCA GCG GC	
as	ACT GAG CTA GCA GAT GCC GGA T	
Quantitative PCR		5'- 3'
IFN- γ		
s	GCG TCA TTG AAT CAC ACC TG	
as	TGA GCT CAT TGA ATG CTT GG	
IL-4		
s	TCA ACC CCC AGC TAG TTG TC	
as	TGT TCT TCG TTG CTG TGA GG	
IL-5		
s	AGC ACA GTG GTG AAA GAG ACC T	
as	TCC AAT GCA TAG CTG GTG ATT T	
IL-13		
s	CCA TCC CAT CCC TAC AGA AA	
as	ATA GGC AGC AAA CCA TGT CC	

(Continued)

TABLE E2. (Continued)

Quantitative PCR		5'- 3'
CD3d		
s	ACA CTC AAC TTG GGC AAA GG	
as	CAG AGT TGC GAT GAG GTC AA	
STAT4		
s	ACT ACA GCT CCC AGC CGT GC	
as	CCG TGG AAT CGC TTC GGA TTG TT	

as, Anti-sense *s*, sense *STAT4*, signal transducer and activator of transcription 4.